DEVELOPMENTALLY REGULATED RIBOSOMAL RNA GENES IN *PLASMODIUM VIVAX*: BIOLOGICAL IMPLICATIONS AND PRACTICAL APPLICATIONS

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ABSTRACT

Title of Dissertation: Developmentally controlled ribosomal RNA genes in *Plasmodium vivax*: Biological implications and practical applications [1]

Jun Li, Doctor of Philosophy, 1994

Dissertation directed by: L. Lance Sholdt, Ph.D., Chief of Staff, Department of Preventive Medicine and Biometrics, USUHS

Ribosomal RNA (rRNA) is an essential determinant of the structure and function of the ribosome, a ubiquitous apparatus for protein synthesis. In contrast to other eukaryotes, the rRNA genes in *Plasmodium* species are unique in terms of their genomic arrangement and transcriptional regulation during development; further investigation may elucidate the functional significance of the gene organization to parasite development and evolution. In this study, three structurally distinct rRNA genes, including one novel type, have been characterized from the genomic DNA of the human malaria parasite, *Plasmodium vivax*. Comparison of the sequences coding for small subunit rRNA (SSUrRNA) indicates that the type A gene seems to be closely related to the type C gene with an overall similarity of 85.5%; while the type B gene, the novel type, appears more distantly related to either the A or C gene. It has an overall similarity to the A and C genes of 52% and 62%, respectively. The sequence differences are not randomly distributed but tend to cluster into several regions known to diverge rapidly in all eukaryotic SSUrRNAs. Comparative analysis of the secondary structures suggests that all three transcripts retain the essential conformation conserved in eukaryotic rRNAs. The structural differences occurring between the genes are localized to three predicted variable regions and appear to be characteristic of the gene type rather than the species of origin. This is also true in the corresponding genes from closely related species of the genus *Plasmodium*. The existence of these structurally distinct rRNAs within all

[1] The dissertation research was supervised by Dr. Thomas F. McCutchan, and performed in the Laboratory of Malaria Research, National Institute of Allergy and Infectious Disease, NIH.

Plasmodium species studied may indicate a functional significance to maintaining the multi-rRNA system.

Transcription of the three distinct genes is differentially regulated during the discrete stages of *P. vivax* development. The A gene transcript is predominant during erythrocytic schizogony in the human host and disappears after 24 hours in engorged mosquitoes. The B gene transcript appears in mosquitoes as early as 48 hours after the infectious blood meal and remains during oocyst development. Transcription of the C gene starts at day 4 after mosquito engorgement and corresponds to the differentiation of sporozoite in maturing oocyte. The sequential expression of structurally distinct rRNA genes seems to correspond to the cytological phases of the parasite differentiation and development. In addition, this unique process suggests that the evolution of novel types of rRNA may have an important role in adoption of the complex parasitic life cycle for the genus *Plasmodium*.

A reverse transcriptase mediated PCR was established for both qualitative and quantitative assessment of parasite development. The assay is based on identified rRNA structures and the changing pattern of rRNA transcription during discrete stages of the parasite's life cycle. The intrinsic design of the assay involves directly competitive amplification of distinct rRNAs using genus conserved primers in a manner that relates the ratio of different rRNA transcripts to the stage of parasite development. This method can be used to determine average susceptibility of a vector population and the extent of multi-species infection. In addition, the high sensitivity of the method permits detection of parasites preserved on stained slides or in dried mosquitoes, thus allowing analysis of historical materials. Hence, this assay is not only useful to evaluate the effects of vaccines and antimalarial drugs in the laboratory but also can be applied in field epidemiological studies to determine the structure of the parasite population and the dynamics of parasite transmission.

DEVELOPMENTALLY REGULATED RIBOSOMAL RNA GENES IN *PLASMODIUM VIVAX*: BIOLOGICAL IMPLICATIONS AND PRACTICAL APPLICATIONS

by

Jun Li

Thesis/dissertation submitted to the Faculty of the Department of Preventive Medicine & Biometrics Graduate Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy 1994

Dedicated to

My father: **MR. LI, ZHONG-HAN**

May 18, 1924 to March 23, 1993

for

his consistent support and inspiration

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ABBREVIATIONS

The followings are abbreviations used in the text:

rRNA - ribosomal ribonucleic acid

rDNA - a segment of deoxyribonucleic acid sequence coding for rRNA or rRNA gene

SSUrRNA - small subunit rRNA

LSUrRNA - large subunit rRNA

ITS - internal transcribed spacer in rRNA gene units

PCR - polymerase chain reaction

RT - reverse transcriptase

bp - base pair

Kb - kilobase pair

INTRODUCTION

Ribosomal RNA (rRNA) is an essential determinant of the structure and function of the ribosome, the central apparatus for protein synthesis [Woese et al., 1983]. As it has equivalent functions and conserved structures in all organisms, rRNA sequence analyses and comparisons are often used to establish relationships among organisms. The small subunit rRNA (SSUrRNA) sequence in particular is considered to be an important indicator of both phylogeny and population structure [Olsen et al., 1986; Woese, 1987]. This approach is extremely useful for distinguishing unicellular organisms, such as bacteria, fungi, and protozoa, since their simple morphology provides few clues for identification and further categorization. Application of this comparative analysis has been expanded by technological advances, especially DNA polymerase chain reaction (PCR), molecular cloning and rapid nucleotide sequencing. These advances have allowed the study of a wide variety of organisms including those resistant to *in vitro* cultivation, such as *Plasmodium vivax*.

Plasmodium species are unique among eukaryotic organisms in expression of structurally distinct rRNA genes during different stages of their life cycle [McCutchan, 1986; Gunderson et al., 1987; Waters et al., 1989a]. This has stimulated an interest in understanding the correlation between rRNA gene expression and parasite development. The study described here is directed towards defining and understanding of this relationship and, in turn, using this information to gain insight into parasite biology and evolution. Furthermore, a detailed understanding of the structure of distinct rRNA genes and their regulation of transcription in malaria parasites should lead to innovative approaches for analyzing population genetics and antimalarial drug intervention.

In the following parts of this introduction, the uniqueness of the ribosomal system in the *Plasmodium* species will be reviewed as fundamental information for the presented research. The cytological stages of malaria parasite development are described to facilitate the explanation of differential transcription of rRNA genes. The genomic organization and transcriptional regulation of both eukaryotic or prokaryotic rRNA genes are also described in a comparative manner so that the *Plasmodium* rRNA gene can be

appreciated as an exception to the standard rules. The deviations in the structural arrangement and transcriptional regulation of the *Plasmodium* rRNA genes may have direct biological significance for parasite development and evolution. The mitochondrial and other extrachromosomal rRNA genes, which exist in most eukaryotic organisms as well as *Plasmodium* species [Feagin, et al., 1991; 1992; Gardner et al., 1988; 1991; 1993], will not be discussed.

1. Four reproductive phases in the *Plasmodium* life cycle:

Members of the genus *Plasmodium*, as unicellular eukaryotes and obligate intracellular parasites, exhibit a very complex life cycle [Garnham, 1966; Coatney et al., 1971]. The parasite undergoes a dozen or more distinguishable stages of development as it travels between the invertebrate vector and the vertebrate host. The entire life cycle may be divided into four continuous but distinct reproductive phases, including three asexual and one sexual. Each phase exhibits a remarkable progression of differentiation and development (Fig.1). With human malaria parasites, the first asexual reproduction phase begins when the female anopheline mosquito inoculates the parasite (sporozoite) into the bloodstream of the human host during a blood meal. The sporozoite invades the liver cells and initiates an exoerythrocytic schizogony. Over a period of 5-15 days depending on the parasite species, the sporozoite differentiates into the trophozoite and then the schizont, which contains as many as 30,000 liver-stage merozoites. The second asexual reproduction phase, erythrocytic schizogony, starts when the released merozoites enter the bloodstream and invade the red blood cells. The parasites proceed through three stages in the red blood cells, the ring form, the trophozoite, and the schizont. The mature schizont contains 8 to 24 merozoites depending on the species. The entire erythrocytic cycle takes 2 to 3 days. Once the infected erythrocyte ruptures, the merozoites are released into the plasma; attach to other erythrocytes and either repeat the cycle, or for reasons that are not clear, differentiate into sexual forms, the gametocytes (macrogametocyte and microgametocyte).

Sexual reproduction takes place in the midgut of the mosquito after the gametocytes are ingested during the blood meal. The gametocytes mature rapidly in the

Fig 1. Summary of the *Plasmodium* life cycle: Four reproductive phases

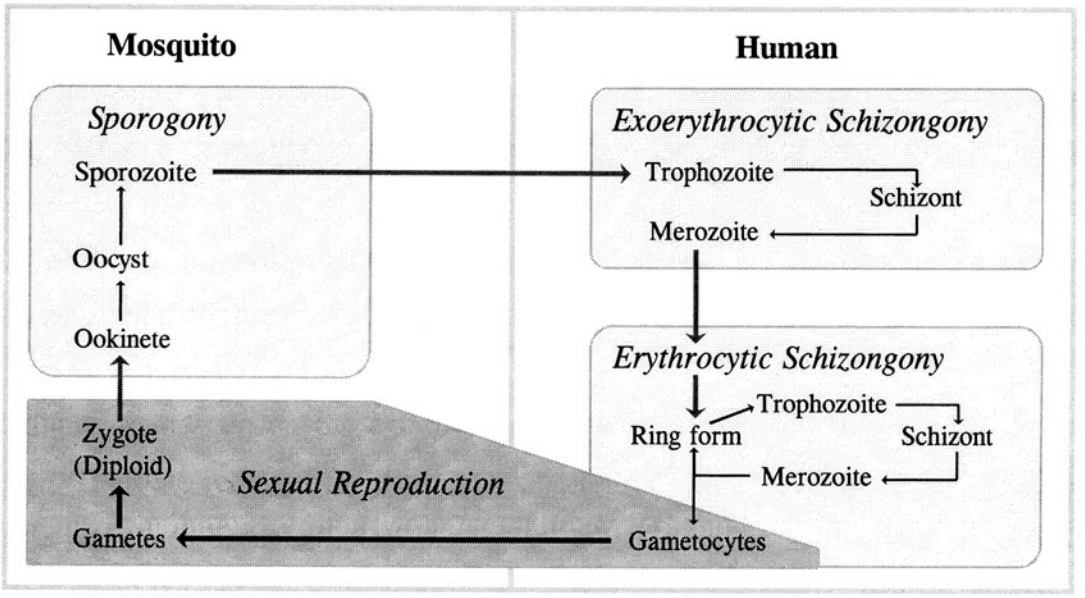
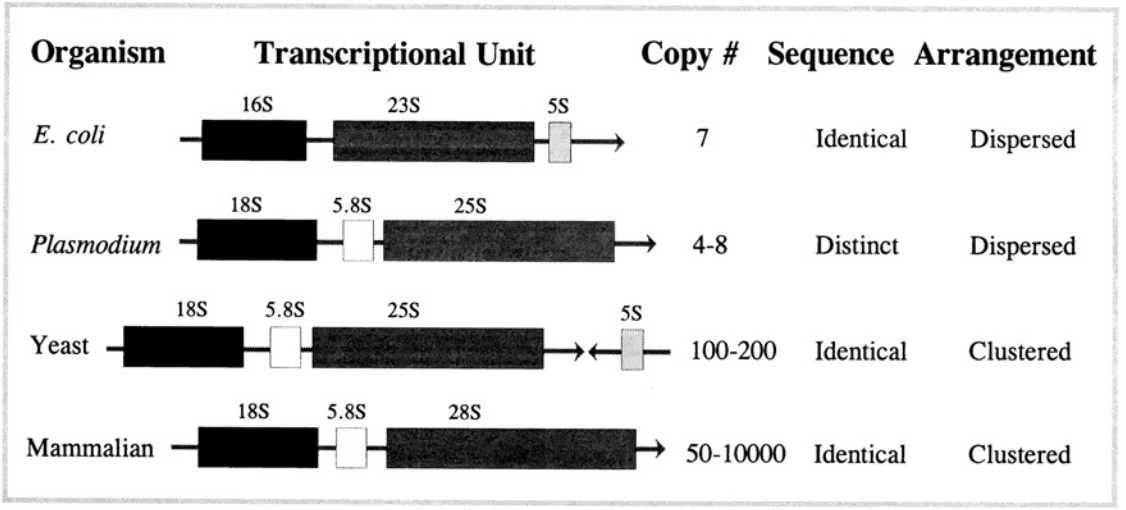


Fig 2. Comparison of rRNA Gene Structure and Genomic Organization



midgut and become extracellular under the stimulation of the changes in temperature, oxygen and carbon dioxide concentrations, and pH. About eight male microgametes are released from one microgametocyte during exflagellation while only one female macrogamete differentiates from a macrogametocyte. Fertilization of the macrogamete occurs within a few minutes after the feeding. The diploid stage begins when a macrogamete fuses with a microgamete to form a zygote. In 12-18 hours, the zygote transforms into a motile organism called the ookinete, which possesses a invasive apparatus similar to that of the sporozoite and merozoite [Sinden, 1978]. The ookinete migrates to the wall of the mosquito midgut, penetrates the peritrophic membrane and epithelium, rounds up on the external surface of the midgut, and develops into the oocyst. The time required for parasite sexual reproduction is 1 to 2 days. When the parasite returns to a haploid state is unclear, but this occurs in the later stage of the ookinete when the and the early stage of the oocyst. Differentiation of the sporozoite in the maturing oocyst is called sporogony. This is the only asexual reproductive phase in the mosquito vector. As many as 10,000 motile sporozoite can be produced from one oocyst during a 9-15 day period. The actual number of sporozoite produced depends on the parasite species and the ambient temperature [Rosenberg and Rungsiwongse, 1991]. After the oocyst ruptures, the sporozoite enter the mosquito hemolymph and invade the salivary glands, where they are ready for transmission to new human hosts.

In summary, *Plasmodium* species are "termly" differentiated organisms; they have to adapt to different environments at various stages of the life cycle and undergo a multi-step development. The protein synthesis and other metabolic machinery may vary both quantitively and qualitatively during the different phases of the parasite's life cycle. The regulated expression of structurally and functionally distinct rRNA genes, which will be discussed in detail later, could be a coarse control for *Plasmodium* development. The evolution of the novel rRNA genes is presumably an important mechanism for the genus *Plasmodium* adopting such a complex life cycle.

2. Unique organization of *Plasmodium* rRNA genes:

Knowledge of consistent features of the ribosome is necessary to understand why

the departures from the norm in *Plasmodium* species have such significance. The cytoplasmic ribosomes from various organisms have very similar architecture, in which there are two major subunits, each with different ribosomal RNAs and numerous ribosomal proteins [Hillis and Dixon, 1991]. The small subunit contains a single type of rRNA (16-18S or SSUrRNA) and about 20 ribosomal proteins in prokaryotes or 30 proteins in eukaryotes. The large subunit is more complex; in the prokaryote there are two types of rRNA (23S or LSUrRNA and 5S rRNA) and about 30 proteins, while in the eukaryote three types of rRNA (25-28S or LSUrRNA, 5.8S and 5S rRNA) and about 40 proteins. The eukaryotic 5.8S rRNA, whose sequence is complementary to the region near the 5' end of the LSUrRNA, is a part of the 23S rRNA in prokaryotes [Cox and Kelly, 1981; Jacq, 1981].

As there are common features of ribosome architecture among organisms, there are also common features attributed to the genes that encode ribosomal RNA. The genomic organization of rRNA genes has been extensively studied in a wide variety of organisms [Hillis and Dixon, 1991; Neefs et al., 1990; 1991]. In prokaryotic organisms, all three rRNA genes are organized as a single transcription unit, in which the 16S, 23S and 5S rRNAs are separated by two internal transcribed spacers (ITS1 & 2) and linearly arranged from 5' to 3' ends (Fig.2). The entire unit is transcribed by RNA polymerase I as a single precursor molecule, which is later processed to yield the three mature transcripts. The prokaryotic rRNA gene units are usually distributed near the replication origin in the genome. This arrangement is thought to allow rRNA genes to be transcribed more efficiently during the rapid DNA replication. For example, the eubacteria, *Escherichia coli*, usually contains seven rRNA transcription units (*rrnA* to *rrnG*), which are all arranged around the origin of replication [Nomura et al., 1977; Ingraham et al., 1983]. Compared with the prokaryotic rRNA gene, the rRNA transcription unit of the eukaryote retains both SSUrRNA and LSUrRNA genes. In addition a 5.8S gene, which is part of LSUrRNA gene in prokaryotes, is inserted in the region between the two genes; while 5S rRNA tends to separate as an independent gene in both physical location and transcriptional regulation from the main transcriptional unit (Fig.2). An apparent intermediate type is seen in the yeast, *Saccharomyces cerevisiae*

[Peters, 1979], in which the 5S rRNA gene, although arranged with the 18S, 5.8S and 25S rRNA genes in one transcription unit, is transcribed by RNA polymerase III while the other genes are transcribed by RNA polymerase I from the opposite strand (Fig. 2). For most higher eukaryotes, the 5S gene is completely separated from other rRNA genes and becomes an independent transcription unit. Furthermore, rRNA transcription units of each type tend to cluster together forming tandem repeated arrays at different chromosome locations. The transcription of the two units by different RNA polymerases is still maintained. The reasons for eukaryotes adopting this tandem arrangement are not clear, although it may have the advantage of enhancing rRNA transcription and regulation by concentrating RNA polymerase and related co-factors and maintaining the sequence identity of this multi-copy gene family [Tartof, 1975; Lewin, 1980; Gonzalez and Schmickel, 1986].

In contrast to most eukaryotes, *Plasmodium* species exhibit a very limited number of rRNA genes, and the main transcription units do not appear to be linked in the genome. The arrangement of individual genes in the main unit is the same as that of other eukaryotes; the SSUrRNA gene is followed by the 5.8S and then the LSUrRNA gene (Fig.2). The studies on genomic DNA from rodent malaria, *Plasmodium berghei* [Dame and McCutchan, 1983a], avian malaria *Plasmodium lophurae* [Unnasch and Wirth, 1983a], and human malaria *Plasmodium falciparum* [Langsley et al., 1983], suggest that the rRNA gene units are not tandemly arranged or clustered in a specific location of the genome. For example, none of the four main rRNA gene units, identified from *P. berghei*, are linked by restriction mapping within a range of 150 Kb of the genome. Further hybridization of rRNA probe to a chromosome blot reveals that distinct rRNA gene units of *Plasmodium cynomolgi* and *P. falciparum* are dispersed on numerous different chromosomes [Van der Ploeg et al., 1985; McCutchan, 1986; Langsley et al., 1987; Langsley and Ponnulurai, 1988; Sharkey et al., 1988; Weber, 1988]. However, the distribution pattern of the 5S rRNA genes is somewhat different. Restriction analysis of *P. berghei* genomic DNA indicates that the 5S rRNA genes, which are not associated with the main transcription unit, tend to be localized to a 1.5 Kb fragment of the genomic DNA [Dame and McCutchan, 1984]. A detailed Southern blots analysis suggests that

all three 5S rRNA genes identified from *P. falciparum* are clustered within a 2.86 Kb fragment of the genomic DNA [Shippen-Lentz and Vezza, 1988]. In *Plasmodium*, the internal arrangement of the individual transcription units resembles those of eukaryotes, however the copy number and nontandem arrangement of the rRNA gene units in the genome is actually reminiscent of the eubacteria, for example *E. coli*. This unique organization of the rRNA genes may represent an intermediate evolutionary status or reflect a genetic solution responding to the complexities of translational control faced by this organism.

3. Low copy number of the rRNA gene in *Plasmodium*:

The rRNA genes (rDNA) in all examined eukaryotes are present in a relatively high copy number to meet variable needs for ribosome biogenesis during different periods of development. Some organisms have only a single copy of the rRNA gene, but usually this copy can be amplified either chromosomally or extrachromosomally when dramatically increased amounts of rRNA are required [Long and Dawid, 1980]. The number of the rDNA copies within a given species is consistent, and the range in most eukaryotes is 50 to 10,000. Significant reduction of the gene number may affect the normal process of development and growth [Tartof, 1975; Shermoen and Kiefer, 1975; Ritossa, 1976]. However, for *Plasmodium* species, the copy number of rRNA genes per haploid genome is relatively low and may represent a rate limiting event in development. The copy number estimation for various species of malaria parasites, including *P. berghei* [Dame and McCutchan, 1983a; b; Dame et al., 1984], *P. lophurae* [Unnasch and Wirth, 1983a; b], and *P. falciparum* [Langsley et al., 1983; McCutchan et al., 1988; Shippen-Lentz et al., 1990], indicates that *Plasmodium* species contain only about 4 to 8 copies per haploid genome.

Furthermore, *Plasmodium* species do not appear to amplify their rRNA transcription units either chromosomally or extrachromosomally during development in either the vertebrate or the mosquito host [Dame and McCutchan, 1983b; McCutchan, 1986; Cornelissen et al., 1985]. This is unlike some protozoa which have few rRNA genes; the developmentally-controlled amplification of extrachromosomal rDNA units

occurs in these organisms when tremendous amounts of the rRNA transcripts are required for ribosome biogenesis [Hadjiolov, 1985]. For example, *Tetrahymena*, which has one extrachromosomal rRNA gene in a germinal micronucleus, amplifies its rRNA gene up to about 600 copies during growth [Engbert and Pearlman, 1972; Yao and Gall. 1977]. Similar amplification also occurs in the amphibian, *Xenopus laevis*, and in the insect, *Drosophila melanogaster*, during oogenesis [Ritossa, 1976].

Considering the lack of amplification, the absolute copy number of the rRNA gene seems low for *Plasmodium* species. The relative number of the genes in terms of total genome size appears not so unique, as the estimated number of rDNA units for *P. berghei* is 4 per 2-5 x 10^7 bp of haploid genome [Dame and McCutchan, 1983a; Dore et al., 1980], about one rDNA unit per 5-12 x 10^6 bp of genomic DNA. This is close to higher eukaryotes, such as mice and humans, which have approximately one rDNA per 1.6 x 10^7 bp of haploid genome. However, the proportion of the genome occupied by rDNA genes of *Plasmodium* species is small compared to other lower organisms. For example, *Dictyostelium discoideum* has one rDNA per 7.0 x 10^5 bp and *Leishmania donovani* has one rDNA unit per 3.3 x 10^5 bp [Lewin, 1980; Long and Dawid 1980]. The low proportion of rDNA units is noted to be consistent with the early cytological observations that *Plasmodium* species lack a defined nucleolus [Aikawa et al., 1969], a complex structure of chromatin consisting of rRNA gene clusters [Dagmar et al., 1991].

The copy number of the 5S rRNA gene, examined from some *Plasmodium* species, also seems lower than those of other eukaryotes. For most eukaryotes, the number of 5S rRNA genes correlates with the abundance of the main rRNA transcription units. The ratio may vary with different organisms, ranging from one-half to many times the main unit number [Hadjiolov, 1985; Selker et al., 1981]. Like the main rRNA transcription units, Plasmodial 5S rRNA genes are present in exceptionally low copy number. Only three copies of 5S genes are identified from the genome of *P. falciparum*, the lowest number reported for any organism [Shippen-Lentz and Vezza, 1988].

With regard to the developmental process for *Plasmodium* species, the estimation based on the assumption of a similar rRNA transcription rate as other eukaryotes suggests

that *P. berghei* requires only two active genes for producing rRNA during a 24-hour erythrocytic cycle, the most active stage for parasite multiplication [Dame and McCutchan, 1983a; McCutchan, 1986]. Malaria parasites are "termly" differentiated organisms; the development during many stages of the life cycle is slower than in the erythrocytic stage. Moreover, recent studies have indicated that the efficiency of rRNA transcription could be greatly modulated by the regulatory structure of the gene as well as various transcription factors, which are involved in the initiation, elongation and termination of transcription [Reeder, 1993]. Thus, the low copy number of the rRNA genes may be an unique feature of *Plasmodium* species.

4. Structurally distinct rRNA genes in *Plasmodium*:

The internal arrangement of rRNA genes in individual transcription units for *Plasmodium* species resembles the structure of other eukaryotes; the SSUrRNA gene, the 5.8S rRNA gene and LSUrRNA gene are linearly organized from 5' to 3' terminus with two internal transcribed spacers, ITS1 and ITS2, separating the three coding genes [McCutchan, 1986]. The coding region of rRNA genes in most eukaryotes is not interrupted by introns, but some insects and unicellular organisms contain intervening sequences, particularly in the coding region for LSUrRNA [Long and Dawid, 1980]. For example the fruit fly, *Drosophila melanogaster*, has an intron in the LSUrRNA gene, though it is not transcribed; *Tetrahymena*, a unicellular eukaryote, has a similar intron, which is transcribed in the precursor and spliced to yield the mature rRNA [Lewin, 1980]. Some previous studies based on DNA restriction mapping and R-loop analysis indicated that both SSUrRNA and LSUrRNA genes of *P. lophurae* contained intron-like sequences [Unnasch and Wirth, 1983b; Unnasch et al., 1985]. Similar interruptions were also reported for *P. falciparum* [Langsley et al., 1983]. However, further sequence analysis indicated that the *P. lophurae* SSUrRNA gene, which is actively expressed in the blood stage is not interrupted [Waters et al. 1989b]. This holds true for the LSUrRNA gene from *P. falciparum* [McCutchan et al., unpublished data]. It seems clear now that, like most eukaryotes, *Plasmodium* species probably have no introns in their rRNA genes. The intervening sequences proposed in earlier studies could have resulted

from the heterogeneity subsequently found between different transcriptional units, the unique feature as yet only found in *Plasmodium*. This could not have been expected at that time or predicted from the research on other eukaryotic rRNA genes.

Mature rRNAs are remarkably conserved presumably because of their fundamental role in protein synthesis. The sequences of transcripts from a single organism are usually identical, although they are transcribed from many different rDNA units. In contrast, structurally distinct transcription units are maintained by individual species in the *Plasmodium* genus. This is the only exception as yet found for the main rRNA gene units in all organisms examined. The original restriction and heteroduplex analysis of the genomic DNA from *P. berghei* demonstrates four types of the main rRNA gene unit, which are different in both the coding regions and the spacers [Dame and McCutchan, 1983a]. Subsequent hybridization analysis of these units with the isolated rRNA suggested that only one of them is actually transcribed in the erythrocytic stage [Dame and McCutchan, 1983b]. Similar results are also found in other species of *Plasmodium*, for example in *P. lophurae* and *P. falciparum*; 6 to 8 rRNA gene units are differentiated by restriction pattern [Unnasch and Wirth, 1983a, b; Langsley et al., 1983]. Further studies have confirmed at least two types of the main rRNA gene unit, designated as type A for those transcribed in the erythrocytic stage and type C for the sporozoite stage [Gunderson et al., 1986, 1987; McCutchan et al., 1988; Corredor and Enea, 1994].

The complete sequences of SSUrRNA genes have been obtained from various species of *Plasmodium*, including avian, rodent, human and other primate malaria parasites (Table 1). The coding region for mature SSUrRNAs ranges from 1.9 to 2.2 Kb, which is similar to most eukaryotes, but the average C/G content is around 38%, which is significant lower than the mosquito and human host. Alignment analysis of these primary sequences demonstrates the typical pattern of several highly conserved regions interspersed with less conserved regions (Fig. 3). The majority of differences, including both composition and size, are localized in the variable areas, which are known to diverge rapidly in most eukaryotic rRNA genes. A similar pattern is also observed from the comparison of three pairs of the stage-specific SSUrRNA genes obtained from

P. berghei, *P. falciparum* and *P. cynomolgi* [Gunderson et al., 1986, 1987; McCutchan et al., 1988; Corredor and Enea, 1993]. The overall divergence between the A and C gene ranges from 7 to 18 %, which is significant for a gene that is usually highly conserved. For example, the difference between SSUrRNA genes from nine species of *Leishmania* (vector borne protozoa) is less than 1%, including a total of 9 point mutations in a 2.3 Kb gene [van Eys et al., 1992]. It is noted that the sequences within conserved regions of rRNA contribute to maintaining the core configuration in the higher-order structure (Fig. 3), which is closely related to the fundamental function of the ribosome in protein synthesis. The significance of the variable regions, containing the unique signature for rRNA genes as well as for species, is under investigation.

Table 1. Comparison of *Plasmodium* and Some Eukaryotic SSUrRNA Genes

Species*	Type	Size (bp)	GC%	Intraspecies Similarity (%)	Reference
P. falciparum	A C	2090 2146	35.6 33.0	*82.2%*	McCutchan et al., 1988
P. malariae	A	2146	34.5	------	Goman et al., 1991
P. vivax	A C	2063 2147	38,3 38.4	*85.5%*	Li et al., 1994
P. cynomolgi	A C	2072 2167	37.9 38.0	*85.0%*	Corredor & Enea, 1994
P. knowlesi	A	2096	36.6	------	Waters et al., 1993
P. fragile	A	2071	37.8	------	Waters et al., 1991b
P. berghei	A C	2059 2059	37.2 36.9	*95.5%*	Gunderson et al., 1986 Gunderson et al., 1987
P. lophurae	A	2118	34.4	------	Waters et al., 1989b
P. gallinaceum	A	2102	34.6	------	Waters et al., 1991a
P. mexicanum	A	1978	31.9	------	Dame et al., 1993**
G. lamblia (protozoan)		1453	74.7		Sogin et al., 1989
S. cerevisiae (yeast)		1798	44.9		Rubtsov et al., 1980
E. coli (eubacteria)		1541	54.4		Ehresmann et al., 1975
A. albopictus (mosquito)		1950	47.9		Baldridge & fallon, 1991**
H. sapiens (human)		1870	56.1		Gonzalez & Schmickel, 1986

Note: * *P = Plasmodium, G = Giardia, S = Saccharomyces, E = Escherichia, A = Aedes, H = Homo*
** only partial sequence available

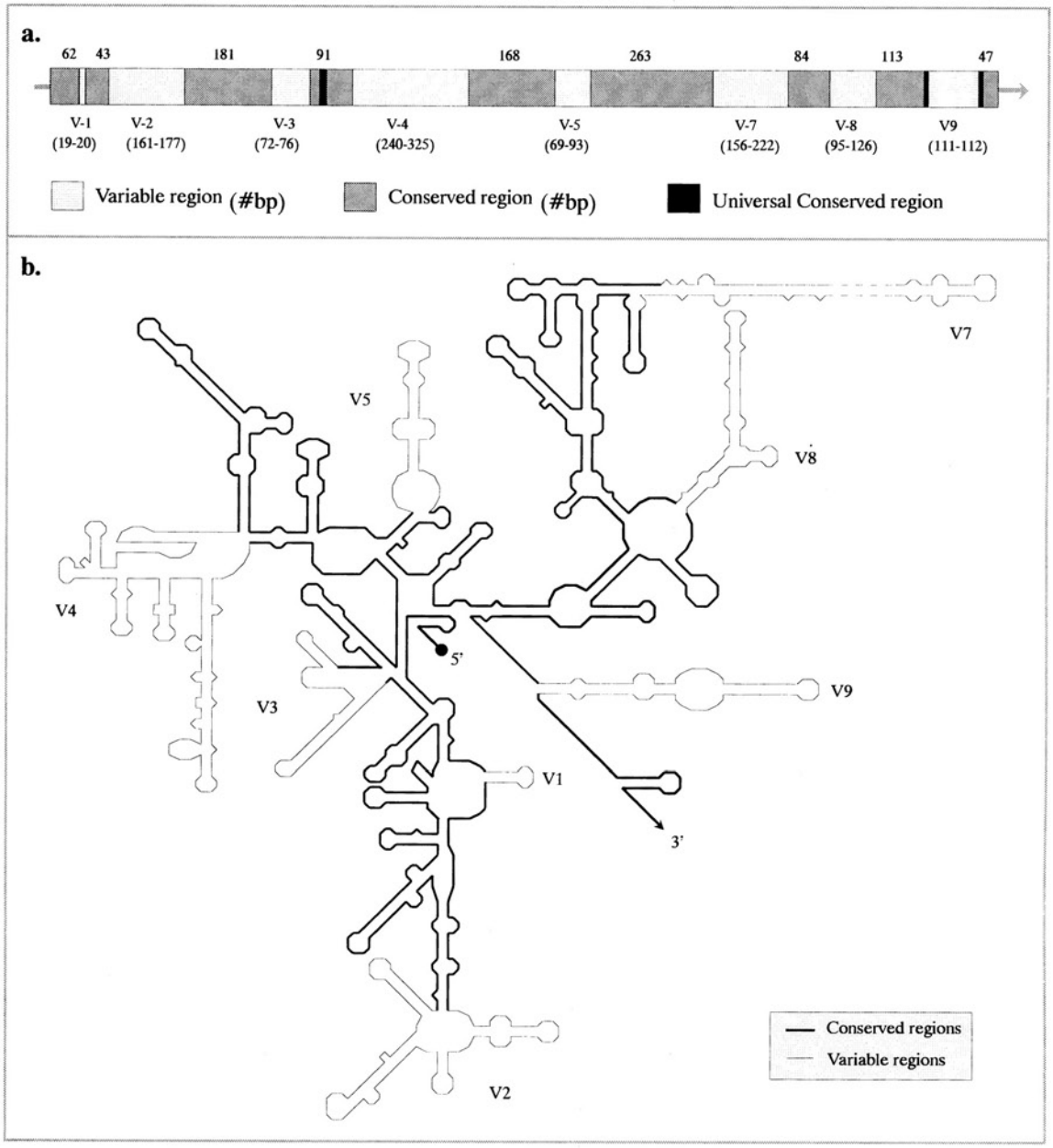


Fig. 3. Schematic representation of conserved and variable regions for *Plasmodium* SSUrRNA and the coding genes. (a)14 *Plasmodium* SSUrRNA genes from GenBank are aligned by the Clustal method. The length of conserved and variable regions are showed by number of base pairs above and below the box, respectively. (b) Secondary structural model for *Plasmodium* SSUrRNAs: sequence starts from 5' end (filled circle) to 3' end (arrowhead). Variable regions are numbered from V1-5 and V7-9 according the standard nomenclature [Neffs et al., 1991]. The V6 region is not present in eukaryotic SSUrRNAs. The broken line in the V7 region indicates significant length variation among *Plasmodium* SSUrRNA genes.

The coding regions for the 5.8S rRNA gene from *P. falciparum* also exhibit significant divergence among various different transcription units [Shippen-Lentz et al., 1987, 1990]. Based on sequence differences, at least four types of 5.8S genes are characterized from the *P. falciparum* genome, though only one of them is actually transcribed in the erythrocytic stage. The sequence comparison of the transcribed gene with the other three untranscribed genes indicates a similarity of 79%, 85% and 98% respectively. The majority of sequence differences, including both substitutions and insertion of nucleotides, are localized within a 30 bp region near the 3' terminus. Little information is available for the heterogeneity of the 5S rRNA gene. A 5S rRNA component of approximately 120 nucleotides has been determined from *P. berghei* RNA [Dame and McCutchan, 1984]. Three *P. falciparum* 5S rRNA genes, about 119 nucleotides in length, have the identical coding sequences, although one or two more uridine residues may be added at the 3' end. This difference may result from inaccurate termination of transcription caused by RNA polymerase III or other termination factors [Shippen-Lentz and Vezza, 1988]. However, structurally distinct 5S rRNAs have been described in the African toad, *Xenopus laevis* [Wolffe and Brown, 1988]. The *Xenopus* genome contains two types of tandem repeated 5S rRNA genes; one is designated as the somatic type containing about 500 copies and the other as the oocyte type containing 2000 copies. The two genes differ only in a few nucleotides, but all reside within a region for binding of a transcription factor. The resulting change of the binding capacity has been shown to be responsible for the differential expression of the two genes during discrete stages of the toad development. This is an example of how the structurally-distinct rRNA genes affect function.

5. Differential expression of rRNA genes in *Plasmodium*:

The transcription of the main rRNA genes in eukaryotes resembles that occurring in prokaryotes; the 18S, 5.8S and 28S rRNAs are generated through processing the single precursor transcribed by RNA polymerase I. The 5S rRNA gene in eukaryotes is transcribed by RNA polymerase III; the resulting RNA usually needs little or no processing. The transcription of rRNA genes in *Plasmodium* species appears similar to

that found in most eukaryotes [McCutchan, 1986]. The large, apparently unprocessed pre-rRNAs were observed from various species of *Plasmodium*, including *Plasmodium knowlesi* [Trigg et al., 1975] and *P. falciparum* [Waters et al., 1989a]. The 18S, 5.8S and 28S rRNAs may also be first transcribed into a single pre-rRNA and then processed in a similar manner. The polarity of transcription for the main unit have been determined for several different species, including *P. berghei* [Dame and McCutchan, 1983b], *P. falciparum* [Langsley et al., 1983] and *P. lophurae* [Unnasch et al., 1985]. Transcription starts before the SSUrRNA gene, through the 5.8S rRNA and then the LSUrRNA gene. The transcriptional control for the *P. falciparum* 5S rRNA gene is not clear, but the lack of precursor in the RNA preparation perhaps indicates that little or no processing is required by the 5S rRNA maturation [Vezza and Trager, 1981; 1982; Shippen-Lentz and Vezza, 1988].

