1987

Characterization Of The 5S And 5.8S Ribosomal Rna Species And Their Genes From The Human Malaria Parasite Plasmodium Falciparum.

Dorothy Elaine Shippen-Lentz
University of Alabama at Birmingham

INFORMATION TO USERS

The most advanced technology has been used to photograph and reproduce this manuscript from the microfilm master. UMI films the original text directly from the copy submitted. Thus, some dissertation copies are in typewriter face, while others may be from a computer printer.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyrighted material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each oversize page is available as one exposure on a standard 35 mm slide or as a 17″ × 23″ black and white photographic print for an additional charge.

Photographs included in the original manuscript have been reproduced xerographically in this copy. 35 mm slides or 6″ × 9″ black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

Accessing the World's Information since 1938

300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA

Order Number 8809574

Characterization of the 5S and 5.8S ribosomal RNA species and their genes from the human malaria parasite *Plasmodium falciparum*

Shippen-Lentz, Dorothy Elaine, Ph.D.

The University of Alabama in Birmingham, 1987

U·M·I
300 N. Zeeb Rd.
Ann Arbor, MI 48106

PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark __✓__.

1. Glossy photographs or pages _____
2. Colored illustrations, paper or print __✓__
3. Photographs with dark background __✓__
4. Illustrations are poor copy __✓__
5. Pages with black marks, not original copy __✓__
6. Print shows through as there is text on both sides of page _____
7. Indistinct, broken or small print on several pages _____
8. Print exceeds margin requirements _____
9. Tightly bound copy with print lost in spine _____
10. Computer printout pages with indistinct print _____
11. Page(s) _____ lacking when material received, and not available from school or author.
12. Page(s) _____ seem to be missing in numbering only as text follows.
13. Two pages numbered _____. Text follows.
14. Curling and wrinkled pages _____
15. Dissertation contains pages with print at a slant, filmed as received _____
16. Other _____

_____

_____

CHARACTERIZATION OF THE 5S AND 5.8S RIBOSOMAL RNA SPECIES AND THEIR GENES FROM THE HUMAN MALARIA PARASITE PLASMODIUM FALCIPARUM

by

DOROTHY E. SHIPPEN-LENTZ

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in the Department of Biology
in the Graduate School, The University
of Alabama at Birmingham

BIRMINGHAM, ALABAMA

1987

ABSTRACT OF DISSERTATION
GRADUATE SCHOOL, UNIVERSITY OF ALABAMA AT BIRMINGHAM

Degree Ph.D Major Subject Biology

Name of Candidate Dorothy E. Shippen-Lentz

Title Characterization of the 5S and 5.8S ribosomal RNA species and their genes from the human malaria parasite Plasmodium falciparum

The ribosomal RNA (rRNA) genes of eukaryotes typically form large multigene families, consisting of hundreds of tandemly arranged units. Previous studies indicate that the rRNA genes of Plasmodium falciparum, the human malaria parasite, are exceptionally low in copy number, heterogeneous in sequence and dispersed in the genome. This dissertation describes experiments designed to investigate the structure and expression of rRNA genes in P. falciparum. In particular, the 5S and 5.8S rRNA molecules and their genes have been characterized. The plasmodial 5S and 5.8S RNA species are typically eukaryotic; however, these components are only distantly related to the 5.8S and 5S rRNA molecules from other organisms. This finding may reflect the lack of sufficient sequence data from other protist groups and, more importantly, other species of the class Sporozoa.

The 5S rRNA genes from P. falciparum are present in only three copies, the lowest number reported for any organism. These domains are clustered at a single chromosomal locus and are abutted by typical Polymerase III (Pol III) termination signals (6-8 consecutive thymidine residues on the non-coding strand). The internal control region of the P. falciparum 5S rRNA gene was recognized by factors in a Pol III cell extract derived from 293 cells and a transcript of approximately 120 nucleotides generated. This finding indicates that the P. falciparum 5S rRNA promoter element and possibly the Pol III transcription apparatus may be similar to those found in other organisms.

The 5.8S rRNA of P. falciparum is encoded by approximately 30 heterogeneous genes which form a predominant transcriptionally active class, and 3-4 low-copy variant gene classes. These minor rDNA components contain multiple point mutations and insertions and appear to be transcriptionally silent pseudogenes. Several lines of evidence suggest that the inactive rRNA genes arose from duplication and translocation of a functional ancestral rRNA transcription unit.

These studies may lead to greater understanding of the mechanisms involved in transcription regulation in P. falciparum.

Abstract Approved by: Committee Chairman Anne Carmel Vezza

Program Director Daniel D. Jones

Date 12/7/87 Dean of Graduate School Kathryn J. Hamel

ACKNOWLEDGMENTS

I feel very fortunate to have been a graduate student at UAB, not only for the excellent scientific training I have received, but also for the opportunity I have had to work with many very fine people. I am very grateful to the members of my committee, Dr. Anne Vezza (my chairperson), Dr. Dunne Fong, Dr. Gary Poirier, Dr. Steve Hajduk and Dr. George Cline for their guidance and assistance during the last four years.

The opportunity to work in the Microbiology Department while maintaining my ties with the Biology Department was made possible by Dr. Daniel Jones, Chairman of the Biology Department. Dr. Jones has been involved with almost every aspect of my graduate career, providing both financial support and the encouragement I needed to succeed.

Also in the Biology Department, I would like to acknowledge Doloris Davis and Madalene Grayson for their help and friendship.

In the Microbiology Department, Dr. Gayle Knapp shared her knowledge of Polymerase III transcription and also provided many helpful discussions concerning my research as well as a great deal of encouragement and enthusiasm about my results.

I have been privileged to be associated with the virologists at UAB who generously provided many helpful discussions and some of the equipment and materials for my research. I am grateful to Dr. Casey Morrow, Dr. Eric Hunter, Dr. Gillian Air, Dr. Dick Compans and all of the members of their groups for their friendship and stimulating conversations during my graduate study.

I would also like to thank Ed Clayson for his tireless instruction and assistance with the Macintosh computer.

In Anne's lab, I have been assisted by an excellent technician and one of my closest friends, Carolyn Grice. I will always be grateful to Anne for making my graduate research

a fulfilling and happy experience. She has given me constant inspiration and encouragement and made me realize that disappointments and setbacks will never defeat me. I feel very fortunate to have been her student.

Finally, I would like to thank my parents for their love and encouragement; and my husband Mike for his support, love and patience.

TABLE OF CONTENTS

	Page
ABSTRACT	ii
ACKNOWLEDGMENTS	iv
LIST OF TABLES	viii
LIST OF FIGURES	ix
LIST OF ABBREVIATIONS	xi
I. INTRODUCTION	1
The life cycle of the malaria parasite	2
Eukaryotic rRNA genes	7
Plasmodial DNA	14
Plasmodial rRNA genes	15
Experimental approach	20
References	22
II. THE THREE 5S rRNA GENES FROM THE HUMAN MALARIA PARASITE PLASMODIUM FALCIPARUM ARE LINKED. Dorothy E. Shippen-Lentz and Anne Carmel Vezza	29
SUMMARY	30
INTRODUCTION	31
MATERIALS AND METHODS	33
Cultivation of parasites	33
Nucleic acid isolation	33
End-labelling and purification of 5S rRNA	34
Southern blot analyses and cloning of 5S rDNA	34
Nucleic acid sequence analyses	35
Copy number analysis	35
Computer analyses	36
RESULTS	36
The plasmodial 5S rRNA transcripts contain heterogeneous 3' termini.	36
The three plasmodial 5S rRNA gene classes are linked	37
P. falciparum contains only three 5S rRNA genes	38
DISCUSSION	40
ACKNOWLEDGEMENTS	43
REFERENCES	56
III. CHARACTERIZATION AND COMPLETE NUCLEOTIDE SEQUENCE OF A 5.8S RIBOSOMAL RNA GENE FROM PLASMODIUM FALCIPARUM. Dorothy Shippen-Lentz, Ratna Ray, John G. Scaife, Gordon Langsley, and Anne Carmel Vezza	59
SUMMARY	61
INTRODUCTION	62

TABLE OF CONTENTS (Continued)

	Page
MATERIALS AND METHODS	63
Cultivation of parasites	63
Preparation of parasitized erythrocytes for RNA isolation	63
End-labelling and purification of 5.8S RNA	64
Plasmid and lambda genomic clones	64
Nucleic acid sequence analyses	64
Analyses of nucleotide sequence data	65
RESULTS	65
Resolution of small molecular weight rRNA species	65
Analysis of the 5.8S rRNA component	66
Nucleic acid sequence analyses	67
Computer and comparative analyses	68
DISCUSSION	68
ACKNOWLEDGEMENTS	71
REFERENCES	81
IV. ANALYSES OF THE 5.8S rRNA CODING DOMAIN OF PLASMODIUM FALCIPARUM rRNA TRANSCRIPTION UNITS: SEQUENCE HETEROGENEITY AND EVIDENCE FOR GENE INACTIVATION.	
Dorothy Shippen-Lentz and Anne Carmel Vezza	84
SUMMARY	85
INTRODUCTION	86
MATERIALS AND METHODS	87
Cultivation of parasites	87
Nucleic acid isolation	88
Isolation of λ clones containing the plasmodial rRNA transcription unit	88
Southern blot analyses	89
Northern blot analyses	89
5.8S rDNA sequence analyses	89
RESULTS	89
Five classes of P. falciparum 5.8S rDNA	89
Heterogeneous 5.8S rRNA coding regions	91
Minor 5.8S rRNA gene classes are not expressed during erythrocyte development	92
DISCUSSION	93
ACKNOWLEDGEMENTS	96
REFERENCES	108
V. DISCUSSION AND SUMMARY	111
The 5S rRNA	114
The 5S rRNA genes	118
The 5.8S rRNA	125
The 5.8S rRNA genes	126
References	137
VI. APPENDIX. 3' TERMINAL NUCLEOTIDE SEQUENCE OF THE 17S RIBOSOMAL RNA SPECIES FROM PLASMODIUM FALCIPARUM	
Dorothy E. Shippen and Anne C. Vezza	141

LIST OF TABLES

Table | Page

I. INTRODUCTION

1 MULTIPLICITY OF rRNA GENES IN SOME EUKARYOTES (per haploid genome) 9

CHARACTERIZATION AND COMPLETE NUCLEOTIDE SEQUENCE OF A 5.8S RIBOSOMAL RNA GENE FROM PLASMODIUM FALCIPARUM

1 D_{AB} VALUES FOR THE 5.8S RNAs of P. FALCIPARUM AND OTHER KNOWN ORGANISMS 80

VI. APPENDIX. 3' TERMINAL NUCLEOTIDE SEQUENCE OF THE 17S RIBOSOMAL RNA SPECIES FROM PLASMODIUM FALCIPARUM

1 3' TERMINAL SEQUENCES OF SOME EUKARYOTIC SMALL rRNAs 148

LIST OF FIGURES

Figure — Page

I. INTRODUCTION

1 The life cycle of Plasmodium 4

2 Eukaryotic rDNA 11

3 Restriction maps of two P. falciparum rRNA transcription units 19

II. THE THREE 5S rRNA GENES FROM THE HUMAN MALARIA PARASITE PLASMODIUM FALCIPARUM ARE LINKED

1 Polyacrylamide gel electrophoresis of the pCp end-labelled low molecular weight RNA species. 44

2 5S rDNA sequences 46

3 Southern Blot analysis of 5S rDNA 48

4 5S clones 50

5 Restriction map of P. falciparum 5S rDNA 52

6 Copy number analysis 54

III. CHARACTERIZATION AND COMPLETE NUCLEOTIDE SEQUENCE OF A 5.8S RIBOSOMAL RNA GENE FROM PLASMODIUM FALCIPARUM

1 Polyacrylamide gel electrophoresis of [^{32}P] pCp end-labelled RNA 72

2 Polyacrylamide gel electrophoresis of purified 5.8S and 5S rRNAs 74

3 Dideoxy sequence analysis of the pGEM-2/rib-2 construct using an oligo-deoxyribonucleotide primer specific for the 3' terminal residues 132 to 152 76

4 Nucleotide sequence alignment 78

IV. ANALYSES OF THE 5.8S rRNA CODING DOMAIN OF PLASMODIUM FALCIPARUM rRNA TRANSCRIPTION UNITS: SEQUENCE HETEROGENEITY AND EVIDENCE FOR GENE INACTIVATION

1 Southern blot analysis of Dra I or Ssp I digested genomic DNA 97

2 Scanning densitometry of genomic Ssp I cleavage products 99

LIST OF FIGURES (Continued)

3 Southern blot analysis of Dra I or Ssp I digestions of the λ recombinants 101

4 Alignment of the 5.8S rRNA coding regions of the λ recombinants 103

5 Northern blot analysis of erythrocytic stage RNA..... 104

6 Southern blot analysis of a Ssp I digestion of the λ recombinants..... 106

V. DISCUSSION AND SUMMARY

1 Agarose gel electrophoresis of P. falciparum RNA 112

2 Possible folding of the P. falciparum 5S rRNA based on the Erdmann 5S rRNA secondary structure model..... 115

3 Nucleotide sequence alignment..... 116

4 Ribosomal RNA biosynthesis 119

5 In vitro transcription analysis of the N2 recombinant..... 123

6 The 5.8S rRNA coding domains of two Dipteran insects..... 128

7 Southern hybridization of restriction digests of P. falciparum chromosomes 130

8 Two correction mechanisms postulated to maintain sequence homogeneity within large multigene families 134

VI. 3' TERMINAL NUCLEOTIDE SEQUENCE OF THE 17S RIBOSOMAL RNA SPECIES FROM PLASMODIUM FALCIPARUM

1 Hairpin structure as proposed by Van Knippenberg et al..... 149

LIST OF ABBREVIATIONS

A	adenine base of a nucleotide
bp	base pair(s)
C	cytidine base of a nucleotide
cDNA	complementary deoxyribonucleic acid
D_{AB}	dissimilarity value
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
EDTA	disodium ethylenediamine-tetraacetic acid
EtBr	ethidium bromide
ETS	external transcribed spacer
G	guanosine base of a nucleotide
HEPES	4-(2-Hydroxyethyl)-1-piperazine ethanesulfonic acid
HSB	high salt buffer
hr	hour
HRPAGE	high resolution polyacrylamide gel electrophoresis
ICR	internal control region
ITS	internal transcribed spacer
LSB	low salt buffer
L-rRNA	large ribosomal RNA species (23-28S)

M	molar
mA	milliampere(s)
min	minute(s)
mM	millimolar
MW	molecular weight
nm	nanometer(s)
pCp	cytidine 3', 5' [^{32}P] biphosphate
PFG	pulsed field gradient
Pol I	RNA polymerase I
Pol II	RNA polymerase II
Pol III	RNA polymerase III
rDNA	ribosomal DNA
RNA	ribonucleic acid
RNase	ribonuclease
r-protein	ribosomal protein
rRNA	ribosomal ribonucleic acid
SDS	sodium dodecyl sulfate
S-rRNA	small ribosomal RNA species (16-18S)
T	thymidine base of a nucleotide
TBE	tris/borate/EDTA buffer
TE	tris-EDTA buffer
tRNA	transfer RNA
U	unit(s)
U	uridine base of a nucleotide
v/w	volume to weight ratio
v/v	volume to volume ratio

µg	microgram
µl	microliter
µM	micromolar

I. INTRODUCTION

Malaria is one of the most devastating human diseases, causing more morbidity and mortality than any other parasitic organism (Beaver et al., 1984). The earliest recorded malaria infections occurred in ancient Egypt, where intermittent fevers were associated with the flooding of the Nile (Halawani and Shawarby, 1957). Hippocrates is credited with the first accurate clinical description of the disease; however, the first successful treatment did not appear until the 1600's when the bark of the cinchona tree was recognized as an antimalarial compound (Wernsdorfer, 1980). The tree's therapeutic action was later attributed to the alkaloid quinine. Since then a variety of synthetic drugs including pyrimethamine and chloroquine have been developed to treat the disease.