The regulation of eukaryotic rRNA gene transcription is an extremely complex process, which involves cooperative interactions among the regulatory elements of rRNA genes and various transcription factors. However, many detailed mechanisms, in terms of precise regulatory pathways for responding to different developmental stages and various environmental modulators, remain controversial [Larson et al., 1991; Reeder, 1993]. As rRNAs are important determinants of both structure and function of the ribosome, the central apparatus for protein biosynthesis, the regulation of 18S, 5.8S and 28S rRNA transcription is considered to be a limiting step in ribosome biosynthesis. A close correlation between the rate of rRNA transcription and cell proliferation was noted in early studies [Maaloe and Kjeldgaard, 1966; Nomura et al., 1984]. The changes in RNA transcription rate could be induced by modulation of cell growth, either through enrichment of culture medium or in terminal differentiation [reviewed by Larson et al., 1991]. Regulation of transcription could involve changing the number of active transcription units and/or transcription efficiency in individual units, which probably depend upon available RNA polymerase and various associated transcription factors. In addition, the level of the rRNAs may also be regulated by the processing of the primary rRNA transcript and degradation of the mature 18S, 5.8S and 28S rRNAs. Several small nuclear ribonucleoproteins (snRNPs) and small nuclear RNAs (snRNA) are involved in

these processes, and the mechanisms are being studied [Larson et al., 1991; Reeder, 1993].

With regard to the unique arrangement of the rRNA gene and presence of the structurally distinct transcription units within the same genome, the regulation of *Plasmodium* rRNA transcription could be expected to be different from most other eukaryotes. The differential expression of the alternative rRNA genes was first indicated in *P. berghei* and *P. falciparum* by the exhibition of different types of cytoplasm SSUrRNAs during discrete stages of the parasite development. The rRNA isolated from the parasite at the erythrocytic stage can only protect the type A gene in a S1 RNase protection assay; while the C gene only hybridized with RNA prepared from sporozoite [Gunderson et al., 1987; McCutchan et al., 1988]. The study of rRNA from *in vitro* cultivated *P. falciparum* indicates that the switch of cytoplasmic rRNA from the A to C types could be regulated by selectively increased processing of the type C pre-RNA transcripts and site-specific degradation of the type A mature rRNAs during development from the gametocyte to the ookinete [Waters et al., 1989a]. In cultured *P. bergei*, the transition of rRNA from type C to A was demonstrated to occur during differentiation of merozoites in exoerythrocytic schizogony [Zhu et al., 1990; Li et al., 1991]. However, the precise mechanism and chronology for the switch during development in the natural host are still open for investigation.

There is little information about the regulation of transcription of *Plasmodium* 5S RNA. The differential expression of the 5S rRNA gene occurs in the Africa toad. As described above, the *Xenopus* genome contains two sets of distinct 5S rRNAs, which differ in 6 nucleotides, all in the binding site for a transcription factor (TF-III). The factor binds about 5 to 10 times better to the somatic 5S genes than to the oocyte 5S genes. In addition, the transcription complexes, a functional organization for binding of RNA polymerase III to the 5S-rRNA gene, are more stable than are oocyte 5S genes. The oocyte 5S genes are transcribed only during oogenesis, while the somatic 5S genes are expressed in all other stages of development [Peterson et al., 1980]. It is not known whether these differences are the only determinative factors in the expression of 5S rRNA genes during development, but they are at least part of the basis for the differential

transcription of 5S rRNA genes in somatic cells and oocytes.

6. Conclusions and perspectives:

In spite of many common features found in rRNA gene structure and transcription, different genomic organizations and regulatory strategies are adopted by various organisms for rRNA synthesis during development. The rRNA genes of the *Plasmodium* species are obviously distinguished from those organisms examined to date. The arrangement of the 18S, 5.8S and 28S rRNA genes within one transcription unit, and the independence of the 5S rRNA unit, are common to the general scheme of eukaryotic rRNA genes. However, the low-copy and nontandem arrangement of rRNA genes in the genome appear to be reminiscent of some prokaryotic pattern or reflect a unique status of the gene evolution. The most remarkable feature is the presence of structurally distinct transcription units, which are differentially expressed during discrete stages of the life cycle. The ramification for *Plasmodium* species maintaining such arrangements probably relates to the unusual biological pressures confronted by these intercellular parasites during their cycling through various cells or tissues in the vertebrate host and the invertebrate vector. Because of their essential role, the rRNA genes and associated regulatory elements have been considered as an ideal system for studying the evolutionary process at the molecular level. With regard to the uniqueness of *Plasmodium* rRNA genes, further examination of the novel members of the gene family, identification of the structural constraints (particularly those involved in transcriptional regulation) and determination of the unique functions adopted by alternative rRNA forms on development, will indeed help to establish a clearer picture of the structure and function of the rRNA genes in *Plasmodium* species. In addition, this knowledge will contribute to the understanding of the evolutionary process of the translational apparatus.

SPECIFIC AIMS

Plasmodium vivax is the most widely distributed human malaria parasite, most commonly found in the tropics but also in some temperate areas. In contrast to *P. falciparum*, *P. vivax* seems to have had a much longer co-evolutionary history with humans and does not usually cause a fatal infection. Overall morbidity from *P. vivax* is still an important health problem in many developing countries. Moreover, *P. vivax* exhibits obvious strain variation with respect to the incubation period and pattern of relapse. These differences seem to relate to local climatic conditions. The strains from tropical areas, where the climate is favorable for continuous transmission, have short incubation, infection, and latent periods; while strains from temperate zones can have long incubation and latent periods [Bruce-Chatt, 1985]. More recently, the circumsporozoite (CS) protein, a major antigenic protein on the surface of the sporozoite, has been reported to be polymorphic in 27% of the cases in Southeast Asia [Rosenberg et al., 1989; Wirtz et al., 1990]. The study of the *P. vivax* rRNA and the coding gene will provide an alternative approach for advancing knowledge on the population structure and genetic diversity of the species. This should be relevant to the development of antimalarial vaccines and other control strategies. The presented studies are designed toward the following goals:

(a) Characterization of rRNA genes in *P. vivax*: Determination of rRNA gene sequences and exploration of possible novel members in the gene family will contribute to the general basis for fully exploiting rRNA-based diagnosis [Waters and McCutchan, 1989a]. Benefits of this approach will include the design of species- or stage-specific oligonucleotide probes or primers for quantitative measurement of the rRNA gene expression during parasite development.

(b) Establishment of the rRNA secondary structural model: The secondary structures of *Plasmodium* rRNAs, deduced from comparative analysis of the available rRNA sequences and the established eukaryotic model, will facilitate characterization of

the structural constraints for individual rRNA types. This approach will also help to elucidate the phylogenetic relationships among distinct rRNA genes and a possible evolutionary pathway for *Plasmodium* species.

(c) Examination of the pattern of the rRNA gene expression: The chronological relationship between the switch of rRNA types and progression of parasite development in the natural host system will indicate possible biological significances of the structurally distinct rRNAs. This will advance the knowledge of molecular mechanisms adopted by the parasite during the host transition, and also provide a fundamental frame to pinpoint potential interruptions of parasite development.

(d) Evaluation of the potential of the rRNA based assays: *Plasmodium* rRNAs are the target of choice for species identification mainly because they are the only sequences that allow amplification of species- or stage-specific sequences with genus specific primers. Therefore, determination of appropriate procedures in terms of target region, sample preparation and quantitative detection is crucial for practical application of this method to study parasite population dynamics and vector susceptibility.

MATERIALS AND METHODS

1. *Plasmodium vivax* isolates: Infected blood samples were collected from four Thai patients on whom mosquitoes had been fed. The sporozoites produced from these mosquitoes were screened for the presence of Pv210 circumsporozoite (CS) protein by ELISA [Wirtz et al., 1991] and by oligo-probe hybridization (Table 2.). Two isolates, which were negative for the Pv210 CS protein polymorph, were found to contain the Pv247 polymorph [Rosenberg et al., 1989]. The heparinized blood samples were stored in 5% glycerolyte at -70°C. The *P. vivax* SAL-1 strain, originally isolated from El Salvador and maintained in the rhesus monkey model for 20 years, was provided by Dr. W. Collins from The Center for Disease Control and Prevention (Atlanta, GA). The *P. vivax* infected blood smears and dried mosquitoes were provided by Dr. R. A. Wirtz from Walter Reed Army Institute of Research (Washington, DC).

Table 2. Features of the *Plasmodium vivax* isolates

Isolates	ELISA[a]	Hybridization[b]	Origin
PVK1294	Normal CS	P. vivax	Thailand
PVK1290	..	..	..
PVK 112	Variant CS	..	..
PVK 115	..	..	..
SAL-1	Not Test		El Salvador

a: Enzyme linked immunosorbent assay (ELISA) based on monoclonal antibody against Pv210 circumsporozoite protein (CSP).

b:Dot blot hybridization with oligoprobe complementary to the specific sequence of *P. vivax* small subunit ribosomal RNA

2. Generation of *P. vivax* infected Mosquitoes: Laboratory reared mosquitoes, *Anopheles dirus*, were fed on *P.vivax* infected Thai patients. The engorged mosquitoes were held at 26°C and 10 mosquitoes were removed and frozen (-70°C) at intervals beginning 2 hours after the blood meal and thereafter every 2 days. The development of the parasites in the mosquito was monitored by microscopic examination of midguts for oocysts and salivary glands for sporozoite.

3. Isolation of genomic DNA: The frozen blood samples were thawed on ice and washed twice with phosphate buffered saline (PBS). Parasite-infected erythrocytes were recovered by centrifugation at 5000 g for 15 minutes. The compacted cells were resuspended in 1.5 volume of PBS containing 0.15% saponin and set on ice for 2 minutes. After washing and centrifugation twice, as described above, the pellets were dissolved in the lysing solution containing 0.1 M NaCl / 50 mM EDTA / 10 mM Tris-HCl, pH 8.0 / 1% sodium dodecyl sulfate (SDS) / 1.5 μg ml^{-1}, pronase K and then incubated at 37°C for 60 minutes. DNA was further processed by standard procedures [Maniatis et al., 1982]. Briefly, the lysed sample was extracted with TE (50 mM Tris-HCl and 10 mM EDTA, pH8.0) saturated phenol-chloroform-isoamyl alcohol (24:24:1, v/v/v). After vortexing and centrifugation, the aqueous phase was precipitated with one volume of isopropanol for 2 hours and then centrifuged at 10,000 g for 30 minutes. The DNA pellets were washed with 75% ethanol and dried. The recovered DNA was resuspended in water and stored at 4°C.

4. Isolation of total RNA: Preparation of total RNA from the *P. vivax* infected blood and mosquitoes was based on the method of Chomczynski and Sacchi [1987] with reagents from Promega Incorporation (Madison, WI). The frozen blood samples and blood smears were directly lysed with a solution containing 4 M guanidine thiocyanate, 0.5% sarcosine, 0.1 M β-mercaptoethanol, and 25 mM sodium citrate, pH7. The mosquito samples were triturated in the same lysing buffer with a pellet pestle (Kontes, Vineland, NJ) in a 1.5-ml microfuge tube. After adding 1/10 volume of 2 M sodium acetate, pH4, the mixture was extracted with a mixture of water saturated

phenol/chloroform/isoamyl alcohol (24:24:1, v/v/v). The aqueous phase was precipitated overnight in an equal volume of isopropanol and centrifuged at 10,000 g for 20 minutes. After washing with 80% ethanol, the recovered RNA was dried and dissolved in RNase free water.

5. Synthesis of Oligonucleotides: Oligonucleotides used as probes or primers were synthesized in an Applied Biosystems DNA Synthesizer (Foster City, CA). The synthesized oligonucleotides were deblocked by adding NH_4OH at 65°C overnight and dried by vacuum centrifugation. The recovered oligonucleotides were redissolved in water and the concentration was determined by measuring A_{260}. The concentrations for PCR, sequencing primers and hybridization probes were 100, 20, and 5 pmol per μl, respectively. The sequence of oligonucleotides and their relative position at the corresponding rRNA genes are listed in Appendix 1.

6. Amplification of SSUrRNA genes: The complete SSUrRNA gene, including the 3' end ITS1 region, was amplified by PCR [Saiki, 1990] from the genomic DNA of *P. vivax* SAL-1 strain with oligonucleotides #705 and #573 as the 5' and 3' end primers. Both primers are known to be conserved for the genus *Plasmodium*. A slightly smaller SSUrDNA fragment was also amplified from the four Thailand isolates with a 5' end primer #566 and a 3' end primer #570. The resulting fragment included all the coding region except 140 bases downstream from the 5' end and 40 bases upstream of the 3' end. The PCR reaction was set up in a 100 μl reaction volume containing 20-50 ng genomic DNA, 200 μM of each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH8.3), 2 mM $MgCl_2$, and 2.5 U *Taq* DNA polymerase. All reagents except target DNA were obtained from Perkin Elmer Cetus (Norwalk, CT). The reaction was processed in a Perkin Elmer DNA Thermal Cycler with the following parameters: 94°C/1 minute for denaturation, 55°C/1 minute for annealing, 72°C/3 minutes for elongation and a total of 30 cycles.

7. Cloning of the SSUrRNA gene: The double strand DNA fragments obtained from either PCR or RT/PCR, which are described later, were purified with the Magic PCR

Preps DNA Purification System (Promega Corp., Madison, WI). The recovered DNA was treated with Klenow fragment of *E. coli* DNA polymerase (BRL, Gaithersburg, MD) to form blunt ends, and then inserted into the *Sma*I site of the vector, pBluescript SK (Stratagene, La Jolla, CA) in the presence of T4 DNA ligase and *Sma*I (BRL). The recombinant plasmids were transformed into *E. coli* DH5α strain (BRL) and ampicillin resistant (50 μg/ml) colonies were selected by colony filter hybridization with the *Plasmodium* genus specific rRNA oligo-probe #569 or by restriction endonuclease analysis for inserts with the expected size.

8. Sequence analysis of SSUrRNA genes: The cloned inserts were sequenced by the dideoxynucleotide chain termination method [Sanger et al., 1977] with the Sequenase Kit (USB, Cleveland, OH). The primers used in the sequencing reaction included two external universal primers, T7 and M13 (USB), and two sets of internal oligonucleotides which are complementary to corresponding conserved regions on the coding and template strands, respectively [See the sequence in Appendix I]. The complete sequences of the three SSUrRNA genes and the ITS regions were determined in both directions from the SAL-1 strain and partial sequences were determined from several clones of each of the Thai isolates. The sequence alignment and analyses were done with the Lasergene software supplied by DNASTAR Corporation (Madison, WI). The two dimensional structures were generated by comparative analysis of the primary sequence alignment for available SSUrRNA sequences and the established secondary structural model for eukaryotic SSUrRNA, particularly for yeast SSUrRNA. The structures, which originate from within variable regions of rRNAs, were assisted by a computer folding program, MulFold, supplied by the NIAID network.

9. Agarose gel electrophoresis: DNA fragments were routinely electrophoresed at 2-10 V/cm for 3-12 hours through 1% agarose gels containing 50 mM Tris/ 20 mM boric acid/10 mM EDTA, pH8.0. The gel was stained with ethidium bromide at 0.05 μg/ml and photographed under ultraviolet illumination. RNA electrophoresis was also performed in a 1% agarose gel, containing 2.2 M formaldehyde, 0.02 M MOPS

(Morpholineo-propanesulonic acid), 0.1 M EDTA, and 8-10 mM sodium acetate, pH 7.0. The RNA samples were denatured before loading on the gel under the condition described for RNA dot blots. The running buffer was similar to the gel solution except formaldehyde was eliminated.

10. Preparation of Southern and Northern Blots: The agarose gel, containing fractionated DNA or RNA, was first denatured in 0.5 N NaOH, 1.5 M NaCl for 30-60 minutes and then neutralized in 0.5 M Tris-HCl, pH 7.5, 1.5 M NaCl for another 30 minutes. The DNA was transferred to Genescreen (Dupont NEN Research Products, PA), a modified Nylon membrane, by the capillary diffusion method (Southern, 1975). Finally, the membrane containing transferred DNA was exposed to UV-irradiation at 1200 $\mu W/cm^2$ for 1.2 minutes and dried in air; the membrane containing RNA was baked *in vacuo* at 80°C for 60 minutes.

11. Preparation of RNA dot blot: The isolated total RNA was denatured in 20 mM MOPS, 8 mM sodium acetate (pH 7.0), 1 mM EDTA, 50% formamide and 2 M formaldehyde at 65°C for 15 min and then immobilized on Genescreen Plus (Dupont, PA) by the minifold procedure (Schleicher & Schuell Inc, Keene, NH). The membrane containing transferred RNA was then baked *in vacuo* at 80°C for 2 hours.

12. Labeling oligonucleotides and hybridization: The oligonucleotides for probe hybridization were labeled by T4-kinase (BRL) with [γ-^{32}P] ATP (Amersham, Arlington Heights, IL) and purified on a Sephadex G25 column (5'-3' Inc., West Chester, PA). The hybridization for Southern and Northern blots, as well as RNA dot blots, were processed using similar conditions. Briefly, the membrane was prehybridized at 55°C for 1 hour in 6 × SSC (1 × SSC = 0.15 M NaCl and 0.015 M sodium citrate) / 10 × Denhardt's solution (0.2% Ficoll, 0.2% polyvinylpyrrolidone, 1% SDS), 20 mM sodium phosphate, 0.05% sodium pyrophosphate, and 100 μg/ ml sonicated salmon sperm DNA. Hybridization was under continuous agitation at an appropriate temperature, ususally 5 °C below the melting temperature of the oligonucleotide overnight. The hybridization

buffer is similar to the solution for prehybridization, containing 50 μg/ ml yeast tRNA and the [^{32}P] end-labeled oligonucleotide, but eliminating Denhardt's solution and salmon sperm DNA. Finally, the membrane was washed once in pre-hybridization solution, three times with 4 × SSC / 0.1% SDS at 30°C and once in 1 × SSC / 1% SDS at 55°C. Autoradiographs were prepared by exposing the hybridized membrane to X-ray film at -70°C with the use of an intensifying screen.

13. Reserve transcriptase mediated PCR: About 5-10 μg of the total RNA isolated from *P. vivax*-infected blood or mosquitoes were digested with 2 units RNase free DNase I (BRL) in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$ and 100 μg/ml BSA to eliminate possible DNA contamination. The reaction was terminated by adding 1/10 volume of 20 mM EDTA and heating at 65°C for 15 minutes. The DNase treated RNA was then annealed with 20 pmol antisense primer at 70°C for 15 minutes. The first strand of cDNA was synthesized with 200 units of Superscript II (BRL), a genetically engineered RNA dependent DNA polymerase without RNase H activity, in 20 μl of reaction buffer (similar to DNase digestion) plus 200 μM of each dNTP and 5 μM DTT. The resulting products were digested with *E. coli* RNase H at 37°C for 15 minutes, and further amplification of the cDNA was accomplished by routine PCR with *Taq* DNA polymerase (Perkin Elmer Cetus).

RESULTS

1. Characterization of *P. vivax* SSUrRNA Genes:

Amplification of structurally distinct SSUrRNA genes by PCR using pairs of oligonucleotide primers complementary to flanking sequences known to be conserved in *Plasmodium* rRNA genes (Fig. 4a). Two partial rDNA fragments, 1.9 Kb and 1.7 Kb, were amplified and isolated from the genomic DNA of *P. vivax* isolates from Thailand (Fig. 4b). The larger fragment, resulting from the primer pair of #566-#570 and covering most of the coding region from 140 bp downstream of the 5' end to 40 bp upstream of the 3' end, was then inserted into the *Sma*I site of plasmid pBluescript. After being transformed into *E. coli* DH5α strains, the recombinant plasmids were purified and digested with restriction endonucleases, *Eco*R1 and *Bam*H1. The resulting banding pattern indicated at least two major types of rRNA genes: the A gene, which is transcribed in the erythrocytic stage (see below and accompanying manuscripts), was cut into three fragments by the two enzymes; whereas the C gene, which is transcribed in the sporozoite stage (see below), was not cleaved (See Fig. 5 for restriction sites). In addition, further sequence analysis of the cloned C gene-like fragments, as will be described later, indicated that a third type of SSUrRNA gene, designated as B gene, existed and was also expressed in a stage-specific manner.

The B gene was originally identified from one of the four Thai isolates (PVK1294); further examination of recombinant plasmids with the B gene-specific oligonucleotide probe (#683) indicated that only one B gene out of 52 randomly selected recombinant plasmids was of this type (Data not shown). Thus, it was possible that the B gene could have resulted from a coinfection of another *Plasmodium* species. To determine whether this was the case, the Southern blot prepared from the original PCR products was hybridized with the B gene-specific oligo-probe #683 (located in 5' end of the 1.9 Kb fragment but not included in 1.7 Kb fragment). These results clearly suggested that the B gene existed in every *P. vivax* isolate from Thailand (Fig. 4c).

In order to confirm the association of the B gene with *P. vivax*, amplification of the complete SSUrRNA genes was carried out on an independently derived strain

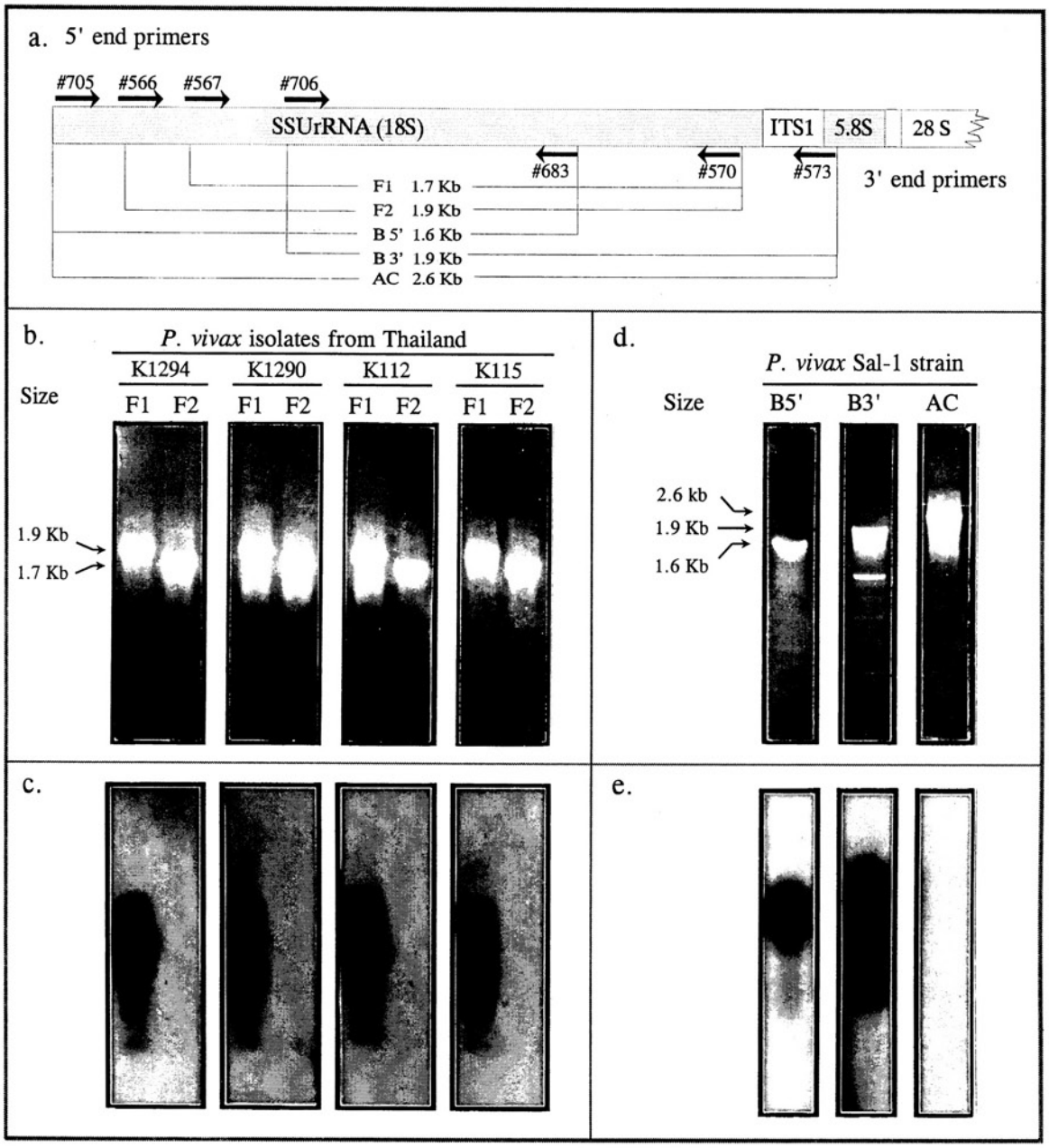


Fig. 4 Characterization of SSUrRNA genes from the genomic DNA of *P. vivax*. Structurally distinct SSUrRNA genes were amplified by PCR with the primers conserved in the genus *Plasmodium* (a). Two partial fragments (F1 and F2) of the gene were produced from the Thai isolates with expected sizes on an agarose gel electrophoresis (b). After cloning and sequencing, three types of SSUrRNA genes (Type A, B and C) were identified from the F1 fragment, including a novel gene (Type B), which was shown to present in all isolates from Thailand by Southern blot hybridization with a B gene specific oligo-probe, #683 (c). The complete A and C genes, including 3' end flanking region (ITS1), were amplified from the Salvador strain (Sal-1) with a genus conserved primer pair, #705-573(a); while the B gene was generated in two overlapping fragments (B5' and B3') and confirmed by probing with a B gene specific probe (#743) against the common regions (d & e).

(SAL-1), which was originally collected from El Salvador and maintained in a monkey model by blood and mosquito passage for more than twenty years. With a pair of the genus conserved oligo-primers (#705-#573), a 2.6 Kb fragment was amplified by PCR and expected to cover the entire SSUrRNA gene and the ITS1 region for all three types of rRNA gene (Fig. 4d). However, the 2.6 Kb fragment did not hybridize with the B gene specific probe #683. Considering the possibility that the ratio of different rRNA genes in the genome could bias the PCR reaction, two B gene specific oligo-primers, #706 and #683, were used in PCR to favor the B gene amplification (Fig. 4a). The completed B gene was produced in two overlapping fragments (1.6 Kb and 1.9 Kb), which were amplified by two primer pairs, #705-#683 and #706-#573, respectively (Fig. 4a & d). Southern blot hybridization suggested that both fragments could be recognized by the B gene specific oligo-probe, #743 (Fig. 4e). Finally, all the fragments, including 3 types of SSUrRNA genes and their ITS1 regions, were cloned by the method described above for further sequence analysis.

2. Primary sequences of the SSUrRNA genes:

The clones, pVSA53, pVSA5-21 and pVSA46, are representatives of the plasmids containing the entire A, B and C SSUrRNA genes from *P.vivax* SAL-1 strain. The inserted genes were sequenced on both strands. Figure 5 and 6 show a clustal alignment of the three genes and their ITS sequences, respectively. The SSUrRNA coding regions are 2063, 2231 and 2147 bp in length for the A, B and C genes, respectively. The overall similarity between the sequences of the A and C genes is approximately 85.5% and the G/C content for both genes is 38%, which is quite typical for *Plasmodium* species. The B gene seems more distant and the overall similarities comparing to the A and C gene are 52% and 62%, respectively, suggesting that the B gene is more similar to the C gene than to the A gene. The G/C content for the B gene is 40%.

The sequence differences, determined by the Clustal alignment of the three genes (Fig. 5), tend to localize in variable regions (V1-5 & V7-9) known to diverge rapidly in other eukaryotic SSUrRNAs [Neefs et al., 1991]. However, the differences between A and C genes seem to be limited to three regions, V2, V4 and V7 (See accompanying

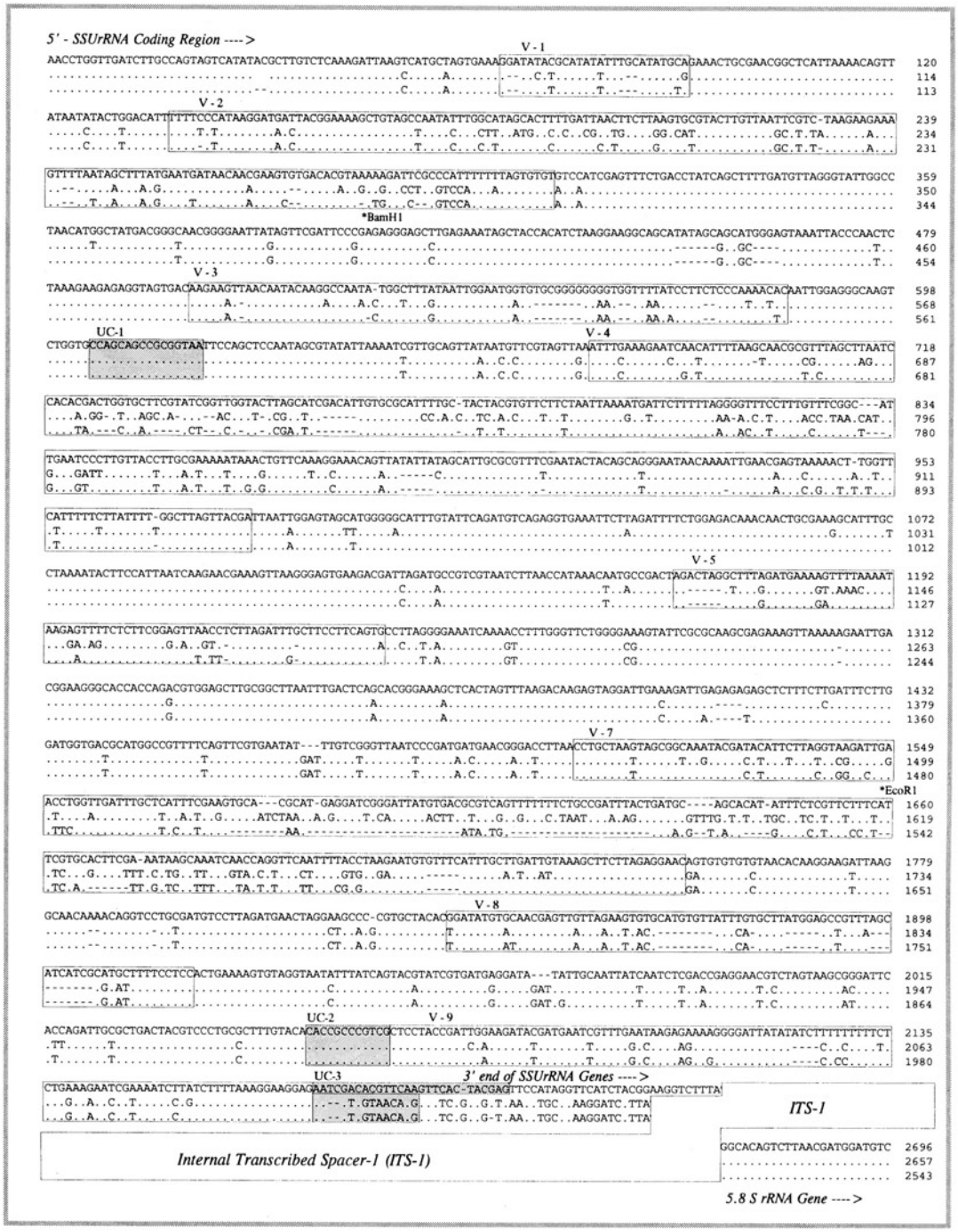


Fig.5. Sequence alignment of three SSUrRNA genes from *P.vivax* SAL-1 strain. The coding region for mature SSUrRNAs starts from the 5' end and is followed at the 3' end internal transcribed spacer (boxed area) ,as well as the 5' end of 5.8S rRNA genes. The upper line shows the B gene sequence which is expressed in the oocyst stage; the middle and lower lines represent the C and A genes, which are expressed in the sporozoite and erythrocytic stages, respectively, and shown only where the sequences differ from the B gene. Dashes represent gaps where the sequences cannot be directly aligned. The variable regions (lightly shaded box), universally conserved regions (darkly shaded box), and position of restriction sites (*Bam*H1 and *Eco*R1) in the A gene are marked.

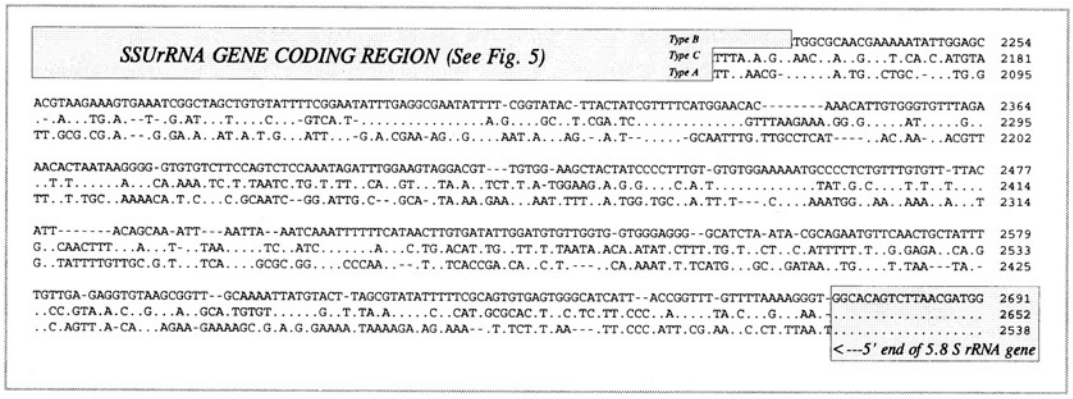


Fig.6. Sequence alignment of the internal transcribed spacer (ITS-1) from three rRNA genes of *P. vivax* SAL-1 strain. The upper line shows the B gene sequence; the middle and lower lines represent the C and A genes, respectively, and are shown only where the sequences differ from the B gene. Dashes represent gaps where the sequences can not be directly aligned. The number shown on right side follows that of Figure 5, representing contiguous positions of the genes.

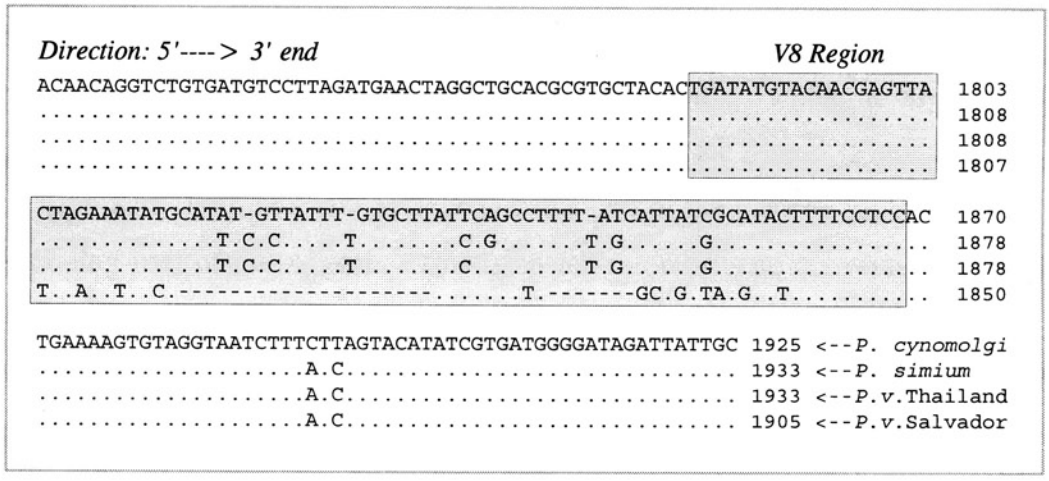


Fig. 7. Sequence comparison of the V8 (shaded) region of the SSUrRNA genes (Type C) from the vivax-like malaria parasites. The upper line shows the C gene sequence of *P. cynomolgi*; the lower lines represent, from top to bottom, the C genes from *P. simium*, Thailand and El Salvador isolates of *P.vivax*, respectively, and shown only where the sequences differ from the B gene. Dashes represent gaps where the sequences cannot be directly aligned. The number shown on right side follows actual positions of the gene sequences.

manuscript in appendix IV). Size differences were also observed in variable regions, particularly in V7. In comparison to the A gene, 64 and 87 nucleotides were added to the C and B genes, respectively. The conserved regions are usually interspersed with the sequence variable regions, as is seen in other eukaryotic SSUrRNA genes. Among these regions, three universally conserved sequences, UC-1, UC-2 and UC-3, are completely maintained by the A and C gene; whereas, only UC-1 and UC-2 are present in the B gene (Fig.5), as is true for the B gene identified from the Thailand isolate. These sequences are known to be integral features involved in higher-order structure and catalytic activities for protein translation.