In the late nineteenth century, the mosquito vector was identified (reviewed in Wernsdorfer, 1980) and the process of vector control as a means of combating the transmission of malaria was initiated. Early eradication attempts were confined mainly to the aquatic stages of the mosquito life cycle, but later, as more sophisticated insecticides became available, vector control was accomplished by the use of DTT and other chlorinated hydrocarbons. These compounds have been replaced by orthophosphates and cabamates.

Before the widespread use of insecticides, malaria was endemic to every continent except Antarctica; however, eradication efforts continue to be complicated by the emergence of drug resistant strains of the parasite and insecticide resistant mosquito vector. At the present time, at least 106 countries are considered malarious and more than 22% of the total population is at risk for the disease (Wernsdorfer, 1980). Recent statistics indicate that 200 million people are infected annually, and at least two million of these cases result in death (Sturchler, 1984). Children appear to be particularly susceptible; more than one million infants and young children living in tropical Africa die each year from malaria or its complications.

The major focus of malaria research in recent years has been directed toward the development of antimalarial vaccines. A number of potential target antigens have been identified which correspond to the sporozoite, merozoite and gametocyte stages of the parasite life cycle (reviewed in Sturchler, 1984; Newbold, 1984). The host's response to these proteins and their effectiveness as vaccine components are currently under investigation; however, it is evident that a basic understanding of the molecular biology of the parasite will be an integral part of the eradication effort.

The life cycle of the malaria parasite. Malaria is caused by obligate intracellular protozoa of the genus Plasmodium. Plasmodia have a very wide host range and are known to infect at least 100 different animal species including more than twenty non-human primate species as well as rodents, birds and reptiles (reviewed in Garnham, 1980). The four plasmodia which infect humans are P. vivax, P. ovale, P. malariae and P. falciparum. Approximately 90% of the human malarial infections are caused by P. vivax and P. falciparum, although P. falciparum is generally regarded as the most lethal, causing acute severe disease which is often fatal.

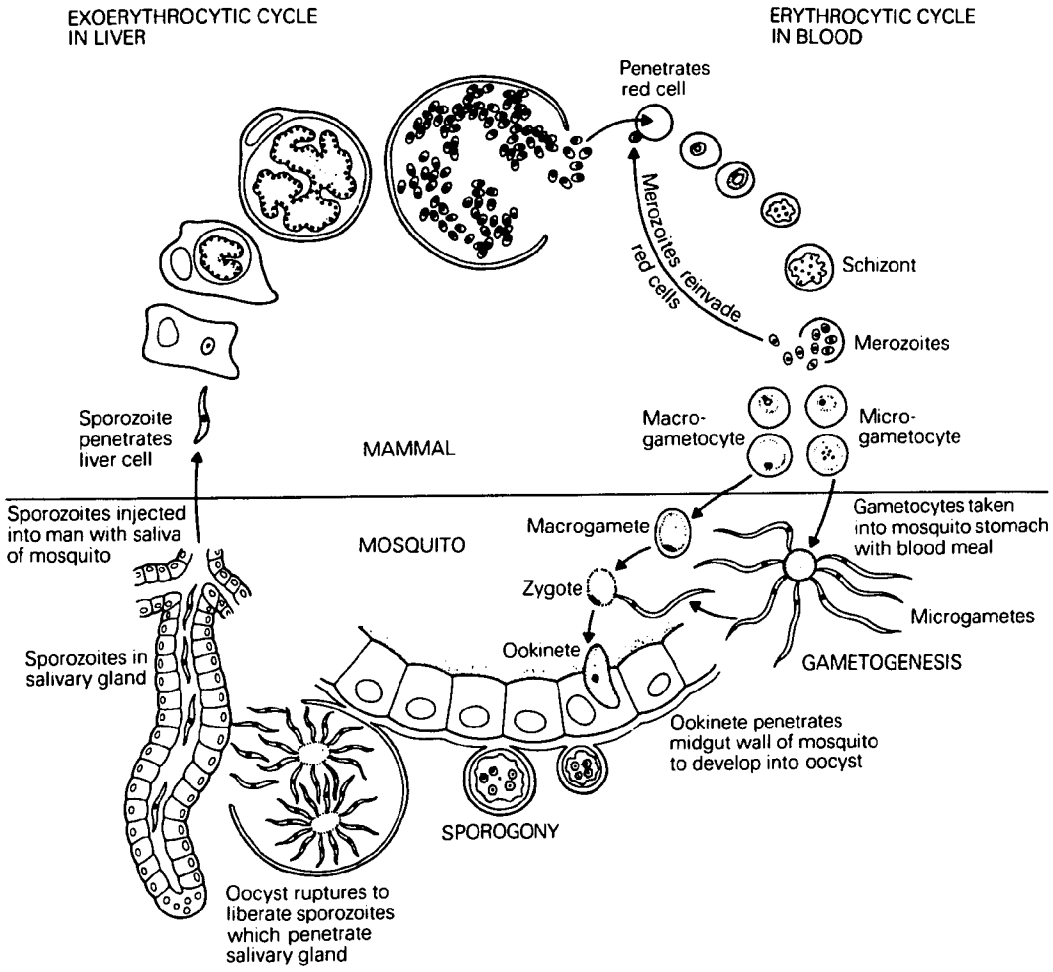
The pathology of malaria is associated exclusively with the erythrocytic stages of parasite development. The characteristic fever and chill cycles correspond directly to the release of merozoites from infected erythrocytes and their subsequent reinvasion into other red cells. In P. falciparum infections, parasitized erythrocytes often adhere to each other and to the endothelial cells lining the capillaries, blocking the circulation in small vessels and limiting the flow of blood to the viscera and other organs. This combination of erythrocyte destruction and obstructed capillaries leads to anemia, anoxia and edema. In acute disease, the liver and spleen become enlarged and congested as parasites accumulate. The lungs, kidneys, and bone marrow may also be affected. One of the most severe manifestations of the disease is cerebral malaria which is characterized by a progressive rise in body temperature followed by delirium and convulsive seizures. Death often occurs within a few hours (Beaver et al., 1984).

Certain genetic conditions, such as sickle cell anemia (Allison, 1963), glucose-6-phosphate-dehydrogenase deficiency (Luzzatto et al., 1969) and the absence of the Duffy blood group factor (Mathews and Armstrong, 1981), appear to influence the severity of malarial infections in humans, although the mechanisms involved remain obscure.

All species of plasmodia have very complex sexual and asexual life cycles, requiring a vertebrate host and an arthropod vector (Figure 1). In the human, the parasite is introduced by an infected female anopheles mosquito during the course of a blood meal. Sporozoites entering the bloodstream will migrate to the liver, invading the parenchyma cells. Here, the sporozoite develops into an exoerythrocytic schizont and its nucleus rapidly divides mitotically to produce many merozoites. For P. falciparum, a single sporozoite will give rise to approximately 40,000 merozoites within 5 to 7 days. These progeny merozoites invade erythrocytes. The duration of this cycle varies from approximately 24 to 72 hours depending on the plasmodial species. In P. falciparum infections the erythrocytic cycle is complete within 48 hours. As the parasite matures within the erythrocyte, several cytologically distinct developmental stages become apparent. Soon after merozoite invasion, a ring-shaped uninucleated structure or early trophozoite appears. This ring stage is characterized by a conspicuous vacuole. In addition, the early trophozoite is capable of ameboid-like movement within the host erythrocyte. As it grows, the parasite feeds on hemoglobin, producing a partially metabolized hemazoin granule. In time, the ameboid movement ceases, the vacuole disappears and the nucleus divides several times, marking the onset of the intraerythrocytic schizont stage. Schizogony occurs and the nuclei are partitioned off into 12 to 24 merozoites. The erythrocyte lyses, releasing merozoites which invade other red cells to perpetuate the cycle.

After several erythrocytic cycles, some of the intracellular merozoites develop into cytologically distinct male microgametocytes or female macrogametocytes. The stimulus for gametocytogenesis is unknown; however, gametocyte formation does not usually occur in vitro. In natural malaria infections, gametocytes are ingested by a susceptible mosquito

Figure 1. The life cycle of Plasmodium. (Cox, 1982).


EXOERYTHROCYTIC CYCLE
IN LIVER
ERYTHROCYTIC CYCLE
IN BLOOD
Penetrates
red cell
Merozoites reinvade
red cells
Schizont
Merozoites
Sporozoite
penetrates
liver cell
MAMMAL
Macro-
gametocyte
Micro-
gametocyte
Sporozoites injected
into man with saliva
of mosquito
MOSQUITO
Macrogamete
Gametocytes taken
into mosquito stomach
with blood meal
Zygote
Microgametes
Ookinete
GAMETOGENESIS
Sporozoites in
salivary gland
Ookinete penetrates
midgut wall of mosquito
to develop into oocyst
SPOROGONY
Oocyst ruptures to
liberate sporozoites
which penetrate
salivary gland

during feeding and are transported to the midgut. The gametocytes rapidly mature into gametes which undergo fertilization to form a diploid zygote. The zygote elongates into a motile ookinete which migrates through the wall of the gut to form a spherical oocyst. A reduction division occurs at some point prior to oocyst maturation such that the thousands of sporozoites developing within the cyst are haploid. Approximately 10 days later, the oocyst bursts releasing sporozoites which disperse through the insect's body. Sporozoites encountering the salivary glands bore through the cells to the acinal ducts. The parasites are then released as the female feeds, thus completing the cycle.

In all, the sexual and asexual life cycles of plasmodia are comprised of at least 11 different developmental stages. Cytologically distinct, these stages are also metabolically distinguishable (Sherman, 1979). The asexual exoerythrocytic development of the parasite within the hepatocyte and sporozoite formation within the oocyst during the sexual life cycle are two stages of parasite development marked by rapid cell division as thousands of progeny are generated within a relatively short period of time. Electron microscopy studies indicate that these stages are characterized by a well-developed endoplasmic reticulum and many ribosomes (Sherman, 1979). Presumably, the rate of protein synthesis increases to meet the demand for cell components. By comparison, parasites living within the host erythrocyte do not appear to be as metabolically active. This stage-specific morphological and physiological diversity makes plasmodia an attractive candidate for the analysis of cellular differentiation and the regulation of gene expression; however, the complex life cycle has not been easily studied in vitro. It was not until 1976 that P. falciparum infected erythrocytes were successfully maintained in vitro (Trager and Jensen, 1976). At the present time no method is available for the in vitro cultivation of gametocytes or sporozoites.

However, the ability to culture P. falciparum erythrocytic stages in the laboratory has been a tremendous technological achievement, paving the way for the analyses of molecular mechanisms involved in replication, transcription and translation of the

plasmodial genes required for growth and multiplication within the host. As a first step toward understanding transcription regulation in plasmodia, the ribosomal RNA (rRNA) genes were chosen for analyses. Ubiquitous and well characterized in many eukaryotic organisms, rRNA genes are a logical choice for studying the structure and transcription of different gene classes in P. falciparum.

Eukaryotic rRNA genes. Eukaryotic ribosomes are composed of four distinct rRNA molecules and approximately 80 ribosomal proteins (r-proteins). Relatively little information is available concerning the eukaryotic r-proteins. In contrast, the rRNA species have been well studied in many eukaryotic organisms. These components consist of a 25-28S molecule, also referred to as the L-rRNA; a 17-18S species or S-rRNA; and two small rRNA components with sedimentation coefficients of 5.8S and 5S. The size of the L-rRNA molecule varies from about 1.2 to 1.7 x 10^6 (3,400 to 5,000 nucleotides) while the S-rRNA species is approximately 0.7 x 10^6 or 2000 nucleotides in length (Attardi and Amaldi, 1970). For most eukaryotes, the 5.8S and 5S rRNA species are about 160 and 120 nucleotides in length, respectively. Analysis of the primary structure of rRNA molecules from a wide variety of organisms reveals a complex pattern of evolutionary conservation reflecting the common functional role of rRNA within the ribosome. For some regions of the rRNA molecules, this sequence conservation is remarkably extensive and has provided a framework for the assessment of phylogenetic relationships among different organisms. The 5S and 5.8S rRNA sequences have been particularly well studied in this regard (Kuntzel et al., 1981; Walker, 1985). The 3' and 5' terminal regions of both the L-rRNA and S-rRNAs also exhibit a high degree of sequence homology among different species (Hadjiolov, 1985).

Ribosome biogenesis requires the coordinated yet independent transcription of three multigene families: one comprised of large transcription units which code for the 25-28S, 17-18S and 5.8S rRNAs; a second gene family for 5S rRNA; and a third set of genes encoding the r-proteins. Each multigene family is transcribed by a different RNA

polymerase, the large rRNA transcription unit by RNA polymerase I, the 5S rRNA genes by RNA polymerase III and, like other protein coding genes, the r-protein genes by RNA polymerase II. All three multigene families must be expressed synchronously for the coordinated assembly of ribosomes. The mechanisms governing this global gene regulation are not known.

Only limited information is available concerning the r-protein genes; however, a few genes have been identified and characterized (Bollen et al., 1981; Vaslet et al., 1980; Meyuhas and Perry, 1980; Bozzoni et al., 1982). These studies suggest that the number of r-protein genes varies for different organisms. Generally speaking, there appears to be an overall increase in copy number of the r-protein genes with evolution (Monk et al., 1981), lower eukaryotes having single copies and mammals having multiple copies. The r-protein gene family does not contain hundreds of tandemly arranged members as is typical for the rRNA gene families (Hyman et al., 1980; D'Eustachio et al., 1981). It is clear, however, that further studies are necessary before general characteristics can be assigned to the genes encoding r-proteins.

The rRNA genes, on the other hand, have been well characterized in a wide variety of organisms. These genes comprise very large families, consisting of hundreds to thousands of tandemly repeated units (Long and Dawid, 1980). This clustering of rRNA genes may enhance their transcriptional productivity by concentrating the appropriate polymerase and cofactors required for rRNA biosynthesis. Typically, rRNA genes are located at specific chromosome loci. The rRNA transcription units form nucleolar organizer regions which constitute the nucleolus, an intranuclear organelle marking the site of rRNA transcription.

The number of rRNA genes roughly correlates with the size of the genome (Table 1); however, rRNA gene copy number is highly variable even among closely related species. Moreover, there appears to be a minimum number of rRNA genes necessary for

TABLE 1

MULTIPLICITY OF rRNA GENES IN SOME EUKARYOTES (per haploid genome)

Species	rRNA Transcription Units	5s rRNA Genes
Physarum polycephalum	80 / 280	690
Neurospora crassa	100 / 180	100
Saccharomyces cerevisiae	100 / 140	150
Saccharomyces carlsbergensis	140	200
Tetrahymena pyriformis	200 / 290	330 / 780
Drosophila hydei	280	320
Drosophila melanogaster	120 / 180 / 100 / 230 / 240	170 / 200 / 180 / 100 / 160
Ambystoma mexicanum	4,100 / 4,500	61,000
Notophtalmus (Triturus) viridescens	5,800 / 5,100	300,000
Triturus cristatus carnifex	5,400	32,000
Xenopus borealis	500	9,000
Xenopus laevis	500 / 600 / 760	24,000 / 9,000
Rattus norvegicus	150 / 170	830
Homo sapiens	200 / 160 / 190 / 50	2,000

survival. Studies with the "bobbed" mutants of Drosophila, flies which possess less than the normal rRNA gene complement, indicate that a critical threshold exists for rRNA gene number. When rRNA gene copy number falls below this level, detrimental phenotypic changes occur which affect growth and development (Tartof, 1975).

In some organisms, a developmentally controlled amplification of the large rRNA transcription units occurs resulting in significant copy number magnification. This differential replication of rRNA genes may be chromosomal or extrachromosomal. rRNA gene amplification has been observed in the oocytes of reptiles (MacGregor, 1982), amphibians (Pardue and Gall, 1969) and insects (Gall, 1969; Bird, 1980) as well as several protozoan species (Gall and Rochaix, 1974; Prescott et al., 1973; Cockburn et al., 1978). The mechanisms involved in rRNA gene amplification and copy number control are poorly understood; however, the differential replication of rRNA genes appears to function in maintaining a critical genetic balance of these genes necessary to accommodate the fluctuating demand for ribosome components (Hadjiolov, 1985).

The organization of the large rRNA transcription unit has been conserved throughout evolution. For all organisms studied to date, the polarity of transcription is 5' end - external transcribed spacer (ETS) - S-rRNA - internal transcribed spacer (ITS) - 5.8S RNA - (ITS)- L-rRNA - 3' end (Figure 2). The size of the rRNA transcription unit can vary from 8 to 13 kb (Long and Dawid, 1980) with the more evolutionarily advanced species having larger rRNA transcription units. Further analyses indicate that the transcribed spacer regions account for the heterogeneity in length (Hadjiolov, 1985).

The signals regulating expression of the rRNA transcription units have not been clearly defined; however, this situation is likely to change rapidly as Pol I in vitro transcription systems become available for a wide variety of eukaryotic organisms. Preliminary studies indicate that like Pol II transcribed genes, Pol I promoter sequences are upstream from the transcription initiation site (reviewed in Mandal, 1984; Hadjiolov, 1985). Putative enhancer sequences have also been identified which appear to increase the

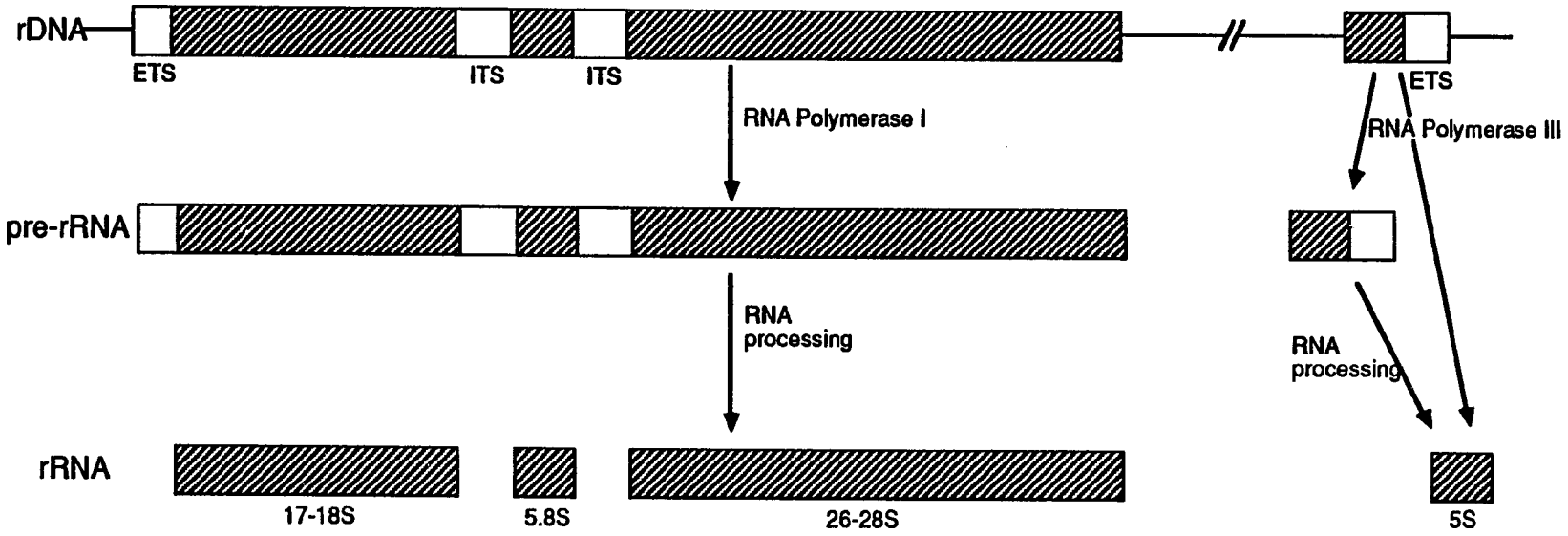


Figure 2. Eukaryotic rDNA.

level of transcription (Labhart and Reeder, 1984; Reeder, 1984). The in vivo product of the rRNA transcription unit is a large precursor molecule which is subsequently processed by endonucleatic cleavage, ligation and nucleotide modification into the mature 25-28S, 17-18S and 5.8S rRNA components.

The rRNA transcription units of some organisms contain introns. In Tetrahymena, the insertions present in the rRNA transcription units are removed by an autocatalytic self-splicing mechanism (Kruger et al., 1982). Similar insertion elements in Drosophila, however, appear to inactivate the rRNA transcription units (Levis and Penman, 1978).

The genes coding for the 5S rRNA species represent a separate multigene family, not usually associated with the large rRNA transcription unit. For most eukaryotes, 5S rRNA gene copy number corresponds to the abundance of the large rRNA transcription units; however, the proportion many vary from one half to many times the number of rRNA transcription units (Selker et al., 1981; Hadjiolov, 1985). Gene amplification has not been demonstrated for any 5S rRNA genes.

The 5S rRNA genes are often located on chromosomes which do not contain nucleolar organizer regions (Hadjiolov, 1985). An exception to this general organization scheme is the 5S rRNA genes of yeast and Dictyostelium which are associated with the large rRNA transcription unit, but are transcribed from the opposite strand (Kramer et al., 1978; Maizels, 1976). Like the rRNA transcription units, 5S rRNA genes are usually arranged in tandem repeats; however, the 5S rRNA genes of Neurospora (Selker et al., 1981) and Schizosaccharomyces (Tabata, 1981) exist as single dispersed genes. Several organisms contain heterogeneous 5S rRNA genes which may be expressed simultaneously, resulting in a mixed population of 5S rRNA molecules (Selker et al., 1985; Piper et al., 1984), or regulated differentially so that only one type is active during a particular stage of development (Peterson et al., 1980; Brown, 1984).

Pol III in vitro extracts have been prepared from many different eukaryotes. Consequently, the signals regulating 5S rRNA transcription have been more precisely

defined than those governing Pol I transcription. In contrast to Pol I or Pol II transcribed genes, the transcriptional control sequence for Pol III genes is located within the coding region (reviewed in Brown, 1984). This signal is not a promoter in the traditional sense and has been designated as an internal control region (ICR). Pol III does not bind directly to the DNA, but interacts instead with a transcription complex which includes a portion of the 5S rRNA coding region and at least three additional transcription factors. One of these proteins, denoted TFIIIA, is required for the formation of a stable transcription complex. The signal for Pol III transcription termination appears to be a stretch of four or more thymidine residues on the non-coding strand (Bogenhagen and Brown, 1981); however, other termination factors may also be required for accurate transcription termination (Rinke and Steitz, 1982). Generally, 5S rRNA genes are transcribed directly into the functional RNA (Figure 2). In some organisms, a precursor 5S rRNA is produced which is processed, usually at the 3' terminus, to yield the mature 5S rRNA molecule (Tekamp et al., 1980).

Both 5S rRNA genes and rRNA transcription units show a high degree of sequence homogeneity within their respective gene families. The conserved function of the rRNA appears to warrant this degree of sequence integrity since a single point mutation can have drastic consequences for the cell (Spangler and Blackburn, 1985). A mechanism which maintains sequence homogeneity among the multiple rRNA gene repeats is therefore not only desirable, but may be mandatory for survival. It has been postulated that such a correction mechanism might involve unequal crossing-over or gene conversion. Both phenomena have been observed with the tandem rRNA repeats of yeast (Petes, 1980; Klien and Petes, 1981).

Presumably, a breakdown in this correction mechanism would result in the accumulation of aberrant rRNA genes. Such divergent genes, termed pseudogenes, have been identified for both 5S rRNA genes (Jacq et al., 1977; Rosenthal and Doering, 1983; McMahon et al., 1984; Reddy et al., 1986) and the large rRNA transcription units (Childs

et al., 1981). Characterized by point mutations, deletions and insertions, rRNA pseudogenes may lie within the tandem repeat (Jacq et al., 1977) or be dispersed as single copies at different chromosome loci (Childs et al., 1981; Rosenthal and Doering, 1983; Reddy et al., 1986). Pseudogenes are normally not transcribed in vivo; however, several 5S rRNA pseudogenes have been transcribed in vitro (Miller and Melton, 1981; Piper et al., 1984). These data suggest that the inactivation of pseudogenes is not entirely the result of an aberrant nucleotide sequence, but may also be influenced by the higher order chromatin structure.

Plasmodial DNA. DNA was identified in the malaria parasite more than forty years ago (Chen, 1944; Deane, 1945). The early studies of plasmodial DNA identified a major and minor component with different bouyant densities and base composition. Analysis of the major DNA component from P. falciparum indicated a bouyant density of 1.697 g/ml and a base composition of 37-38% G+C, values identical to those of the host leukocyte DNA. The bouyant density of the minor component was estimated to be 1.679 gm/ml and a base composition of 19% G+C (Gutteridge et al., 1971). Subsequent experiments demonstrated that with the careful removal of leukocytes from culture material, a single DNA component of 1.673 - 1.680 g/ml (Pollack et al., 1982) and an 18-19% G+C content remained (Pollack et al., 1982; McCutchan et al., 1984). These values are typical of parasitic protozoa (Dore et al., 1980).

The size of the P. falciparum genome has also been disputed. Estimates of 1-2 x 10^7 and 3 x 10^7 bp have been reported which indicates that the genome of P. falciparum is between 3 and 90 times the size of the E. coli genome (Hough-Evans and Howard, 1982; Goman et al., 1982; Pollack et al., 1982). The lower estimate correlates well with the size reported for the genome of the rodent malaria parasite, P. berghei (3.8 times the size of E. coli) (Dore et al., 1980). It seems likely that the larger estimation may be artifactually high as a result of contaminating host cell material.

The DNA of P. falciparum and P. chabaudi have been analyzed by pulsed-field gradient (PFG) gel electrophoresis (Kemp et al., 1985; Van der Ploeg et al., 1985; Langsley et al., 1987), a technique which separates chromosome sized DNA molecules to generate a unique molecular karyotype. Although the size of the chromosomes varied, fourteen chromosomes were identified for both species (Langsley et al., 1987). PFG analyses have revolutionized the study of the plasmodial genome. Already, more than thirty loci have been mapped, including the genes encoding numerous infected erythrocyte surface antigens (Coppel et al., 1984), the secreted (S) antigen (Coppel et al., 1983), and two different classes of P. falciparum rRNA transcription units (Van der Ploeg et al., 1985; Langsley et al.,1987). PFG studies have also revealed that the P. falciparum chromosomes exhibit size polymorphisms. Chromosomes isolated from in vitro culture material and from parasites present in natural malarial infections appear to show dramatic size differences. It has been postulated that these changes reflect large scale genomic rearrangements, particularly deletions and duplications which can result in the alteration of repetitive DNA as well as structural genes (Corcoran et al., 1986).

Plasmodial rRNA genes. Early studies demonstrated that the plasmodial RNA content is approximately five times greater than the DNA content and that rRNA constitutes the majority of the cellular RNA (Whitfeld, 1953; Gutteridge and Trigg, 1970; Vezza and Trager, 1981). One of the first reports on the characterization of plasmodial ribosomes suggested that the large ribosomal subunit was donated by the host cell and the small subunit by the parasite (Tokuyasu et al., 1969). Subsequent analyses disputed this finding demonstrating that plasmodial ribosomes are, in fact, typically eukaryotic in nature with both small and large subunits derived from the parasite (Vezza and Trager, 1981). More recent studies indicate that the plasmodial ribosome is an 80S monomer which can be dissociated in the presence of magnesium into a large 50-60S particle and a small 40-44S particle (Cook et al., 1971; Trigg et al., 1975; Sherman et al., 1975). Preliminary analyses of the rRNA species isolated from plasmodial ribosomes revealed that these components

have relatively low G+C contents of 37-43% when compared to the host rRNA which may be greater than 60% G+C (Sherman et al., 1975; Sherman and Jones, 1977; Miller and Ilan, 1978; Vezza and Trager, 1981). The L-rRNA from P. falciparum has an estimated molecular weight of 1.3-1.49 x 10^6 and the S-rRNA, 0.72-0.78 x 10^6 (Vezza and Trager, 1981; Hyde et al., 1981). Interestingly, the L-rRNA of P. yoelii and P. berghei appear to be specifically nicked to generate two species of 1.2 x 10^6 and 0.3 x 10^6 molecular weight (Miller and Ilan, 1978; Dame and McCutchan, 1983a). The cleavage of the L-rRNA into two fragments has been observed in several other organisms, including trypanosomes and Tetrahymena (Ishikawa, 1977; Eckert et al., 1978). In these species, the two molecules are hydrogen bonded together within the ribosome. A 5S and 5.8S rRNA species have been identified in plasmodial RNA preparations (Dame and McCutchan, 1984b); however, these molecules have not been characterized further.