Comparison of *P.vivax* SSUrRNA genes between the field isolates and SAL-1 strain reveals that the A and B genes are almost identical; whereas, the C genes are different, particularly within the V8 region including extensive substitutions and gaps (Appendix II). Further sequence alignment (Fig. 7) indicated that the diverged region from the C gene of the field isolates was almost identical to corresponding areas of the C genes from *P. simium*, and also similar to the C gene from *P. cynomolgi* (both of these monkey malarias are known to be closely related to *P. vivax*), while the SAL-1 C gene is identical to the A gene in this region and extending to the 3' end (Fig.5). Thus, the C gene from SAL-1 strain seems to have lost this part of the gene by replacement of the corresponding part of the A gene. This is an indicator of gene conversion, as will be discussed below.

The internal transcribed spacer (ITS1) regions between the SSUrRNA coding region and the 5.8S rRNA gene are 454, 465 and 484 bp in length for the A, B and C gene, respectively. No significant similarity was found among the spacers of the three SSUrRNA genes (Fig. 6), suggesting that they originated from distinct transcription units and probably evolved under different selection constraints.

3. Secondary structures of the SSUrRNAs:

The secondary structure for SSUrRNA is very complex, and cannot be predicted by any available computer programs. It may be modelled following known physical-chemical parameters and phylogenetic comparisons compiled from the study of rRNA

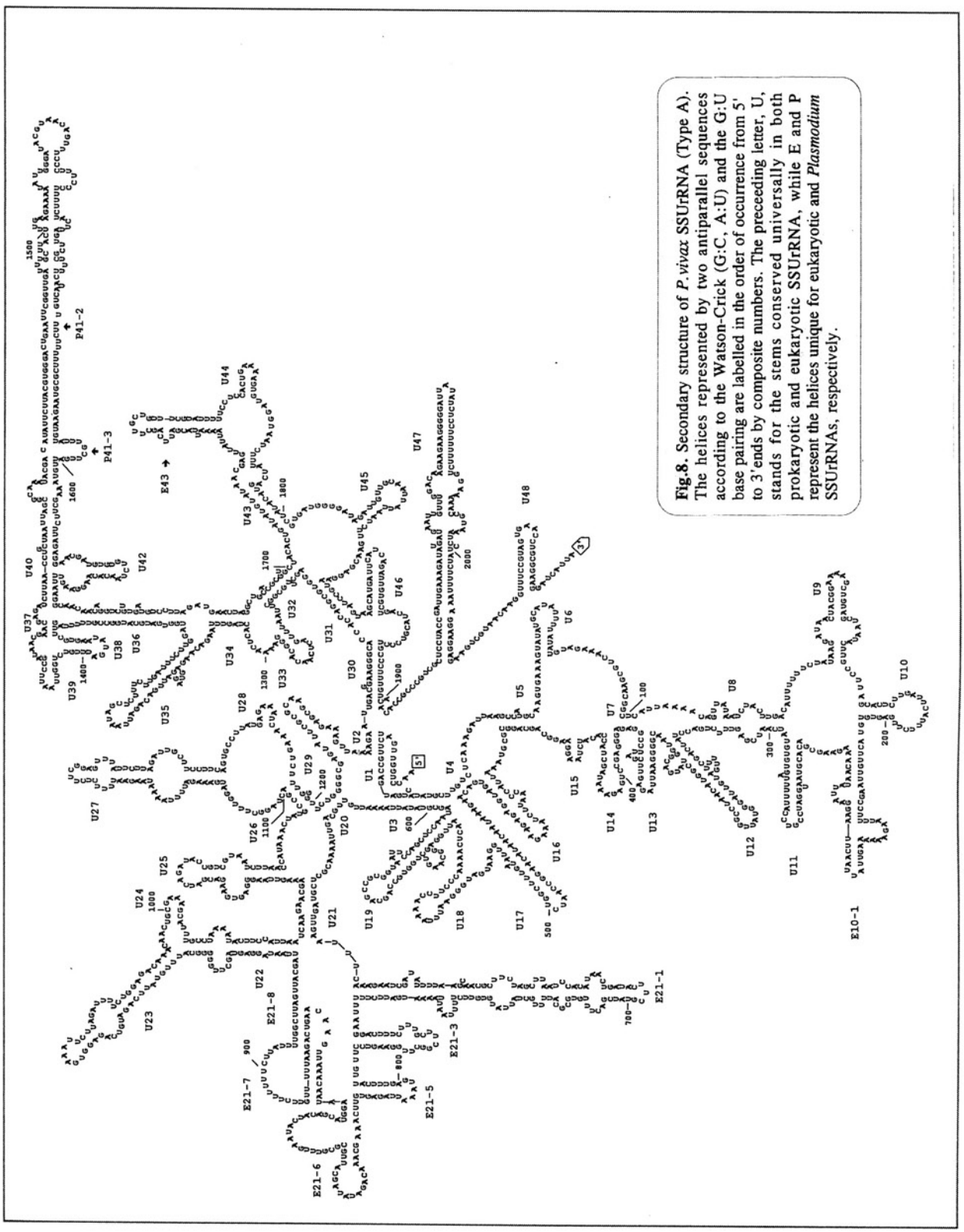


Fig.8. Secondary structure of *P.vivax* SSUrRNA (Type A). The helices represented by two antiparallel sequences according to the Watson-Crick (G:C, A:U) and the G:U base pairing are labelled in the order of occurrence from 5' to 3' ends by composite numbers. The preceeding letter, U, stands for the stems conserved universally in both prokaryotic and eukaryotic SSUrRNA, while E and P represent the helices unique for eukaryotic and *Plasmodium* SSUrRNAs, respectively.

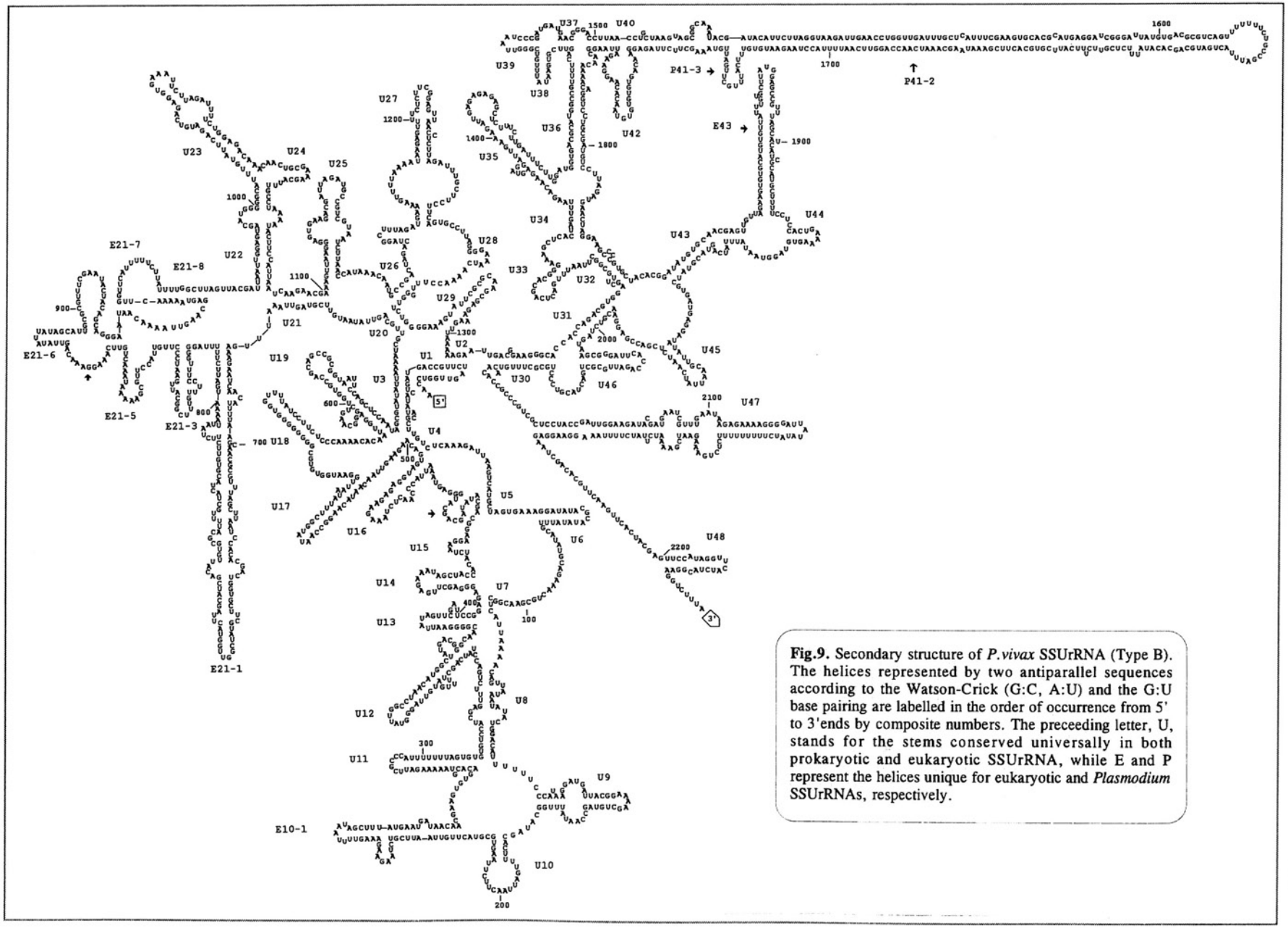


Fig.9. Secondary structure of *P.vivax* SSUrRNA (Type B). The helices represented by two antiparallel sequences according to the Watson-Crick (G:C, A:U) and the G:U base pairing are labelled in the order of occurrence from 5' to 3'ends by composite numbers. The preceeding letter, U, stands for the stems conserved universally in both prokaryotic and eukaryotic SSUrRNA, while E and P represent the helices unique for eukaryotic and *Plasmodium* SSUrRNAs, respectively.

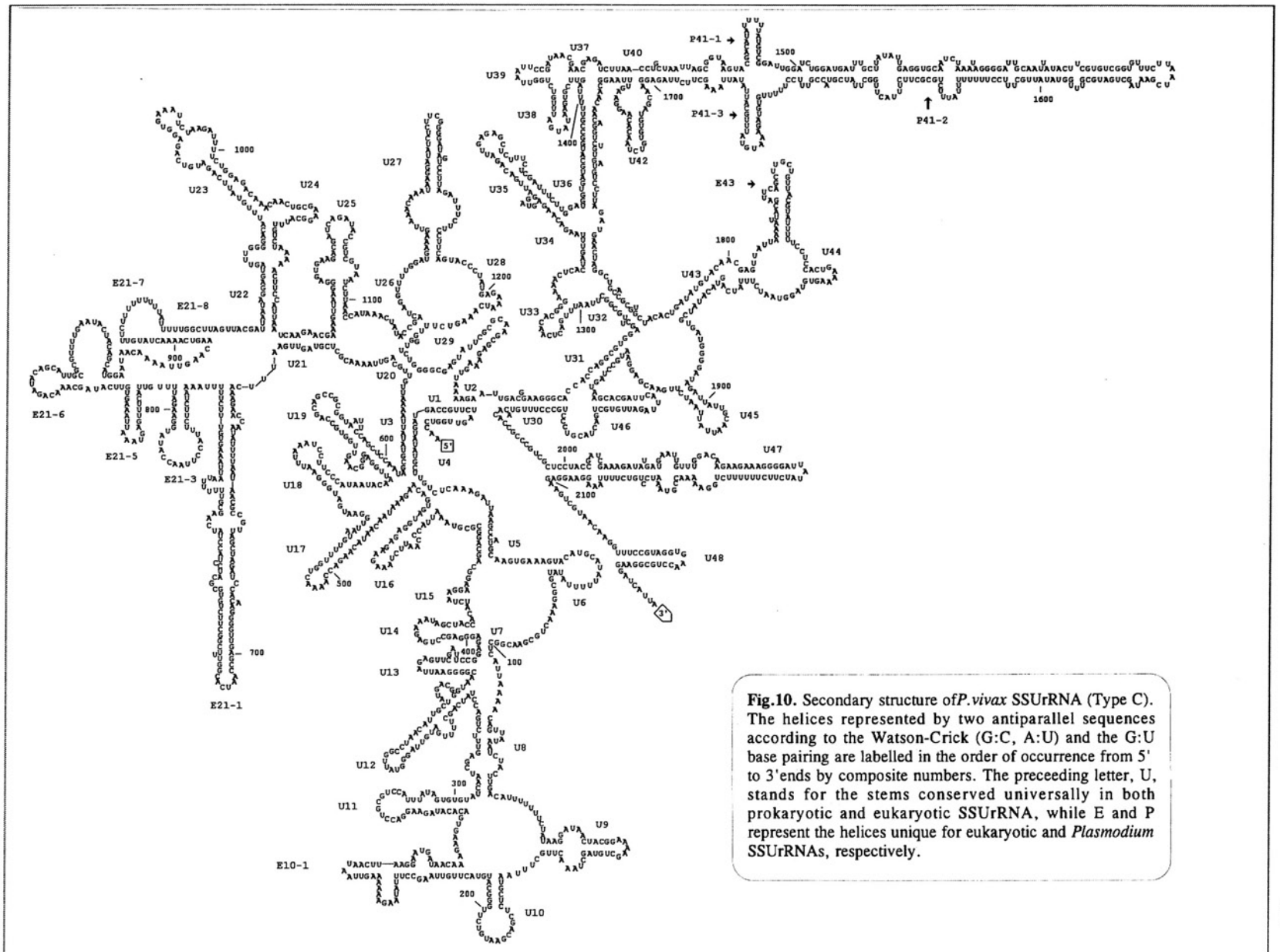


Fig.10. Secondary structure of*P.vivax* SSUrRNA (Type C). The helices represented by two antiparallel sequences according to the Watson-Crick (G:C, A:U) and the G:U base pairing are labelled in the order of occurrence from 5' to 3'ends by composite numbers. The preceeding letter, U, stands for the stems conserved universally in both prokaryotic and eukaryotic SSUrRNA, while E and P represent the helices unique for eukaryotic and *Plasmodium* SSUrRNAs, respectively.

in other systems. The proposed models for *P. vivax* SSUrRNAs (Fig. 8-10) were deduced by comparing their sequences with those of other organisms and confirmed by showing a phylogenetic consistency among the *Plasmodium* species. Most highly conserved regions of the parasite sequences could be unambiguously aligned to the corresponding places in a yeast 18S rRNA model [Neefs et al., 1990, 1991]. Within only moderately conserved regions where the primary sequences are different, simulations of corresponding yeast structures were developed by taking into account compensatory base changes at the corresponding positions. Non-Watson-Crick pairs like the G:U pair, which allow the switch between A and G or C and U to occur without destroying the base pair interactions, were also considered. For most variable regions, which occur exclusively in the places where diverse conformations are found in all the examined eukaryotic models, the structures were predicted with a computer-assisted folding program and comparison with available eukaryotic structures.

The secondary structures for the three distinct SSUrRNAs of *P. vivax* seem to share similar conformations, although the primary sequences are only moderately homologous (Fig. 8-10). All three models maintain the core structures, including 48 universally conserved stem-loops (U1-48) and several eukaryotic conserved ones (E10-1; E21-1, 3, 5, 6, 7, 8; E43). The structures that may vary between the distinct SSUrRNAs are localized in U6, U9-11 and E10-1, U17 and U18, E21, U27, U41, E43, and U47; which correspond to the eight variable regions (V1-V5 and V7-V9) in the primary sequences. Most differences seem to localize within the eukaryotic stem-loops designated E21 (V4) and E43 (V8). In addition, the most characteristic differences between the gene types include extensions or branches within U43; these appear to be *Plasmodium* specific stem-loops and are designated as P41-1, P41-2 and P41-3 according to the direction of primary sequence from 5' to 3' ends. The type A rRNA model has a short P41-2 stem-loop and a stable pseudoknot around position 900, including two joined stem-loops (E21-7 and E21-8). This pseudoknot structure is highly conserved in both conformation and sequence for all the A genes examined in the *Plasmodium* but not for the B or C genes (Fig. 11). The type B rRNA structure exhibits an extra loop in the middle of E21-5, an elongated E43, and a long extension on U41 (P41-2), which is

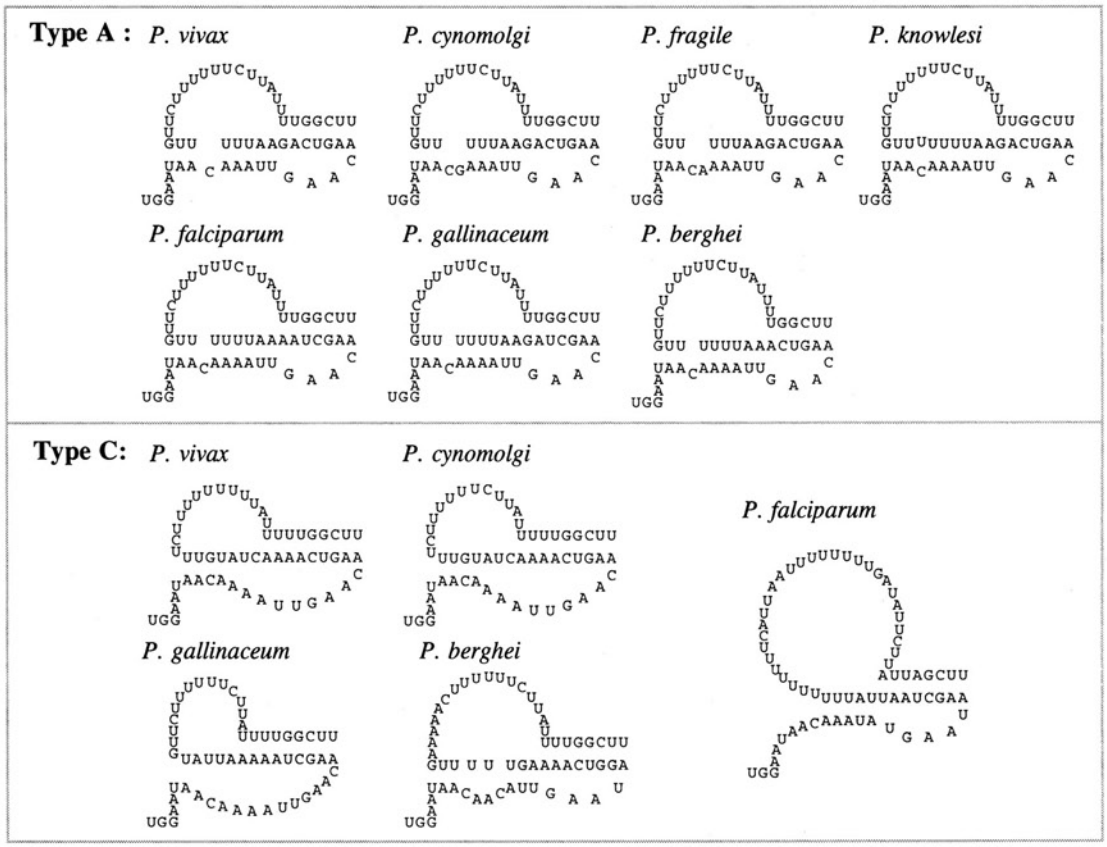


Fig. 11. Comparison of pseudoknot structures of *Plasmodium* SSUrRNAs. The structures are deduced from the sequences around position 900 of the genes according to a secondary structural model for eukaryotic SSUrRNA (Neefs et al., 1990).

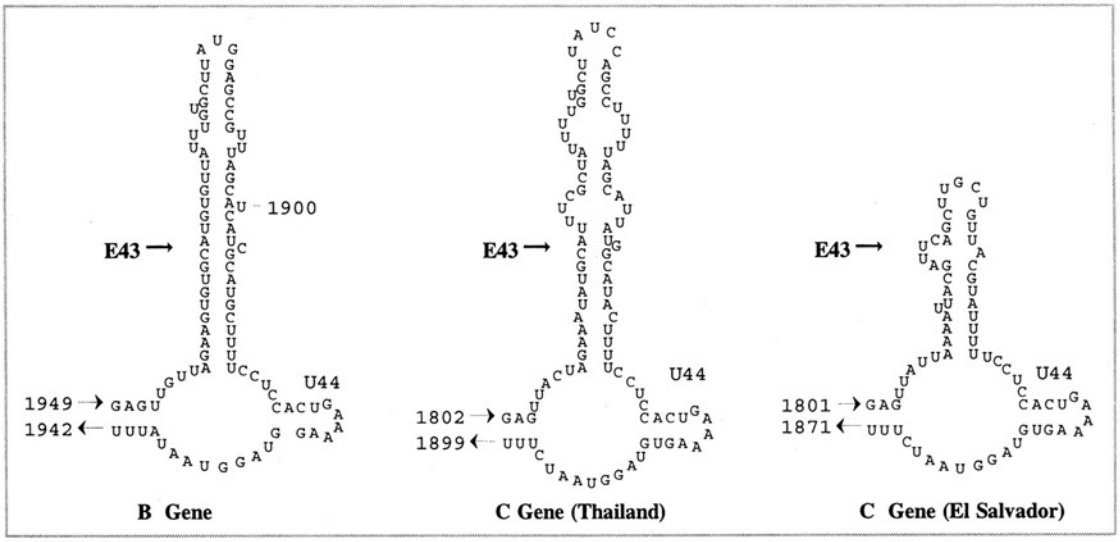


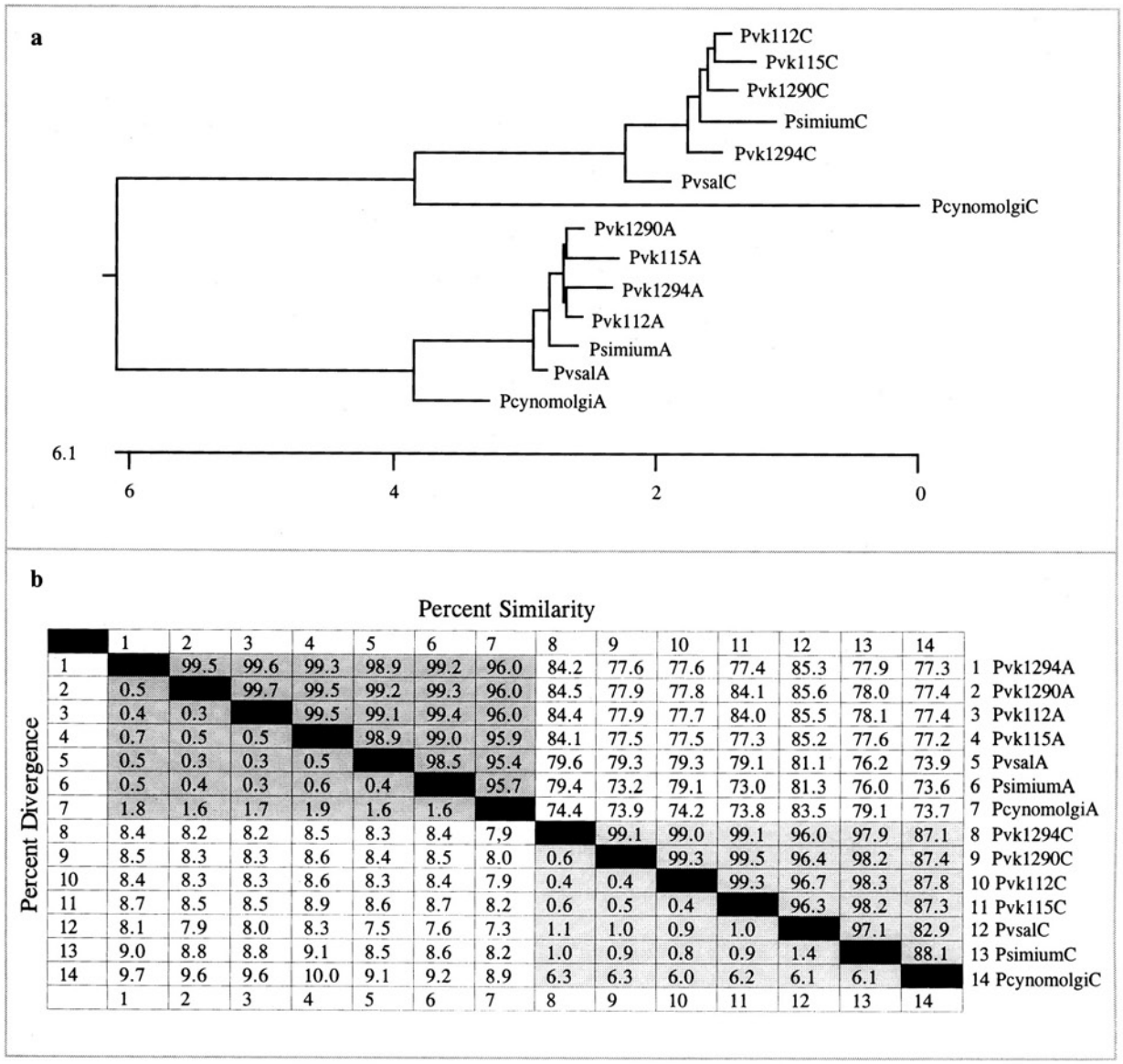
Fig.12. Comparison of secondary structures in V8 regions of *P.vivax* SSUrRNAs. Thailand isolates and the El Salvador strain are identical in type A and B SSUrRNAs; while the type C transcripts differ dramatically from each other in the V8 region where the structure from the Thai isolates resembles the corresponding part of the type B gene transcript, while the sequence from the Salvador strain is identical to that of the type A gene transcripts.

characterized as a long stem with approximately 200 anti-parallel paired bases. The type C rRNA has a unique stem-loop (P41-1) branched from its moderately extended P41-2 and an extended P41-3. In addition, the C gene SSUrRNA from the Thailand isolates has an extended E43 stem-loop resembling the corresponding place of the B gene (Fig. 12), suggesting that this part of the gene could possibly have arisen from the B gene.

4. Comparative analysis of *P. vivax* rRNA structures:

In order to determine detailed relationships between each SSUrRNA gene and its counterparts from other related species, the sequences of both type A and C genes from *P. simium* and *P. cynomolgi* were aligned with the corresponding genes from *P. vivax*. The phylogenetic tree generated by the Clustal sequence analysis indicates that these sequences were clearly grouped by the type of gene, rather than origin of the species (Fig. 13). The similarity between the same types of the gene from different species is much higher than that between the different types of same species. The average homology among the A and C genes (only SAL-1 is included for *P. vivax*) are 96.5% (95.4-98.5) and 89.4% (82.9-97.1), respectively; however, the overall similarity between the different genes in same species are 73.9% (73.7-81.1). This pattern suggests that some sequence arrangement or structures could be particularly restrained by each type of the SSUrRNA genes.

A search for particular constrained nucleotides in the sequence alignment indicated that most nucleotide substitutions among different genes were far from random. Beside some randomly distributed mutations, the majority of the changes, in both variable and conserved regions of the genes, were correlated to the gene type (Appendix III). Further analysis of these variable positions based on the proposed secondary structures indicates that the gene-specific substitutions are responsible for maintaining the unique conformation for each type of SSUrRNA. The secondary structure established for the *P. vivax* type A SSUrRNA is almost exactly maintained by the counterparts from *P. simium* and *P. cynomolgi*, and a similar situation is also found for the type C rRNA. In the latter case, a few localized changes are observed with the *P. cynomolgi* secondary



b

Percent Similarity

Percent Divergence

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
1		99.5	99.6	99.3	98.9	99.2	96.0	84.2	77.6	77.6	77.4	85.3	77.9	77.3	1 Pvk1294A
2	0.5		99.7	99.5	99.2	99.3	96.0	84.5	77.9	77.8	84.1	85.6	78.0	77.4	2 Pvk1290A
3	0.4	0.3		99.5	99.1	99.4	96.0	84.4	77.9	77.7	84.0	85.5	78.1	77.4	3 Pvk112A
4	0.7	0.5	0.5		98.9	99.0	95.9	84.1	77.5	77.5	77.3	85.2	77.6	77.2	4 Pvk115A
5	0.5	0.3	0.3	0.5		98.5	95.4	79.6	79.3	79.3	79.1	81.1	76.2	73.9	5 PvsalA
6	0.5	0.4	0.3	0.6	0.4		95.7	79.4	73.2	79.1	73.0	81.3	76.0	73.6	6 PsimiumA
7	1.8	1.6	1.7	1.9	1.6	1.6		74.4	73.9	74.2	73.8	83.5	79.1	73.7	7 PcynomolgiA
8	8.4	8.2	8.2	8.5	8.3	8.4	7,9		99.1	99.0	99.1	96.0	97.9	87.1	8 Pvk1294C
9	8.5	8.3	8.3	8.6	8.4	8.5	8.0	0.6		99.3	99.5	96.4	98.2	87.4	9 Pvk1290C
10	8.4	8.3	8.3	8.6	8.3	8.4	7.9	0.4	0.4		99.3	96.7	98.3	87.8	10 Pvk112C
11	8.7	8.5	8.5	8.9	8.6	8.7	8.2	0.6	0.5	0.4		96.3	98.2	87.3	11 Pvk115C
12	8.1	7.9	8.0	8.3	7.5	7.6	7.3	1.1	1.0	0.9	1.0		97.1	82.9	12 PvsalC
13	9.0	8.8	8.8	9.1	8.5	8.6	8.2	1.0	0.9	0.8	0.9	1.4		88.1	13 PsimiumC
14	9.7	9.6	9.6	10.0	9.1	9.2	8.9	6.3	6.3	6.0	6.2	6.1	6.1		14 PcynomolgiC
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	

Fig.13. Phylogenetic tree and sequence pair distance of the SSUrRNA genes from *P.vivax* and two closely related species. The sequences of both the A and C genes from *P. vivax*, including four Thailand isolates (Pvk1294, 1290, 112 and 115 and one El Salvador strain (Pvsal), as well as *P. simium* and *P. cynomolgi,* are aligned with the Clustal method. The algorithm groups sequences into clusters by examining the distance between all pairs. The clusters are aligned, first individually, then collectively to produce an overall alignment. (a) Phylogenetic relationships between the sequences are represented by a series of jointed branches. The length of each pair of branches represents the distance between sequences pairs. The scale beneath the tree measures the distance (numbers of mutations per 100 nucleotides) between sequences. (b) The sequence distances are shown in a table of calculated divergence and similarity between each sequence pair, which are used in the first step of the multiple alignment. The percent divergence is calculated by comparing sequence pairs in relation to the phylogeny reconstructed by the Megalign program, while the similarity index compares sequences directly, without accouting for phylogenetic relationships. The highlighted areas represent the comparisons for the type A (dark) and the type C (light) genes, respectively.

structure on U11 and P41-2, which correspond to the sequence variable regions V2 and V7 (Data not shown). The true random substitutions, which do not relate to any gene type, seem not to influence the conformation of the secondary structures. The reason for maintaining similar secondary structures even when the primary sequences are different could be any or all of the following: (1) Mutations occur in single stranded loops or bulges of the secondary structure where random substitutions do not influence the neighboring conformation; (2) Compensatory substitutions in corresponding positions occur and, in so doing, maintain the stem structure; (3) Switches between the Watson-Crick pairing (A:U, G:C) and the G:U pairing, serve to maintain similar stem structure.

The amount of intra-species diversity of the SSUrRNA gene within different isolates of *P. vivax* from Thailand was also determined by sequence analysis (Fig. 13b; Appendix II). The A genes from different isolates are highly conserved and the average homology is 99.5% (99.3-99.7%). The differences among genes in this case are limited to single substitutions. The C genes consistently appear to maintain more diversity than the A genes. The C gene diversity includes both multiple substitutions and gaps, although the overall degree of similarity is still very close to that seen in the A gene group 99.2% (99.0-99.5%). In addition, all the C genes from the Thailand isolates differ from the C gene of SAL-1 strain in the V8 region as described above. Thus, the overall tendency of intra-species diversity is consistent with inter-species diversity, suggesting that the C gene is more divergent than the A gene.

5. Evaluation of rRNA gene specific oligonucleotides:

Based on primary sequence alignments of the A, B and C SSUrRNA genes of *P. vivax*, specific oligonucleotides complementary to the unique sequences of each gene were synthesized for the purpose of distinguishing transcripts derived from the three genes. The oligonucleotide probes (Appendix I) were evaluated for their specificity and sensitivity by RNA dot blot hybridization. Equivalent amounts of total RNA prepared from both *P. vivax* infected human blood and sporozoite were covalently linked to membrane strips and hybridized with [^{32}P] end-labelled oligo-probes. Uninfected human blood cells or mosquitoes, and the plasmid clones containing SSUrRNA genes served as

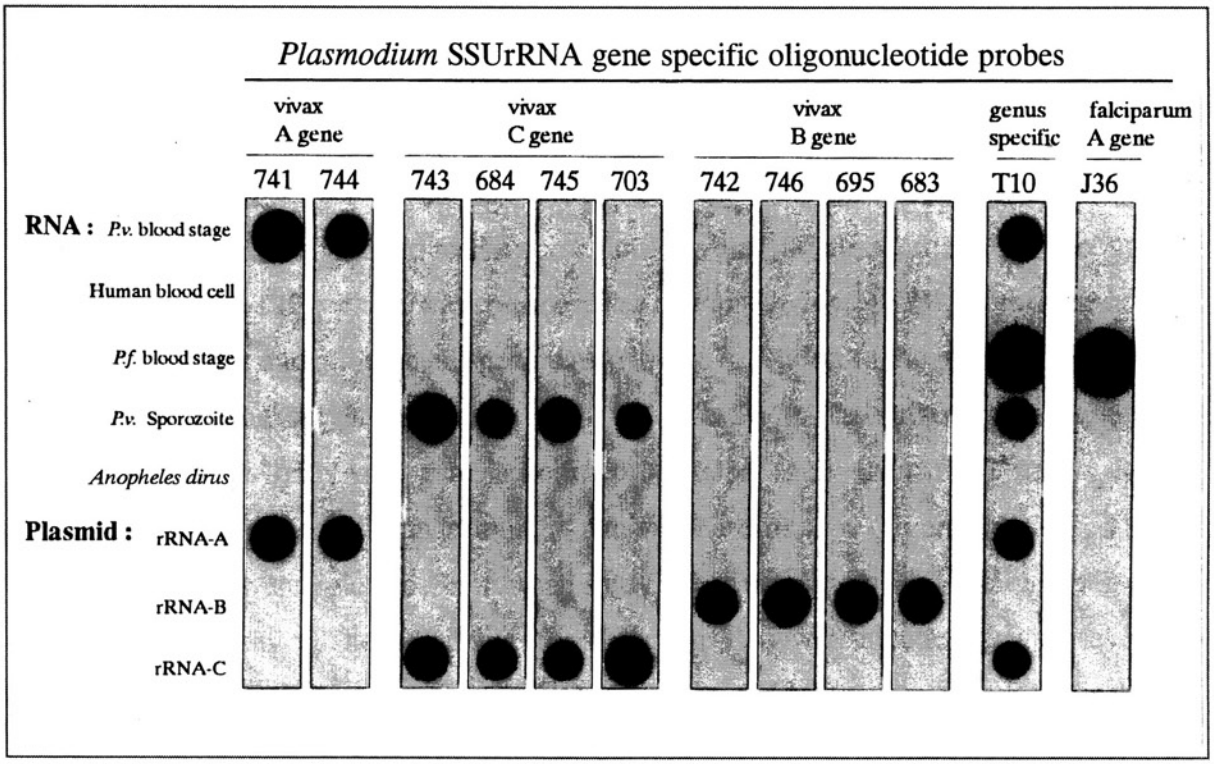


Fig. 14. Specificity of the rRNA gene specific oligonucleotides in detection of distinct SSUrRNAs of *P.vivax*. The rRNA from infected and uninfected human blood cells and mosquitoes, and plasmid DNA containing the type A, B and C genes, as positive controls, were blotted on nylon membrane. The membrane strips containing identical sets of samples were hybridized with each of the [^{32}P] end-labelled oligo-probes at 55 °C overnight, and the autoradiography was processed at -20 °C for 3 hours.