Analyses of the rRNA genes from plasmodia suggest that these components are unlike other eukaryotic rRNA genes. The first indication that the plasmodial rRNA genes might be unusual came in the late 1960's when electron microscopy showed that plasmodia do not have a well-defined nucleolus (Aikawa et al., 1969). An exception is the avian malaria parasite P. lophurae which appears to contain a compact nucleolus. Several possible explanations for this finding were proposed: 1) rRNA gene copy number is low; 2) the rate of rRNA gene transcription is slow compared to the processing time of the rRNA precursor molecule into mature rRNA; 3) the supply of r-proteins synthesized in the cytoplasm is rate-limiting; 4) the nucleoli are dispersed and therefore not detectable (Sherman, 1979).

The first of these possibilities has been confirmed. For P. berghei (Dame and McCutchan, 1983b), P. lophurae (Unnasch and Wirth, 1983a) and P. falciparum (Langsley et al., 1983), the number of rRNA transcription units has been estimated to be 4 to 8 per haploid genome, the lowest number of rRNA genes reported for any eukaryotic organism. In contrast to other protozoa which have very few rRNA genes, plasmodia do

not appear to amplify their rDNA. Analysis of the asexual erythrocytic life cycle of P. berghei failed to detect the presence of either chromosomal or extrachromosomal amplified rRNA genes (Dame and McCutchan, 1983b). A similar examination of the micro- and macro-gametocytes (Cornelissen et al., 1985; McCutchan, 1986), zygotes and ookinetes also indicated a lack of rRNA gene amplification (McCutchan, 1986). It is important to note, however, that the most metabolically active stages of parasite development, sporozoite formation and exoerythrocytic development within the hepatocyte, have not been assayed for rRNA gene amplification due to the lack of sufficient material for analysis.

In addition to their exceptionally low copy number, the plasmodial rRNA transcription units are also atypical in their genomic arrangement. An examination of the flanking regions surrounding the rRNA transcription units of P. berghei (Dame and McCutchan, 1983b) and P. lophurae (Unnasch and Wirth, 1983) revealed that these genes are not organized in recognizable tandem repeats. In fact, the plasmodial rRNA transcription units appear to be dispersed on numerous chromosomes (Van der Ploeg et al., 1985; Langsley et al., 1987). rRNA genes have been identified on chromosomes 5, 6, 8 and 9 in P. chabaudi and chromosomes 1, 5, 6, 7, 11 and 13 in P. falciparum (Langsley et al., 1987; Wellems et al., 1987).

A further indication of the unusual nature of the plasmodial rRNA transcription units was discovered when the rDNA from several species of plasmodia was isolated and characterized by restriction endonuclease digestion analyses. For each species examined, the rRNA genes exhibited sequence heterogeneity in both spacer and coding region. The four rRNA transcription units of P. berghei could be distinguished by their restriction endonuclease digestion profiles and were categorized into two transcription classes (Dame and McCutchan, 1983b). The rRNA transcription units of P. lophurae which also showed marked sequence heterogeneity have been placed into four gene classes (Unnasch and Wirth, 1983a). The rRNA transcription units of P. falciparum form two to

threetranscriptional classes (Langsley et al., 1983). A restriction map for a portion of two P. falciparum rRNA genes is shown in Figure 3.

The extent of heterogeneity between the different plasmodial rRNA gene classes has not been determined; however, it has been reported that some gene classes contain regions of nonhomology within the mature rRNA species which may constitute introns. These insertions have been identified in both the S-rRNA and L-rRNA coding regions of P. lophurae (Unnasch and Wirth, 1983b). DNA sequence analyses of the large insertion element present in the L-rRNA coding region detected some homology with the Drosophila transposable element copia (Unnasch et al., 1985). It is not known whether the transcription units containing these insertions are active, although it has been suggested that not all of the plasmodial rRNA genes are expressed. Of the four rRNA transcription units identified in P. berghei, only two appear to be transcribed as shown by S_1 nuclease protection studies (Dame et al., 1984a).

The structure of the plasmodial rRNA genes is similar to other eukaryotic rRNA transcription units with the polarity of transcription being 5' end - S-rRNA - ITS - 5.8S rRNA - ITS - L-rRNA - 3' end (Dame and McCutchan, 1983b; Unnasch et al., 1985). However, the size of the plasmodial rRNA transcription units has not been determined and nothing is known about the transcription initiation or termination signals. An early report suggests that a large rRNA molecule, similar to the rRNA precursors found in other eukaryotes, is present in P. knowlesi and P. falciparum RNA preparations (Trigg et al., 1975; Hyde et al., 1981). The nature of these putative precursor rRNAs has not been determined nor have any processing intermediates been reported.

In summary, the previous studies indicate that the plasmodial rRNA transcription units are unlike their eukaryotic counterparts in terms of copy number, arrangement and sequence homogeneity. One of the more intriguing aspects to emerge from these findings is the idea that not all of the plasmodial rRNA transcription units may be expressed. If this is true, then the actual number of rRNA genes which contribute to the rRNA pool is lower

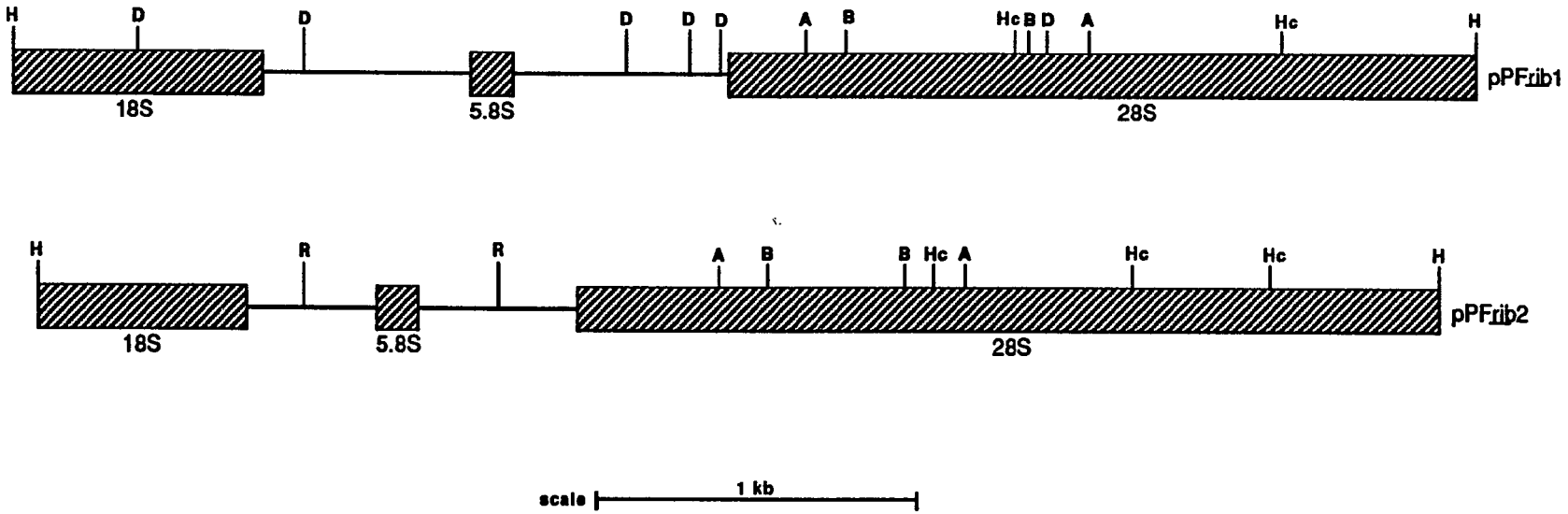


Figure 3. Restriction maps of two P. falciparum rRNA transcription units (adapted from Langsley et al., 1983).

than previously reported. The reasons one gene should be transcribed and another silent are not clear. Perhaps the expression of the plasmodial rRNA genes is differentially regulated, such that one gene class is transcribed during asexual development and another during the sexual life cycle. On the other hand, if all of the rRNA genes are transcriptionally active, one would predict that the rRNA species produced would be heterogeneous, since the genes from which they are derived exhibit extensive sequence variation.

Nothing is known about the 5S rRNA genes from plasmodia. One report has been published which indicates that 5S rRNA genes of P. berhgei is not closely associated with the large rRNA transcription units (Dame and McCutchan, 1984b). However, these 5S rRNA genes have not been isolated to determine if they also exhibit exceptionally low copy number, dispersion and sequence heterogeneity.

Experimental approach. To study the rRNA transcription units of P. falciparum, the 5.8S rRNA and its coding region were chosen for analyses. The 5.8S rRNA species is easily isolated and has been well characterized from a variety of eukaryotic organisms. A comparative analysis of the 5.8S rRNA sequence with the 5.8S rRNA coding regions from different classes of rRNA genes may help to identify which rRNA transcription units are expressed. In addition, these studies may also provide some information on the extent of heterogeneity between the rRNA transcription units. Like the 5.8S rRNA, a great deal is known about the 5S rRNA species and its genes from many different organisms. The characterization of these components in P. falciparum will provide important information about the gene expression of an independent yet coordinately regulated multigene family.

This dissertation is divided into several sections. Following the introduction are three manuscripts, the first of which describes the isolation and characterization of the 5S rRNA and the 5S rRNA genes from P. falciparum. The second manuscript details the analysis of the 5.8S rRNA of P. falciparum and demonstrates how this RNA species is related to other 5.8S rRNA molecules. The third manuscript concerns the characterization

of the 5.8S rRNA coding region from different classes of P. falciparum rRNA transcription units and the identification of transcriptionally active and inactive rRNA genes. A summary and discussion of the results follow the third manuscript. A fourth manuscript, added as an appendix, describes the characterization of the 3' terminal region of the P. falciparum 17S rRNA, one of the most evolutionarily conserved regions identified in eukaryotic rRNAs.

References

Aikawa, M., Huff, C. G., and Spinz, H. (1969). Comparative fine structure study of the gametocytes of avian, reptilian, and mammalian malarial parasites. J. Ultrastruct. Res. 26, 316-331.

Allison, A. C. (1963). Inherited factors in blood conferring resistance to protozoa. In "Immunity to Protozoa: A Symposium" (Garnham, P. C. C., Pierce, A. E., and Roitt, A., eds.) Blackwell Scientific Publications, Oxford.

Attardi, G., and Amaldi, F. (1970). Structure and synthesis of ribosomal RNA. Ann. Rev. Biochem. 39, 183-226.

Beaver, P. C., Jung, R. C., and Cupp, E. W. (1984). Malaria parasites and piroplasms. In "Clinical Parasitology" Lea and Febiger Press, Philadelphia.

Bird, A. P. (1980). Gene reiteration and gene amplification. In "Cell Biology" Vol. 3. (Goldstein, L., and Prescott, D. M., eds.) pp. 62-113. Academic Press, New York.

Bogenhagen, D. F., and Brown, D. D. (1981). Nucleotide sequences in Xenopus 5S DNA required for transcription termination. Cell 24, 261-270.

Bollen, G. H., Choen, L. H., Mager, W. H., Jenneskens, W. H., Planta, L. W., and Planta, R. J. (1981). Isolation of cloned ribosomal protein genes from the yeast Saccharomyces carlsbergensis. Gene 14, 279-287.

Bozzoni, I., Tognoni, A., Pierandrei-Amaldi, P., Beccari, E., Buongiorno-Nardelli, M., and Amaldi, F. (1982). Isolation and structural analysis of ribosomal protein genes in Xenopus laevis. J. Mol. Bio. 161, 353-371.

Brown, D. D. (1984). The role of stable complexes that repress and activate eukaryotic genes. Cell 37, 359-365.

Chen, T. T. (1944). The nuclei in avian malaria parasites. I. The structure of nuclei in Plasmodium elongatum with some considerations on technique. Am. J. Hyg. 40, 26-34.

Childs, G., Maxson, R., Cohn, R. H., and Kedes, L. (1981). Orphons: Dispersed genetic elements derived from tandem repetitive genes of eucaryotes. Cell 23, 651-663.

Cockburn, A. F., Taylor, W. C., and Firtel, R. A. (1978). Dictyostelium rDNA consists of non-chromosomal palindromic dimers containing 5S and 36S coding regions. Chromosoma 70, 19-29.

Cook, R. T., Rock, R. C., Aikawa, M., Fournier, M. J. (1971). Ribosomes of the malarial parasite, Plasmodium knowlesi - 1. Isolation, activity and sedimentation velocity. Comp. Biochem. Physiol. 39B, 897-913.

Coppel, R. L., Cowman, A. F., Lingelbach, K. R., Brown, G. V., Saint, R. B., Kemp, D. J., and Anders, R. F. (1983). Isolate-specific S antigen of Plasmodium falciparum contains a repeated sequence of eleven amino acids. Nature 306, 751-756.

Coppel, R. L., Cowman, A. F., Anders, R. F., Bianco, A. E., Saint, R. B., Lingelbach, K. R., Kemp, D. J., and Brown, G. V. (1984). Immune sera recognize on erythrocytes a Plasmodium falciparum antigen composed of repeated amino acid sequences. Nature 310, 789-791.

Corcoran, L. M., Forsyth, K. P., Bianco, A. E., Brown, G. V., and Kemp, D. J. (1986). Chromosome size polymorphisms in Plasmodium falciparum can involve deletions and are frequent in natural parasite populations. Cell 44, 87-95.

Cornelissen, A. W. C. A., Langsley, G., Walliker, D., and Scaife, J. (1985). Gametocytogenesis and ribosomal rRNA gene organisation in rodent malarias Plasmodium chabaudi and Plasmodium berghei. Mol. Biochem. Parasitol. 14, 165-174.

Cox, F. E. G. (1982). Parasitic protozoa. In "Modern Parasitology" (Cox, F. E. G., ed) p. 23. Blackwell Scientific Publications, Oxford.

Dame, J. B., and McCutchan, T. F. (1983a). Cloning and characterization of a ribosomal RNA gene from Plasmodium berghei. Mol. Biochem. Parasitol. 8, 263-279.

Dame, J. B., and McCutchan, T. F. (1983b). The four ribosomal DNA units of the malaria parasite Plasmodium berghei: Identification, restriction map and copy number analysis. J. Biol. Chem. 258, 6984-6991.

Dame, J. B., Sullivan, M., and McCutchan, T. F. (1984). Two major sequence classes of ribosomal RNA genes in Plasmodium berghei. Nucl. Acids Res. 12, 5943-5952.

Dame, J. B., and McCutchan, T. F. (1984). Identification of 5S and 5.8S ribosomal RNA molecules and their genes in Plasmodium berghei. Mol. Biochem. Parasitol. 11, 301-307.

Deane, H. W. (1945). Studies on malarial parasites. II. The staining of two primate parasites by the Feulgen technique. J. Cell. Comp. Physiol. 26, 139-145.

D'Eustachio, P, Meyuhas, O., Ruddle, F., and Perry, R. P. (1981). Chromosomal distribution of ribosomal protein genes in the mouse. Cell 24, 307-312.

Dore, E., Birago, C., Frontali, C., and Battaglia, P. A. (1980). Kinetic complexity and repetivity of Plasmodium berghei DNA. Mol. Biochem. Parasitol. 1, 199-205.

Eckert, W. A., Daffenberger, W., Krohne, G., and Franke, W.W. (1978). Introduction of hidden breaks during rRNA maturation and ageing in Tetrahymena pyriformis. Eur. J. Biochem. 87, 607-616.

Francoeur, A. M., Gritzmacher, C. A., Peebles, C. L., Reese, R. T., and Tan, E. M. (1985). Synthesis of small nuclear ribonucleoprotein particles by the malarial parasite Plasmodium falciparum. Proc. Natl. Acad. Sci. U.S.A. 82, 3635-3639.

Gall, J. G. (1969). The genes for rRNA during oogenesis. Genetics 61 (suppl.), 121-132.

Gall, J. G., and Rochaix, J. D. (1974). The amplified rDNA of Dytiscid beetles. Proc. Natl. Acad. Sci. U.S.A. 71, 1819-1823.

Garnham, P. C. C. (1980). Malaria in various vertebrate hosts. In "Malaria" (Kreier, J. P., ed.) pp. 95-144. Academic Press, New York.

Goman, M., Langsley, G., Hyde, J. E., Yankovsky, N. K., Zolg, J. W., and Scaife, J. G. (1982). The establishment of genomic DNA libraries for the human malaria parasite Plasmodium falciparum and identification of individual clones by hybridisation. Mol. Biochem. Parasitol. 5, 391-400.

Gutteridge, W. E., and Trigg, P. I. (1970). Incorporation of radioactive precursors into DNA and RNA of Plasmodium knowlesi in vitro. J. Protozool. 17, 89-96.

Gutteridge, W. E., Trigg, P. I., and Williamson, D. H. (1971). Properties of DNA from some malarial parasites. Parasitology 62, 209-216.

Hadjiolov, A. A. (1985). "The Nucleolus and Ribosome Biogenesis." Springer-Verlag/Wein Press, New York.

Halawani, A., and Shawarby, A. A. (1957). Malaria in Egypt. J. Egypt. Med. Assoc. 40, 753-792.

Hough-Evans, B. R., and Howard, J. (1982). Genome size and DNA complexity of Plasmodium falciparum. Biochimica et Biophysica Acta. 698, 56-61.

Hyde, J. E., Zolg, J. W., and Scaife, J. G. (1981). Isolation and characterization of ribosomal RNA from the human malaria parasite Plasmodium falciparum. Mol. Biochem. Parasitol. 4, 283-290.

Hyman, L. E., McCusker, J., Hereford, L., Haber, J. E., and Rosbash, M. (1980). Mapping of ribosomal protein genes in the yeast Saccharomyces cerevisiae. Eur. J. Cell Biol. 22, 11-18.

Ishikawa, H. (1977). Evolution of rRNA. Comp. Biochem. Physiol. 58, 1-7.

Jacq, C., Miller, J. R., and Brownlee, G. G. (1977). A pseudogene structure in 5S DNA of Xenopus laevis. Cell 12, 109-120.

Kemp, D. J., Corcoran, L. M., Coppel, R. L., Stahl, H. D., Bianco, A. E., Brown, G. V., and Anders, R. F. (1985). Size variation in chromosomes from independent cultured isolates of Plasmodium falciparum. Nature 315, 347-350.

Klien, H. L., and Petes, T. D. (1981). Intrachromosomal gene conversion in yeast. Nature 289, 144-148.

Kramer, R. A., Philippsen, P., and Davis, R. W. (1978). Divergent transcription in the yeast rRNA coding region as shown by hybridization to separated strands and sequence analysis of cloned DNA. J. Mol. Biol. 123, 405-416.

Kruger, K., Grabowski, P. J., Zaug, A. J., Sands, J., Gottschling, D. E., and Cech, T. R. (1982). Self-splicing RNA: Autoexcision and autocyclization of the ribosomal RNA intervening sequence of Tetrahymena. Cell 31, 147-157.

Kumazaki, T., Hori, H., and Osawa, S. (1982). The nucleotide sequence of 5S ribosomal RNA from a sea anemone, Anthopleura japonica. FEBS Lett. 146, 307-310.

Kuntzel, H., Heidrich, M., and Piechulla, B. (1981). Phylogenetic tree derived from bacterial, cytosomal and organelle 5S rRNA sequences. Nucl. Acids Res. 9, 1451-1461.

Labhart, P., and Reeder, R. H. (1984). Enhancer-like properties of the 60/81 bp elements in the ribosomal gene spacer of Xenopus laevis. Cell 37, 285-289.

Langsley, G., Hyde, J. E., Goman, M., and Scaife, J. G. (1983). Cloning and characterization of the rRNA genes from the human malaria parasite Plasmodium falciparum. Nucl. Acids Res. 11, 8703-8717.

Langsley, G., Sibilli, L., Mattei, D., Falanga, P., and Mercereau-Puijalon, O. (1987). Karyotype comparison between P. chabudi and P. falciparum: analysis of a P. chabuadi cDNA containing sequences highly repetitive in P. falciparum. Nucl. Acids Res. 15, 2203-2211.

Levis, R., and Penman, S. (1978). Processing steps and methylation in the formation of the rRNA in cultured Drosophila cells. J. Mol. Biol. 121, 219-238.

Long, E. O., and Dawid, I. B. (1980). Repeated genes in eucaryotes. Ann. Rev. Biochem. 49, 727-764.

Luzzatto, L., Usanga, E. A., and Reddy, S. (1969). Glucose-6-phosphate-dehydrogenase deficient red cells: Resistance to infection by malaria parasites. Science 164, 839-841.

MacGregor, H. C. (1982). Ways of amplifying ribosomal genes. In "The Nucleolus" (Jordan, E. G., and Cullins, C. A., eds.) pp. 129-153. Univ. Press, Cambridge.

Maizels, N. (1976). Dictyostelium 17S, 25S and 5S rDNAs lie within a 38,000 base pair repeated unit. Cell 9, 431-438.

Mandal, R. K. (1984). The organization and transcription of eukaryotic ribosomal RNA genes. Progress in Nucleic Acid Research and Molecular Biology 31, 115-159.

Mathews, H. M., and Armstrong, J. C. (1981). Duffy blood groups and vivax malaria in Ethiopia. Am. J. Trop. Med. Hyg. 30, 299-303.

McCutchan, T. F. (1986). The ribosomal RNA genes of Plasmodium. Int. Rev. Cyt. 99, 295-309.

McCutchan, T. F., Dame, J. B., Miller, L. H. and Bainwell, J. (1984). Evolutionary relatedness of Plasmodium species as determined by the structure of DNA. Science 225, 808-811.

McMahon, M. E., Stamenkovich, D., and Petes, T. D. (1984). Tandemly arranged variant 5S ribosomal RNA genes in the yeast Saccharomyces cerevisiae. Nucl. Acids Res. 21, 8001-8016.

Meyuhas, O., and Perry, R. P. (1980). Construction and identification of cDNA clones for mouse ribosomal proteins: Application for the study of r-protein gene expression. Gene 19, 113-129.

Miller, F. W., and Ilan, J. (1978). The ribosomes of Plasmodium berghei: Isolation and ribosmal ribonucleic acid analysis. Parasitology 77, 345-350.

Miller, J. R., and Melton, D. A. (1981). A transcriptionally active pseudogene in Xenopus laevis oocyte 5S DNA. Cell 24, 829-835.

Monk, R. J., Meyuhas, O., and Perry, R. P. (1981). Mammals have multiple genes for individual ribosomal proteins. Cell 24, 301-306.

Newbold, C. I. (1984). Intraerythrocytic development and antigenicity of asexual malaria parasites. Mol. Biochem. Parasitol. 11, 1-22.

Pardue, M. L., and Gall, J. G. (1969). Molecular hybridization of radioactive RNA to the DNA of cytological preparations. Proc. Natl. Acad. Sci. U.S.A. 64, 600-608.

Peattie, D. (1979). Direct chemical method for sequencing RNA. Proc. Natl. Acad. Sci. U.S.A. 76, 1760-1764.

Peterson, R. C., Doening, J. L., and Brown, D. (1980). Characterization of two Xenopus somatic 5S DNAs and one minor oocyte-specific 5S DNA. Cell 20, 131-141.

Petes, T. D. (1980). Unequal meiotic recombination within tandem arrays of yeast ribosomal RNA genes. Cell 19, 765-774.

Piper, P. W., Patel, N., and Lockheart, A. (1984). Processing of the 3' sequence extensions upon the 5S rRNA of a mutant yeast in Xenopus laevis germinal vesicle extract. Eur. J. Biochem. 141, 115-118.

Pollack, Y., Katzen, A. L., Spira, D. T., and Golenser, J. (1982). The genome of Plasmodium falciparum. I: DNA base composition. Nucl. Acids Res. 10, 539-546.

Prescott, D. M., Murti, K. G., and Bostock, C. J. (1973). Genetic apparatus of Stylonichia. Nature 242, 597-600.

Ravetch, J. V., Young, J., and Poste, G. (1985). Molecular genetic strategies for the development of anti-malarial vaccines. Biotechnology 3, 729-740.

Reddy, R., Henning, D., Rothblum, L., and Busch, H. (1986). Some gene variants for 5S RNA are dispersed in the rat genome. J. Biol. Chem. 261, 10618-10623.

Reeder, R. H. (1984). Enhancer and ribosomal gene spacers. Cell 38, 349-351.

Reese, R. T., Langreth, S. C., and Trager, W. (1979). Isolation of stages of the human malaria parasite Plasmodium falciparum from culture and from animal blood. Bull. W.H.O. 57 (suppl. 1), 53-61.

Rinke, J., and Steitz, J. A. (1982). Precursor molecules of both human 5S ribosomal RNA and transfer RNAs are bound by a cellular protein reactive with anti-La lupus antibodies. Cell 29, 149-159.

Rosenthal, D. S., and Doering J. L. (1983). The genomic organization of dispersed tRNA and 5S RNA genes in Xenopus laevis. J. Biol. Chem. 258, 7402-7410.

Selker, E. U., Stevens, J. N., and Metzenberg, R. L. (1985). Heterogeneity of 5S RNA in fungal ribosomes. Science 227, 1340-1343.

Selker, E. U., Yanofsky, C., Driftmier, K., Metzenberg, R. L., Alzner-DeWeerd, B., and RajBhandary, U. (1981). Dispersed 5S RNA genes in N. crassa: Structure, expression and evolution. Cell 24, 819-828.

Sherman, I. W. (1979). Biochemistry of Plasmodium (malaria parasites). Microbiol. Rev. 43, 453-495.

Sherman, I. W., Cox, R. A., Higginson, B., McLaren, D. J., and Williamson, J. (1975). The ribosomes of the simian malaria, Plasmodium knowlesi. Isolation and characterization. J. Protozool. 22, 568-572.

Sherman, I. W., and Jones, L. A. (1977). The Plasmodium lophurae (avian malarial) ribosome. J. Protozool. 24, 331-338.

Sinden, R. E. (1978). Cell biology. In "Rodent Malaria". (Killick-Kendrich, R., and Peters, W., eds.) pp. 85-168. Academic Press, London.

Southern, E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-517.

Spangler, E. A., and Blackburn, E. H. (1985). The nucleotide sequence of the 17S ribosomal RNA gene of Tetrahymena thermophila and the identification of point mutations resulting in resistance to the antibiotics paromomycin and hygromycin. J. Biol. Chem. 260, 6334-6340.

Sturchler, D. (1984). Malaria prophylaxis in travellers: The current position. Experientia 40, 1357-1362.

Tabata, S. (1981). Nucleotide sequences of the 5S ribosomal RNA genes and their adjacent regions in Schizosaccharomyces pombe. Nucl. Acids Res. 9, 6429-6437.

Tartof, K. D. (1975). Redundant genes. Ann. Rev. Genet. 9, 355-385.

Tekamp, P. A., Garcea, R. L., and Rutter, W. J. (1980). Transcription and in vitro processing of yeast 5S rRNA. J. Biol. Chem. 255, 9501-9506.

Tokuyasu, D., Ilan, J., and Ilan, J. (1969). Biogenesis of ribosomes in Plasmodium berghei. Military Medicine 134, 1032-1038.

Trager, W., and Jensen, J. B. (1976). Human malaria parasites in continuous culture. Science 193, 673-675.

Trigg, P. I., Shakespeare, S. B., and Kyd, S. I. (1975). Ribonucleic acid synthesis in Plasmodium knowlesi maintained both in vivo and in vitro. Parasitology 71, 199-209.

Unnasch, T. R., and Wirth, D. F. (1983a). The avian malaria Plasmodium lophurae has a small number of heterogeneous rRNA genes. Nucl. Acids Res. 11, 8443-8459.

Unnasch, T. R., and Wirth, D. F. (1983b). The cloned genes of P. lophurae: a novel rDNA structure. Nuc. Acids Res. 11, 8460-8472.

Unnasch, T. R., McLafferty, M., and Wirth, D. F. (1985). The primary structure of the rRNA insertions of Plasmodium lophurae. Mol. Biochem. Parasitol. 16, 149-161.

Van der Ploeg, L. H. T., Smits, M., Ponnudurai, T., Vermeulen, A., Meuwissen, J. H. E. T., and Langsley, G. (1985). Chromosome-sized DNA molecules of Plasmodium falciparum. Science 229, 658-661.

Vaslet, C., O'Connell, P., Izquierdo, M., and Rosbash, M. (1980). Isolation and mapping of a cloned ribosomal protein gene of Drosophila melanogaster. Nature 285, 647-676.

Vezza, A. C., and Trager, W. (1981). Preliminary characterization of the major RNA species from the human malaria parasite Plasmodium falciparum. Mol. Biochem. Parasitol. 4, 149-162.

Walker, W. F. (1985). 5S and 5.8S Ribosomal RNA sequences and protist phylogenetics. BioSystems 18, 269-278.

Wellems, T. E., Wallider, D., Smith, C. L., de Rosario, V. E., Maloy, W. L., Howard, J., Carter, R., and McCutchan, T. F. (1987). A histidine-rich protein gene marks a linkage group favored strongly in a genetic cross of Plasmodium falciparum. Cell 49, 633-642.

Wernsdorfer, W. H. (1980). The importance of malaria in the world. In "Malaria" (Kreier, J. P., ed.) pp. 1-79. Academic Press, New York.

Whitfeld, P. R. (1953). Nucleic acids in erythrocytic stages of a malaria parasite. Nature, 169, 751-752.

Wilde, D. C. (1986). Pseudogenes. CRC Critical Reviews in Biochemistry 19, 323-352.