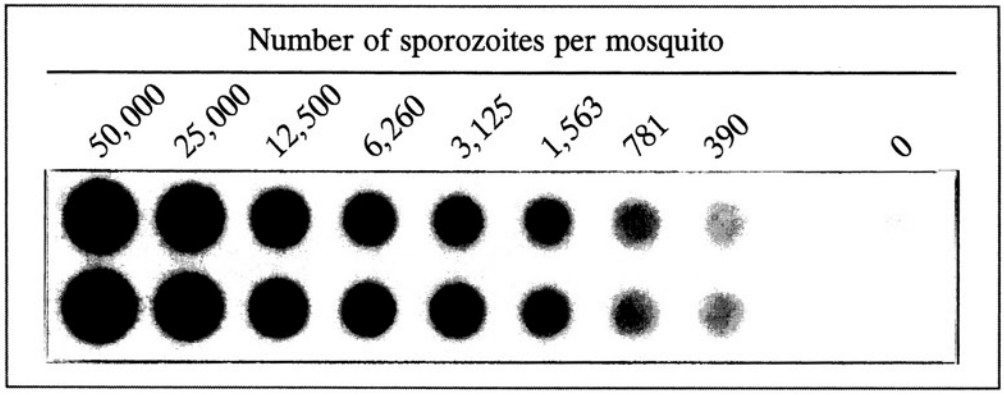


Fig. 15. Sensitivity of dot blot hybridization in sporozoite detection. The total RNA prepared from a known number of *P. vivax* sporozoites and one mosquito (*Anopheles dirus*) was probed with oligonucleotide #743, which is complementary to the type C SSUrRNA of *P. vivax*.

negative and positive controls, respectively. The results shown in Figure 14 indicate that the A gene specific probes exclusively hybridized to RNA from the *P. vivax* infected human blood and C gene specific probes to the RNA from the salivary gland sporozoite. The B gene specific probes did not hybridize to the RNA from these stages of the parasite, but selectively bound to the corresponding plasmid clone, the positive control. The rRNA probe T10, which was considered to be *Plasmodium* specific, hybridized with both the plasmids and all infected specimens with the exception of the plasmid containing the B gene. The sequence of probe T10 is different to that of the B gene clone. *P. falciparum* SSUrRNA A gene specific probe (J36) only hybridized to the RNA isolated from the infected human blood. Among the evaluated oligonucleotides, #741, #742 and #743 were selected for further study because of their high efficiency in the hybridization assay. The sensitivity for detecting *P. vivax* infected mosquitoes was determined by hybridizing the C gene specific probe, #743 (Fig. 15) with RNA isolated from a single uninfected mosquito that was mixed with a known number of salivary gland sporozoite. The lower limit of sensitivity was as few as 400 sporozoite per mosquito. This is well below the average number of sporozoite per mature oocyst, estimated to be between 2000-6000 for *P. vivax* [Rosenberg and Rungsiwongse, 1991]. This presumably assures detection of the C gene transcripts even if only a single oocyst completes development in mosquito. The correlation between the strength of the hybridization signal and the number of sporozoite also confirms the sporozoite origin of the C gene transcripts (Fig. 15).

6. Developmental switch of rRNA forms:

The differential expression of the structurally distinct rRNAs in *P. vivax* was first followed by probing total RNA isolated from the mosquitoes collected at different intervals after the infectious blood meal. One tenth of the sample isolated from 10 pooled mosquitoes was used to probe so that hybridization results shown in Figure 16 represent the average signal from one infected mosquito. The A gene transcripts predominated in the infected blood and engorged mosquitoes, but disappeared within 24 hours after the blood meal. The positive signal at day 1, two hours after the feeding,

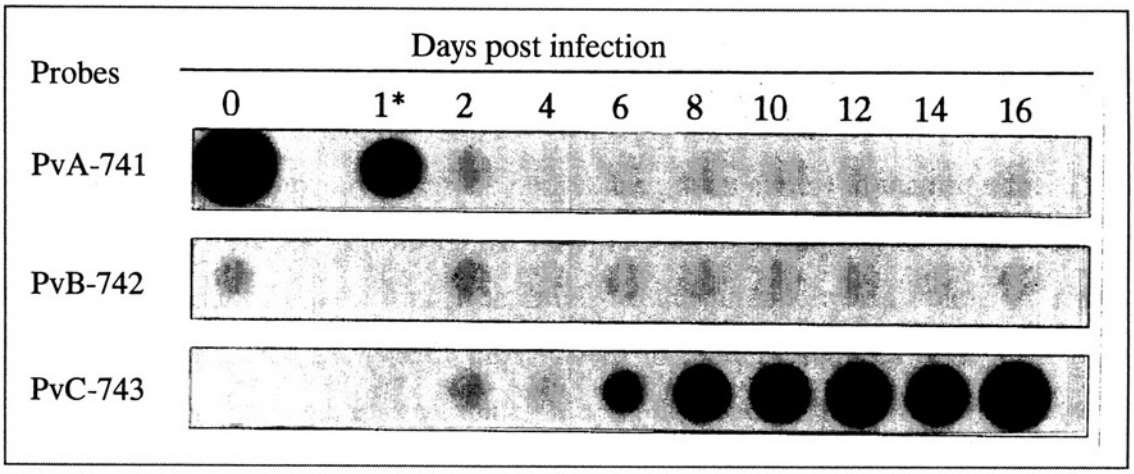


Fig. 16. Transition of types of rRNA gene transcripts during *P. vivax* development in mosquitoes. The total RNA isolated from *P. vivax*-infected mosquitoes at different intervals (Day 1 to 16) after the infectious blood meal was probed with oligomer #741 #742 and #743, which are specific for the type A, B and C genes, respectively. Each dot represents the average signal from one tenth of a 10-mosquito pool collected at each time point. The sample at day 0 represents the RNA prepared from blood stage parasites, while the sample of day 1 was collected two hours after the mosquitoes fed on the infected patient.

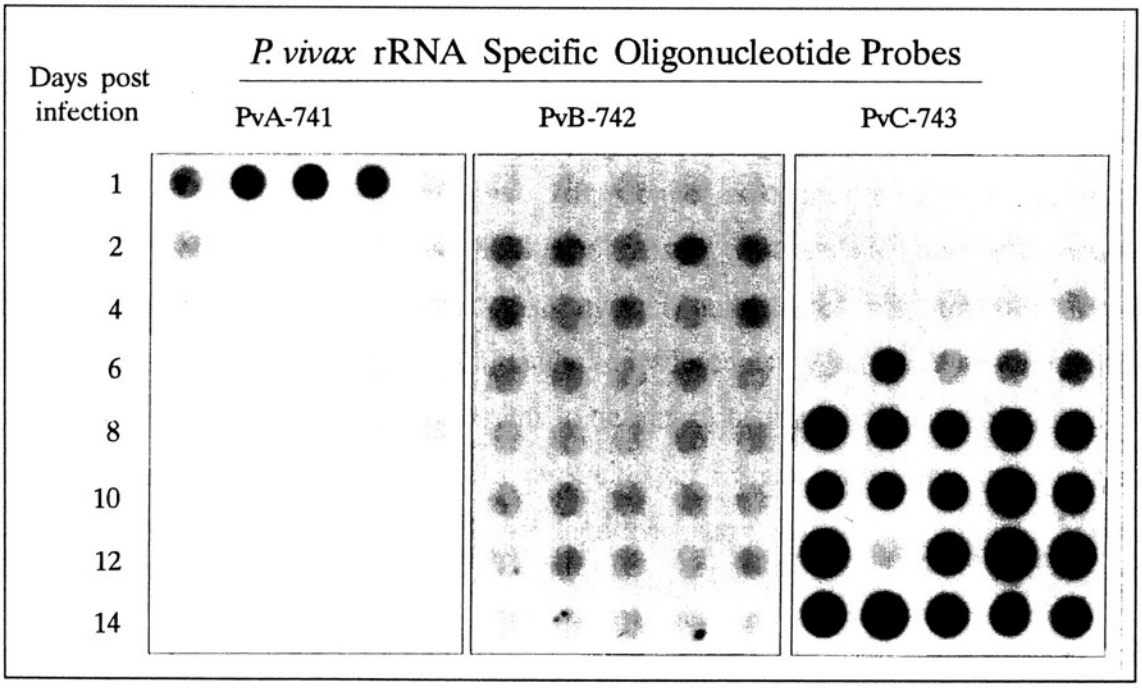


Fig 17. Individual variability of rRNA transition in *P. vivax* infected mosquitoes. The total rRNA from individual mosquitoes collected at different intervals after the infectious blood meal was hybridized with the rRNA gene specific probes, #741, #742 and #743, which are complementary to the A, B and C gene transcripts, respectively.

presumably represented undigested blood stage parasites. The C gene transcripts were not detected in infected mosquitoes until day 6; thereafter, the signal sharply increased and then leveled off at about day 10-12. This rapid accumulation of C gene transcripts occurs during late sporogony when the oocyst has grown almost to mature size. The B gene transcripts were not detected under these conditions during the entire time course. This possibly results from the limited sensitivity of the dot blot hybridization. A similar procedure was repeated using individual *P. vivax* infected mosquitoes (Fig. 17). Some variation in signal strength existed among the samples collected at different time points and is probably related to variability in the number of oocysts that develop in the mosquito gut. The results were, as an average, equivalent with those shown in Figure 16. In addition, the B gene transcript seems to be present at a lower level; however it is not substantial compared to the Northern analysis described later.

The same set of the RNA samples, described above for dot blot analysis, were also analyzed using Northern blots. Each sample was separated by electrophoresis and then linked to a membrane following hybridization with a gene specific probe (Fig. 18). The mature transcripts of the A genes were detected as a 2.0 Kb fragment in the infected human blood, while the C gene transcripts appeared as a 2.2 Kb fragment during the period of development in the mosquitoes. After longer exposure (16 hours compared to 1 hour for the other two transcripts) the B gene transcript was detected during the period from day 2 to day 14. This transcript was, however, not detected in sporozoite that had been isolated directly from salivary glands and subsequently mixed with uninfected mosquitoes. Thus, the B transcript seems to be transcribed during the period of oocyst development after encapsulation on the mosquito gut. Since only a few parasites, as compared with a total number of the ingested parasites, could complete sexual reproduction and develop to oocysts in the mosquito, the number of the B gene transcripts could be very limited and hence difficult to detect by the dot blot hybridization as described above. In addition, the size of the B gene transcript is about 200 to 300 bp shorter than the size expected from the cloned sequence, suggesting that part of the transcript may be processed during rRNA maturation. Further hybridization of the same blot with oligonucleotide probes complementary to the common region of the template

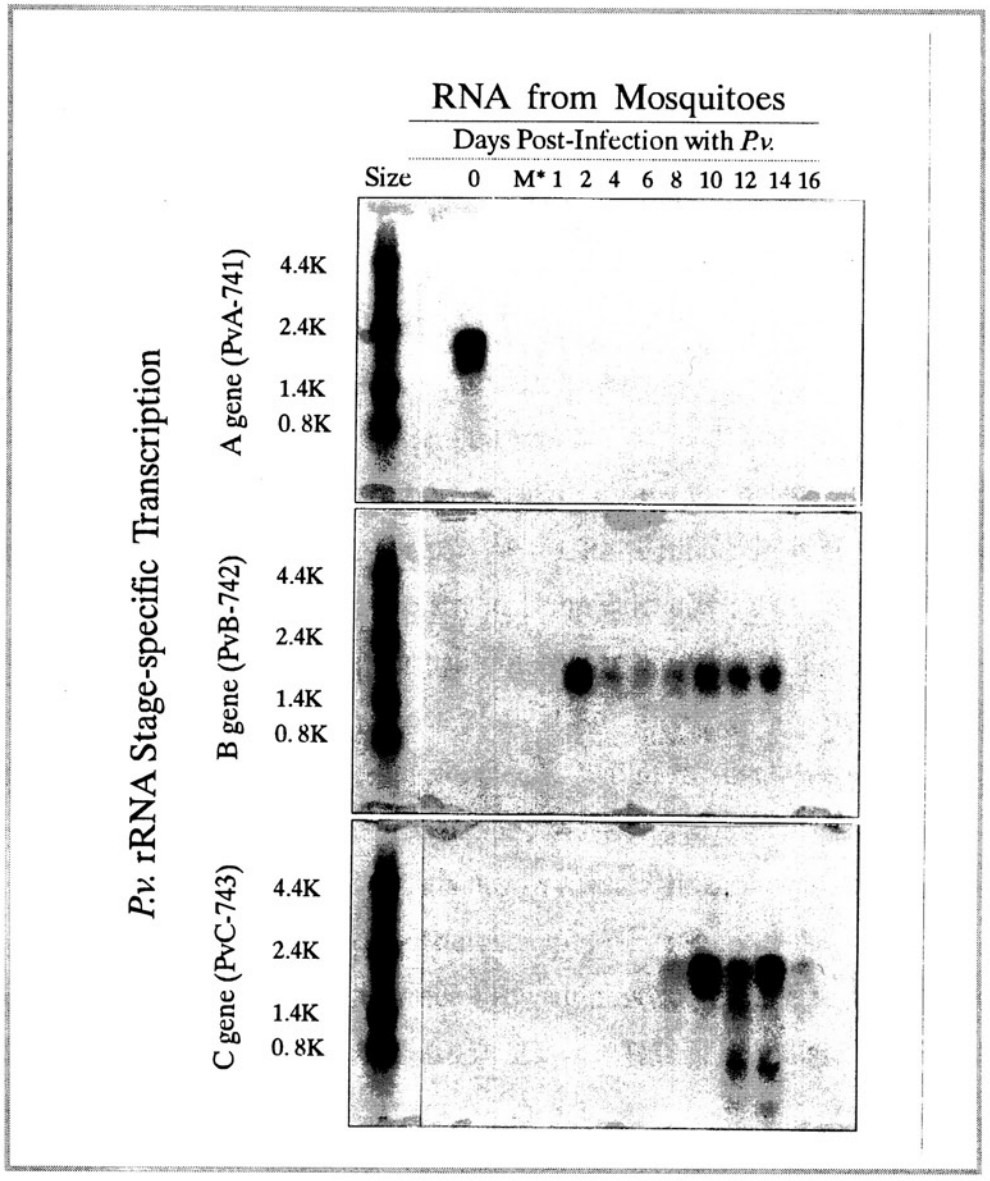


Fig. 18. Northern blot analysis of developmentally controlled transcription of rRNA genes in *Plasmodium vivax*. The total RNA prepared from the infected mosquitoes (*Anopheles dirus*) at different intervals after an infectious blood meal was first separated by agarose gel electrophoresis and immobilized on nylon membrane. Then, the blots were individually hybridized with three distinct oligo-probes that can differentiate the type A, B and C gene transcripts, respectively. *The sample "M" represents the RNA from uninfected mosquitoes.

strand of the three genes also revealed similar results, with no signal for either A or C gene transcripts (data not shown). This suggests that the B gene is possibly transcribed from both strands. Alternatively, the fast migration of the B transcripts on the gel could have resulted from the formation of transcripts into stable duplexes which are not denatured under the conditions of electrophoresis.

7. Establishment of a competitive RT/PCR assay:

The development of an RNA amplification system was pursued because it offered greater sensitivity, fewer possibilities for contamination, and data that could be quantified. RT/PCR is one of the most sensitive assays for detecting gene transcripts [Kawasaki, 1990], in which RNA or part of the RNA molecule is first transcribed by reverse transcriptase (RT) into single strand complementary DNA, and then amplified by routine PCR. Thus, by selecting pairs of conserved primers, the part of the RNA transcript containing unique sequences can be amplified and serve as a target for diagnosis or monitoring parasite development. Based on the diverse pattern of the *Plasmodium* SSUrRNA genes, the primer pair #841-844 was selected as it covers the two most variable regions (V7 and V8). This design assures the differentiation both of genes from different species as well as of different types of gene from the same species. In addition, the internal conserved sequence between the two variable regions can serve as a genus marker in subsequent hybridization assays. This is of practical importance for the detection of multi-species infections.

In order to access the accuracy of quantitation, the competitive nature of RT/PCR had to be established. The RNA isolated from known numbers of *P. vivax* sporozoite (10240 to 40; 1:2 dilution) was co-amplified with a known amount (6 pg) of competitive template, the RNA from *P. berghei* infected blood. A reverse situation was also tested, in which a fixed amount of RNA from approximately 750 *P. vivax* sporozoite was competitively amplified against a serial dilution (1:4) of the *P. berghei* RNA (1280 to 0.005 pg). The results (Fig. 19a) show that both the *P. vivax* type C rRNA (0.44 Kb) and the *P. berghei* type A rRNA (0.39 Kb) were amplified in a competitive fashion; the strength of the signal is directly related to the ratio of the two target molecules rather

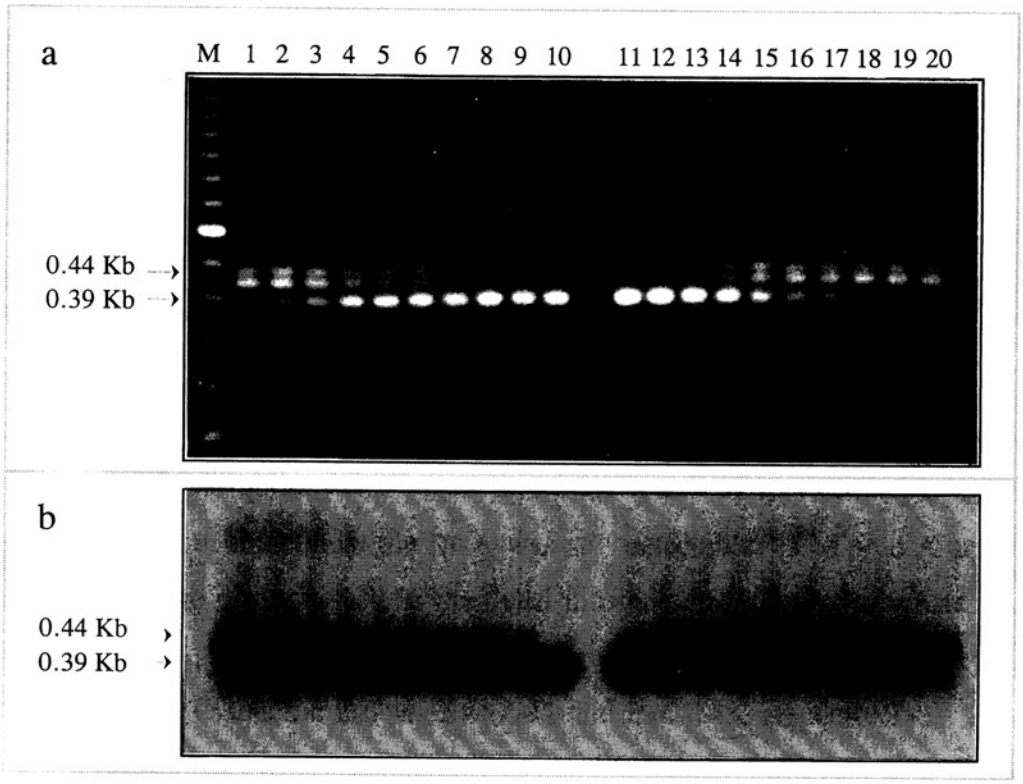


Fig .19. Competitive amplification of the type C rRNA from *P.vivax* against the type A rRNA from *P. berghei*. (a) Ethidium bromide-stained gel: lanes 1-10 contain a series dilution (1:2) of RNA from *P.vivax* sporozoites(10,240 to 40) in the mosquitoes against a fixed amount of rRNA from *P.berghei* blood stage (6 pg); lanes 11-20 contains a fixed amount of RNA from 750 sporozoites versus a serial dilution (1:4) of the *P. berghei* RNA (1,280 to 0.005 pg). (b) Autoradiogram of the Southern blot hybridized with a genus specific oligo-probe, #842. The upper band (0.44 Kb) represents the type C rRNA from *P.vivax* sporozoites and the lower band shows the type A rRNA from *P. berghei* blood stage parasites.

than the absolute amount of each target. This relationship was confirmed by hybridization of the RT/PCR product with a genus specific oligo-probe #842, which is complementary to the internal conserved sequence described above (Fig. 19b). The sensitivity of the assay is able to detect as few as 40 sporozoite, the lowest dilution in this test, suggesting at least a 10-fold increase in the sensitivity as compared with dot blot hybridization.

The stability of the rRNA target was also investigated, especially as it relates to sample storage. The high sensitivity of RT/PCR also has the advantage of being useful for detecting parasites preserved on stained blood slides and in dried mosquitoes. The results (Fig. 20) show that the type A rRNA was detected from *P. vivax*-infected blood smears prepared from patients two years ago. Similar results were obtained from *P. vivax*-infected mosquitoes that were dried ten years ago. Although the sample material is no longer infectious, the RNA was still detectable. The type A and C gene transcripts could be distinguished in the dried mosquitoes. The approximate length of time that the mosquito had been infected still related to the RNA transcript level as detected by A and C specific probes, PvA-741 and PvC-743, respectively. The signal from the dried mosquitoes was generally weak and only the type A transcript was visible on the ethidium bromide stained gel, however both transcripts were detected by subsequent hybridization (Fig. 20a, b). These results indicate that rRNA is more stable than expected and this approach will permit analysis of historical material.

The usefulness of RT/PCR to follow developmental transitions was also investigated. For further determination of the transition of structurally distinct rRNAs during parasite development, the total RNA from *P. vivax* infected human blood cells and mosquitoes (the same set of samples used in previous dot blot hybridization and the Northern analysis) was amplified by RT/PCR. The reaction products were separated on an agarose gel and transferred to membrane for probe hybridization. The results shown in Figure 21 demonstrate a clear transition between the distinct types of SSUrRNAs. The A gene transcripts, which were dominant in infected blood and freshly engorged mosquitoes, were gradually replaced by the C gene transcripts during parasite development in the mosquito. Presence of both transcripts from day 4 to day 6 indicated

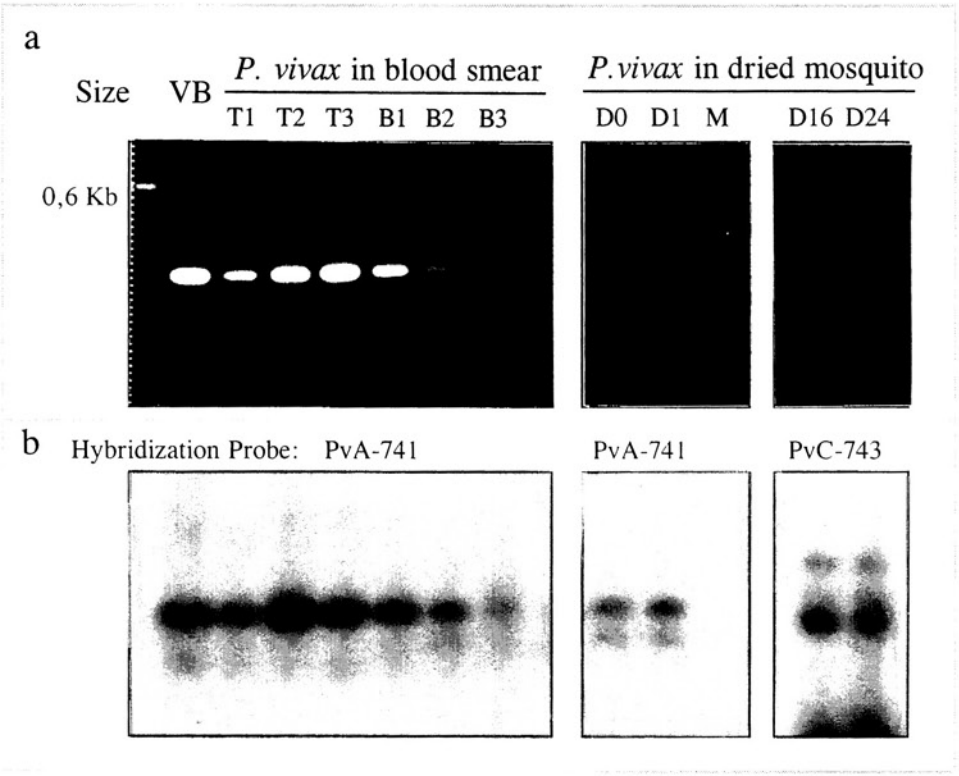


Fig. 20. Detection of *P. vivax* preserved on stained blood smears and in dried mosquitoes. Total RNA prepared from the Giemsa stained slides and dried mosquitoes containing *P. vivax* was first transcribed into cDNA and then amplified by PCR. The product was fractioned by electrophoresis on a 1% agarose gel and stained with ethidium bromide (a). After transfer to nylon membrane, the Southern blot was sequentially probed with two *P. vivax* rRNA probes specific to the A (PvA-741) and C (PvC-743) genes, respectively. The sample T1-3 and B1-3 represent the infected smears made 2 years ago from Thailand and Brazil, respectively. The *P. vivax* infected blood (VB) served as a positive control. The dried mosquitoes were from laboratory-infected mosquitoes (*Anopheles dirus*) that were preserved at room temperature for almost 10 years; D0 (2 hours), D1, D16 and D24 represent days post infection and M represents an uninfected mosquito.

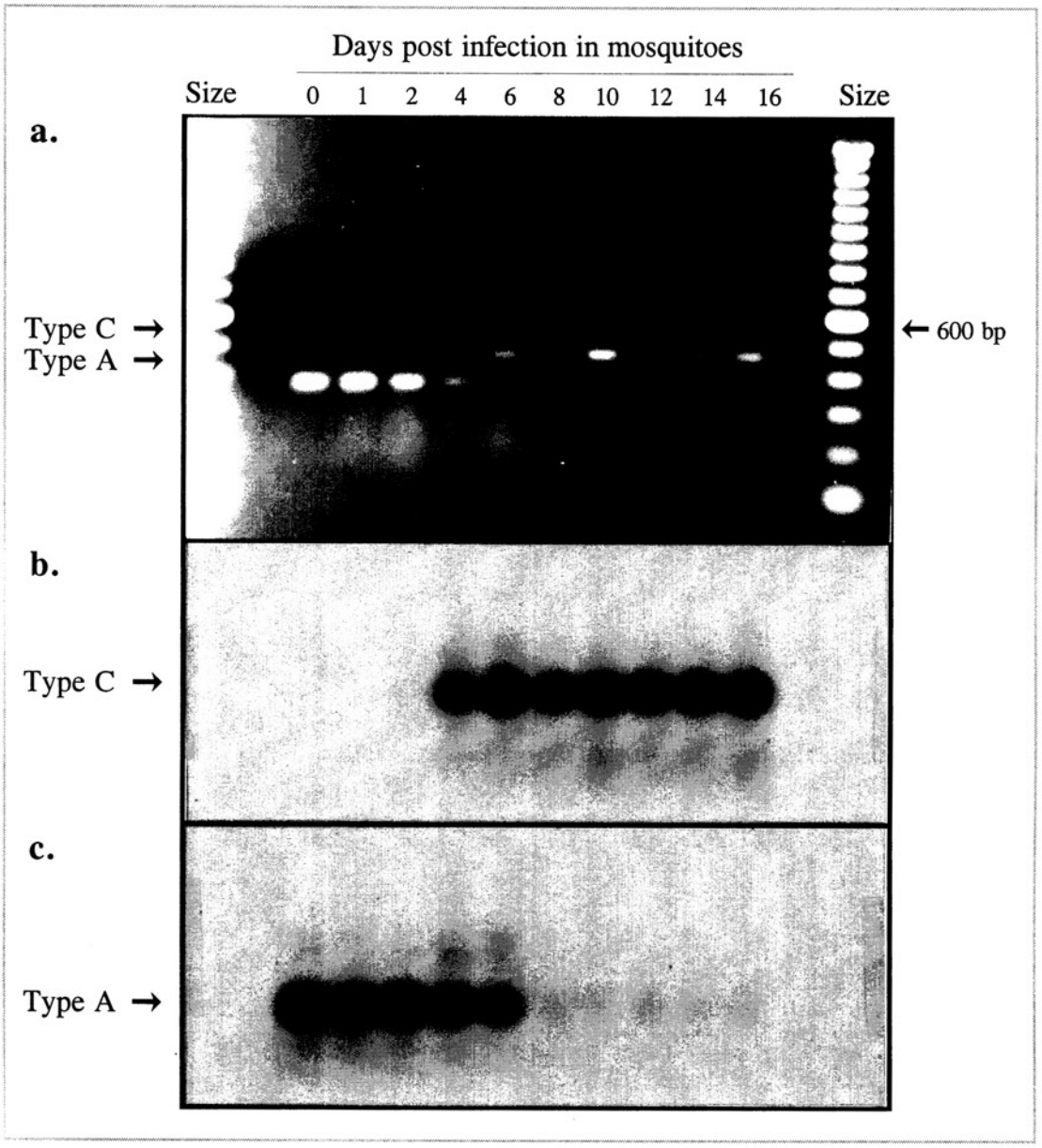


Fig. 21. *P.vivax* switches ribosomal RNA type during the developmental transition from human to mosquito. The total RNA isolated from the mosquitoes collected at different intervals after the infectious blood meal was first transcribed into cDNA by reverse transcriptase (RT) and then amplified by *Taq* DNA polymerase in the polymerase chain reaction (PCR). The primer pair (#841-#844) covers the variable regions (V7 and V8) of SSUrRNA sequences where a 60 base difference exists between the erythrocytic stage specific (type A) and sporozoite stage specific (type C) gene transcripts. The RT/PCR products were separated on a 1% agarose gel (a) and, after being transferred onto nylon membrane, were probed with the type C (b) and A gene (c) specific oligonucleotides.

a transitional period when the C gene transcripts began to accumulate while the A gene expression decreased. However, this primer pair, #841-#844, covering about 0.45 Kb of sequence, including variable regions V7 and V8, did not support amplification of the B gene transcripts. This was possibly due to the unique conformation of the V7 region on the B gene transcripts or self-splicing that occurred in this region. The B gene transcripts were successfully amplified from these RNA samples with another primer pair, #836-#845, covering about 1 Kb of sequence on 5' end of the B gene transcript (Fig. 22). This band could be hybridized with the B gene specific probe #706. Cloning and sequencing of the amplified products confirmed the identification of this as a transcription product of the B gene (data not shown).

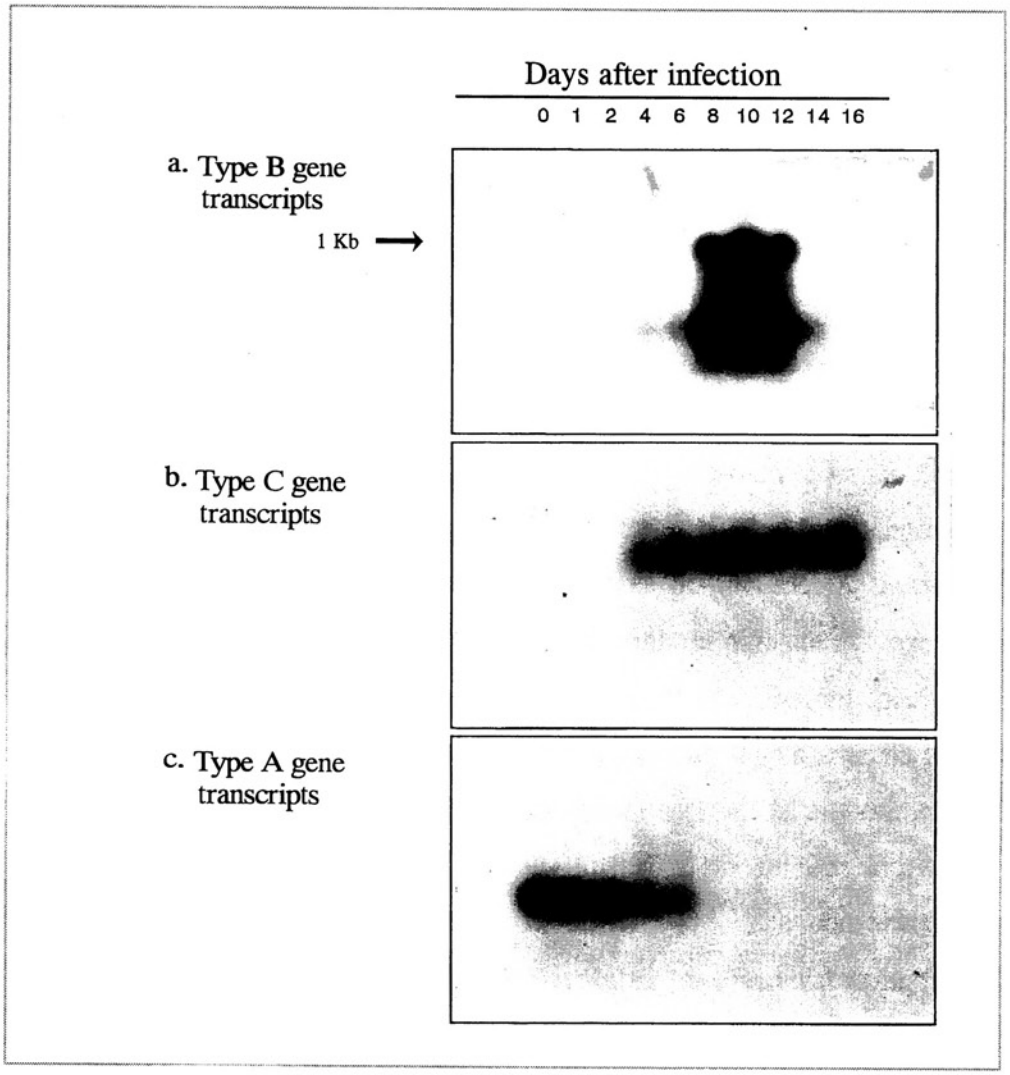


Fig. 22. Amplification of the SSUrRNA B gene transcripts during *P. vivax* development in mosquitoes. (a) The total RNA from the infected mosquitoes at different time points after the blood meal was first transcribed into cDNA by reverse transcriptase (RT) with a *Plasmodium* specific antisense-primer, #836. The product was then amplified with a B gene specific sense-primer, #845 by PCR. After separation by agarose gel electrophoresis, the PCR product was transfered onto nylon membrane and hybridized with an internal B gene specific probe, #706. (b) and (c) The RT/PCR products with a pair of genus specific primers (See Fig. 21) were processed in the same fashion to show the time frame of the B gene transcription.

DISCUSSION

1. The stage specific rRNA genes in *Plasmodium vivax*:

Previous studies have suggested that structurally distinct rRNA genes are maintained in *Plasmodium* species as a unique feature of the genus [McCutchan, 1986]. These alternative genes are dispersed on several different chromosomes, suggesting that *Plasmodium* species do not employ all of the standard mechanisms adopted by most eukaryotes for homogenizing the sequences of rRNA genes. The reason for *Plasmodium* maintaining such a unique multi-rRNA system is not clear, but a functional significance seems to be indicated by the differential expression of alternative genes during discrete stages of the parasite's development [Gunderson et al., 1987; McCutchan et al., 1988; Waters et al., 1989b]. In the presented study, three distinct rRNA transcription units were characterized from the genome of the human malaria parasite *P. vivax*. The A and C genes, whose counterparts have been found in other *Plasmodium* species, are active in erythrocytic and sporogonic stages, respectively. The B gene seems to function in oocyst development and no analogous gene has been described in any other species. The coding sequences for three SSUrRNA genes differ in the regions known to diverge rapidly in most eukaryotes [Neefs et al., 1990; 1991]. The conserved regions are localized in the predicted regions and help maintain a structural core that is almost identical to that found in the ribosomal rRNA of other eukaryotic organisms. The presence of a common eukaryotic architecture of the SSUrRNA, which is supported by the proposed secondary structures, suggests the equivalent roles of *Plasmodium* rRNAs as central components of ribosome in protein synthesis. In addition, the presence of distinct ITS1 regions in each transcription unit probably indicates that the three rRNA genes have independently evolved under different selective constraints.

In most organisms examined to date, rRNA genes are identical within the species or individual organisms because of their equivalent function in protein synthesis. The presence of structurally distinct rRNA genes in *Plasmodium* species may reflect a unique status of rRNA gene evolution. This multi-rRNA system is probably adopted by the parasite to facilitate their complex life cycle, which includes four contiguous but discrete

reproductive phases, involving various tissues and cells in both vertebrate and invertebrate hosts. Our comparative analysis indicates that the A gene of *P. vivax* is closely related to the analogous genes isolated from primate vivax-like malaria parasites, *P. simium* [Lal et al., unpublished data] and *P. cynomolgi* [Corredor and Enea, 1994], and a similar situation is also found for the C gene. In a pair-wise sequence comparison, the average similarity among the analogous (orthologous) genes from the three species could be as high as 96.5% for the A gene and 89.4% for the C gene (Fig. 13b); whereas the mean identity between the A and C genes (paralogous genes) from the same species is only about 76.7%. The reversed pattern of the orthologous versus paralogous homology suggests that the A gene diverged from the C gene at a time before process of the speciation, and the two genes are specifically maintained in the contemporary species under different selective constraints. In addition, the majority of the sequence differences between corresponding gene types contribute to forming the unique parts of the rRNA gene structures. Hence, these distinct structures are likely to have been part of a specialized process selected during the evolution of *Plasmodium* species.