II. THE THREE 5S rRNA GENES FROM THE HUMAN MALARIA PARASITE PLASMODIUM FALCIPARUM ARE LINKED

Dorothy E. Shippen-Lentz[1] and Anne Carmel Vezza[2]

[1]Department of Biology, University College, University of Alabama at Birmingham, Birmingham, AL USA 35294; [2]Department of Microbiology, Schools of Medicine and Dentistry, University of Alabama at Birmingham Birmingham, AL USA 35294

Molecular and Biochemical Parasitology, in press

SUMMARY

The 5S rRNA and rDNA from Plasmodium falciparum have been characterized. The 5S rRNA transcripts isolated from erythrocytic stage parasites are composed of three distinct subclasses, 117 to 119 nucleotides in length, which are identical in sequence with the exception of one or two additional uridine residues at the 3' terminus. Southern blot analysis of genomic DNA identified three 5S rRNA gene classes which are clustered within 1.5 kb of DNA. Cloning and sequence analyses of the 5S rDNA revealed identical coding regions surrounded by divergent A+T rich flanking sequences (>90%). Typical Pol III termination signals (6-8) T residues abut each coding region. Copy number analysis indicates that P. falciparum contains only three 5S rRNA genes, the lowest number reported for any organism.

INTRODUCTION

The eukaryotic ribosomal RNA components are encoded by two distinct multigene families (Long and Dawid, 1980; Hadjiolov, 1985). The 25-28S, 17-18S and 5.8S rRNA species are transcribed from one family as a single rRNA precursor which is subsequently processed to yield the mature rRNA components. In contrast, 5S rRNA is transcribed from a separate multigene family usually not associated with the large transcription units. Ordinarily, both types of genes are present in hundreds to thousands of tandemly arrayed repeating units. In some organisms, developmentally controlled amplification of the large rRNA transcription units occurs resulting in significant copy number magnification. This "dosage repetition" is presumably necessary to meet the tremendous demand for components during ribosome biogenesis. The clustering of rRNA genes may also enhance rRNA production by concentrating the appropriate RNA polymerase and co-factors required for transcription. When the number of rRNA genes falls below a critical threshold, phenotypic changes affecting cell growth and development occur, suggesting that a minimum species specific number of rRNA genes is required for survival (Tartof, 1975).

The number of 5S rRNA genes usually correlates with the the abundance of the large rRNA transcription units; however, the proportion varies in different organisms, ranging from one-half to many times the number of large rRNA transcription units (Hadjiolov, 1985; Selker et al., 1981). In any case, the transcription rate of the 5S rRNA genes must be sufficient to keep pace with that of the large rRNA transcription units, including amplified copies, to ensure that enough 5S rRNA is available for assembly into ribosomes.

The rRNA genes of the malaria parasite, plasmodia, are atypical in many respects (McCutchan, 1986). Studies with the rodent and avian malaria parasites, Plasmodium berghei (Dame and McCutchan, 1983) and P. lophurae (Unnasch and Wirth, 1983a), and

the human malaria parasite, P. falciparum (Langsley et al., 1983), indicate that only four to eight rRNA transcription units are present. These are dispersed throughout the genome on several chromosomes (Van der Ploeg et al., 1985). Although low in copy number, plasmodial rRNA genes do not appear to be amplified, either chromosomally or extrachromosomally during development within the host erythrocyte or the mosquito vector (Dame and McCutchan, 1983; McCutchan, 1986). These data correlate with the finding that plasmodia lack a clearly defined nucleolus. Restriction endonuclease cleavage and Southern blot analyses show that the large rRNA transcription units form two to six distinct classes which are heterogeneous in both spacer and coding regions (Dame and McCutchan, 1983; Unnasch and Wirth, 1983a; Langsley et al., 1983). In addition, intron-like elements have been identified in both the 17S and 26S rRNA coding regions of P. lophurae rRNA genes (Unnasch and Wirth, 1983b). The transcriptional capability of these "intron" containing rRNA transcription units is unknown; however, not all of the plasmodial rRNA transcription units appear to be expressed (Dame et al., 1984). These studies indicate that plasmodia have fewer transcriptionally active rRNA genes than any other organism examined thus far.

Little information is available concerning the 5S rRNA genes or transcripts from plasmodia. A 5S rRNA component of approximately 120 nucleotides has been identified in P. berghei RNA preparations. Southern blot analysis indicated that, like most eukaryotic 5S rRNA genes, plasmodial 5S genes are not associated with the large rRNA transcription unit (Dame and McCutchan, 1984). The 5S rRNA genes have not been previously identified or characterized in any species of plasmodia. To determine whether the malarial 5S rRNA genes exhibit similar dispersion, low copy number and sequence heterogeneity as the large rRNA transcription units, we have isolated and characterized the 5S rRNA genes and transcripts from P. falciparum.

MATERIALS AND METHODS

Cultivation of parasites. The Gambian isolate of P. falciparum (FCR-3) was maintained by serial passage in type A Rh positive human erythrocytes as described previously (Trager and Jensen, 1976) with some modifications (Shippen-Lentz et al., 1987). Parasitized erythrocytes, containing predominantly trophozoites and schizonts, were collected by the gelatin flotation method (Reese et al., 1979).

Nucleic acid isolation. Trophozoite/schizont infected cell pellets were resuspended in 5 volumes of low salt buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4). The nucleic acids were extracted by the addition of 0.1 volume 10% SDS and an equal volume of a phenol/m-cresol/chloroform mixture (Vezza and Trager, 1981). After the addition of 4M NaCl to a final concentration of 400 mM, the nucleic acids were recovered by ethanol precipitation. The majority of the DNA was removed from the sample by spooling onto a pasteur pipet. The remaining nucleic acids, containing the majority of the RNA and some DNA, were precipitated at -20°C overnight. Following centrifugation the pellet was resuspended in sterile water.

High molecular weight DNA was extracted from parasitized erythrocytes by disrupting the cell pellet in DNA lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8, 5 mM EDTA) containing 200 µg/ml predigested Pronase and 1% SDS. Following incubation at 37°C for 15 to 30 min, the sample was extracted with phenol and chloroform. The DNA was precipitated with ethanol, spooled onto a pasteur pipet and immediately resuspended in 2 ml of 10 mM Tris-HCl (pH 8), 5 mM EDTA. After an overnight incubation at 4°C, the preparation was adjusted to 10 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl (pH 8) and digested at 37°C for 45 min with 20 µg/ml DNase-free RNase A. Following extraction with phenol and chloroform, the DNA was precipitated with ethanol, spooled onto a pasteur pipet, resuspended in 10 mM Tris-HCl (pH 8), 1 mM EDTA and stored at 4°C.

End-labelling and purification of 5S rRNA. Approximately 15 to 30 µg of total cellular RNA was 3' end-labelled using T4 RNA ligase (Pharmacia) and 100 µCi of [^{32}P] pCp (Amersham) in a total reaction volume of 30 µl as described previously (Shippen and Vezza, 1986). Following an overnight ligation at 4°C, the sample was diluted with 200 µl of High Salt Buffer (400 mM NaCl, 10 mM Tris-HCl, pH 7.4), phenol extracted, ethanol precipitated and recovered by centrifugation. The pellet was washed with 80% ethanol and redissolved in either 10 mM Tris-HCl (pH 7.4), 1 mM EDTA or sterile water.

The 5S RNA species was purified from other cellular RNA components on a 40 cm x 20 cm x 1.5 mm 10% (w/v) polyacrylamide gel (acrylamide to bis-acrylamide ratio of 38:2) containing 8 M urea in TBE buffer (100 mM Tris-borate, pH 8.2, 2 mM EDTA). The 5S RNA band, visible after autoradiography, was excised from the gel and eluted into 10 mM Tris-HCl (pH 7.6), 500 mM NaCl and 1 mM EDTA (Kumazaki et al., 1982) by first crushing the gel slice through a syringe and then shaking the gel slurry mixture overnight at 37°C. The RNA was recovered by ethanol precipitation of the gel slice supernatant. The sample was recovered by centrifugation, washed with 80% ethanol and resuspended in 100 µl sterile water. The 5S RNA species was further analyzed and purified by high resolution polyacrylamide gel electrophoresis (HRPAGE). Aliquots of 5S RNA, prepared as above, were resolved on a 90 cm x 20 cm x 0.35 mm 15% (w/v) polyacrylamide gel (acrylamide to bis-acrylamide ratio of 29:1) containing 3 M urea in TBE (Selker et al., 1985). The individual 5S RNA species, located by autoradiography, were excised, eluted from the gel and precipitated as stated above.

Southern blot analyses and cloning of 5S rDNA. Approximately 10 µg of plasmodial genomic DNA was digested to completion with an assortment of restriction endonucleases. Following resolution by agarose gel electrophoresis, the DNA was denatured, transferred to nitrocellulose paper (Southern, 1975) and subsequently probed with pCp end-labelled 5S RNA or radiolabelled 5S cDNA. To clone the 5S rDNA, plasmodial genomic DNA, digested to completion with Alu I, was resolved on an agarose

gel and the appropriate sized restriction fragments electroeluted and ethanol precipitated. A partial genomic library was constructed from these fragments which were blunt-end ligated into the Sma I cloning site of the alkaline phosphatase-treated cloning vector pGEM-1 (Promega Biotec, Milwaukee, WI). E. coli MC 1061 transformed cells were screened with radiolabelled 5S cDNA, using the colony hybridization technique (Grunstein and Hogness, 1975).

Nucleic acid sequence analyses. Aliquots of approximately 50 to 100 x 10^3 cpm of purified 5S RNA were cleaved using either the base specific chemical cleavage (Peattie, 1979) or enzymatic method (D'Alessio, 1982). The reaction products were analyzed by polyacrylamide gel electrophoresis. Recombinant DNA inserts were sequenced using the GemSeq dideoxy chain termination sequencing kit (Promega Biotec) and protocol. Since the pGEM vector contained a multiple cloning site flanked by SP6 and T7 RNA polymerase promoters, primers specific for these regions were used in the dideoxy sequencing reactions.

Copy number analysis. Gene copy number was determined as described by Lis and associates (1978). As the copy number control, the A-960 clone, which contains a single 5S RNA gene, was digested with Pvu II and then diluted in TBE containing salmon sperm DNA to give concentrations of 1, 3, 6, 9 or 12 copies of the 5S RNA gene per genome equivalent. The concentration of plasmid standard required to yield the appropriate number of 5S genes was calculated based on a P. falciparum genome size of 2.2 x 10^7 (Goman et al., 1982) base pairs and a 5S RNA gene length of 119 nucleotides. Plasmodial genomic DNA, digested with either Hpa II or Dra I, and the plasmid standards were fractionated on an agarose gel and the fragments transferred to nitrocellulose filters and then probed with radiolabelled 5S cDNA. The degree of hybridization, determined visually and by scanning densitometry, was used to estimate the number of 5S rRNA genes.

Computer analyses. Computer analyses of the 5S rRNA and DNA were performed using an IBM-PC/XT microcomputer and the Beckman Microgenie Sequence analysis program (Queen and Korn, 1984).

RESULTS

The plasmodial 5S rRNA transcripts contain heterogeneous 3' termini. Preliminary electrophoretic analyses of total cellular RNA extracted from erythrocytic stage parasites resolved three to four low molecular weight RNA size classes (Figure 1A). Comparison of their electrophoretic mobilities to those of known RNA markers indicated three of these migrated in positions similar to 5.8S, 5S and tRNAs. The P. falciparum 5.8S rRNA species has been characterized (Shippen-Lentz et al., 1987). The fourth RNA size class which migrates slightly slower than the tRNA species has not been analyzed further; however, it may represent another class of tRNA molecules or a previously unidentified small RNA component.

Heterogeneous 5S rRNA genes have been identified in several organisms. A notable example is the 5S rRNA genes of Xenopus which form three distinct multigene families (Peterson et al., 1980). The two major gene families encode the somatic and oocyte type 5S RNAs which differ by only a few nucleotides (Brown, 1984). In some organisms, heterogeneous 5S rRNA genes are transcribed simultaneously resulting in a heterogeneous population of 5S rRNA transcripts (Selker et al., 1985; Piper et al., 1984). 5S rRNAs may also be heterogeneous in length as a result of inaccurate transcription termination (Bogenhagen and Brown, 1981) or aberrant 5S rRNA precursor processing (Tekamp et al., 1980). To ascertain if plasmodial 5S rRNA transcripts may be the products of different 5S genes, we analyzed gel purified 5S rRNA by high resolution polyacrylamide gel electrophoresis (HRPAGE). Three RNA subclasses were resolved (Figure 1B). Scanning densitometry of in vitro labelled 5S RNA indicated a ratio of 12 : 3 : 1 for 5S 1, 5S 2 and 5S 3, respectively (data not shown). Similar analyses of [^{32}P] Orthophosphate in vivo labelled RNA also identified three 5S RNA subclasses (data not

shown). The three in vitro labelled RNA species were subsequently isolated and compared by RNA sequence analysis. With the exception of one to two additional uridine residues at the 3' terminus, the RNA sequences were identical (data not shown). The plasmodial 5S RNA sequence is shown in boldface type in Figure 2, Panel A. The smallest of the three 5S species, 117 nucleotides in length, terminated at the A residue while the longest, most abundant transcript of 119 nucleotides contained two additional uridine residues.

The three plasmodial 5S rRNA gene classes are linked. Although single dispersed 5S rRNA genes have been identified (Selker et al., 1981; Tabata, 1981), the 5S rRNA genes of eukaryotic organisms are usually arranged in multi-copy tandem arrays. To study the organization of plasmodial 5S rRNA genes, genomic DNA was digested with restriction endonucleases predicted to cleave outside the 5S rRNA coding region (Alu I, Dra I, Hpa II, EcoR I, etc.) based on the RNA sequence data. Southern blot analysis of the digestion products identified three distinct 5S rRNA gene classes, shown by the three small Dra I fragments (Figure 3, Lane C) of approximately 350-400bp. Scanning densitometry of these three fragments revealed that they were present in equal proportion (data not shown). This result was supported by scanning densitometry of the 960bp and 1900bp Alu I fragments (Figure 3, Lane B) and the 6000bp Hpa II fragment (Figure 3, Lane A) from several autoradiograms which indicated a ratio of 1 : 2 : 3, respectively (data not shown). The presence of a single Hpa II fragment suggested that all three 5S rRNA gene classes may be linked.

To analyze these gene classes in greater detail, the 1900 and 960bp Alu I restriction fragments were cloned into pGEM-1. Two recombinant plasmids, designated 10-1 and A-960, respectively, were isolated, digested with an assortment of restriction endonucleases and examined by Southern blot analysis (Figure 4). Dra I digestion of the 10-1 clone resolved two 5S rDNA fragments, designated L9 and S8 (Figure 4, Lane B), while a single band, N2, was resolved from the A-960 clone (Figure 4, Lane F). The electrophoretic mobility of these segments was identical to those identified previously in genomic blots.

The resolution of the L9 and S8 genes from the 10-1 clone demonstrated that at least two of the plasmodial 5S genes are linked. The linkage of the N2 gene remained tentative; however, further evidence for the clustering of all three 5S gene classes was obtained by Southern blot analysis of genomic DNA digested with enzymes predicted to cleave within the 5S coding region, Hae III, Dde I, Ava I (Figure 3, Lanes D, E, F), Mnl I and Sau 3A (data not shown). Two common restriction fragments of 1000 and 650bp were identified in all digests, which suggested that these segments contain the 5S intergenic spacer regions. Restriction endonuclease digestion of the 10-1 recombinant resolved the 1000bp fragment (Figure 4, Lane C), indicating that this segment spanned the S8 and L9 genes and that N2 was separated from its nearest neighbor by approximately 650 bp.