Comparative analysis of rRNA gene sequences can be used to define the constraints or patterns from which the molecular structure could be inferred. A comparison of corresponding structures can reveal functional and regulatory information. Simple sequence alignment, however, without considering the phylogenetic relationship of the species, is not sufficient to define certain types of structures. The primary sequences of rRNAs from distant species may differ dramatically but retain similar structures for their ultimate function. Previous analysis of the SSUrRNA gene sequences from *P. berghei*, *P. falciparum*, and *P. cynomolgi* raised a confusing situation in which the paralogous genes from the same species were more similar to each other than the orthologous genes from different species, suggesting that no specific sequences are maintained for the stage-specific rRNA genes and that gene conversion is dominant in *Plasmodium* rRNA gene evolution [Enea and Corredor, 1991; Corredor and Enea, 1993; 1994]. This explanation seems to be debatable in several aspects based on the feature of the SSUrRNA gene structures and is not supported by this study.

First, the *Plasmodium* species used in the analysis are from three defined groups,

rodent, primate and bird-related malaria, and they clearly diverged as early as the parasites adapted into the different vertebrate hosts, although the exact time of the divergence and precise phylogenetic relationships are still unclear [Garnham, 1966; Waters et al., 1993]. As a consequence of biological adaptation of the parasites, the rRNA genes among these distant species could differ remarkably as long as the ultimate function of the gene in protein synthesis is maintained. Since the translational function is probably determined by the high-order structure of rRNA, the primary sequences could diverge very rapidly, especially in the regions that do not influence the core structure. The truly conserved sequences are very limited, and only three stretches of the sequences, approximate 60 nucleotides in total, are actually maintained within all the SSUrRNAs examined to date [Ofengand et al., 1993]. Most of the positions are subject to variation but the rate of divergence may depend on the region of the gene and its corresponding functional or structural purpose. Hence, certain patterns or constraints on the structures may not be demonstrated by analysis of the sequences from distantly related species. Second, the structural constraints for stage specific genes may vary with the species and may not be conserved at the genus level. It is likely that the unique structures defined by the highly diverged regions of the gene are integrated parts of the parasite adaptation to facilitate efficient protein synthesis in different environments. This pattern could be maintained only in closely related species, as described in the results, since they still share most of the common features from a recent ancestor. Third, the pattern of molecular changes in *Plasmodium* rRNA genes and the presence of distinctive ITS1 regions seem unlikely to be the result of gene conversion, one of the important mechanisms responsible for homogenizing the repeated rRNA genes in most eukaryotes. Gene conversion is a nonreciprocal recombination process in which usually the entire gene, or a significant part, along with the flanking region is converted [Li and Graur, 1991]. In the case of *Plasmodium* rRNA genes, sequence variation appears to have a scattered distribution throughout the coding sequence of the gene and the distinct ITS regions of individual transcription units. This is more likely to be caused by mutations being accumulated during DNA replication or possibly even errors resulting from reverse transcription during the gene transposition.

2. Phylogenetic implications of the rRNA genes

Comparative analysis of rRNA sequences has been used to reconstruct the evolutionary history of organisms, especially for unicellular bacteria and protists [Woese, 1987]. For *Plasmodium* species, this approach could be extended to explore the evolutionary path of parasite history since divergence of the stage-specific rRNA genes presumably corresponds to adaptation to different hosts during the course of evolution. The evolutionary history of malaria parasites is extremely complex and two controversial scenarios have been proposed based on the biological relationships between the parasites and their hosts [reviewed by Coatney et al., 1971]. Both of the scenarios suggests that *Plasmodium* species descended from a coccidian ancestor but differ whether this ancestor was parasitic in an insect host or in a vertebrate host. Our results, based on structural analysis of the stage specific rRNA genes seems to favor the insect origin of the malaria parasites.

A knowledge of the time frame in which the stage-specific rRNAs arose might give important information as to when the developmentally distinct forms of the parasite were incorporated into the life cycle of *Plasmodium* as a whole. Among the three distinct rRNA genes identified from *P. vivax*, the B gene, which is transcribed during oocyst development after fertilization, is structurally distant to the other two genes and may also be the most archaic of the genes. The overall homologies of the B gene to the A and C genes are 52% and 62%, respectively; whereas the similarity between the latter two genes is 85%. This suggests that the A and C genes are much more closely related to each other than either is to the B gene and probably are the result of a more recent gene duplication. Presumably, the ancestor of malaria parasites adopted the contemporary style of parasitism in a step-wise fashion. Emergence of novel genes probably facilitated the adaption of *Plasmodium* species to intra-tissue or intracellular environments. It seems reasonable to propose that the B gene was the most ancestral gene since adaptation to intracellular development is obviously a recent event in evolutionary terms. This is also supported by the high homology among the A genes from closely related species and from different isolates of *P. vivax*.

Other features of the sequences also support the evolution described above.

Comparison of the SSUrRNA secondary structures within the region of each, corresponding to the V7 area, suggests that the B gene transcript possesses the most stable stem-loop structure. It is composed of about 200, almost non-interrupted, antiparallel paired nucleotides. In the same location, the other two genes exhibit various bulges caused by unpaired nucleotides and branches. Interestingly, in this region, the A gene sequence could be derived from the corresponding region of the C gene after 5 deletions and 1 duplication (Fig. 23). This is as if the A gene had been created from the C gene by an already established and accepted mechanism; the slipped-strand mispairing that often occurs during gene duplication [Li and Graur, 1991]. Hence the derivation of the A gene can be explained as being directly derived from the C gene, indicating that the insect form of the parasite predated the vertebrate form. One then must wonder whether or not the B gene is ancestral to the C gene. An event that suggests this may in fact be the case is revealed by analysis of B and C genes from different sources. An indicative structure is present in the V8 region of the genes (Fig. 12), where the C gene from Thai *P. vivax* isolates, as well as other primate malarias, have a structure similar to the corresponding region of the B gene, while the region of the C gene from the New World *P. vivax* strain, SAL-1, seems to be altered so that it resembles the A gene in that area. This suggests that B type sequences can be altered to the C type and then to the A type. Changes of this type are then possible and perhaps occur frequently and would also indicate a direction in the gene evolution. One can propose that the B gene was carried by a coccidian ancestor of malaria parasites in the insect gut. It subsequently underwent a gene duplication. In this scenario, a duplicated B gene would diverge by mutation from its present sequence to the C type. This would in turn favor speciation by favoring the life stage and environment of contemporary malaria parasites. Further duplication and divergence of the C gene would then give rise to an A gene favoring success in vertebrate tissue and establishing the intracellular parasitic cycle for *Plasmodium* species. However, more conclusive evidence is obviously required to substantiate this evolutionary scheme for *Plasmodium* species, and will depend on more rRNA genes being sequenced, especially for the B gene from other species of the genus *Plasmodium*.

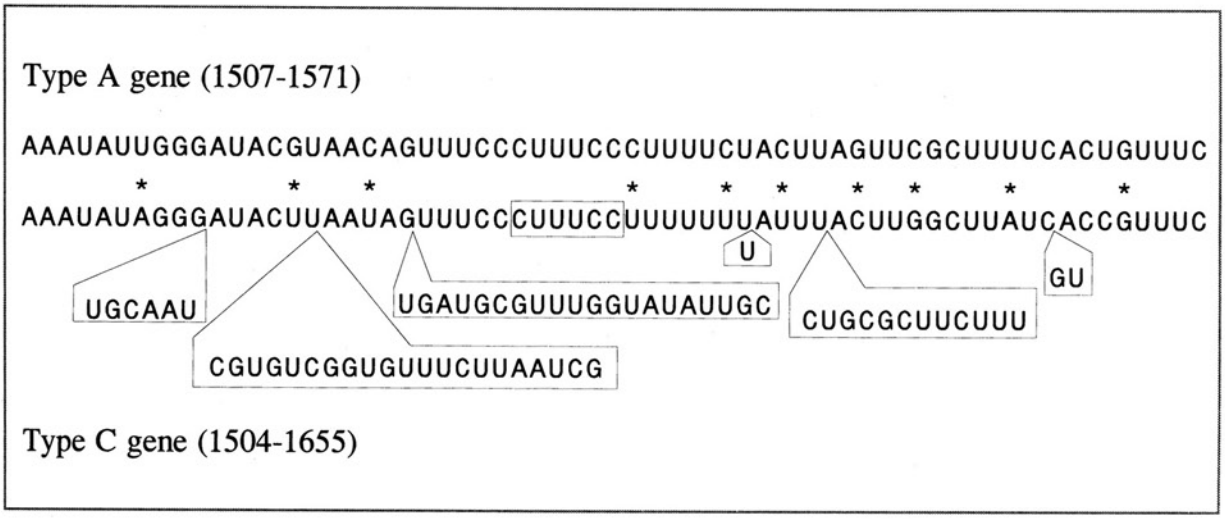


Fig. 23. Rearrangment of the sequence segments in V7 region of *P.vivax* SSUrRNA genes. In this region, the A gene may be derived from the C gene through five deletions and one duplication .

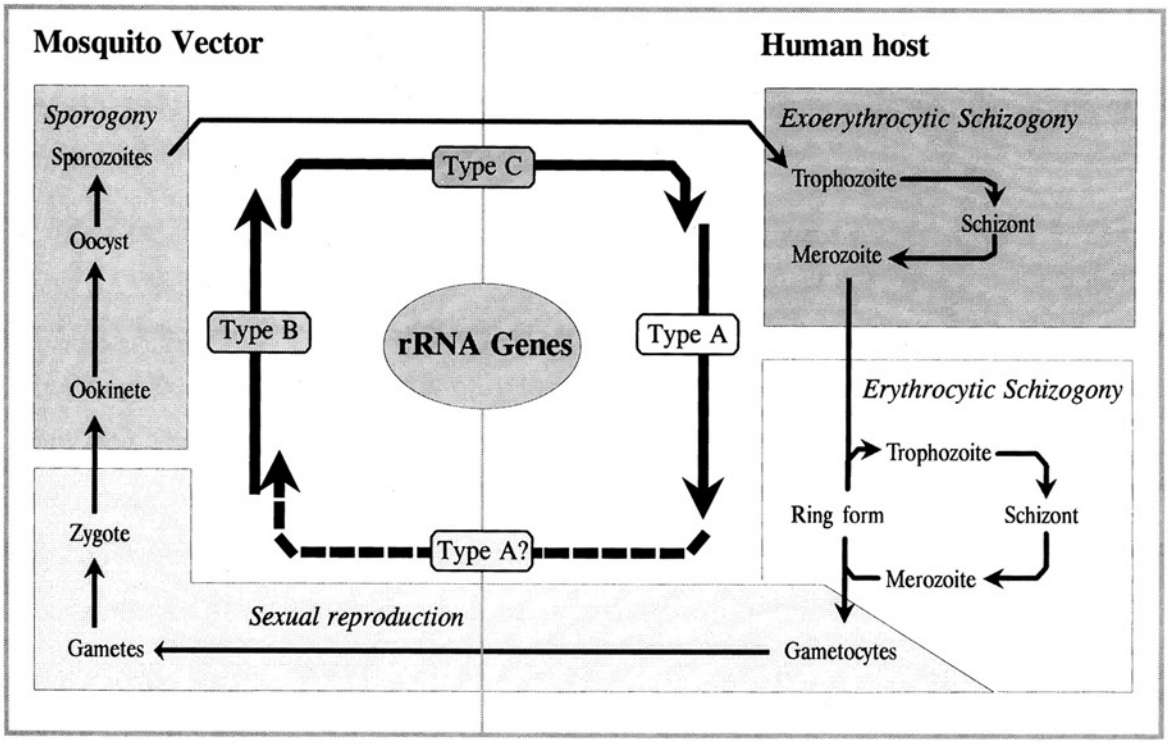


Fig. 24. Summary of *Plasmodium* ribosomal RNA gene expression. Three structurally distinct rRNA genes are differentially transcribed according to discrete reproductive stages during parasite development. The question mark represents the possibility that a type A-like rRNA gene is responsible for parasite sexual reproduction

Another interesting fact warranting comment is that the C gene of a South American strain, SAL-1, differs dramatically from those of Thai *P. vivax* isolates at the V8 region. This is a region of the SSUrRNA that corresponds to regions of the *E. coli* SSUrRNA that influences temperature sensitivity of the rate of protein synthesis [Noller et al., 1990]. All other regions of the C gene sequences from various sources are identical. Since the A genes, as well as the B genes, are almost identical among these isolates of *P. vivax*, the rapid divergence of the C gene, which is exclusively transcribed during sporogony in mosquitoes, could relate to changes of the vectorial and ecological system. To determine whether this gene type has been fixed in the New World *P. vivax* population requires further investigation of the C genes from field isolates of South America. In addition, sequence analysis indicates that *P. simium*, the sole vivax-like parasite found in New World primates, seems to be more similar to human vivax isolates from Thailand than to some other *P. vivax* isolates. This probably substantiates the human origin of New World primate malaria previously proposed [reviewed by Coatney et al., 1971] and may lead to the proposal that *P. vivax* from Asia was the origin of this infection.

3. Developmentally controlled expression of the rRNA genes

The regulation of expression of rRNAs, the central components of the ribosome, has been noted to be crucial not only for the translational function in protein biosynthesis but also as a coarse control for development and differentiation [Ramagopal, 1992]. In this study, three structurally distinct rRNA genes identified from *P.vivax* are shown to be differentially transcribed during the contiguous but discrete stages of the parasite development (Fig. 24). The A gene is predominantly transcribed during erythrocytic schizogony in the vertebrate host, while the C gene is expressed during sporogony in the maturing oocyst on the mosquito midgut. Transcription of the B gene seems to be limited to oocyst development. In addition, distribution of the B gene transcripts seems to be localized within the oocyst since they are not detectable in salivary gland sporozoite of infected mosquitoes. This transition of alternative rRNA forms seems to correlate better with the progression of distinct stages of development than to the presence of the

parasite in one host or the other, suggesting that the regulation of the differential expression of rRNA is an integrated part of the developmental control for the *Plasmodium* species.

The mechanisms involved in the turnover of alternative RNA types may be complex since both the degradation of one type of transcript and the concomitant expression of the alternative rRNA genes must be involved. Previous studies based on *in vitro*-cultured *P. falciparum* suggested that the transition of major rRNA forms during the development from gametocyte to ookinete was assisted by selective degradation of the A gene transcripts and accelerated processing of the C gene precursors [Waters et al., 1989b]. The specific cleavage of the functional region of rRNA seems to be an efficient way to eliminate the pre-existing transcripts. However, accumulation of the C gene precursor in gametocytes and early ookinetes was not detected in the present study where *P. vivax* was examined *in vivo* within its natural host system. This confusion could be explained by differences in transcriptional control between the species. Alternatively, given that the *in vitro* development of *P. falciparum* ookinetes is incomplete and ceases within about 48 hours, it is possible that the accumulation of the C gene precursors resulted from the lack of essential nutrients in culture medium. Ribosomal RNA precursor accumulation does occur in bacteria cultures when deprived of essential nutrients, and is much less likely to accumulate during normal development since processing of the rRNA precursor is usually coupled with transcription [reviewed by Larson, et al., 1991]. Nevertheless, the regulation of transcription seems to be essential for the transition of rRNA types, and this control mechanism should be able to selectively access the genes localized on different chromosomes during discrete stages of parasite development [Dame and McCutchan, 1983b].

Plasmodium species, as intracellular parasites, must undergo development within a number of different living situations. The process of distinct differentiation and morphogenesis is probably the consequence of long-term adaptation. The rRNA with their function may have been modulated by structure to meet the requirement of protein synthesis. The A gene transcripts, the major rRNA in the erythrocytic stage, is replaced by the B gene transcripts during the transition period from vertebrate host to invertebrate

vector. The transcription of the B gene starts when the parasite encapsulates on the mosquito midgut and continues throughout the period of the oocyst development. The rapid accumulation of the C gene transcripts, the sporozoite-specific rRNA, begins at a time when the oocyst has grown nearly to its maximum size and nuclear division has been completed [Garnham, 1966]. In addition, distribution of the different rRNA transcripts within the maturing oocyst seems to be directed by a mechanism that separates the transcripts of the B gene from those of the C gene, which represent the major, and possibly the only type of cytoplasmic rRNA in malaria sporozoite. Physical separation of individual sporozoite during this period has been investigated by observation of the cytological process of sporozoite production [Terzakis et al., 1967; Sinden, 1979; 1982]. The switch of transcription of the alternative rRNA genes is, at least temporally, correlated to the stage-specific progression of development and differentiation of the parasites. One possible implication of these results is that protein synthesis in early development of the oocyst is spatially separated and distinct from that in the sporozoite. Hence, the transition between ribosomal types in *P. vivax* appears to reflect its state of differentiation rather than the host in which the parasite resides.

4. Practical considerations and future perspectives

The study of the ribosomal RNA of *Plasmodium* species should be considered not only as an academic exercise for understanding developmental biology of the parasites but also as an unique approach for investigating evolutionary history and population structure. The stage-specific rRNAs and the coding genes are considered in several aspects as the target of choice for species identification [Waters and McCutchan, 1989b; Lal et al., 1989; Li et al., 1991; 1993;]. First, the rRNA is one of the most abundant macromolecules in cells and very stable and easily preserved. The presented results suggest that the sensitivity of RNA dot blot hybridization and RT/PCR can detect infection in a single mosquito. In addition, the rRNA-based competitive PCR can differentiate parasites from the infected blood smear and dried mosquitoes which have been preserved for years, suggesting that historical materials could be analyzed by this modern technology. Secondly, the mosaic structure of the molecule, including the

alternative arrangement of the variable and conserved sequences, allows one to amplify stage- or species-specific sequences with the genus specific primers. Therefore, many species of malaria can be distinguished using this evolutionarily stable marker; since differential diagnosis is not always easy on the basis of parasite morphology alone, especially for those vivax-like malarias that infect both humans and primates. The determination of the phylogenetic relationships may also yield clues to the dynamics of parasite populations. Moreover, parasite infection in the mosquito can be followed by RT/PCR since the ratio of distinct rRNAs reflects the state of the parasite development. The competitive design of the assay can add a quantitative aspect to the measurement of the out- and in-put of the parasite in a local vector system. Hence, this unique approach allows an accurate assessment of vectorial susceptibility that could be underestimated by other methods based on the parasite morhpology and surface antigens. Third, since the polymorphic rRNA genes reside on different chromosomes, DNA-based competitive PCR can be used to assess the extent of chromosome segregation among field isolates. Determination of clonal variation and other modeling factors of the parasite population structure could be crucial for vaccine design and dissemination, as well as for choosing the appropriate antimalarial drug and other control stratgeies.

It will be difficult to determine why the multi-ribosomal RNA system arose until we understand the functional significance of the variation among the different genes. It may be that the different ribosomes have functional differences pertaining to protein synthesis, as translational control can be associated with variability in ribosome structure. For example, even though *Plasmodium* species are unique in making distinct types of rRNA, they are perhaps not unique in maintaining heterologous populations of ribosomes. Ribosome heterogeneity has recently been discovered in diverse organisms ranging from protozoa to humans [reviewed by Ramagopal, 1992]. In these cases, the modulation of ribosomes is associated with changes in the quality or quantity of certain ribosomal proteins or the 5S rRNA. The alteration of these molecules subsequently results in modification of ribosomal form and function. It is likely that different approaches to ribosome heterogeneity could serve similar functions in the modulation of the translation apparatus and, hence, the heterogeneity seen in *Plasmodium* species may

be reflective of a broader, biological phenomenon.

Further investigation of *Plasmodium* rRNA genes should focus on details of regulatory structures and mechanisms. For example, the presence of different rDNA loci may relate to mechanisms of transcriptional control which access different regions of the chromosome to transcribe at different times during development. In addition, an assessment of the different subset of macromolecules that associate with the different rRNAs during protein synthesis will also provide insight into the function of the eukaryotic ribosome. Since numerous events occur during the course of ribosome biosynthesis, the rRNA must interact with many other molecules, including ribosomal proteins and translation accessory factors. Hence, the coarse control over development could be regulated by the ribosome at many different levels including the obvious possibility that different ribosomes preferentially translate different subsets of messenger RNAs.

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Appendix I: List of synthetic DNA oligonucleotides

Code	Sequence (5'--->3')	Bp	Position[a]
PCR primers for *Plasmodium* SSUrRNA genes:			
#566[c]	GGATAACTACGGAAAAGCTGTAGC	24	149 -> 172
#567[c]	TGGCTATGACGGGTAACGGGGAATTAG	27	365 -> 391
#570[b]	CGACTTCTCCTTCCTTTAAAAGATAGG	26	2168 -> 2143
#573[b]	GACATCCATCGTTAAGACTGTGC	23	2696 -> 2674
#683[b]	GTGCACGAATGAAAGAACG	19	1669 -> 1651
#705[c]	AACCTGGTTGATCTTGCCAG	20	1 -> 20
#706[c]	CGACTGGTGCTTCGTATCGG	20	723 -> 742
Sequencing Primers for *Plasmodium* SSUrRNA gene:			
#599[c]	GACCTATCAGCTTTTGATGTTAG	23	327 -> 349
#600[b]	CTAACATCAAAAGCTGATAGGTC	23	349 -> 327
#601[c]	GCCGCGGTAATTCCAGCTCCAAT	23	608 -> 630
#602[b]	ATTGGAGCTGGAATTACCGCGGC	23	630 -> 608
#603[c]	GCTTAGTTACGATTAATAGGAGT	23	970 -> 992
#604[b]	ACTCCTATTAATCGTAACTAAGC	23	992 -> 970
#605[c]	GAGAAATCAAAGTCTTTGGGTTC	23	1247 ->1269
#606[b]	GAACCCAAAGACTTTGATTTCTC	23	1269 ->1247
#607[c]	GAGATCTTAACCTGCTAATTAGC	23	1495 ->1517
#608[b]	GCTAATTAGCAGGTTAAGATCTC	23	1517 ->1495
#609[c]	GGCAACAACAGGTCTGTGATGTC	23	1779 ->1801
#610[b]	GACATCACAGACCTGTTGTTGCC	23	1801 ->1779
Hybridization probes for *Plasmodium* SSUrRNA genes:			
T10[b]	ACTCGATTGATACACACT	18	Genus *Plasmodium*
#569[b]	GTTCAAGATTAATAATTGCAA TAATCTATCCC	32	1991 -> 1959
#881[c]	AGGTAGTGACAAGAAGTT	18	490 -> 507
#741[b]	AACCGAATTCAGTCCCACGT	20	1488 -> 1469
#744[b]	GTACACACTCAAGAAATGAATC	22	208 -> 187
#684[b]	CATGCCCAAGACATTCGTCG	20	207 -> 188
#703[b]	GCCAAGTAAAGAAGCGCAG	19	1641 -> 1623
#743[b]	ATCCAGATCCAATCCGACATA	21	1506 -> 1486
#745[b]	CGAAACCGTGATTGGCTCAA	20	716 -> 697
#683[b]	GTGCACGAATGAAAGAACG	19	1669 -> 1651
#695[b]	CCGATACGAAGCACCAGTCG	20	742 -> 723
#742[b]	CAACAAGGTTCAATCTTACCT	21	1557 -> 1537
#746[b]	GAAGTTAATCAAAAGTGCTATG	22	204 -> 183
#842[c]	TAACACAAGGAAGTTTAAGGC	21	1761 -> 1781
RT/PCR primers for *P. vivax* SSUrRNA:			
#836[b]	AACTTTCTCGCTTGCGCGAATAC	23	1279 -> 1301
#841[c]	GAACGAGA(C/T)CTTAACCTGC	19	1491 -> 1509
#844[b]	TACTGATAAA(T/G)ATTACCTA	19	1948 -> 1930
#845[c]	ACTTTTGATTAACTTCTTAAGTGCG	25	189 -> 213

Note: a: Corresponding positions of DNA oligonucleotides on *P. vivax* SSUrRNA gene sequences (see Fig.5). **b:** DNA oligonucleotides complementary to the coding strand of the small subunit rRNA gene (antisense oligonucleotides). **c:** DNA oligonucleotides complementary to the non-coding strand of the small subunit rRNA gene (sense oligonucleotides).

Appendix II.

Sequence alignment of SSUrRNA genes from different isolates of *P.vivax*

(1) Type A Genes:

```
Sal-1   AACCTGGTTGATCTTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCAAGTGAAAGTATATGCATATTTTATA  80
K1294   ================================================================================  80
K1290   ================================================================================  80
K-112   ================================================================================  80
K-115   ================================================================================  80

Sal-1   TGTAGAAACTGCGAACGGCTCATTAAAACAGTTATAATCTACTTGACATTTTTTCTATAAGGATAACTACGGAAAAGCTG  160
K1294   ============================================================.....................  160
K1290   ============================================================.....................  160
K-112   ============================================================.....................  160
K-115   ============================================================.....................  160
                                                  ************ V2 ************

Sal-1   TAGCTAATACTTGCCTTAGCACTCTTGATTCATTTCTTGAGTGTGTACTTGTTAAGCCTTTTAAGAAAAAAGTTATTAAC  240
K1294   ..............T.................................................................  240
K1290   ..............T.................................................................  240
K-112   ..............T.................................................................  240
K-115   ..............T................................T.................................  240
        ********************************************************************************

Sal-1   TTAAGGAATTATAACAAAGAAGCGACACGTAATGGATCCGTCCATTTTTAGTGTGTATCAATCGAGTTTCTGACCTATCA  320
K1294   ...G..................T.........................................................  320
K1290   .....................................................................................  320
K-112   .....................................................................................  320
K-115   .....................................................................................  320
        ******************************************************
Sal-1   GCTTTTGATGTTAGGGTATTGGCCTAACATGGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAGAGGGAGCCT  400
K1294   .....................................................................................  400
K1290   .....................................................................................  400
K-112   .....................................................................................  400
K-115   .....................................................................................  400

Sal-1   GAGAAATAGCTACCACATCTAAGGAAGGCAGCAGGCGCGTAAATTACCCAATTCTAAAGAAGAGAGGTAGTGACAAGAAA  480
K1294   .....................................................................................  480
K1290   .....................................................................................  480
K-112   .....................................................................................  480
K-115   .....................................................................................  480
                                                                                     *****

Sal-1   TAACAATACAAGGCCAATCTGGCTTTGTAATTGGAATGATGGGAATTTAAAACCTTCCCAAAACTCAATTGGAGGGCAAG  560
K1294   .....................................................................................  560
K1290   .....................................................................................  560
K-112   .....................................................................................  560
K-115   .....................................................................................  560
        ********** V3 ************************************************

Sal-1   TCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAATTGTTGCAGTTAAAACGCTCGTAGTTGAAT  640
K1294   .....................................................................................  640
K1290   .....................................................................................  640
K-112   .....................................................................................  640
K-115   .....................................................................................  640
                                                                                       **

Sal-1   TTCAAAGAATCGATATTTTAAGCAACGCTTCTAGCTTAATCCACATAACTGATACTTCGTATCGACTTTGTGCGCATTTT  720
K1294   .....................................................................................  720
K1290   .....................................................................................  720
K-112   .....................................................................................  720
K-115   .....................................................................................  720
        ********************************** V4 ****************************************
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```
Sal-1   GCTATTATGTGTTCTTTTAATTAAAATGATTCTTTTTAAGGACTTTCTTTGCTTCGGCTTGGAAGTCCTTGTTACTTTGA  800
K1294   ................................C.....................T.....................  800
K1290   ..................................................................G.............  800
K-112   ................................................................................  800
K-115   ................................................................................  800
        ********************************************************************************

Sal-1   GTAAATTAGAGTGTTCAAAGCAAACAGATATAGCATTGCGCGTTTGAATACTACAGCATGGAATAACAAA-TTGAACAAG  879
K1294   ...................................................................A.........  880
K1290   ...................................................................A.........  880
K-112   ...................................................................A.........  880
K-115   ..........................................C.........................A.........  880
        ********************************************************************************

Sal-1   TCAGAATTTTTGTTCTTTTTTCTTATTTTGGCTTAGTTACGATTAATAGGAGTAGCTTGGGGGCATTTGTATTCAGATGT  959
K1294   ..........-.....................................................................  959
K1290   ..........-.....................................................................  959
K-112   ..........-.....................................................................  959
K-115   ..........-.....................................................................  959
        ******************************************

Sal-1   CAGAGGTGAAATTCTTAGATTTTCTGGAGACAAACAACTGCGAAAGCATTTGCCTAAAATACTTCCATTAATCAAGAACG  1039
K1294   ................................................................................  1039
K1290   ................................................................................  1039
K-112   ................................................................................  1039
K-115   ................................................................................  1039

Sal-1   AAAGTTAAGGGAGTGAAGACGATCAGATACCGTCGTAATCTTAACCATAAACTATGCCGACTAGGCTTTGGATGAAAGAT  1119
K1294   ................................................................................  1119
K1290   ................................................................................  1119
K-112   ................................................................................  1119
K-115   ................................................................................  1119
                                                                 ******** V5 ******

Sal-1   TTTAAAATAAGAATTTTCTCTTCGGAGTTTATTCTTAGATTGCTTCCTTCAGTGCCTTATGAGAAATCAAAGTCTTTGGG  1199
K1294   ................................................................................  1199
K1290   ................................................................................  1199
K-112   ................................................................................  1199
K-115   ............G..................C.................................................  1199
        ************************************************

Sal-1   TTCTGGGGCGAGTATTCGCGCAAGCGAGAAAGTTAAAAGAATTGACGGAAGGGCACCACCAGGCGTGGAGCTTGCGGCTT  1279
K1294   ........T.......................................................................  1279
K1290   ................................................................................  1279
K-112   ................................................................................  1279
K-115   ................................................................................  1279

Sal-1   AATTTGACTCAACACGGGAAAACTCACTAGTTTAAGACAAGAGTAGGATTGACAGATTAATAGCTCTTTCTTGATTTCTT  1359
K1294   ................................................................................  1359
K1290   ................................................................................  1359
K-112   ................................................................................  1359
K-115   ................................................................................  1359

Sal-1   GGATGGTGATGCATGGCCGTTTTTAGTTCGTGAATATGATTTGTCTGGTTAATTCCGATAACGAACGAGATCTTAACCTG  1439
K1294   ................................................................................  1439
K1290   ................................................................................  1439
K-112   ................................................................................  1439
K-115   ................................................................................  1439
                                                                                    ****

Sal-1   CTAATTAGCGGCAAATACGACATATTCTTACGTGGGACTGAATTCGGTTGATTTGCTTACTTTGAAGAAAATATTGGGAT  1519
K1294   ..................T.............................................................A  1519
K1290   ..................T.............................................................  1519
K-112   ..................T.............................................................A  1519
K-115   ..................T.........................................C...................  1519
        ********************************** V7 ******************************************
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Sal-1   ACGTAACAGTTTCCCTTTCCCTTTTCTACTTAGTTCGCTTTTCATACTGTTTCTTTTTCGCGTAAGAATGTATTTGCTTG  1599
K1294   ........................................................................................  1599
K1290   ........................................................................................  1599
K-112   ..........................................................T.............................  1599
K-115   ..........................................................C.............................  1599
        ****************************************************************************************

Sal-1   ATTGTAAAGCTTCTTAGAGGAACGATGTGTGTCTAACACAAGGAAGTTTAAGGCAACAACAGGTCTGTGATGTCCTTAGA  1679
K1294   ........................................................................................  1679
K1290   ........................................................................................  1679
K-112   ........................................................................................  1679
K-115   ........................................................................................  1679
        ************************

Sal-1   TGAACTAGGCTGCACGCGTGCTACACTGATATGTATAACGAGTTATTAAAATTACGATTCAGCTTGCTGTTTCGTATTTT  1759
K1294   ........................................................................................  1759
K1290   ..........................................................A.............................  1759
K-112   ........................................................................................  1759
K-115   ......G.................................................................................  1759
                            **************************** V8 ****************************

Sal-1   TCCTCCACTGAAAAGTGTAGGTAATCTTTATCAGTACATATCGTGATGGGGATAGATTGTTGCAATTATTAATCTTGAAC  1839
K1294   ..........................................................A.............................  1839
K1290   ..........................................................A.............................  1839
K-112   ..........................................................A.............................  1839
K-115   ..........................................................A.............................  1839
        ******

Sal-1   GAGGAATGCCTAGTAAGCATGATTCATCAGATTGTGCTGACTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTA  1919
K1294   ........................................................................................  1919
K1290   ........................................................................................  1919
K-112   ..............................................C.........................................  1919
K-115   ........................................................................................  1919
                                                                                      ******

Sal-1   CCGATTGAAAGATATGATGAATTGTTTGGACAAGAAGAAGGGGGATTATATCTCCTTTTTTCTGGAAAAACCGTAAATCC  1999
K1294   ..................................A.....................................................  1999
K1290   ........................................................................................  1999
K-112   ........................................................................................  1999
K-115   ........................................................................................  1999
        ******************** V9************************************************************

Sal-1   TATCTTTTAAAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAGTGAACCTGCGGAAGGATCATTA  2063
K1294   ......................==========================================  2063
K1290   ......................==========================================  2063
K-112   ......................==========================================  2063
K-115   ......................==========================================  2063
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(2) Type C genes:

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Sal-1   AACCTGGTTGATCTTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCAAGTGAAAGTACATGCATATTTTTAT  80
K1294   ================================================================================  80
K1290   ================================================================================  80
K-112   ================================================================================  80
K-115   ================================================================================  80

Sal-1   ATGCGGAAACTGCGAACGGCTCATTAAAACAGTTATAATCTACTTGACATTTTTTTTCTATAAGGATAACTACGGAAAAGC
K1294   ================================================================.....  160
K1290   ================================================================.....  160
K-112   ================================================================.....  160
K-115   ================================================================.....  160
                                                        ************ V2 ************
```

```
Sal-1   TGTAGCTAATACTTGCTTTAATGCTCTCGACGAATGTCTTGGGCATGTACTTGTTAAGCCTTTATAAGAAAAAAGTTAAT
K1294   ...........................................................T..............  240
K1290   ...........................................................................  240
K-112   ...........................................................................  240
K-115   ...........................................................................  240
        ***************************************************************************

Sal-1   AACTTAAGGAATGATAACAAAGAAGTGACACATAGAAGGACCTGCGTCCATTTATAGTGTGTATCAATCGAGTTTCTGAC  320
K1294   ...........................................................................  320
K1290   ...........................................................................  320
K-112   ...........................................................................  320
K-115   ...........................................................................  320
        ************************************************************

Sal-1   CTATCAGCTTTTGATGTTAGGGTATTGGCCTAACATTGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGAGAGG  400
K1294   ...........................................................................  400
K1290   ...........................................................................  400
K-112   ...........................................................................  400
K-115   ...........................................................................  400

Sal-1   GAGCCTGAGAAATAGCTACCACATCTAAGGAAGGCAGCAGGCGCGTAAATTACCCAATTCTAAAGAAGAGAGGTAGTGAC  480
K1294   ...........................................................................  480
K1290   ...........................................................................  480
K-112   ...........................................................................  480
K-115   ........................C..................................................  480

Sal-1   AAGAAATAACAATACAAGACCAAAACTGGTTTTGTAATTGGAATGATGGGAATTTAAATCCTTCCCATAATACAATTGGA  560
K1294   ..................G....T-....C.............................................  559
K1290   ...........................................................................  560
K-112   ...........................................................................  560
K-115   ...................................C.......................................  560
        ********************** V3 ********************************************

Sal-1   GGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAATTGTTGCAGTTAAAACGCTCGTA  640
K1294   ...........................................................................  639
K1290   ...........................................................................  640
K-112   ...........................................................................  640
K-115   ...........................................................................  640

Sal-1   GTTGAATTTCAAAGAACCAATATTTTAATAACGCCGTTAGCTAGATCCACAAGGGGTTGAGCCAATCACGGTTTCGGCTT  720
K1294   ...........................................................................  719
K1290   ...........................................................................  720
K-112   ...........................................................................  720
K-115   ...........................................................................  720
             *************************** V4 ***********************************

Sal-1   CTGTGCGCATCCTACCTATCAAGCGTTTTTTTAATTAAAGTGTTTCTTTTTAAAATCTTCTTTACCTTAACCATATGGAA  800
K1294   ........................T..................................................  799
K1290   .......T.....................................................G..........  800
K-112   ........................T..................................................  800
K-115   ...........................................................................  800
        ***************************************************************************

Sal-1   GATTTTGTTACTTTGAGTAAATTAAAGTGTTCATAGCAAACAGATACAGCATTGCGCGTTTTGAATACTACAGCATGGAA  880
K1294   ...........................................................................  879
K1290   ...........................................................................  880
K-112   ...........................................................................  880
K-115   ...........................................................................  880
        ***************************************************************************

Sal-1   TAACAAAATTGAACAAGTCAAAACTATGTTTCTTTTTTTTTATTTTTGGCTTAGTTACGATTAATAGGAGTAGTTTGGGG  960
K1294   ...........................................................................  959
K1290   ...........................................................................  960
K-112   ...........................................................................  960
K-115   ...........................................................................  960
        ************************************************************
```

```
Sal-1   ACATTTGTATTCAGATGTCAGAGGTGAAATTCTAAGATTTTCTGGAGACAAACAACTGCGAAGGCATTTGTCTAAAATAC  1040
K1294   ..............................C.....................................................  1039
K1290   ......................G.....................................................  1040
K-112   ..........................................................................  1040
K-115   .........C.....................................................................  1040

Sal-1   TTCCATTAATCAAGAACGAAAGTTAAGGGAGTGAAGACGATCAGATACCGTCGTAATCTTAACCATAAACTATACCGACT  1120
K1294   ..........................................................................  1119
K1290   ..........................................................................  1120
K-112   ..........................................................................  1120
K-115   ..........................................................................  1120

Sal-1   AGGTTTTGGATGAAAGTTAAACAAATAAGGATAGTCTCTTCGGGGATAGTCCTTAGATTTCTTCCTTCAGTACCCTATGA  1200
K1294   ..........................................................................  1199
K1290   ..........................................................................  1200
K-112   ..........................................................................  1200
K-115   ..........................................................................  1200
        ********************************** V5 ***********************************

Sal-1   GAAATCAAAGTCTTTGGGTTCTGGGGCGAGTATTCGCGCAAGCGAGAAAGTTAAAAGAATTGACGGAAGGGCACCACCAG  1280
K1294   ..........................................................................  1270
K1290   ..........................................................................  1280
K-112   ..........................................................................  1280
K-115   ..........................................................................  1280

Sal-1   GCGTGGAGCTTGCGGCTTAATTTGACTCAACACGGGAAAACTCACTAGTTTAAGACAAGAGTAGGATTGACAGATTGAGA  1360
K1294   ..........................................................................  1359
K1290   ..........................................................................  1360
K-112   ..........................................................................  1360
K-115   ..........................................................................  1360

Sal-1   GCTCTTTCTCGATTTCTTGGATGGTGAT-GCATGGCCGTTTTTAGTTCGTGAATATGATTTGTCTGGTTAATTCCGATAA  1439
K1294   .........T................................................................  1439
K1290   .........T................................................................  1440
K-112   .........T................................................................  1440
K-115   .........T................................................................  1440

Sal-1   CGAACGAGATCTTAACCTGCTAATTAGCGGTAAGTACGACATATTTTTATGTCGGATTGGATCTGGATGATTTGCTTATA  1519
K1294   ..........................................................................  1519
K1290   ..........................................................................  1520
K-112   ..........................................................................  1520
K-115   ..........................................................................  1520
                  ********************** V7 ************************************

Sal-1   TTGAGGTGCAATCTAAATAGGGGATTGCAATTATACTTCGTGTCGGTGTTTCTTAATCGAATAGCTGATGCGTTTGGTAT  1599
K1294   ..........................................................................  1599
K1290   ..........................................................................  1600
K-112   ..........................................................................  1600
K-115   ........................................C.................................  1600
        ******************************************************************************

Sal-1   ATTGCTTTCCTTTTTTTT-ATTTCTGCGCTTCTTTACTTGGCTTATCGTACCGTTTCCTTTTTGTGTAGAAATGTATTTG  1678
K1294   ..........................................................................  1679
K1290   ..........................................................................  1680
K-112   ................-..C.....................................................  1679
K-115   ..........................................................................  1680
        ******************************************************************************

Sal-1   CATTATATTAAAGCTTCTTAGAGGAACGATGTGTGTCTAACACAAGGAAGTTTAAGGCAACAACAGGTCTGTGATGTCCT  1758
K1294   ..........................................................................  1759
K1290   ..........................................................................  1760
K-112   ..........................................................................  1759
K-115   ..........................................................................  1760
        **************************
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Sal-1   TAGATGAACTAGGCTGCACGCGTGCTACACTGATATGTACAACGAGTTATTAAAATTACG------------------  1818
K1294   ..............................................C..G..A..T.CATTTCGCTATTTTGTGCTT  1839
K1290   ..............................................C..G..A..T.CATTTCGCTATTTTGAGCTT  1840
K-112   ..............................................C..G..A..T.CATTTCGCTATTTTGTGCTT  1839
K-115   ..............................................C..G..A..T.CATTTCGCTATTTTGTGCTT  1840
                                  ********************  V8  **************************

Sal-1   ATTCAGCTT-------GCTGTTACGTATTTTTCCTCCACTGAAAAGTGTAGGTAATCTTTATCAGTACATATCGTGATGG  1891
K1294   ..C....C.TTTTAGCAT.A.GG.A..C.....................................................  1919
K1290   ..C....C.TTTTAGCAT.A.GG.A..C.....................................................  1920
K-112   ..C....C.TTTTAGCAT.A.GG.A..C.....................................................  1919
K-115   ..C....C.TTTTAGCAT.A.GG.A..C.....................................................  1920
        ************************************

Sal-1   GGATAGATTATTGCAATTATTAATCTTGAACGAGGAATGCCTAGTAAGCACGATTCATTAGATTGTGCTGACTACGTCCC  1971
K1294   ..........................................................C.......................  1999
K1290   ..........................................................C.......................  2000
K-112   ..........................................................C.......................  1999
K-115   ..........................................................C.......................  2000

Sal-1   TGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATCGAAAGATATGATGAATTGTTTGGACAAGAAGAAAGGGGATTAT  2051
K1294   ..........................................................C.......................  2079
K1290   ..........................................................C.......................  2080
K-112   ..........................................................C.......................  2079
K-115   ..........................................................A.......................  2080
                             ************************** V9 **************************

Sal-1   ATCTTCTTTTTTCTGGAAAAACCGTAAATCCTGTCTTTTAAAGGAAGGAGAAGTCGTAACAAGGTTTCCGTAGGTGAACC  2131
K1294   ................................A.....................=========================  2159
K1290   ................................A.....................=========================  2160
K-112   .....T.........................A.....................=========================  2159
K-115   ................................A.....................=========================  2160
        ************************************************

Sal-1   TGCGGAAGGATCATTA  2147
K1294   ================  2175
K1290   ================  2176
K-112   ================  2175
K-115   ================  2176
```

Note: The alignment of the coding sequence for *P. vivax* SSUrRNA was produced by the Clustal method. The first line represents the sequence of the Salvador strain; the lower lines represent the genes of four Thailand isolates and are shown only where the sequences differ from the Salvador strain. Dashes represent gaps where the sequences can not be directly aligned and "=" represent the 5' and 3'end of the gene that has not been cloned and sequenced. The sequence alignment underlined with stars represent regions that diverged rapidly in eukaryotic SSUrRNA genes.

Appendix III.

Sequence Alignment of SSUrRNA genes from *P. vivax, P. simium* and *P. cynomolgi*

```
Pv-A    AACCTGGTTGATCTTGCCAGTAGTCATATGCTTGTCTCAAAGATTAAGCCATGCAAGTGAAAGTATATGCATAT-TTTAT  79
Ps-A    ..........................................................................-.....  79
Pc-A    ..........................................................................-.....  79
Pv-C    ..........................................................C.........T.....  80
Ps-C    ..........................................................C.........T.....  80
Pc-C    ..........................................................C.........A..C..  80
                                                                  ******* V1 *******

Pv-A    ATGTAGAAACTGCGAACGGCTCATTAAAACAGTTATAATCTACTTGACATTTTTTCT-----ATAAGGATAACTACGGAA  154
Ps-A    ..................................................------.................T.  154
Pc-A    ..................................................T----.....................  155
Pv-C    ...CG..............................................T----CT...................  156
Ps-C    ...CG..............................................T----CT...................  156
Pc-C    ...C...G...........................................TTTTTCT...................  160
        *****                                          ****************** V2 ******

Pv-A    AAGCTGTAGCTAATACTTGCCTTAGCACTCTTGATTCATTTCTTGAGTGTGTACTTGTTAAGCCTTT-TAAGAAAAAAGT  233
Ps-A    ..................T.....................................................-.....  233
Pc-A    ..................T.............A.G.....................................-.....  234
Pv-C    ..................T...ATG....C..CGA..G.....G.CA.....................A.....  236
Ps-C    ..................T...ATG....C..CGA..G.....G.CA.....................A.....  236
Pc-C    ..................TT...TT.....C..CGA..G.........AG..........-......A.....  239
        ********************************************************************************

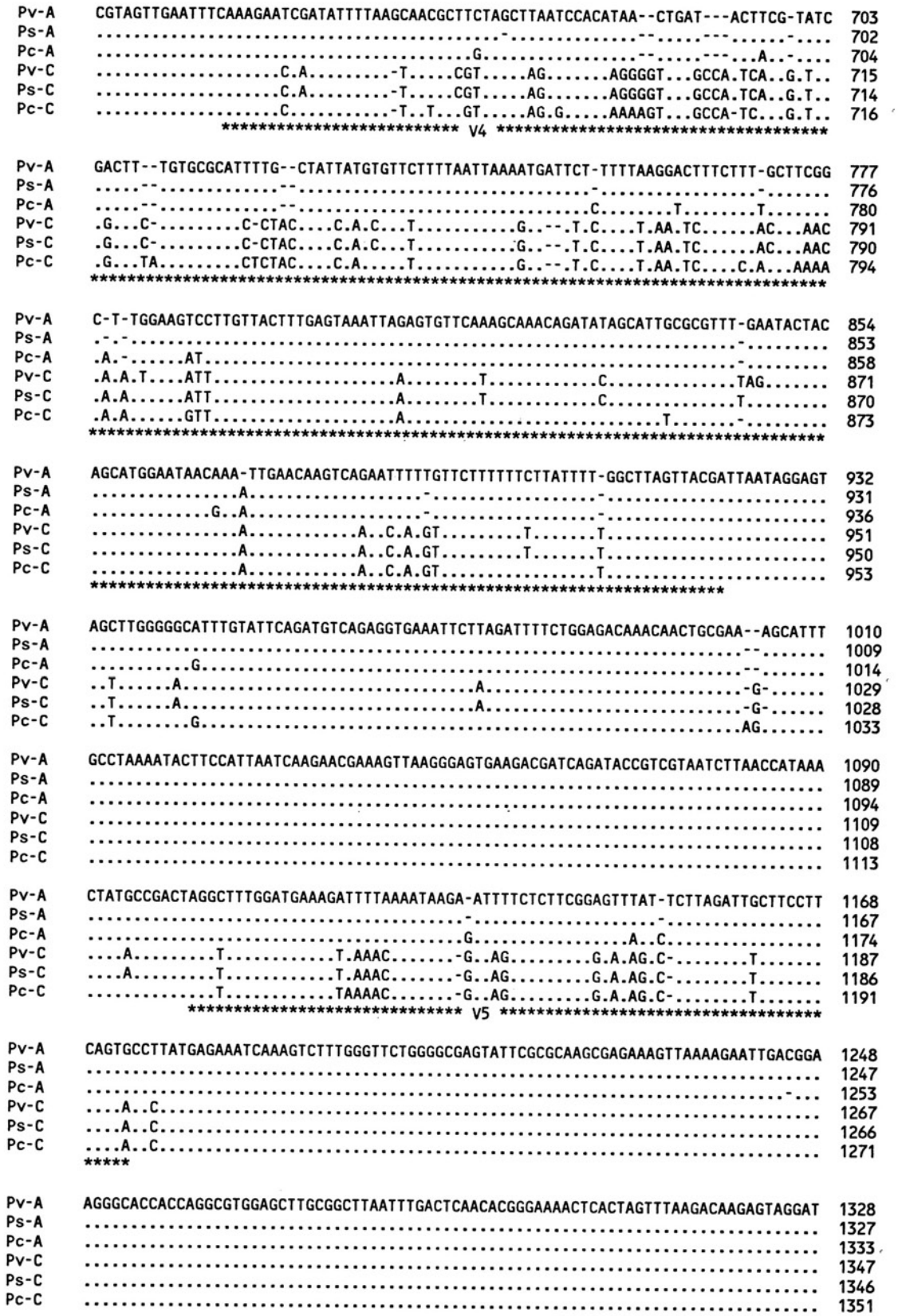
Pv-A    TATTAACTTAAGGAATTATAACAAAGAAGCGACACGTAATGGA---TCCGTCCATTTTTAGTGTGTATCAATCGAGTTTC  310
Ps-A    ..........................................---.....................................  310
Pc-A    ..........................TA............---.....................................  311
Pv-C    ..A.............G.............T.....A..GAA.GACC.G.........A.....................  316
Ps-C    ..A............AGT...........T.....A..GAA.-ACC.G....AC...A.....................  315
Pc-C    ..A.........................A.....AC..CAAAGAT.-.A...CAAAGC.....................  318
        ************************************************************

Pv-A    TGACCTATCAGCTTTTGATGTTAGGGTATTGGCCTAACATGGCTATGACGGGTAACGGGGAATTAGAGTTCGATTCCGGA  390
Ps-A    ..........................................................................  390
Pc-A    ..........................................................................  391
Pv-C    ..........................................T...............................  396
Ps-C    ..........................................T...............................  395
Pc-C    ..................................................................T.....  398

Pv-A    GAGGGAGCCTGAGAAATAGCTACCACATCTAAGGAAGGCAGCAGGCGCGTAAATTACCCAATTCTAAAGAAGAGAGGTAG  470
Ps-A    ..........................................................................  470
Pc-A    ..........................................................................  471
Pv-C    ..........................................................................  476
Ps-C    ..........................................................................  475
Pc-C    ..........................................................................  478

Pv-A    TGACAAGAAATAACAATACAAGGCCAAT-CTGGCTTTGTAATTGGAATGATGGGAATTTAAAACCTTCCCAAAACTCAAT  549
Ps-A    ..........................................................................  549
Pc-A    ..........................................................................  550
Pv-C    ......................A....AA....T.........................T........T..TA....  556
Ps-C    ......................A....AA....T.........................TA.......T..TA....  555
Pc-C    ......................A....AA....T.........................TT.......T..TA....  558
            ************************************ V3 ******************************

Pv-A    TGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAATTGTTGCAGTTAAAACGCT  629
Ps-A    ..........................................................................  629
Pc-A    ..........................................................................  630
Pv-C    ..........................................................................  636
Ps-C    ..........................................................................  635
Pc-C    ..........................................................................  638
```


Pv-A CGTAGTTGAATTTCAAAGAATCGATATTTTAAGCAACGCTTCTAGCTTAATCCACATAA--CTGAT---ACTTCG-TATC 703
Ps-A 702
Pc-A 704
Pv-C .....C.A.....-T.....CGT.....AG.....AGGGGT...GCCA.TCA..G.T.. 715
Ps-C .....C.A.....-T.....CGT.....AG.....AGGGGT...GCCA.TCA..G.T.. 714
Pc-C .....C.....-T..T...GT.....AG.G.....AAAAGT...GCCA-TC...G.T.. 716
V4
Pv-A GACTT--TGTGCGCATTTTG--CTATTATGTGTTCTTTTAATTAAAATGATTCT-TTTTAAGGACTTTCTTT-GCTTCGG 777
Ps-A 776
Pc-A 780
Pv-C .G...C-.....C-CTAC....C.A.C...T.....G..--.T.C....T.AA.TC.....AC...AAC 791
Ps-C .G...C-.....C-CTAC....C.A.C...T.....G..--.T.C....T.AA.TC.....AC...AAC 790
Pc-C .G...TA.....CTCTAC....C.A.....T.....G..--.T.C....T.AA.TC....C.A...AAAA 794
Pv-A C-T-TGGAAGTCCTTGTTACTTTGAGTAAATTAGAGTGTTCAAAGCAAACAGATATAGCATTGCGCGTTT-GAATACTAC 854
Ps-A 853
Pc-A 858
Pv-C .A.A.T....ATT.....A.....T.....C.....TAG..... 871
Ps-C .A.A.....ATT.....A.....T.....C.....T..... 870
Pc-C .A.A.....GTT.....A.....T.....-..... 873
Pv-A AGCATGGAATAACAAA-TTGAACAAGTCAGAATTTTTGTTCTTTTTTCTTATTTT-GGCTTAGTTACGATTAATAGGAGT 932
Ps-A .....A.....-.....-..... 931
Pc-A .....G..A.....-.....-..... 936
Pv-C .....A.....A..C.A.GT.....T.....T..... 951
Ps-C .....A.....A..C.A.GT.....T.....T..... 950
Pc-C .....A.....A..C.A.GT.....T..... 953
Pv-A AGCTTGGGGGCATTTGTATTCAGATGTCAGAGGTGAAATTCTTAGATTTTCTGGAGACAAACAACTGCGAA--AGCATTT 1010
Ps-A .....-- 1009
Pc-A .....G.....-- 1014
Pv-C ..T.....A.....A.....-G-..... 1029
Ps-C ..T.....A.....A.....-G-..... 1028
Pc-C ..T.....G.....AG..... 1033
Pv-A GCCTAAAATACTTCCATTAATCAAGAACGAAAGTTAAGGGAGTGAAGACGATCAGATACCGTCGTAATCTTAACCATAAA 1090
Ps-A 1089
Pc-A 1094
Pv-C 1109
Ps-C 1108
Pc-C 1113
Pv-A CTATGCCGACTAGGCTTTGGATGAAAGATTTTAAAATAAGA-ATTTTCTCTTCGGAGTTTAT-TCTTAGATTGCTTCCTT 1168
Ps-A .....-..... 1167
Pc-A .....G.....A..C..... 1174
Pv-C ....A.....T.....T.AAAC.....-G..AG.....G.A.AG.C-.....T..... 1187
Ps-C ....A.....T.....T.AAAC.....-G..AG.....G.A.AG.C-.....T..... 1186
Pc-C .....T.....TAAAAC.....-G..AG.....G.A.AG.C-.....T..... 1191
V5
Pv-A CAGTGCCTTATGAGAAATCAAAGTCTTTGGGTTCTGGGGCGAGTATTCGCGCAAGCGAGAAAGTTAAAAGAATTGACGGA 1248
Ps-A 1247
Pc-A .....-... 1253
Pv-C ....A..C..... 1267
Ps-C ....A..C..... 1266
Pc-C ....A..C..... 1271
Pv-A AGGGCACCACCAGGCGTGGAGCTTGCGGCTTAATTTGACTCAACACGGGAAAACTCACTAGTTTAAGACAAGAGTAGGAT 1328
Ps-A 1327
Pc-A 1333
Pv-C 1347
Ps-C 1346
Pc-C 1351

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Pv-A    TGACAGATTAATAGCTCTTTCTTGATTTCTTGGATGGTGATGCATGGCCGTTTTTAGTTCGTGAATATGATTTGTCTGGT  1408
Ps-A    ........................................................................................  1407
Pc-A    ........................................................................................  1413
Pv-C    .........G.G..........C.................................................................  1427
Ps-C    .........G.G.....................................................................  1426
Pc-C    ........................................................................................  1431

Pv-A    TAATTCCGATAACGAACGAGATCTTAACCTGCTAATTAGCGGCAAATACGACATATTCTTACGTGGGACTGAATTCGGTT  1488
Ps-A    ..................................................T.....................................  1487
Pc-A    ..................................................T.........T......T.....A.....  1493
Pv-C    ..........................................T..G...........T...T..C...T..G..CT..A.  1507
Ps-C    ..........................................T..G...........T...T..C...T..G..CT..A.  1506
Pc-C    ..........................................T..G...........T...TA.C.A.T..G..AT.TA.  1511
                                  ********************* V7 ****************************

Pv-A    GATTTGCTTACTTTGAAG---------AAA----------------------ATATTGG-------------------G  1517
Ps-A    ..................---------...----------------------.......-------------------.  1516
Pc-A    ..........T.......---------...----------------------.......-------------------.  1522
Pv-C    ..........TA....G.T-GCAATCT...TAGGGGATTGCAATTATACTTCG.G.C..TGTTTCTTAATCGAATAGCT.  1586
Ps-C    ..........TA....G.T-GCAATCT...TAGGGGATTGCAATTATACTTCG.G.C..TGTTTCCTAATCGAATAGCT.  1585
Pc-C    ........A.-A...CG.TCGCAAAT--..T-GAAGATCTTGATTGCTTTTC.CG.CA.TGTTTTCC.---GAATCGTT.  1584
        ********************************************************************************

Pv-A    ATACG--TAACA----GTTTCCCTTTCCCTTT-----------TCTACTTAGTTCGCTTTTCATACTGTTTCTTTTTCGC  1580
Ps-A    .A...--.....----.............-----------.....................................  1579
Pc-A    ..G..--...-.----..G..........-----------........A..T....A.....................  1584
Pv-C    ..G..TT.GGT.TATT.C..T.....TTT..--ATTTCTGCGC.TC-T...C..G....A..G...C.....C....T.T  1663
Ps-C    ..G..TT.GGT.TATT.C..TTT...TTT...TATTTCTGCGC.TC-T...C..G....A..G...C.....C....T.T  1664
Pc-C    .CGT.AC..GT.TAT-AC.CG.TCC.T.TG..C---CCTGGAC.TC....CCC.G....A......C.....C....T.T  1660
        ********************************************************************************

Pv-A    GTAAGAATGTATTTGCTTGATTGTAAAGCTTCTTAGAGGAACGATGTGTGTCTAACACAAGGAAGTTTAAGGCAACAACA  1660
Ps-A    ........................................................................................  1659
Pc-A    ........................................................................................  1664
Pv-C    ...GA...........A.T..AT.................................................................  1743
Ps-C    ...GA...........A.T..AT.................................................................  1744
Pc-C    ...GA.........AT..T..GT-................................................................  1739
        ******************************************

Pv-A    GGTCTGTGATGTCCTTAGATGAACTAGGCTGCACGCGTGCTACACTGATATGTATAACGAGTTATTAAAATTACG-ATTC  1739
Ps-A    ..............................................G.............................-....  1738
Pc-A    ..................................................................C..........-T...  1743
Pv-C    ..............................................................C.....................-....  1822
Ps-C    ..............................................................C.........C..G..A..T.C...T  1824
Pc-C    ..............................................................C.........C..G..A..T.C..AT  1819
                                                  *************** V8 ****************

Pv-A    AGCT------TGCT------GTTT--------------CGTATTTTTCCTCCACTGAAAAGTGTAGGTAATCTTTATCAG  1793
Ps-A    ....------....------....--------------..C.....................................  1792
Pc-A    T...------....TG--CA....--------------....C.....................................A  1801
Pv-C    ....------....----------------GTTACG---.....................................  1876
Ps-C    C...ATTTTG....TATCCGGCCTTTTTAGCATTATGGCA..C.....................................  1904
Pc-C    -.T.ATTT-G....TATTCAGCCTTTT-ATCATTATGGCA..C.....................................C.T..  1896
        ************************************************

Pv-A    TACATATCGTGATGGGGATAGATTGTTGCAATTATTAATCTTGAACGAGGAATGCCTAGTAAGCATGATTCATCAGATTG  1873
Ps-A    ........................A.....................................................  1872
Pc-A    ........................A.....................................................  1881
Pv-C    ........................A.....................................C.....T.....  1956
Ps-C    ........................A.....................................C.....T.....  1984
Pc-C    ........................A.....................................C.....T.....  1976

Pv-A    TGCTGACTACGTCCCTGCCCTTTGTACACACCGCCCGTCGCTCCTACCGATTGAAAGATATGATGAATTGTTTGGACAAG  1953
Ps-A    ........................................................................................  1952
Pc-A    ........................................................................................  1961
Pv-C    ..................................................C.....................................  2036
Ps-C    ..................................................C.....................................  2064
Pc-C    ..................................................C..G...C.....................  2056
                                              ***************** V9 *********************
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```
Pv-A    AAGAAGGGGGATTATATCTCCTTTTTTCTGGAAAAACCGTAAATCCTATCTTTTAAAGGAAGGAGAAGTCGTAACAAGGT  2033
Ps-A    ............................................................................  2032
Pc-A    .....A.............T.....................................................  2041
Pv-C    .....A.............T.....................G.....................................  2116
Ps-C    .....A.............T.....................G.....................................  2144
Pc-C    ..A..A.AA..........A.A.............T.....G.....................................  2136
        ************************************************************