To determine the spatial order of the plasmodial 5S rRNA genes, we first established the orientation of the S8 and L9 genes within the 10-1 clone. This was facilitated by the presence of a single Eco RI site in the 5' flanking region of the L9 gene (Figure 2, Panel B) and a unique Eco RI site in the vector. These sites were also useful in determining the distance of the S8 and L9 coding regions to the terminal Alu I sites. Digestion of the 10-1 clone with Eco RI and subsequent Southern blot analysis resolved two bands of approximately 700 and 4100 bp (Figure 4, Lane D). Of the four possible orientations within the vector, only one, Sp6-S8-L9-T7, could generate this restriction profile. Because the distance between the N2 gene and its nearest counterpart was estimated to be approximately 650 bp, we predicted that the N2 gene must be upstream from the S8 gene and not downstream of L9. Double restriction endonuclease digestion analyses of genomic DNA (Figure 3, Lanes G and H) provided further evidence that N2 was in fact located upstream of S8. The restriction map of the 5S rRNA gene locus in Figure 5 shows the cleavage strategy in greater detail.

P. falciparum contains only three 5S rRNA genes. Previous studies suggest that plasmodia have very few large rRNA transcription units. In this report, we have shown that P. falciparum contains three distinct 5S rRNA gene classes, which are present in equal

proportion and are linked in the parasite genome. To determine whether these three genes actually represent multi-copy gene clusters present at more than one chromosome locus, we estimated the total number of 5S rRNA genes. Known quantities of Dra I (data not shown) or Hpa II digested genomic DNA were compared to A-960 plasmid standards (Figure 6). The A-960 5S clone contains a single gene which was diluted to yield various copy numbers based on a P. falciparum genome size of 2.2×10^7 bp (Goman et al., 1982). Southern blot and scanning densitometry analysis (data not shown) indicated that S8, L9 and N2 are the only 5S genes present.

Because the Dra I restriction fragments containing the S8, L9 and N2 5S genes were small and restriction endonuclease digestion analyses might not determine the full extent of heterogeneity between them, the individual Dra I fragments were subcloned and sequenced. As shown in Figure 2, Panel A, the DNA sequence of all three 5S rRNA coding regions is identical. Although plasmodial genomic DNA is characteristically A+T rich, the 5S flanking regions are exceptionally so (90% A+T) when compared to the coding region. This may be a common feature of plasmodial genes and the genes of other lower eukaryotes, where G+C residues are conserved for coding regions.

Abutting the 3' terminus of each 5S rRNA coding region is a typical Pol III termination signal (Bogenhagen and Brown, 1981), which is six to eight T residues in length. In addition to the polymerase, other termination factor(s) may be required for 5S rRNA transcription termination. A protein, immunoprecipitated by anti-La sera derived from systemic lupus patients, is associated with HeLa cell 5S rRNA transcripts in 7S particles. These transcripts contain one or two additional U residues at their 3' terminus (Rinke and Steitz, 1982). An La-like protein has been immunoprecipitated from P. falciparum cellular extracts and appears to be associated with two low molecular weight RNAs of unknown origin (Francoeur et al., 1985). It is unclear whether these RNAs are related to the 5S RNA species we have identified. Based on these findings, it appears that the 3' terminal heterogeneity observed with the plasmodial 5S rRNA may be a result of Pol

III "stuttering" or inaccurate termination by the polymerase and an additional termination factor, rather than aberrant RNA precursor processing. No evidence for a 5S RNA precursor was found by polyacrylamide gel electrophoresis of either in vitro (Figure 1) or in vivo labelled RNA (data not shown).

The study of 5S rRNA genes has been greatly advanced by the development of Pol III in vitro transcription systems. To determine whether the plasmodial 5S rRNA promoter sequence is recognized by a heterologous RNA polymerase III, the N2 recombinant was tested in Pol III transcription extracts derived from S. cerevisiae or 293 cells, an adenovirus transformed human cell line. The plasmodial 5S rRNA gene was not transcribed in the yeast extract; however, a transcript of approximately 120 nucleotides was generated in the 293 cell reaction (data not shown). This putative 5S rRNA transcript has not been analyzed further. The ability of the plasmodial 5S rRNA gene to be transcribed in a heterologous transcription system suggests that the 5S promoter and possibly the Pol III transcription apparatus of P. falciparum may be similar to those found in other eukaryotes.

DISCUSSION

Plasmodium is a diverse genus of obligate intracellular eukaryotic organisms whose sexual and asexual development requires an insect vector and a vertebrate host. The complex life cycle of the parasite is characterized by many cytologically distinct developmental stages; however, very little is known about the regulation of plasmodial gene expression. The plasmodial genes which encode the 17S, 5.8S and 26S rRNAs are unlike their eukaryotic counterparts. Their dispersion, diversity and exceptionally low copy number appear to place them at a transcriptional disadvantage. In this report, we demonstrate that the 5S rRNA genes of P. falciparum, unlike the large rRNA transcription units, are tightly linked. They exhibit no sequence heterogeneity in their coding regions and appear to contain typical Pol III termination and promoter signals. However, in contrast to other eukaryotic 5S rRNA genes, the 5S rRNA genes of P. falciparum are present in only three copies, the fewest reported for any eukaryotic organism.

Given the paucity of rRNA genes, it is unclear how the parasite is able to meet the demand for 5S rRNA during ribosome biogenesis. The rate of plasmodial protein synthesis varies markedly with the changing environment. In addition, electron microscopy data indicate that the number of ribosomes also varies during parasite development, presumably to accommodate this fluctuation in protein synthesis (McCutchan, 1986). The stability and turnover rate for plasmodial ribosomes is not known. It has been postulated that during the asexual erythrocytic life cycle, transcription of the plasmodial rRNA genes may be greatest after the genome has replicated and that the contribution made by these newly synthesized rRNA genes would be sufficient to meet the requirement for rRNA components (McCutchan, 1986; Dame and McCutchan, 1983). Studies with P. falciparum indicate that rRNA genes are not constitutively expressed during erythrocytic development. [^{32}P] Orthophosphate in vivo labelling studies demonstrate that de novo synthesis of rRNA and tRNA is not initiated until approximately 8-12 hours after merozoites have invaded new erythrocytes. RNA synthesis appears to increase steadily during the remaining 36 hours of the P. falciparum asexual life cycle (Shippen-Lentz and Vezza, unpublished results). It is not clear whether this increase in production results from the activation of newly synthesized rRNA genes or the components required for rRNA transcription are being produced during this 8-12 hour period.

To meet the demand for rRNA it is conceivable that the transcription of plasmodial rRNA genes is magnified by external factors such as enhancer sequences (Labhart and Reeder, 1984; Reeder, 1984), or that the genes are located within "active unlocked" chromatin structure (Hadjiolov, 1985). To address this possibility, the transcription control signals which influence plasmodial rRNA gene expression must first be identified. It is also possible that large quantities of rRNA are not necessary for parasite survival. As with terminally differentiated cells, only a subset of the gene repertoire may be transcribed during a particular developmental stage. The number of transcriptionally active rRNA genes in adult somatic cells is not known and it is possible that a significant proportion may not

be transcribed. Previous studies suggest that a critical threshold exists for the number of rRNA genes required for proper growth and development (Tartof, 1975). The rRNA gene copy number is highly variable among different species (Hadjiolov, 1985) and the acceptable lower limit of rRNA genes for most organisms is unknown. It will be interesting to determine if these few plasmodial rRNA genes represent the absolute minimum number for survival and how the inactivation of one or more rRNA genes affects the growth and development of the parasite.

ACKNOWLEDGMENTS

This study was supported by Public Health Service Grants AI 18767 and AI 20597 from the National Institutes of Health. D.S.-L. is a graduate student supported by a University of Alabama at Birmingham Graduate Fellowship. We thank Dr. Jeff Engler, Director of the Primer Core Facility at UAB, for synthesizing the oligodeoxyribonucleotide primers and Dr. Ranga V. Srinivas for his assistance with scanning densitometry analyses. We also are indebted to Dr. Gayle Knapp at UAB and Dr. Stephen Yoshinaga at U.C.L.A. for their advice and assistance with the in vitro transcription studies.

Figure 1. Polyacrylamide gel electrophoresis of the pCp end-labelled low molecular weight RNA species. Panel A: Total cellular RNA. The arrows indicate the positions of unlabelled E. coli 5S RNA (upper arrow) and yeast tRNAphe (lower arrow) markers. Panel B: Purified 5S RNA resolved by high resolution polyacrylamide gel electrophoresis (HRPAGE).

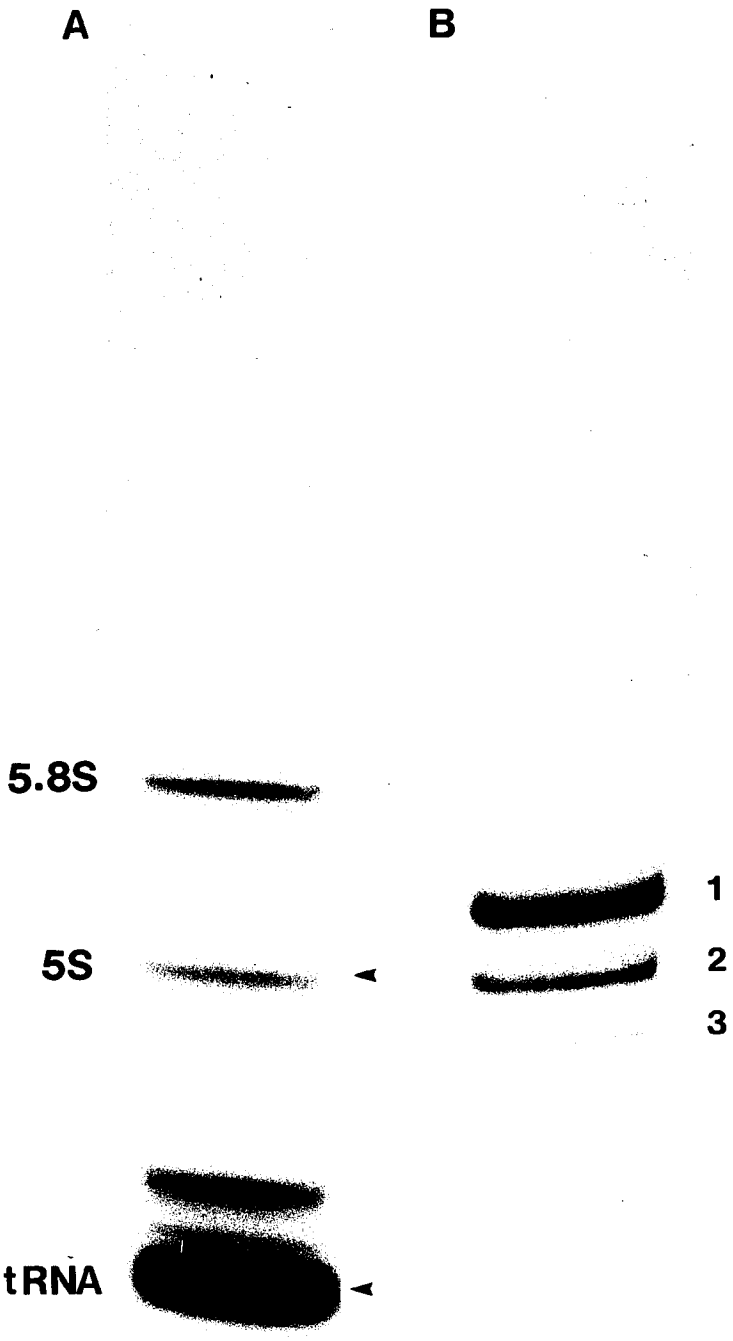

A
B
5.8S
5S
tRNA
1
2
3

Figure 2. 5S rDNA sequences. Panel A. The nucleotide sequence of the three Dra I subclones L9, S8 and N2. Bold nucleotides represent the 5S coding region as determined by RNA sequence analysis. Panel B. Restriction map of the L9, S8 and N2 clones. Bold sites are those found within the coding region. Additional cleavage sites within the gene include Bal I and Cfr I (position 19), Xho II (29), Bin I (30), Rru I (73), Rsa I (74), Mnl I (79), Scr F I (102) and Hph I (106).

A

```
          -140       -130       -120       -110       -100        -90
L9  AAAGGAAATATTTTTTTTGTTCGTAAAATTTTGTATATAATATATATATTATTTATTCAA
S8        AAAATGTATATGTATTAAGAATACAAAATAATTATGCTAATATAAAAGAAGAAA
N2                                 AAATTATTTTTTTTCTTTAACTTATTTAGATA

          -80        -70        -60        -50        -40        -30
L9  TTTTAAGGTCTTATTTTTTTTTATTTTACTATTATTTTAGGAAATTCTTTTTTTATTTTAA
S8  ACTCATTAAATATTTTAATATAGAATGTTTTATATATGATTATTCTTAAATAAATATGAA
N2  TATATTTATACCATATGTACATTAAAAAAATAAATTTATTGTAGAAACAAAAAATTTGAA

          -20        -10          1         10         20         30
L9  TATGGAATTCTATGTACATTATACTTTTGACTCGTTCATACTACAGTGGCCACACCAGAT
S8  TATTAAATTTTAGTGAAGTAAATCTTTTGACTCGTTCATACTACAGTGGCCACACCAGAT
N2  TATTAAATTTTAGTAAAGTAAAACTATTGACTCGTTCATACTACAGTGGCCACACCAGAT

          40         50         60         70         80         90
L9  CCCATCAGAACTCTGAAGTTAAGCACTGTAAGGCTTGGCTAGTACTGAGGTGGGAGACCG
S8  CCCATCAGAACTCTGAAGTTAAGCACTGTAAGGCTTGGCTAGTACTGAGGTGGGAGACCG
N2  CCCATCAGAACTCTGAAGTTAAGCACTGTAAGGCTTGGCTAGTACTGAGGTGGGAGACCG

         100        110          +1        +10        +20        +30
L9  CTCGGGAACACCAGGTGATGAGTCATTTTTTATATAATAAAAATTTTTTTTTTTTTTTTA
S8  CTCGGGAACACCAGGTGATGAGTCATTTTTTTTATATAAAAAAAAATTTTTATTATTTTA
N2  CTCGGGAACACCAGGTGATGAGTCATTTTTTATATAATATAAATTTTTTTTGCAGTAATA

         +40        +50        +60        +70        +80        +90
L9  TTTATATAAAATATATATATATATATATATATATAATATTACATGTAATTAATTGCAATT
S8  TATAATAAAAAAAATAATATATGTTATTATAGATAATTAGTATTTTCATTAATTT
N2  AAAATGTAATATATTTAATGATATAAAATAATAATATTTAATAAATTTATTAATACAAAG

        +100       +110       +120
L9  TATTTAATAAATATATTTTTTTTTATTTAATTT
S8
N2  TACTTTCTAATATGAATTTTTTTTTTTTTTTTTTT
```