Pv-A    TTCCGTAG-TGAACCTGCGGAAGGATCATTA  2063
Ps-A    ........G.....................   2063
Pc-A    ........G.....................   2072
Pv-C    ........G.....................   2147
Ps-C    ........-.....................   2174
Pc-C    ........G.....................   2167
```

Note: The alignment of the coding sequence for SSUrRNA was produced by the Clustal method with weight table. The upper line represents the sequence of *P. vivax* A gene; the following lines represent, from top to bottom, the A gene of *P. simium*, the A gene of *P. cynomolgi*, the C gene of *P. vivax*, the C gene of *P. simium* and the C gene of *P. cynomolgi* show only where the sequences differ from the A gene of *P. vivax*. Dashes represent gaps where the sequences can not be directly aligned. The regions that diverged rapidly in eukaryotic SSUrRNA genes are marked by stars below the sequence alignment.

Appendix. IV. Publications:

Li, J., Wirtz, R.A., McConkey, G.A., Sattabongkot, J., McCutchan, T.F. (1994). Transition of *Plasmodium vivax* ribosomal types corresponds to sporozoite differentiation in the mosquito. *Molecular and Biochemical Parasitology* 65: 283-289.

Li, J., McConkey, G.A., Rogers, M.J., McCutchan, T.F. (1994). *Plasmodium*: The developmentally regulated ribosome. *Experimental Parasitology* 78: 437-441.

Li, J., Wirtz, R.A., Schneider, I., Muratova, O,V., McCutchan, T.F., Appian, A., Hollingdale, M.R. (1993). *Plasmodium falciparum*: Stage-specific ribosomal RNA as a potential target for monitoring parasite development in *Anopheles stephensi*. *Experimental Parasitology* 76: 32-38.

Li, J., Wirtz, R.A., Zhu, J., Appian, A., McCutchan, T.F., Long, G. W., Milbous, W.K., Hollingdale, M.R. (1991). *Plasmodium berghei*: Quantitation of *in vitro* effects of antimalarial drugs on exoerythrocytic development by a ribosomal RNA probe. *Experimental Parasitology* 72: 450-458.

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Molecular and Biochemical Parasitology 65 (1994) 283–289

MOLECULAR AND BIOCHEMICAL PARASITOLOGY

Transition of *Plasmodium vivax* ribosome types corresponds to sporozoite differentiation in the mosquito

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Abstract

Two distinct small subunit ribosomal RNA (SSUrRNA) genes were amplified from the genomic DNA of *Plasmodium vivax*. Comparison of the two coding sequences reveals an overall divergence of 14.5% and most differences are clustered into the regions known to diverge rapidly in all eukaryotic SSUrRNAs. Oligonucleotides complementary to unique sequences of each gene have been used to distinguish the transcripts expressed either at schizogony in human blood (A gene) or at sporogony in the mosquito (C gene). These oligonucleotides were also used to monitor turnover of ribosomes during parasite development in mosquitoes. Transcripts of the A gene were predominant in the infected human blood and engorged mosquitoes but disappeared within 24 h after feeding. Expression of the C gene in mosquitoes was not detected until day 6 after the blood meal. A period of rapid accumulation of the C type rRNA from day 6 to day 8 corresponds to differentiation of individual sporozoites within the oocyst. Possible functional implications relating to the timing of this transition are discussed.

Key words: Plasmodium vivax; SSUrRNA; 18S rRNA; Developmental diagnostics

1. Introduction

The small subunit ribosomal RNA (SSUrRNA) gene sequence is a mosaic of conserved and variable regions [1,2]. The conserved sequences are localized to predictable regions of the gene and contribute to form the highly conserved secondary structure. These core sequences appear to be associated with the universal function of the ribosome in protein synthesis. The variable sequences are interspersed among the conserved regions and contribute the major differences in gene size and composition. The functions of these regions are less well understood. They are, however, of con-

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Note: Nucleotide sequence data reported in this paper have been submitted to the GenBank™ and EMBL data bases with the accession numbers U07367 and U07368.

SSDI 0166-6851(94)00059-V

siderable interest because they retain the unique signature of each species and provide valuable information for taxonomy and diagnosis [1–3]. Interest in variable regions of the gene in *Plasmodium* species potentially extends to understanding the biological role of the heterologous ribosome in parasite development.

In contrast to most eukaryotic organisms, *Plasmodium* rRNA genes are unique with regard to copy number, genomic arrangement and developmental regulation of transcription [4–6]. The copy number of the gene is very limited, about 4–8 per haploid genome, and the sequences of different gene units are not identical. These genes are not arranged in a tandem array as is common in other eukaryotes, but dispersed among different chromosomes. The most unique feature of *Plasmodium* rRNA genes is the differential expression of their alternative forms at discrete stages of the life cycle. In previous studies, at least two types of SSUrRNA genes (types A and C) have been identified from *Plasmodium berghei* and *P. falciparum* [7–10]. The A gene is predominantly transcribed during the parasite blood and tissue schizogony in the vertebrate host while the C gene is expressed during sporogony in the invertebrate vector [5,6,11,12]. For the human malaria parasites, the partial and complete sequences of type A SSUrRNA genes have also been identified from *P. vivax* [13] and *Plasmodium malariae* [14], respectively. Elucidation of the types of *P. vivax* SSUrRNA genes and their transcription pattern will advance the studies mentioned above and provide new insight into the molecular mechanisms involved in parasite development.

2. Materials and methods

PCR amplification of SSUrRNA genes. The genomic DNA of *P. vivax* SAL I strain was kindly provided by W. Collins (Centers for Diseases Control, Atlanta). The complete SSUrRNA gene was amplified from the parasite DNA with oligonucleotides 705 (5′-AACCTGGTTGATCTTGCCAG-3′) and 573 (5′-GACATCCATCGTTAAGACTGTGC-3′) as 5′ and 3′ end primer, respectively. Both of the primers are known to be conserved for the genus *Plasmodium*. The polymerase chain reaction (PCR) was set up in a 100-μl reaction containing 20–50 ng parasite DNA/ 100 pmol of each primer/ 200 μM of each dNTP/ 50 mM KCl/ 10 mM Tris-HCl, pH 8.3/ 2 mM $MgCl_2$/ 2.5 U *Taq* DNA polymerase. All reagents except target DNA and primers were obtained from Perkin Elmer Cetus (Norwalk, CT). The reaction was processed in a Perkin Elmer DNA Thermal Cycler with the following parameters: 94°C/1 min for denaturation, 55°C/1 min for annealing, 72°C/3 min for extension and a total of 30 cycles.

Cloning and sequencing of the SSUrRNA gene. The PCR products were purified with the Magic PCR Preps DNA Purification System (Promega Corp., Madison, WI). The recovered DNA was treated with Klenow DNA polymerase (BRL, Gaithersberg, MD) and inserted into the *Sma*I site of the vector, pBluescript SK (Stratagene, La Jolla, CA) in presence of T4 DNA ligase and *Sma*I enzyme (BRL). The resulting plasmids were transformed into *E. coli* DH5 α strain (BRL) and the desired clones were selected by hybridization with rRNA oligo-probe 569 (5′-GTTCAAGATTAATAATTGCAA-3′), which is conserved for the *Plasmodium* genus. The inserts were sequenced by the dideoxynucleotide chain termination method [15] with a Sequenase Kit (USB, Cleveland, OH). The sequence alignment and other analysis was carried out with the Lasergene software supplied by DNASTAR Incorporation (Madison, WI).

Isolation of total RNA from P. vivax. Infected mosquitoes were produced by feeding laboratory-reared *Anopheles dirus* on *P. vivax*-infected human patients in Thailand. Mosquitoes were held at 26°C and 10 insects were removed 2 h after the blood meal and thereafter every 2 days. Parasite development in the mosquito was monitored by microscopy of midgut oocysts and salivary gland sporozoite. Mosquitoes and patient blood samples were frozen and shipped on dry ice to our laboratory. The isolation of total RNA was as previously described [12].

Oligonucleotide and hybridization. Oligonucleotides used for the hybridization assay were synthesized with the Applied Biosystems 381 DNA synthesizer (Foster City, CA). The sequences of the genus and species stage-specific oligomers and their relative positions in SSUrRNA gene are listed in Table 1. The radioactive labelling of oligonucleotides and the dot blot hybridization were previously described [11,12].

3. Results

Two structurally distinct rRNA gene units. PCR amplification of DNA from *P. vivax*, SAL I strain, with a genus conserved primer pair (nos. 705 and 573) yielded fragments approximately 2.5 kb in length, which contain the entire SSUrRNA gene and the internal transcribed spacer (ITS1) between that gene and the 5.8S rRNA coding region. All fragments were cloned into the *Sma*I site of the pBluescript SK vector. The physical map generated by restriction of purified plasmids with *Eco*RI and *Bam*HI indicated at least two major types of rRNA gene units; one type was not cleaved while the other type was cleaved once by either enzyme (see Fig. 1 for restriction sites).

Characterization of SSUrRNA gene sequences. The clones pVSA53 and pVSA46 are representative of 2.5 kb fragments containing type A and C SSUrDNA genes as distinguished by hybridization (see below). The inserts were sequenced on both stands. Fig. 1 shows a Martinez/Needleman-Wunsch DNA alignment of the two sequences. The mature SSUrRNA coding regions were 2063 and 2147 bp in length for type A and C genes, respectively. The overall similarity between the two SSUrRNA genes is 85.5%. The G/C content for both genes is 38%. The differences are mainly clustered in three regions (positions 170–300, 650–940, 1460–1690) known to diverge rapidly in all eukaryotic SSUrRNAs [1,16]. These regions correspond to the predicted variable region V2, V4 and V7 from a general rRNA secondary structural model [1]. The difference in size maps to these variable regions, particularly in the V7 region where the C gene contains 64 additional bases. No sequence similarity was found between the internal transcribed spacers of the two distinct rRNA gene units (data not shown).

Selection of rRNA gene specific oligo-probes. Oligonucleotides complementary to the unique sequences of each SSUrRNA gene (Table 1) were evaluated for specificity and sensitivity by dot blot hybridization. The SSUrRNA A gene specific probes hybridized exclusively to RNA from *P. vivax* infected human blood and the plasmid clone pVSA53 (Fig. 2). The C gene specific oligomers hybridized solely to RNA from the sporozoite infected mosquitoes and the plasmid clone pVSA46. The *Plasmodium* genus specific rRNA probe (TM10) hybridized with both plasmid clones and all the infected specimens, including *P. falciparum* SSUrRNA. Among the evaluated oligomers, 741 and 743 were the best probes in terms of efficiency of hybridization with the A and C gene

Table 1
P. vivax SSUrRNA gene specific oligonucleotide probes

Name[a]	Code	Sequence (5′ → 3′)	bp	Position[b]	T_m (°C)[c]
PvA	744	GTACACACTCAAGAAATGAATC	22	207 → 186	60
PvA	741	AACCGAATTCAGTCCCACGT	20	1488 → 1469	60
PvC	684	CATGCCCAAGACATTCGTCG	20	207 → 188	62
PvC	745	CGAAACCGTGATTGGCTCAA	20	716 → 697	60
PvC	743	ATCCAGATCCAATCCGACATA	21	1507 → 1487	60
PvC	703	GCCAAGTAAAGAAGCGCAG	19	1640 → 1622	58

[a]PvA and PvC: specific to *P. vivax* type A and C SSUrRNA genes.
[b]Position of the antisense oligonucleotides from original sequences (See Fig. 1).
[c]Melting temperature calculated as $T_m = 4(C+G) + 2(A+T)$.

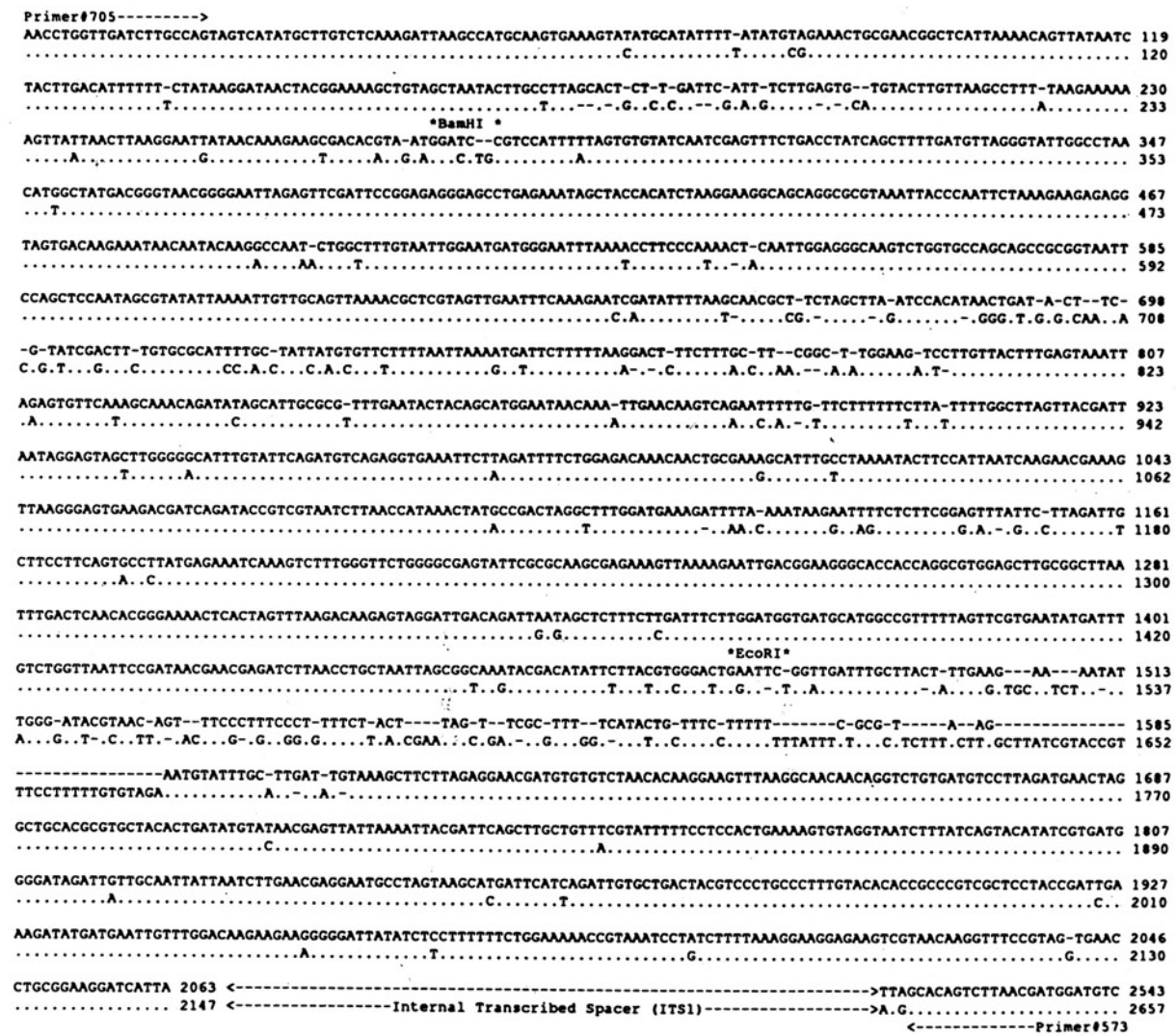

Fig. 1. The sequence comparison of two rRNA gene units amplified from the genomic DNA of *Plasmodium vivax*. The complete sequence of the mature SSUrRNAs starts from the 5'end, and is followed by the 3'end internal transcribed spacer (broken line with arrow) as well as the 5'end of 5.8S rRNA genes. The upper line shows the A gene sequence which is transcribed in the erythrocytic stage. The lower line is the C gene which is expressed in the sporozoite stage and shows only where the sequence differs from the A gene. Dashes represent gaps where the two sequences cannot be directly aligned. The position of restriction sites and the amplification primers are marked in the figure. The GenBank™ accession number for *P. vivax* SSUrRNA gene sequences are: type A, U07367; type C, U07368.

transcripts, respectively. Sensitivity for detecting *P. vivax* infected mosquitoes was determined by hybridizing probe 743 to RNA isolated from a single uninfected mosquito mixed with a known number of salivary gland sporozoite (Fig. 3). The lower limit of sensitivity was fewer than 400 sporozoite. This is well below the average number of sporozoite per mature *P. vivax* oocyst, estimated to be between 2000 and 6000 [17]. This presumably assures detection of the C gene transcripts even if only a single oocyst completes development in the mosquito. The correlation between

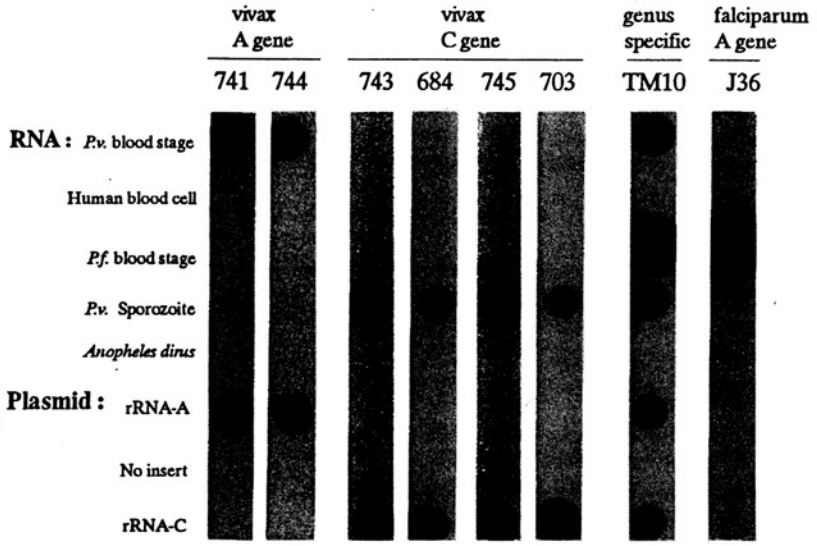

Fig. 2. Dot blot hybridization of *Plasmodium* SSUrRNA specific oligonucleotides to the RNA isolated from infected and uninfected human blood cells and mosquitoes. Plasmid DNAs containing the A gene, the C gene and no insert were used as hybridization controls. The membranes were then hybridized with ^{32}P end-labelled oligonucleotides at 55°C overnight and the autoradiography was processed at −20°C for 3 h [11,12].

the strength of the hybridization signal and the number of the parasites confirmed the sporozoite origin of the C gene transcripts (Fig. 3).

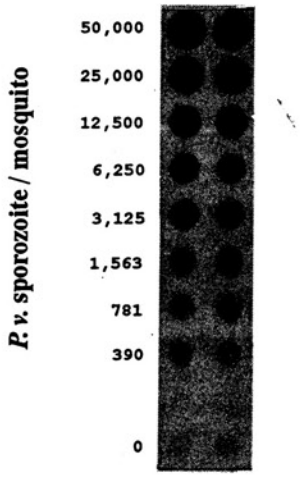

Fig. 3. The sensitivity of sporozoite detection: the total RNA prepared from a mixture of a known number of *P. vivax* sporozoite and one mosquito (*Anopheles dirus*) was probed with oligomer 743, which is complementary to the C type rRNA of *P. vivax*.

Developmental switch of rRNA forms. Differential expression of the structurally distinct rRNAs in *P. vivax* was followed by probing total RNA isolated from mosquitoes at different intervals after the infectious blood meal. One tenth of the sample isolated from 10 pooled mosquitoes was used so that hybridization results shown in Fig. 4 represent the average signal from one mosquito. The A gene transcripts were predominant in infected blood and engorged mosquitoes, but disap-

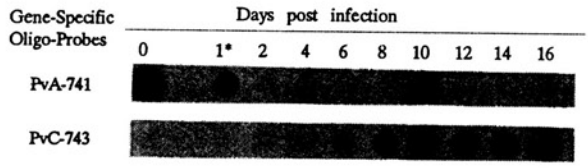

Fig. 4. The accumulation of C gene transcript during parasite development: RNAs isolated from *P. vivax* infected mosquitoes at different time intervals after the infectious blood meal were probed with oligomers 741 (upper stripe) and 743 (lower stripe), which are specific for *P. vivax* SSUrRNA A gene and C gene, respectively. Each dot represents the average signal from one tenth of a 10-mosquito pool collected at each time point. *The sample from day 1 was collected 2 h after the mosquito fed on the infected patient.

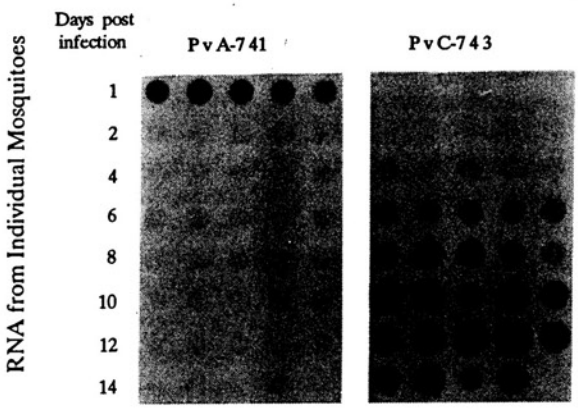

Fig. 5. Individual variability of type C transcripts during development. Total RNA prepared from individual mosquitoes was isolated at different intervals after the blood meal and was probed with oligomers 741 (left panel) and 743 (right panel), which are specific for SSUrRNA A gene and C gene, respectively.

peared within 24 h after the blood meal. The positive day 1 signal, two h after the feeding, presumably represented undigested blood stage parasites. The C gene transcripts were not detected in the infected mosquito until day 6. After day 6, the signal sharply increased and then levels off at about day 10–12. This rapid accumulation of C gene transcripts occurs during late sporogony when the oocyst has grown almost to mature size. This procedure was repeated using individual *P. vivax* infected mosquitoes (Fig. 5). Some variation in signal strength among the samples collected at any one time point exists and probably relates to variability in number of oocysts that develop on the mosquito gut. However, as an average, the results were equivalent with those shown in Fig. 4.

4. Discussion

It has been suggested that the two ribosome system evolved in response to its special environments in a two host system [7]. The change in ribosomal types is shown here to correlate better with a discrete stage of sporozoite development than with one host or the other. It will be difficult to determine why the two ribosome system arose until we understand the functional significance of the variation between the different genes. It may be that the different ribosomes have functional differences pertaining to protein synthesis, as translational control can be associated with variability in ribosome structure. For example, even though *Plasmodium* species are unique in making distinct types of rRNA, they are not unique in maintaining heterologous populations of ribosomes. Ribosome heterogeneity has recently been discovered in diverse organisms ranging from protozoa to humans. In these systems ribosome modulation is associated with changes in the quality or quantity of certain ribosomal proteins or the 5S rRNA [18]. Alteration of ribosomal protein results in modification of ribosomal form and function. Different approaches to ribosome heterogeneity may serve similar functions in the modulation of translation and, hence, the heterogeneity seen in *Plasmodium* species may be reflective of a broader, biological phenomenon. Alternatively, we have suggested that the presence of different ribosomes may relate to mechanisms of transcriptional control which open different areas of the chromosome to transcription at different times during development [7]. This would be consistent with the interesting suggestion put forth by Enea and colleagues [19,20] that, with regard to translational machinery, there may be no functional difference between the two ribosome types.

Two structurally distinct ribosomal RNA genes were isolated from the genomic DNA of *P. vivax* and their transcription pattern studied during parasite development in the mosquito. Two interesting features of the gene expression warrant comment. One is the lack of detectable transcripts in infected mosquitoes between days 2 and 6, and the other is the rapid accumulation of the C gene transcripts between days 6 and 10 (Figs. 4, 5).

Analysis of RNA from *P. falciparum* zygotes and ookinetes developing in culture clearly shows small amounts of a C type transcript [6]. The apparent gap in the detection of transcription immediately following that period of parasite development is difficult to interpret. This gap may simply relate to our inability to detect transcripts from so few organisms. The lack of detectable transcripts

from immature oocysts is somewhat surprising, however, due to the sensitivity of the assay. The notably low concentration of either A or C transcripts during early oogenesis may be an indicator of a period involved in the transition between ribosomal types.

The period of rapid accumulation of sporozoite-specific rRNA begins at a time in parasite development when the size of the oocyst is already close to its maximum and nuclear division has been completed [21]. Physical separation of individual sporozoites during this period has been shown by observation of the cytological process of sporozoite production [17], indicating that up-regulation of transcription of the C gene is, at least temporally, correlated to generation of individual sporozoites within the oocyst. Hence, the transition between ribosomal types in *Plasmodium vivax* appears to reflect its state of differentiation rather than the host in which the parasite resides.

Acknowledgements

Procedures for drawing blood and feeding mosquitoes on patients were approved by the human use committees of the Ministry of Public Health, Thailand, and US Army Medical Research and Development Command. The work was supported by a World Health Organization Grant, No. 890093.

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EXPERIMENTAL PARASITOLOGY 78, 437–441 (1994)

MINIREVIEW

Plasmodium: The Developmentally Regulated Ribosome

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LI, J., MCCONKEY, G. A., ROGERS, M.J., WATERS, A. P., AND MCCUTCHAN, T. F. 1994. *Plasmodium:* The developmentally regulated ribosome. *Experimental Parasitology* 78, 437–441.

INTRODUCTION

Why study the ribosomes of *Plasmodium* species? The central reasons are potent and relate not only to understanding ribosomes but also to investigating the biology of the parasite. It is true that most of the experimental approaches that have led to productive study of the ribosome as the central complex in protein synthesis are presently impossible to apply to *Plasmodium* species. For example, it is unlikely that, in the near future, we will have an easily manipulatable genetics system in any of the *Plasmodium* species or the potential to isolate material suitable for biochemical analyses (for example, an efficient translation system, a ribosome reconstitution procedure, or good systems for structural probing of functional ribosomes). Alternatively, much that can be learned by studying ribosomal RNA (rRNA) rests with the predictable way in which the rRNAs of all eucaryotic organisms are structured and the generally accepted association of certain structural configurations of the rRNA with catalytic mechanisms involved in protein synthesis. Further, several features of the rRNA of *Plasmodium* species set them apart from the rRNAs of other organisms and greatly expand the potential benefits of their study. This review will briefly discuss what is known about the *Plasmodium* small subunit ribosomal RNA (SSU rRNA) and then outline potential ramifications of rRNA studies for taxonomy, population genetics, drug design, developmental biology, drug and vaccine assessment, and the study of ribosome function in eucaryotic organisms.

THE RIBOSOMAL RNA OF *Plasmodium* SPECIES

The SSU rRNA gene sequences of *Plasmodium* have been analyzed from a large number of species, including those that infect humans, nonhuman primates, rodents, birds, and lizards. In general, the results show that the SSU rRNAs of *Plasmodium* species, like all eucaryotic rRNAs, are a mosaic of conserved and variable regions. The conserved sequences are localized to predictable regions of the gene and contribute to form the highly conserved secondary structure of the eucaryotic rRNA (Fig. 1A). These core sequences appear to be associated with the universal function of the ribosome, as in all organisms. As these core regions are considered to be an important indicator of the phylogenetic relationships among organisms, rRNA sequence analysis has been used to explore phylogenetic as-

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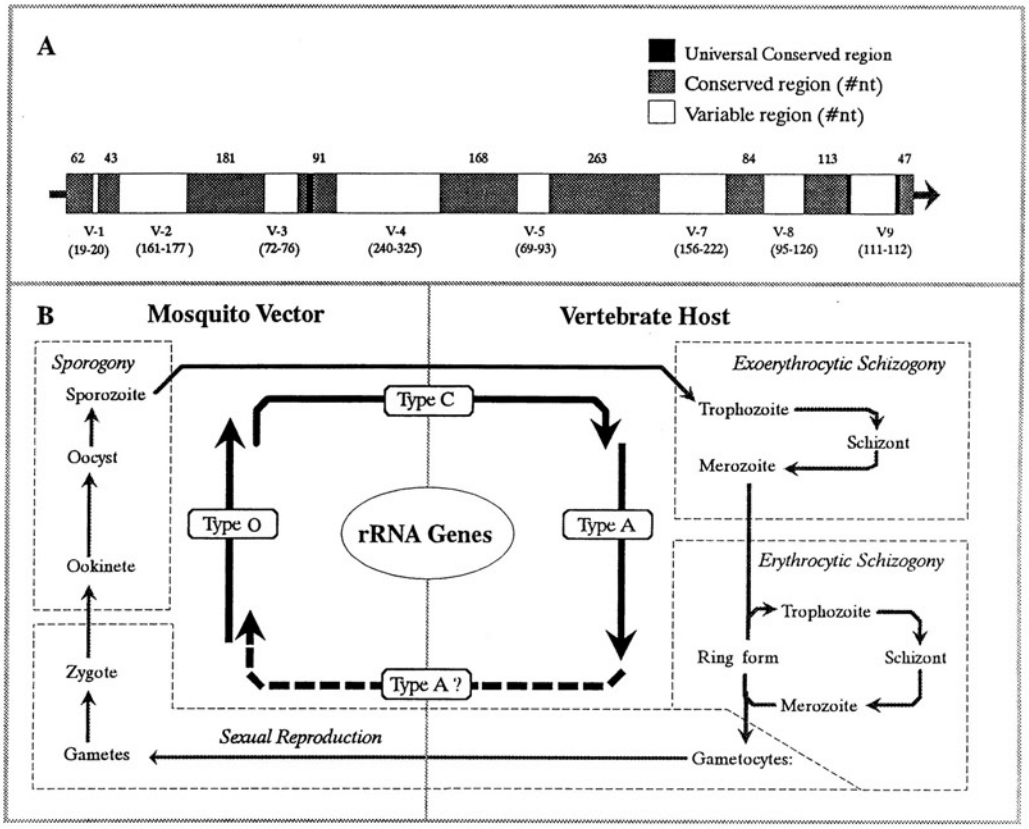

FIG. 1. Structure and expression of *Plasmodium* SSU rRNA genes. (A) A schematic representation of the alignment of all *Plasmodium* SSU rRNA gene sequences available in Gene Bank. (B) Transcription of distinct SSU rRNAs corresponds to the discrete developmental stages of the *Plasmodium* life cycle.

sociations among *Plasmodium* species (Waters *et al.* 1991, 1993). The variable sequences are interspersed among the conserved regions and contribute the major differences in gene size and composition. Although the functions of these regions are less well understood, they are of considerable interest because they retain the unique signature of each species and provide valuable information pertaining to population structure, taxonomy, and diagnosis. As described below, this is being used to gather information about population structure of *Plasmodium* species that was previously inaccessible.

Some features of the sequence and genomic arrangement of rRNA genes of *Plasmodium* are different from those found in other eucaryotic organisms. For example, most eucaryotic organisms have ribosomal genes that are highly repetitive and arranged in tandem arrays. The number of locations of these tandem arrays is usually restricted to one, or a very few regions of the genome. In contrast, *Plasmodium* species have four to seven genes which are dispersed to nearly as many chromosomes (Wellems *et al.* 1987). Further, unlike most eucaryotic organisms, where copies of the rRNA genes within an organism are identical, each rRNA gene of *Plasmodium* seems to retain a unique structure. Analysis from three species of *Plasmodium* showed at least three types of gene broadly defined by re-

striction site and heteroduplex mapping of mature coding sequence (Dame *et al.* 1984; Dame and McCutchan 1983; Unnasch and Wirth 1983; Langsley *et al.* 1983). Hence, these organisms do not employ all the standard eucaryotic mechanisms involved in homogenization of rRNA sequences, although it seems clear that gene conversions have had a significant effect on their evolution (Enea and Corredor 1991).

One of the more interesting features associated with the ribosomal RNA of *Plasmodium* species is that their expression is clearly regulated during the parasite's life cycle, as outlined in Fig. 1B (Gunderson *et al.* 1987; McCutchan *et al.* 1988; Waters *et al.* 1989; Li *et al.* 1994). One type of gene is expressed in the asexual blood stage form of the parasite (the A gene) while it is in the vertebrate host, and another type is expressed in the mature parasite of the mosquito (the C gene). The C gene is not abundantly expressed until about 6 days after fertilization in the mosquito (Li *et al.* 1994). This timing corresponds well with the differentiation of the parasite into individual sporozoites rather than simply with the presence of the parasite in the mosquito as was originally postulated. Further investigations of the *Plasmodium vivax* genome have resulted in the isolation and cloning of a third type of ribosome gene (O gene), which is expressed in the maturing oocysts as early as 2 days after fertilization (Li and McCutchan, unpublished). The O gene transcripts are not detectable when sporozoites are purified away from the mosquito. This may indicate that ribosomes are sorted in the oocysts, directing C ribosomes into the budding sporozoites and excluding the O ribosome even though the cytoplasm of oocyst and sporozoite are interconnected. Another curiosity of the three ribosome system relates to the period of transition between types. The active clearing of one type of ribosome and maturation of another type of ribosome appear to be integral parts of cellular differentiation, both playing important roles in parasite development (Li *et al.* 1994).

The progression of development in the liver also seems to correlate with a transition of ribosome types (Zhu *et al.* 1990). Investigation of the transition between ribosomal types during the invasion process indicated that the "switch signal" is not related simply to temperature changes, attachment to liver cells, or even invasion of liver cells. Irradiated sporozoites, which develop for a period of time in the liver but do not produce active infections, do make the transition from C ribosomes to A ribosomes in a manner indistinguishable from untreated sporozoites. Hence, only successful invasion of the cells results in expression of the A gene.

Applications to Biological Investigation

rRNA genes are the target of choice for species identification because they are the only sequences that allow one to amplify species-specific sequence using genus-specific primers (14). Amplification of all types of *Plasmodium* rRNA genes from the same primers, "competitive" PCR, adds a quantitative aspect to parasite analysis. A few specific applications are described below.

With regard to taxonomy, we consider it important to provide a simple molecular identification mark for *Plasmodium* species (Waters and McCutchan 1989). Many species of malaria cannot be distinguished on the basis of the morphology of the blood stage parasite. Many distinctions must be made on the basis of careful morphological examination of forms collected throughout the complete life cycle and by observing the range of host specificity. Due to the difficulty involved in establishing and characterizing these differences, an understanding of the population dynamics and distribution of many species is not well understood. Even the distribution of the human malaria species and the degree to which people are

infected with more than one species of parasite has been vastly underestimated (Snounou *et al.* 1993).

The fact that the ribosomal genes are all polymorphic in form and reside on different chromosomes allows one to follow the distribution of particular chromosomes. Competitive PCR allows these data to be assessed both qualitatively and quantitatively. Such a procedure can be applied directly to field isolates and the data derived used to determine the extent of chromosome segregation among field isolates. Work of this type should answer a number of questions relating to possible clonal variation and allow a modeling of disease parameters. The extent to which clonal types are maintained, as opposed to being members of one large gene pool, is of importance to vaccine design and dissemination as well as decisions about anti-malarial drug distribution.

Species-specific primers serve to amplify developmentally regulated target sequences in competitive RNA PCR mixes. This is important in monitoring interruptions to development and quantifying development success in either individuals or populations. We have shown that one can quantitatively follow the development of the parasite in the mosquito and in the liver using this technique. Where a quantitative assessment of the effects of anti-malarial drugs or vaccines are considered useful indicators, this approach should have considerable value.

Interaction with Other Molecules

Study of the *Plasmodium* species offers at least two possibilities for gaining insight into the functions of the eucaryotic ribosome. One relates to correlation of sequence differences in rRNA and corresponding differences in the subset of macromolecules that associate with the rRNA during protein synthesis. The other relates to the subcellular distribution of the rRNA.

During the course of ribosome synthesis, assembly, and function, the ribosome interacts with many molecules. As the rRNA may provide the catalytic moiety for protein synthesis, distinct functional differences between ribosomes may relate directly to rRNA sequence differences. The function of the distinct rRNAs is also defined by assembly with other components of the ribosome, in particular the ribosomal proteins and interaction with initiation, elongation, and termination factors. The catalytic activity of the ribosome is also associated with nucleotide and ribose modification of the rRNA and the interaction of the rRNA with mRNA and with aminoacylated tRNA. Course control over development could then, for example, relate to the effects of different ribosomes preferentially translating different subsets of messenger RNAs. Another possibility is that distinct A, O, and C rRNA sequences may provide a way of targeting features of the superstructure involved in ribosome synthesis and assembly by allowing one to follow the fate of the components of the nucleolus throughout development. The life cycle of *Plasmodium* then provides an ideal tool to examine interactions of the ribosome and their possible implications for development.

Plasmodium as a Model for Studying the Eucaryotic Ribosome

One important feature associated with the study of the eucaryotic ribosome in *Plasmodium* species relates to advantages of a system in which one or two rRNA genes can supply all the rRNA needed for growth. This raises the possibility of studying mutagenized populations and populations carrying genetically engineered genes as the sole source of rRNA. Studies of the effects of subtle differences in ribosomal genes (i.e., growth rate, translation efficiency) that are not possible in other eucaryotes due to the large number of ribosomal gene copies would be accessible. Currently, only dominant alteration of ribo-

somes can be studied in eucaryotes. Further, the stage specificity of expression would allow replacement of ribosomal genes during their inactive stage and subsequently following the ramifications of these altered ribosomes through development to the active stage. Development of this model could provide the sole system for which recessive and lethal mutations in rRNAs can be studied. This may mean that *Plasmodium* as a model system for the study of ribosomes will outlast malaria as a human disease.

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Plasmodium falciparum: Stage-Specific Ribosomal RNA as a Potential Target for Monitoring Parasite Development in *Anopheles stephensi*

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LI, J., WIRTZ, R. A., SCHNEIDER, I., MURATOVA, O. V., MCCUTCHAN, T. F., APPIAH, A., AND HOLLINGDALE, M. R. 1993. *Plasmodium falciparum:* Stage-specific ribosomal RNA as a potential target for monitoring parasite development in *Anopheles stephensi*. *Experimental Parasitology* **76**, 32–38. The transcriptional switch of *Plasmodium falciparum* ribosomal RNA A gene to the C gene was demonstrated during the developmental transition from the vertebrate blood stage to the invertebrate sporozoite stage. Expression of the sporozoite specific C gene in infected mosquitoes was not detected until Day 10 postinfectious blood meal, the time of mature oocyst formation on the midgut. As a potential target for monitoring malaria parasite development in mosquitoes, oligonucleotide probes based on sequences of small subunit ribosomal RNA were evaluated for specificity and sensitivity by filter blot hybridization against different species and stages of malaria parasites. Probes PfC02 and PfA02 were selected as the most sensitive for sporozoite and blood stage parasites, respectively. Filter blot hybridization using probe PfC02 resulted in sensitivity comparable with microscopic dissection in single mosquitoes, detecting mosquitoes with an average of 1.2 oocysts per gut or as few as 800 salivary gland sporozoites.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Plasmodium falciparum;* Malaria; rRNA probe; *Anopheles stephensi;* Mosquito; Small subunit ribosomal RNA (SSUrRNA); Hybridization; Standard saline citrate (SSC); Circumsporozoite (CS); Ribosomal ribonucleic acid (rRNA); Red blood cells (RBC).

INTRODUCTION

Malaria remains an important health problem in most developing countries in temperate and tropical areas (World Health Organization 1988). Control programs often require identification of sporozoite-infected mosquitoes for effective vector suppression. In addition, monitoring of sporozoite development is essential to determine the effect of antimalarial drugs on parasite development in mosquitoes. Traditionally, vectors have been identified by dissection of freshly collected mosquitoes and microscopic examination of the salivary glands or midgut to obtain the sporozoite and oocyst rates, respectively (Bruce-Chwatt 1987). Parasite species identification could only be accomplished by *in vivo* experimental infection (Boyd 1949; Bruce-Chwatt 1988). More recently, monoclonal antibody-based methods have been developed to augment the dissection procedure (Wirtz and Burkot 1991). The immunological methods are powerful tools with good sensitivity, however, the recent discovery of *Plasmodium vivax* CS protein heterogeneity (Rosenberg *et al.* 1989) has complicated the use of these monoclonal antibody-based assays.

Ribosomal RNA (rRNA) oligonucleotide

0014-4894/93 $5.00

probes offer an alternative approach for identification of infected mosquitoes because of the unique primary structure and tremendous abundance of the rRNA target. Previous research demonstrated that malaria parasites express distinct rRNA genes during different stages of the life cycle (Gunderson *et al.* 1987; Waters *et al.* 1989; Zhu *et al.* 1990). Oligonucleotide probes, based on the parasite species and stage-specific rRNA sequences, have been developed for diagnosis of blood stage infections (Lal *et al.* 1989; Waters and McCutchan 1989) and for *in vitro* quantitation of parasite exoerythrocyte development to monitor the efficacy of antimalarial drugs (Li *et al.* 1991). In this study, synthetic oligonucleotide probes were evaluated for use in identification of *Plasmodium falciparum*-infected mosquitoes and to monitor sporozoite development within the vector.

MATERIALS AND METHODS

Oligonucleotide probes (17–24 bp) complementary to species or stage-specific sequences in the two 18S rRNA genes of *P. falciparum*, as well as a universal and a *Plasmodium* genera specific rRNA probe, were synthesized (Applied Biosystems DNA Synthesizer, Foster City, CA). Stage-specific probes PfA01 and PfA02 were complementary to the SSUrRNA gene sequences predominantly expressed during parasite blood schizogony, while probes PfC01 and PfC02 contained sequences predominantly expressed during sporogony (Table I). Probe P01 contains the SSUrDNA sequence conserved in the genus *Plasmodium*, while probe VD3 was based on a universal rRNA sequence (McCutchan *et al.* 1988). Probes were labeled by T4-DNA kinase (Bethesda Research Laboratory, Gaithersburg, MD) and γ-[^{32}P]-ATP (Amersham, Arlington Heights, IL) and purified on a Sephadex G25 spun column (5′–3′, Inc., West Chester, PA). Specific activity was determined using autoradiography as described (Zhu *et al.* 1990; Li *et al.* 1991).

Four species of *Plasmodium* were used to evaluate probe specificity. Blood Stage *P. falciparum* (NF54) and *Plasmodium vivax* parasites were harvested from *in vitro* cultures and frozen blood from a Thailand patient, respectively. Blood stage *Plasmodium berghei* (ANKA) and *Plasmodium yoelii* were isolated from laboratory-infected mice. Blood was washed twice with PBS and the cells were packed by centrifugation (1500*g*, room temperature). About 1.5 vol of 0.15% saponin in PBS were added to free parasites from the RBCs. After two additional PBS washes, isolated parasites were aliquoted and frozen at −70°C.

Sporozoite-infected mosquitoes were produced by feeding *Anopheles stephensi* on either *P. falciparum* cultures of *P. berghei* infected mice (Burkot *et al.* 1984). Salivary gland sporozoites were harvested approximately 20 days after the infectious blood meal, counted with a hemocytometer, and used to evaluate probe sensitivity.

Whole mosquitoes, from pools with infection rates ≥60% confirmed by dissection, were frozen at −70°C in 1.5-ml tubes. Groups of *P. falciparum*-infected mosquitoes held at 26° ± 1°C were also harvested at intervals beginning 2 hr after the blood meal and thereafter every 2 or 5 days. Wings and legs were removed and the bodies were frozen at −70°C. Sporozoite development in the mosquitoes was monitored by dissection and oligonucleotide probe hybridization.

Total RNA was isolated using an acid guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi 1987). Briefly, fresh or frozen parasites were lysed with a solution of 4 *M* guanidine isothiocyanate, 0.5% Sarkosyl, 0.1 *M* β-mecaptomethanol, and 25 m*M* sodium citrate, pH 7. Individual mosquitoes were triturated with a pellet pestle (Kontes, Vineland, NJ) in a 1.5-ml tube containing the lysing buffer. Total RNA was isolated by phenol–chloroform extraction and precipitated with isopro-

TABLE I

Plasmodium falciparum: Synthetic Oligonucleotide Probe Sequences Complementary to the 18S rRNA Genes

Name	Sequence (5′ 3′)[a]	Bases	Position[b]
P01	ACT CGA TTG ATA CAC ACT	18	
PfA01	TAG TTC CCC TAG AAT AGT TAC AAT	24	687–664
PfA02	AGG AAG CAA TCT AAA AGT CA	20	1170–1151
PfC01	GGT TTT CCC AAA CCA GT	17	685–669
PfC02	ACT CAA TCA TGA CTA CCC GTC	21	860–840
VD3	TTA CCG CGG(A) CT(G)G CTG GC	17	

[a] McCutchan *et al.* 1988.

[b] PfA01–02 and PfC01–02: Positions on type A and C rDNA genes, respectively.

panol and ethanol. The RNA was denatured in a formaldehyde salt solution at 65°C, and equivalent amounts of RNA were immobilized on a nitrocellulose filter and baked at 80°C for 2 hr. The filter was prehybridized at 42°C for 1 hr in 6×SSC, 0.08‰ polyvinylpyrrolidone, 0.08% Ficoll, 0.1% sodium dodecyl sulfate, 1 mg bovine serum albumin/ml and 33 μg yeast tRNA/ml. Hybridization was conducted using this solution containing the ^{32}P-end-labeled probe at 42°C for 6–8 hr. The filter was then washed three times in 6xSSC containing 0.1% sodium dodecyl sulfate at room temperature and once at 42°C in 2×SSC for 10 min. Autoradiography and liquid scintillation counting of the filter were processed as described by Li *et al.* (1991).

Results

Probes PfA01 and PfA02 detected only blood stage *P. falciparum* rRNA while probes PfC01 and PfC02 hybridized predominantly with sporozoite stage rRNA (Fig. 1). The *Plasmodium* genus-specific probe, P01, hybridized strongly with rRNA from blood of all four species of malaria parasites and to a lesser degree with rRNA from sporozoites of *P. falciparum* and *P. berghei*. No significant signal was detected with RNA isolated from human RBCs or uninfected mosquitoes.

Stage-specific rRNA expression was detected using probes PfA02 and PfC02, selected for their higher hybridization efficiency and specificity. Autoradiography results (Fig. 2) indicated that equal amounts of total RNA were identified on each blot with the universal rRNA probe VD3. Type A rRNA, predominant in the blood stage parasite, strongly hybridized with probe PfA02 in mosquitoes 2 hr (D_0) after the blood meal and disappeared by Day 5 (D_5). Peak parasite C gene transcription occurred on Day 10, the time of mature oocyst formation on the midgut, and remained positive until Day 21, although probe PfC02 signal intensity decreases with the duration of infection. The weak C gene signal detected immediately after the blood meal (D_0) may be due to the large amount of blood stage or gametocyte rRNA, in which C gene rRNA transcription may have started. Assess-

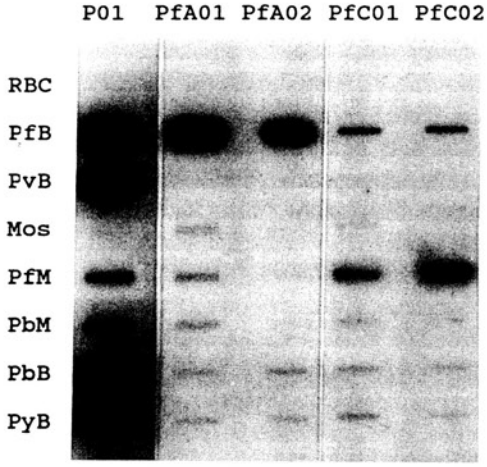

FIG. 1. *Plasmodium* spp.: Comparison of genus and species specific probe hybridization results for rRNA of four different malarias. Probe: P01, *Plasmodium* genus specific; PfA01 and PfA02, *P. falciparum* blood stage specific; PfC01 and PfC02, *P. falciparum* sporozoite stage specific. Parasites: PfB, PvB, PbB, and PyB, *P. falciparum, P. vivax, P. berghei,* and *P. yoelii* blood stage, respectively. PfM and PbM: *P. falciparum*- and *P. berghei*-infected mosquitoes, respectively. Controls: RBC, uninfected human red blood cells; Mos, uninfected mosquitoes.

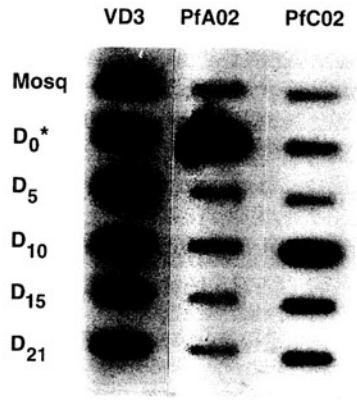

FIG. 2. *Plasmodium falciparum:* Autoradiography of genus, species, and stage-specific probes to the rRNA of *P. falciparum*-infected mosquitoes collected at different times after the blood meal. Probes: VD3, universal rRNA probe; PfA02, *P. falciparum* blood stage specific; PfC02, *P. falciparum* sporozoite stage specific. Mosquitoes: Mosq, before feeding and, D_0, 2 hr or, D_{5-21}, 5, 10, 15, and 21 days after the infectious blood meal.

ment of this process at shorter intervals (2 day) resulted in a similar pattern (data not shown).

Transition of parasite rRNA transcripts from the A to the C gene is shown in Fig. 3. The A gene rRNA was only detected shortly after the infectious blood meal, followed by a 7- to 8-day delay before the C gene rRNA peak on Day 10. This procedure was repeated on three different groups of *P. falciparum*-infected mosquitoes with nearly identical results.

The ability to identify single infected mosquitoes using the species and stage-specific probes was evaluated using counted sporozoites and infected mosquitoes. Serial (1:1) dilutions of known numbers of *P. falciparum* sporozoites were mixed with uninfected mosquitoes, and total RNA was isolated and immobilized on a membrane and hybridized with probe PfC02. Hybridization increased with the number of parasites, with a lower limit of detection between 160–800 sporozoites (Fig. 4).

Results for the comparison of probe hybridization and microscopic dissection in identifying individual infected mosquitoes are given in Fig. 4 and Table II. Cohort *Anopheles stephensi* examined by dissection or tested by dot blot hybridization with probe PfC02 resulted in comparable rates of infection. Probe hybridization results on mosquitoes stored at −20°C for 45 days were similar to those conducted on fresh samples (Fig. 4).

Discussion

Techniques for identifying sporozoite-infected mosquitoes and monitoring the sporogonic development of malaria parasites are crucial for vector epidemiological studies and laboratory evaluation of chemotherapeutics. Dissection is laborious, requires skilled personnel, and does not permit species identification of the parasite.

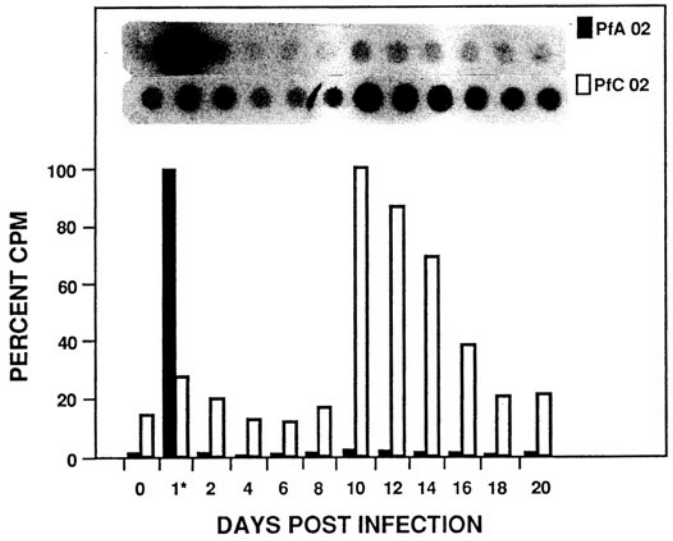

FIG. 3. *Plasmodium falciparum:* Oligonucleotide probe monitoring of the switch regulation of the A to C rRNA gene, associated with parasite transition from the human to mosquito stage. Cohort mosquitoes were randomly sampled before and after the infected blood meal and equal amounts of total rRNA (pool of 10 mosquitoes) were hybridized against blood and sporozoite stage-specific probes PfA02 and PfC02, respectively. Hybridization is presented by dot blot autoradiography and bar graph percentage liquid scintillation CPM (Percent CPM, counts per minute for each sample/the maximum counts for each probe ×100%).

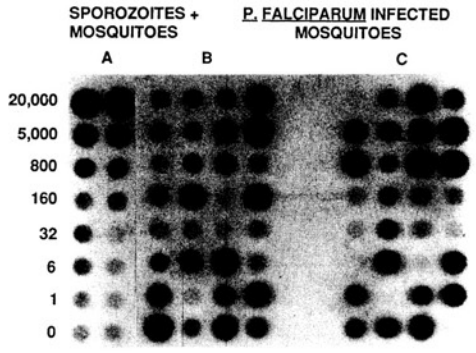

FIG. 4. *Plasmodium falciparum:* Identification of malaria-infected mosquitoes with an oligonucleotide rRNA probe. Total rRNA was isolated from individual mosquitoes, dot blotted onto nitrocellulose membrane, and hybridized with ^{32}P-labeled probe PfC02 (8-hr exposure). (A) Uninfected mosquitoes plus a known number of sporozoites; (B) cohort mosquitoes processed immediately at Day 20 postinfection; (C) cohort mosquitoes frozen at −20°C for 45 days before processing.

However, it remains the only method to obtain salivary gland sporozoite and gut oocyst infection rates. Immunological techniques based on anti-CS protein monoclonal antibodies, e.g., IRMA and ELISA, have overcome many drawbacks associated with dissection, however, the discovery of *P. vivax* CS protein heterogeneity (Rosenberg *et al.* 1989) has complicated the use of these assays. Probes for multiple copy DNA lack the sensitivity required (Delves *et al.* 1989) and the use of polymerase chain reaction amplification on large numbers of field-collected mosquitoes is unrealistic. Neither of these nucleic acid-based methods would distinguish between infectious mosquitoes and nonvectors engorged with infected blood. The species and stage-specific oligonucleotide probes target naturally amplified rRNA and could serve as alternative methods for identification of the sporozoite-infected mosquitoes and quantitation of sporogony.

TABLE II

Plasmodium falciparum: Comparison of Species and Stage-Specific rRNA Probe Hybridization and Microscopic Dissection in the Identification of Individual Infected Mosquitoes

	Probe hybridization[a]		Dissection	
Infection[b]	Group 1	Group 2	Group 1	Group 2
Positive	14	20	15	20
Heavy	(8)	(6)	(8)	(14)
Moderate	(2)	(9)	(5)	(2)
Light	(4)	(5)	(2)	(4)
Negative	16	10	15	10
% Infection:	47	67	50	67

[a] Probe: PfC02, *P. falciparum* C gene rRNA specific.
[b] Degree of infection graded by liquid scintillation CPM or the number of the sporozoites determined by dissection.

In this study, oligonucleotide probes complementary to specific regions of *P. falciparum* SSUrRNA clearly distinguish the stages of parasite development in infected mosquitoes. Probes PfA01 and PfA02 detected blood stage *P. falciparum* while probes PfC01 and PfC02 reacted with sporozoites of this species. The SSUrRNA sequences identified by probe P01, shared by both blood and sporozoite stages, also hybridized with other plasmodia and appear to be conserved within the genus. The switch from A to C gene expression was clearly demonstrated. The type A rRNA, predominantly transcribed in blood stage parasites, was detected in the mosquitoes shortly after ingesting an infectious blood meal and disappeared within 48 hr as digestion continued. Transition to the C gene was sharply defined, with peak probe hybridization always occurring 10 days after gametocyte ingestion and when mature oocysts had formed. The decrease of the C gene signal after Day 10 may be due to the loss of sporozoites during feeding or destroyed during their migration from the oocyst to the salivary glands. The sporozoite is a relatively stable stage and rRNA might be reduced because of a lower requirement for protein synthesis. The 8-day interval when little or no A and C rRNA were detected may be due to probe sensitivity. It is also possible that another type of rRNA is transcribed during the sexual development from gametocyte to ookinete.

The rRNA probe hybridization is suffi-

ciently sensitive to detect between 160–800 sporozoites (Fig. 4). Since the mean number of *P. falciparum* and *P. vivax* sporozoites per oocyst have been reported as 3385 and 3688, respectively (Rosenberg and Rungsiwongse 1991), rRNA probes are probably sufficiently sensitive to detect a single infected mosquito. Infection rates obtained by probe hybridization correlated well with dissection results of cohort mosquitoes which had a mean of 1.2 oocysts per gut. The rRNA probe hybridization method is, however, less sensitive than the ELISA which can detect fewer than 100 sporozoites per mosquito (Wirtz and Burkot 1991), but twice as sensitive as a repetitive genome DNA probe with a sensitivity of approximately 1500 sporozoites per mosquito (Delves *et al.* 1989). There is 0.2–1.0 pg of total RNA per parasite, of which SSUrRNA represents 30%, compared to approximately 0.02 pg of total DNA, of which only 1–10% is composed of any individual repetitive element (Bahr 1969; Draper *et al.* 1986). Theoretically, the rRNA probes would be orders of magnitude more sensitive than any repetitive DNA probe. However, actual sensitivity is affected by sample preparation, efficiency of hybridization, and target rRNA copy number, the latter of which varies with protein synthesis during parasite development. Practical application of rRNA probes in field studies will require simplification of sample processing and hybridization and the use of a nonradioactive label. A promising approach is a liquid "sandwich" hybridization assay in which a genus conserved capture probe, coated on microtiter plate wells, is used in conjunction with a labeled species or stage-specific probe. Theoretically, a significant improvement in assay sensitivity would be achieved. Such experiments are currently in progress.

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EXPERIMENTAL PARASITOLOGY 72, 450–458 (1991)

Plasmodium berghei: Quantitation of *in Vitro* Effects of Antimalarial Drugs on Exoerythrocytic Development by a Ribosomal RNA Probe

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Visiting Scientist, Second Military Medical University, 594 Xiang Yin Road, Shanghai, People's Republic of China; †Biomedical Research Institute, 12111 Parklawn Drive, Rockville, Maryland 20852, U.S.A.; ‡Malaria Unit, Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases, National Institute of Health, Bethesda, Maryland 20892, U.S.A.; §Malaria Division, Infectious Diseases Department, Naval Medical Research Institute, Bethesda, Maryland 20814-5055, U.S.A.; and ‖Department of Parasitology, Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington, DC 20307, U.S.A.

LI, J., ZHU, J., APPIAH, A., MCCUTCHAN, T. F., LONG, G. W., MILHOUS, W. K., AND HOLLINGDALE, M. R. 1991. *Plasmodium berghei:* Quantitation of *in vitro* effects of antimalarial drugs on exoerythrocytic development by ribosomal RNA probe. *Experimental Parasitology* 72, 450–458. A stage-specific ribosomal RNA probe has been used to quantitate exoerythrocytic development of *Plasmodium berghei* in primary cultures of mouse hepatocytes. Parasite rRNA could be detected as soon as 6 hr after sporozoite invasion and was increased during schizogony to a maximum at 48 hr, when mature schizonts were identified by microscopy. As few as 10 exoerythrocytic schizonts could be detected by filter blot hybridization, followed by autoradiography and liquid scintillation counting. By hybridizing the culture rRNA samples with either parasite-specific or universal rRNA probes, the *in vitro* tissue schizonticidal activity and hepatotoxicity of primaquine, two of its analogues, and pyrimethamine, could be assessed. After a 48-hr exposure of the culture to serial dilutions of each drug, a quantitative relationship was demonstrated between the decrease of the parasite rRNA and the increase of the drug concentrations. No significant parasite-specific rRNA could be detected at the concentration achieving complete inhibition of schizont formation but causing no cytotoxic effects on host hepatocytes. In contrast to microscopic-based assays, this molecular approach provides an objective and quantitative *in vitro* method for rapid screening and evaluation of tissue schizonticidal antimalarials.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Plasmodium berghei;* Malaria; rRNA probe; Malarial chemotherapy; *In vitro* drug assay; Ribosomal RNA (rRNA); Exoerythrocytic (EE); 50% Inhibitory concentration (IC_{50}); Primaquine (PQ) Pyrimethamine (PYR); Chloroquine (CQ); Standard saline citrate (SSC).

INTRODUCTION

Primaquine (PQ), the 8-aminoquinoline introduced about 40 years ago, remains the only drug available for clinical causal prophylaxis and radical treatment despite intensive efforts in search of antimalarial compounds with a higher tissue schizonticidal activity and lower side effects (Davidson *et al.* 1981). Because of limitation in methods for determining the infectivity of inoculated sporozoites and for directly quantitating exoerythrocytic (EE) development of malaria parasites, data from existing *in vivo* animal models for causal prophylactic and radical curative testing cannot be easily reproduced, and none of these models are capable of supporting rapid, accurate, and economical assessment (Davidson *et al.* 1981; Heiffer *et al.* 1984). Thus, there is a clear need for improved models and more reliable techniques in tissue schizonticidal assays.

With recent achievements in the cultivation of malaria EE parasites (Hollingdale *et al.* 1981, 1983b, 1985; Landau *et al.* 1984;

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Long *et al.* 1989), *in vitro* evaluation of tissue schizonticidal activity appears to offer a promising alternative for the rapid screening of candidate compounds and for identifying mechanisms of the drug action (Millet *et al.* 1986; Hollingdale 1987; Fisk *et al.* 1989). However, these *in vitro* systems have relied on microscopic examination to enumerate parasites, which is time consuming, and counting is often difficult to standardize, especially in the case of drug-induced parasite morphological changes. We have, therefore, investigated, molecular procedures for objective and quantitative results.

Ribosomal RNA (rRNA) is one of the most abundant cellular macromolecules and as such is a potential target for microbe detection. Recently, two structurally distinct small subunit rRNA genes have been identified in different stages of malaria parasites. Expression of gene A predominates in mammalian exoerythrocytic and blood stage parasites, while expression of gene C predominates in sporozoites within the insect vector (Gunderson *et al.* 1987; Waters *et al.* 1989; Zhu *et al.* 1990). Synthetic oligonucleotide probes, based on the parasite species and stage-specific sequences of rRNA, have already been developed for diagnosis of malaria blood stage infection (Lal *et al.* 1989; Waters and McCutchan 1989) and for *in vitro* quantitation of sporozoite invasion and EE development (Zhu *et al.* 1990). In this study, a stage-specific oligonucleotide probe complementary to the type A small subunit rRNA sequence of *Plasmodium berghei* has been used to quantitate EE development of *P. berghei* in mouse hepatocyte cultures and, in combination with a universal rRNA probe, to evaluate *in vitro* tissue schizonticidal activity and hepatotoxicity of antimalarial drugs.

Materials and Methods

Primary hepatocyte culture. Hepatocytes were isolated from BALB/c mice by enzymatic perfusion (Klauning *et al.* 1981; Long *et al.* 1989). Briefly, mouse liver was perfused *in situ* with 0.5% collagenase (Boehringer Mannheim Biochemicals, Indianapolis, IN) in Hank's balanced salt solution with 25 m*M* Hepes and 5 m*M* calcium chloride. The separated cells were released into cold, 2.5% fatty acid-free bovine serum albumin (Sigma Chemical Co., St. Louis, MO) in Hepes-balanced HBSS and further filtered by nylon mesh. After centrifugation for 5 min at 50*g*, the cells were resuspended in Williams' medium E supplemented with 10% fetal bovine serum, 25 m*M* Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μg/ml fungizone (Flow Laboratories Inc.), 10 μg/ml insulin (Boehringer Mannheim Biomedicals), and 2 μg/ml dexamethasone (Collaborative Research Inc., Lexington, MA). Cells (10^5) were plated onto a 1-cm^2 glass coverslip coated with rat skin collagen (Boehringer Mannheim Biochemicals) and incubated at 37°C in 5% CO_2 in air. The medium was changed 3 hr later and the confluent cell monolayer was incubated for a further 20–30 hr before use.

Cultivation of EE parasites. P. berghei (ANKA strain) sporozoites were isolated from the salivary glands of *Anopheles stephensi* mosquitoes fed 21 days earlier on infected mice. The salivary glands were dissected into complete Williams' medium E, triturated in a glass grinder, and released sporozoites counted in a hemocytometer. Sporozoites were diluted in the culture medium such that a known number were inoculated on each cell monolayer loaded in a glass shell vial. Fresh medium was added 3 hr later and changed daily thereafter (Long *et al.* 1989).

In vitro *drug assay.* The clinical tissue schizonticidal drug primaquine, two of its analogues (WR238605 and WR242511), and pyrimethamine (PYR), as well as the clinical blood schizonticidal agent chloroquine (CQ), were all provided by the Walter Reed Army Institute of Research (Washington, DC). Each compound, except CQ which was dissolved in distilled water, was first dissolved in dimethyl sulfoxide (Sigma Chemical Co.) and then diluted with the complete medium. Three hours after inoculation of the sporozoites, a series of fivefold dilutions of each drug were added to the infected hepatocyte cultures. Medium was replaced with freshly prepared drug dilutions at 24 hr. After 48 hr, two of the triplicate samples in each concentration were frozen at −70°C for probe hybridization; the remaining sample was fixed with methanol and stained by the indirect immunoperoxidase assay with a monoclonal antibody (3D11) (Hollingdale *et al.* 1983a). The cytotoxicity of each drug against primary hepatocytes was tested as above using an universal rRNA probe, except that sporozoites were not added to the cell monolayers.

Hybridization and quantitation. Oligonucleotide probe TM4, complementary to relevant sequence of *P. berghei* type A small subunit rRNA (Gunderson *et al.* 1987), and VD3, a universal rRNA probe (Synthecell Corp., Rockville, MD), were used in slot blot hybrid-

TABLE I
Oligonucleotide Probes Complementary to Small Subunit Ribosome RNA

Name	Sequence	Target
TM4	CATGAAGATATCGAGGCGGAG	*P. berghei* type A rRNA
VD3	TTACCGCGG (or A) CT (or G)GCTGGC	Universal rRNA

ization to detect EE parasite and hepatocyte rRNA, respectively (Table I). They were labeled by T_4-DNA kinase (Bethesda Research Laboratory, Gaithersberg, MD) and γ-^{32}P-ATP (Amersham, Arlington Heights, IL) and purified by a Sephadex G25 spun column (5′-3′ Inc., West Chester, PA). Isolation of total RNA from the culture cells was prepared by the acid guanidinium thiocyanate–phenol–chloroform extraction (Chomczynski and Sacchi 1987). This procedure selectively extracted RNA from DNA and proteins. Briefly, culture samples removed from −70°C were lysed with the guanidine salt solution. The total RNA was isolated by the phenol–chloroform extraction and denatured at 65°C in the formaldehyde salt solution (Zhu *et al.* 1990). After immobilization on a nitrocellulose filter, rRNA was prehybridized at 42°C for 1 hr in 100 ml of 6 × SSC, 0.08% polyvinylpyrrolidone and Ficoll, 0.1% sodium dodecyl sulfate, 1 mg/ml bovine serum albumin, and 33 μg/ml yeast tRNA. Hybridization was processed in 25 ml of the same solution plus the ^{32}P end-labeled oligonucleotide probe at 42°C for overnight and then washed three times at room temperature and once at 42°C in 6 × SSC, 0.1% sodium dodecyl sulfate for 10 min. For quantitation of the detected rRNA, the filter was cut according to the band position recognized by autoradiography and counted on a liquid scintillation counter (1217 Rockbeta, LKB). The 50% inhibitory concentrations (IC_{50}) were calculated by comparing the counts of the hybridized ^{32}P end-labeled probe to the drug concentrations using computerized nonlinear regression analysis (Desjardins *et al.* 1979; Oduola *et al.* 1986).

Results

EE parasite development. Approximately 80% viability of mouse hepatocytes was routinely obtained by the described enzymatic perfusion method, and confluent

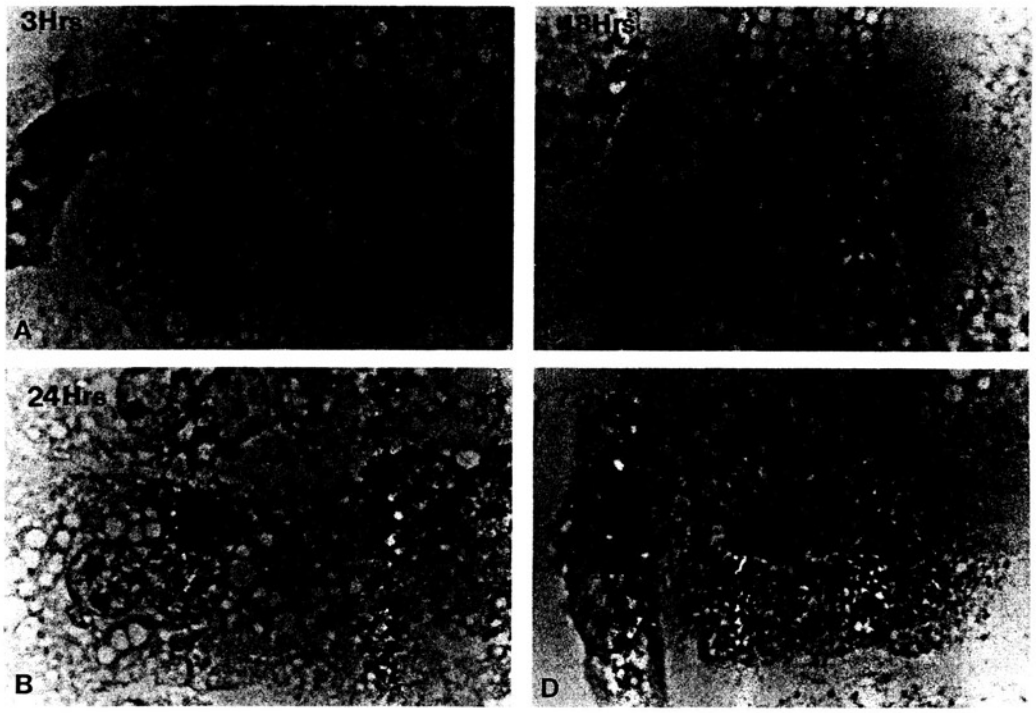

FIG. 1. Light micrographs of *P. berghei* exoerythrocytic development in primary cultures of mouse hepatocytes (×850). The parasite was fixed and stained by an indirect immunoperoxidase assay with a monoclonal antibody (3D11) against the CS protein of *P. berghei* at 3 (A), 24 (B), 48 (C), and 72 hr (D) after inoculation of the sporozoite in mouse hepatocyte culture.

cell monolayers were formed within 20–30 hr. After inoculation, *P. berghei* sporozoites invaded by about 3 hr (Fig. 1A) and early schizonts with several nuclei could be identified by 24 hr (Fig. 1B). Between 24 and 48 hr after infection, the size of EE schizonts dramatically increased, and by 48 hr they had developed into mature schizonts (Fig. 1C), contained hundreds of merozoite nuclei, and averaged 30 μm in diameter. At 72 hr, the number of the schizonts decreased as they ruptured and released EE merozoites (Fig. 1D). Approximately 0.2–0.5% of a standard inoculation of 4.5–5.0 $\times$ 10^4 sporozoites per coverslip developed into morphologically mature schizonts.

Time course of rRNA increase of EE parasites. To identify whether the *in vitro* development of EE parasites could be quantitated by the hybridization of oligonucleotide probes to type A rRNA culture samples were collected at different intervals after inoculation of the sporozoites. The level of EE parasite-specific rRNA slightly increased during the first 24-hr culture, the peak rRNA concentration was reached at 48 hr, and thereafter the signal sharply decreased from 72 to 96 hr (Fig. 2). Thus, the level of hybridization correlated with the morphological development of EE parasites described in Fig. 1.

Sensitivity of rRNA based hybridization assay. In order to determine the sensitivity of the filter blot hybridization test, various numbers of sporozoites were added to the mouse hepatocyte cultures and incubated for 48 hr. These results demonstrated that the hybridization signal increased in proportion to the number of inoculated sporozoites (Fig. 3). Although as few as 2.5–5.0 $\times$ 10^3 inoculated sporozoites, i.e., about 10 mature schizonts in each culture, could be detected, an inoculum of 4.5 $\times$ 10^4 sporozoites, or about 200 mature schizonts, was generally required to obtain quantitative signals in this *in vitro* system.

Hepatotoxicity test. Since hepatotoxicity is one of the major side effects of 8-aminoquinolines, the toxicity of each drug to cultured mouse hepatocytes was determined, to select the appropriate range of drug dilutions for *in vitro* tissue schizonticidal assay. After a 48-hr incubation of mouse primary hepatocytes with each drug at fivefold serial dilutions of 1.5 $\times$ 10^{-4} to 2.4 $\times$ 10^{-7} *M*, samples were processed in

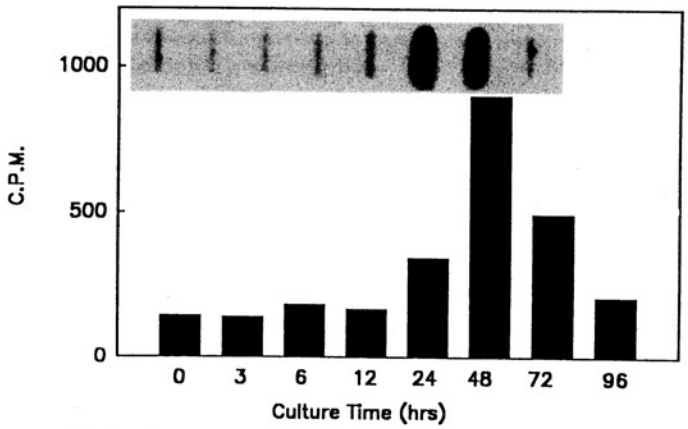

FIG. 2. Increase of *P. berghei* type A rRNA in a 96-hr culture of sporozoite-infected mouse hepatocyte. Culture samples were collected at different intervals after inoculation of 5 $\times$ 10^4 sporozoites. Total RNA of each culture was extracted, immobilized on a nitrocellose filter, and hybridized with a ^{32}P-labeled TM4 probe. The hybridization signal was detected by autoradiography and quantitated by liquid scintillation counting.

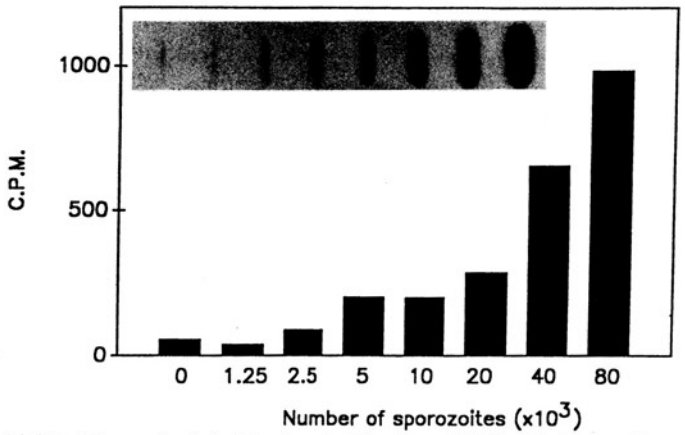

FIG. 3. Sensitivity of the probe hybridization in detection of *P. berghei* exoerythrocytic schizonts. Increasing numbers of sporozoites were added to mouse hepatocyte cultures and incubated for 48 hr. The detection and quantitation of hybridized parasite rRNAs were processed with the same method used in Fig. 1.

the same procedure described above but hybridized with an universal rRNA probe, VD3. These results indicated (Fig. 4) that PQ was about 65% inhibitory at 1.5×10^{-4} *M*, whereas WR238605 and WR242511 were 95% inhibitory at 3×10^{-5} *M*. No significant cytotoxicity was detected at lower concentrations of each drug. In contrast to 8-aminoquinolines, PYR, a dihydrofolate reductase inhibitor, exhibited no detectable cytotoxic effects on the cultured hepatocytes at concentrations up to 1.5×10^{-4} *M* (Fig. 4). Corresponding to the above rRNA probe hybridization results, changes in cellular morphology were observed microscopically, such as poor nuclei staining, hypervacuolation at the cytoplasm, and cell detachment from the coverslip (data not shown).

EE schizonticidal activity assay. Based on the above results, the probe hybridization assay was applied to evaluate the EE schizonticidal effects of PQ, its analogues WR238605 and WR242511, and PYR. In initial experiments, CQ, a typical erythrocytic schizonticide, was also analyzed as a negative control. After a 48-hr exposure, PQ at a concentration of 3×10^{-5} *M* completely inhibited the growth of *P. berghei* EE parasites in mouse hepatocyte cultures as measured by hybridization of parasite rRNA and parasite EE development by microscopy, but had no significant cytotoxicity on host hepatocytes (Fig. 5). In addition, a quantitative relationship was shown between rRNA levels and PQ concentrations, as hybridization of parasite rRNA decreased proportionally to the increase of PQ concentration (Fig. 6). In contrast, CQ showed no inhibitory effect on the EE schizogony, even at those concentrations that caused obvious cytotoxicity on cultured hepato-

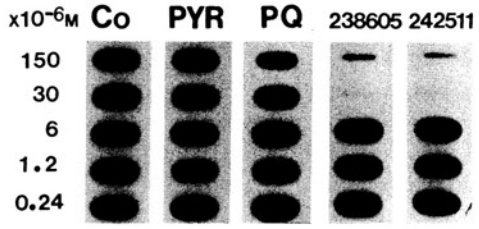

FIG. 4. Autoradiograms of cytotoxic effects of primaquine (PQ), two of its analogues WR238605 and WR242511, and pyrimethamine (PYR) on mouse hepatocyte culture cells. Mouse hepatocytes were incubated with fivefold serial dilutions of drugs (1.5×10^{-4} to 2.4×10^{-7} *M*) for 48 hr. Total rRNA of each culture was determined by slot blot hybridization with a ^{32}P-labeled universal rRNA probe VD3 (Co, drug negative control).

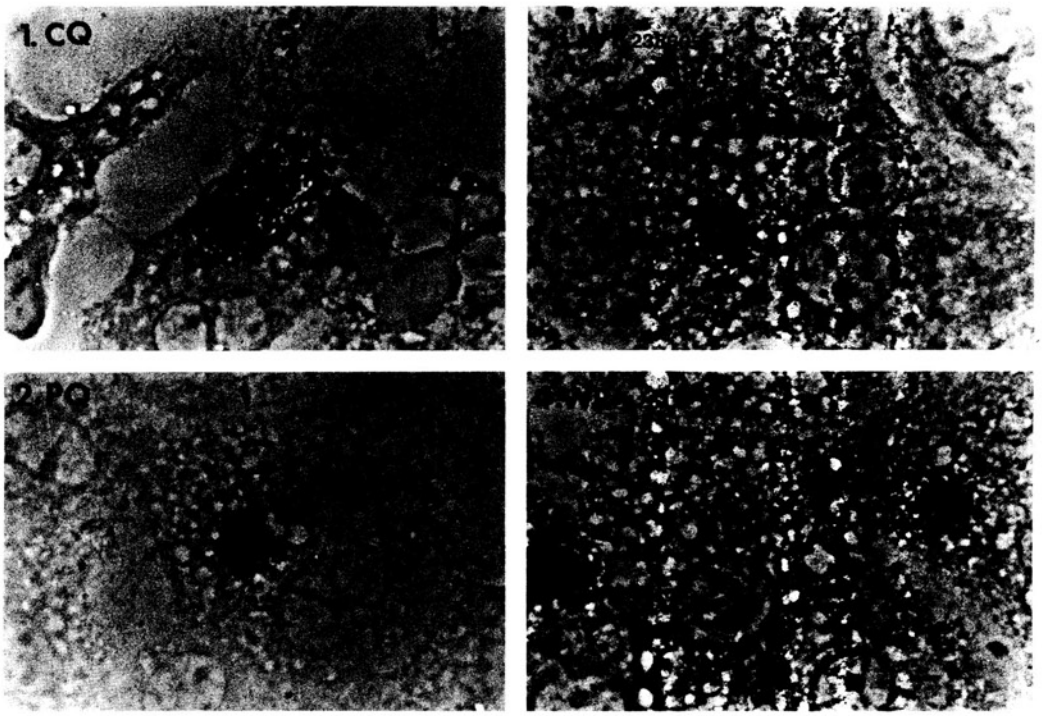

FIG. 5. Light micrographs showing effects of chloroquine (CQ), primaquine (PQ), WR238605, and WR242511 on exoerythrocytic development of *P. berghei* in mouse hepatocytes (×850). Freshly prepared drug dilutions were added at 3 and 24 hr after inoculation of sporozoites. Parasite cultures were fixed at 48 hr and stained by an immunoperoxidase assay with a monoclonal antibody 3D11. Drug concentration for CQ and PQ was 3×10^{-5} *M* and was 6×10^{-6} *M* for WR238605 and WR242511.

cytes (Figs. 5 and 6). The *in vitro* EE IC_{50} activities of each drug are summarized in Table II. The mean IC_{50} of PQ was about 0.256×10^{-6} *M*, and one analogue, WR238605, was slightly less active, whereas analogue WR242511 had approximately sixfold higher activity. IC_{50} of PYR, a dihydrofolate reductase inhibitor (0.045×10^{-6} *M*), was comparable to that of WR242511.

DISCUSSION

These results suggest that the rRNA probe-based hybridization technique quantitatively measures the efficacy of tissue schizontocides. The morphology of parasite development determined by microscopy corresponded to the increase of stage-specific type A rRNAs during the period of cultivation, and the peak level of expression occurred when schizonts reached maximum size at 48 hr. Although as few as 10 EE schizonts could be detected by this molecular technique, a practical inoculum of 5×10^4 sporozoites, in which 0.3–0.5% develop to mature EE schizonts, was routinely used for the determination of drug activity. It appears that drug activity in cultures, when the sporozoite infection rate dropped below 0.2%, was lower than that in cultures with infectivity above 0.3% (Table II).

P. berghei sporozoite-induced rodent malaria in mice has been used as an *in vivo* model for the primary screening of candidate tissue schizonticidal compounds. The *P. berghei*–mouse hepatocyte culture system was chosen for applying this rRNA-

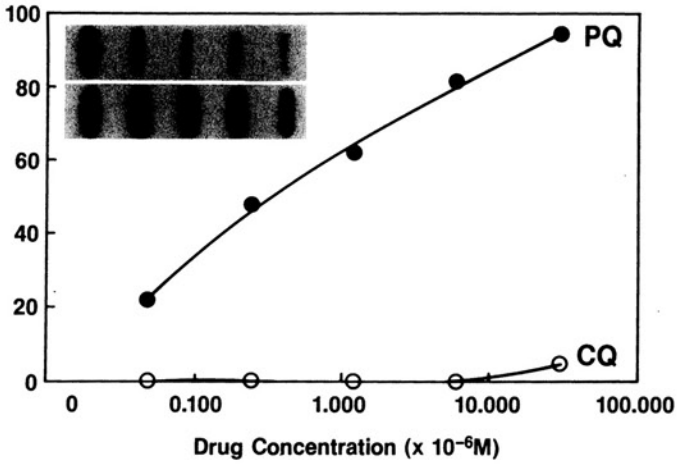

FIG. 6. Inhibitory effects of primaquine (PQ) and chloroquine (CQ) on a type A rRNA increase of *P. berghei* EE parasites in a mouse hepatocyte culture. After a 48-hr exposure of the culture to various drug dilutions, total RNA was hybridized with a ^{32}P-labeled TM4 probe and quantitated by liquid scintillation counting. Percentage inhibition (Y axis) at each concentration (X axis) was compared to control culture without addition of PQ and calculated as: Inhibition rate (%) = $(C_0 - C_d)/C_0 \times 100\%$, where C_0 stands for average counts from infected culture sample without drug, C_d stands for average counts from drug sample, and average background counts from uninfected culture have been subtracted from both C_0 and C_d.

based probe hybridization to evaluate the *in vitro* activity of tissue schizontocides because of the easy maintenance of the infective sporozoites and its short EE cycle which allows culture of functional mouse hepatocytes. By hybridization of culture samples with either parasite-specific or the universal ^{32}P-labeled oligo probe, the EE schizonticidal activity and hepatotoxicity of PQ, WR242511, WR238605, and PYR could be clearly differentiated and quantitated. The EE inhibitory effects obtained by this method were not only comparable with the previous *in vitro* studies on *P. yoelii* (Millet *et al.* 1986), *P. cynomolgi,* and *P. knowlesi* using the microscope-based technique (Fisk *et al.* 1989), but appeared closer to the *in vivo* results. For example, WR242511 exhibited about a 6 times higher schizonticidal effect than PQ in this assay, comparable with an approximate 10-fold higher activity against *P. cynomolgi* in the

TABLE II
IC_{50} of Primaquine (PQ), Two of Its Analogues (WR238605 and WR242511), and Pyrimethamine (PYR) against Exoerythrocytic Stages of *P. berghei* in Mouse Primary Hepatocyte Culture

		IC_{50} ($\times 10^{-6}$ *M*)			
Test	Infectivity[a]	PQ	WR238605	WR242511	PYR
1	0.52%	0.2701	NT[b]	NT	NT
2	0.44%	0.2961	0.6106	0.0618	NT
3	0.18%	0.1280	0.3004	0.0077	NT
4	0.43%	0.3614	0.3468	0.0431	0.0419
5	0.38%	0.2225	0.2963	0.0445	0.0487

[a] Percentage of inoculated sporozoites which grow to mature schizonts.
[b] Not tested.

rhesus monkey model (W. K. Milhous, unpublished data). In contrast with the relative difficulty to standardize the microscopic counting in the presence of the drug-induced parasite morphological changes, the quantitative hybridization signal allows an objective determination of drug activity. In addition, it also allows direct comparison of multiple compounds on the parasite-specific inhibitory activity and host cytotoxicity in the same assessment. Therefore, this rRNA-based *in vitro* drug assay may provide a rapid and accurate way for the primary screening of tissue schizonticidal candidates and for studying potential effective metabolites of PQ and the mechanism of its action for lead-directed chemical synthesis.

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