B

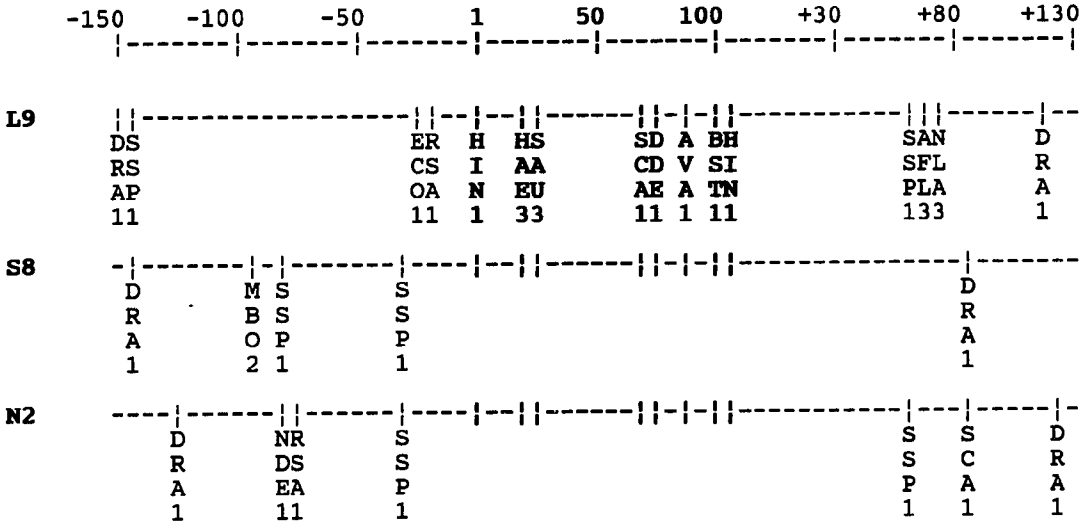


Figure 3. Southern blot analysis of 5S rDNA. Genomic DNA, digested with the following restriction enzymes (A) Hpa II, (B) Alu I, (C) Dra I, (D) Hae III, (E) Dde I, (F) Ava I, (G) Alu I/ Dde I, (H) Alu I/ Hae III, was resolved on a 1.2% agarose gel until the Hind III λ DNA markers (indicated by the arrows) were sufficiently separated.

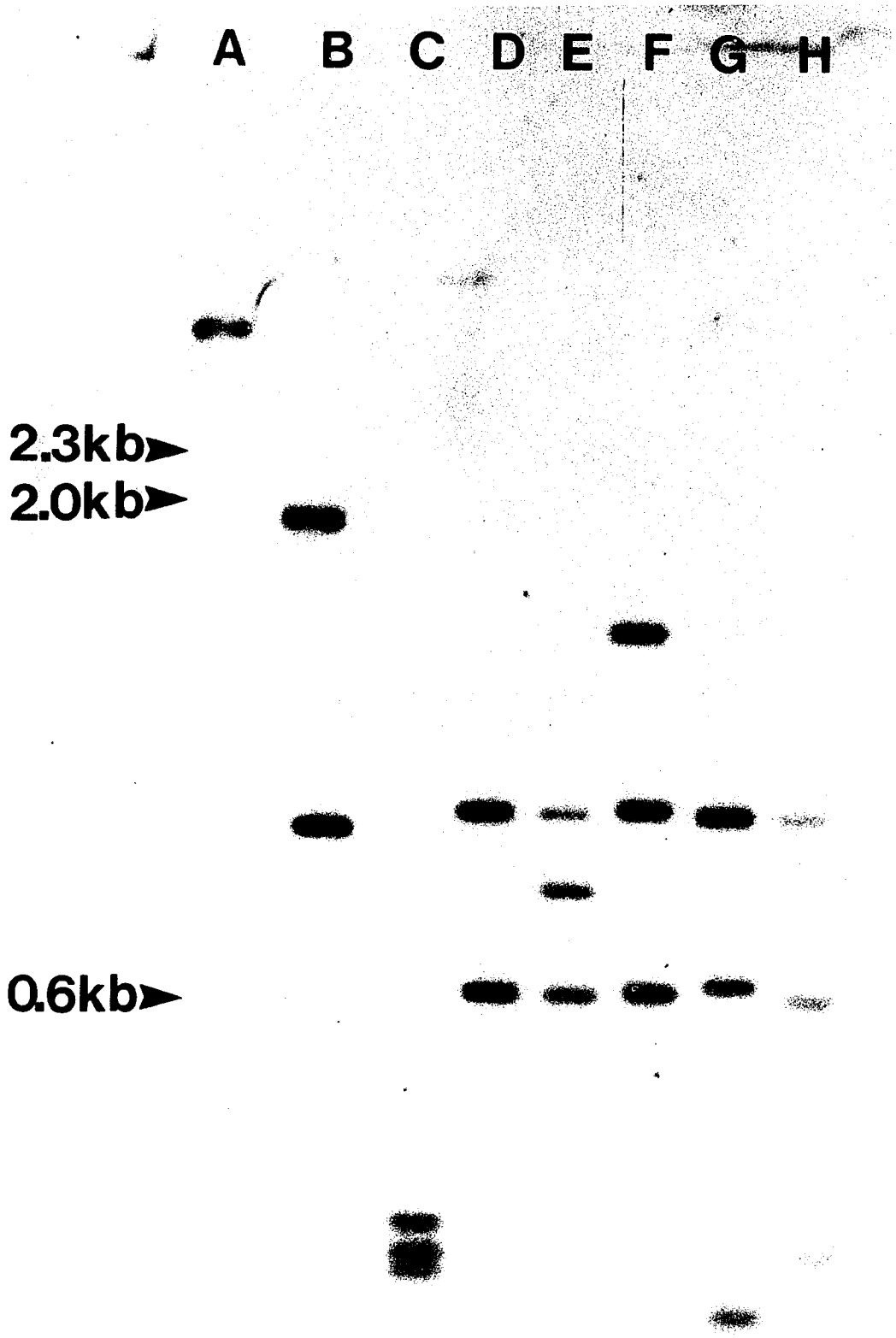

A
B
C
D
E
F
G
H
2.3kb
2.0kb
0.6kb

Figure 4. 5S clones. Recombinant plasmids, 10-1 and A-960, were digested with the following enzymes and examined by Southern blot analysis: (A) Pvu II, 10-1; (B) Dra I, 10-1; (C) Hae III, 10-1; (D) EcoR I, 10-1; (E) Pvu II, A-960; (F) Dra I, A-960. The arrows indicate the position of Hind III λ DNA markers.

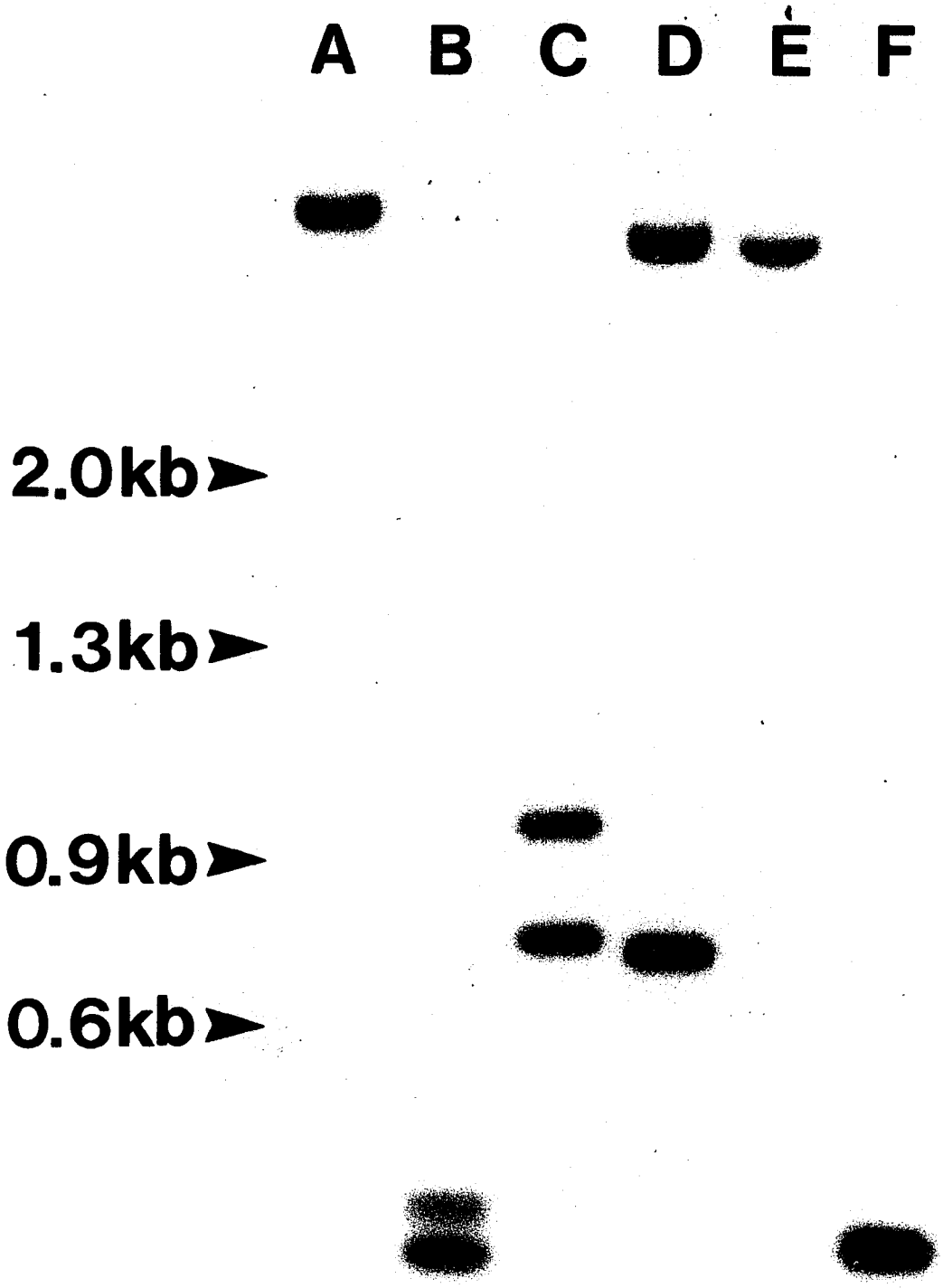

A
B
C
D
E
F
2.0kb
1.3kb
0.9kb
0.6kb

Figure 5. Restriction map of P. falciparum 5S rDNA. Bars below the map indicate the position and length of fragments identified by Southern blot analyses of genomic (Figure 3) and cloned (Figure 4) 5S rDNA.

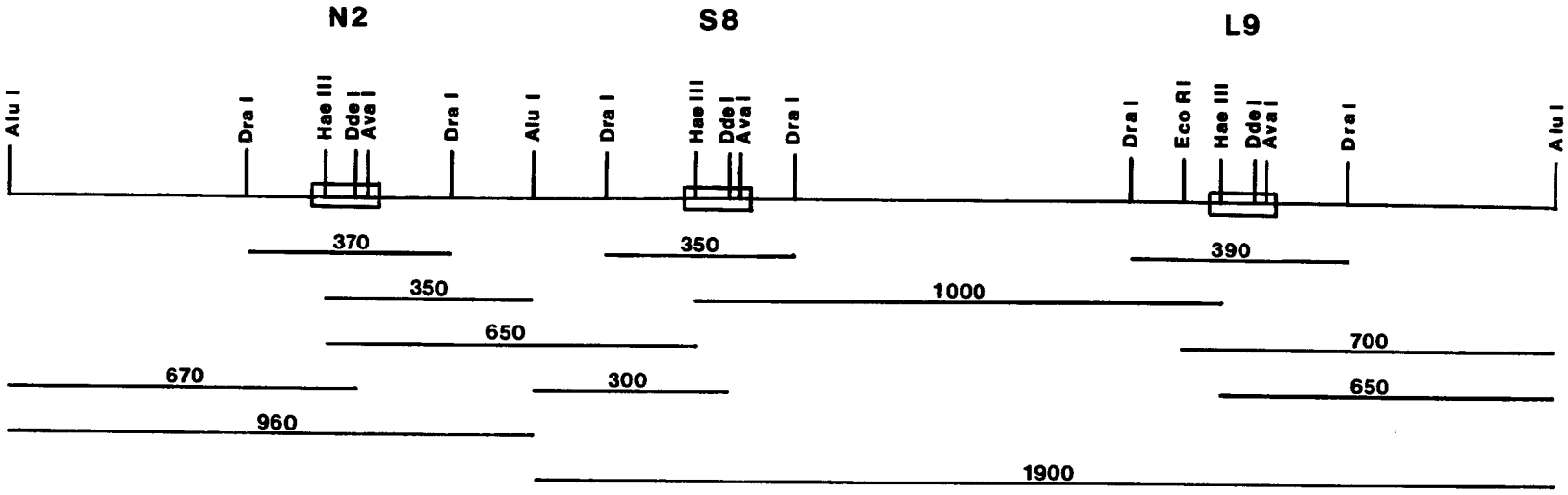
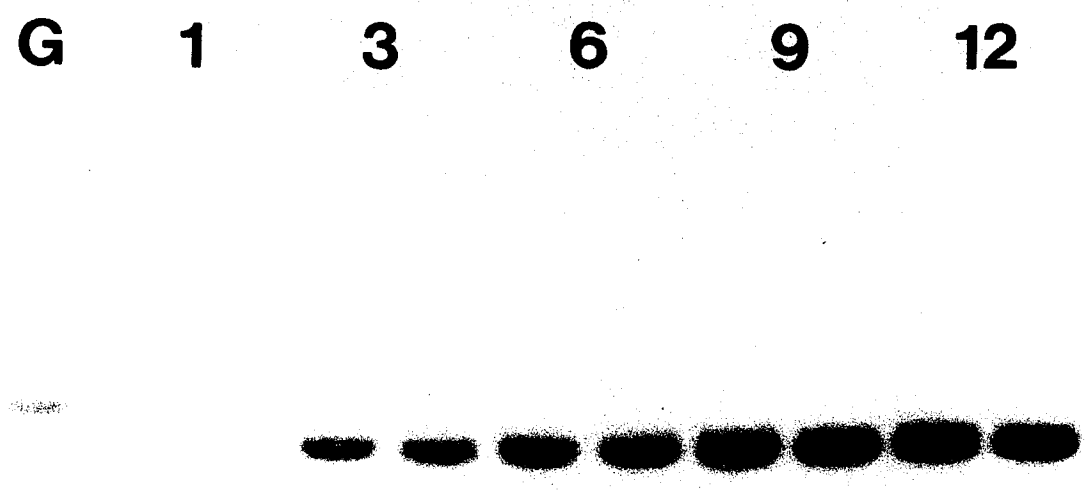

N2
S8
L9
Alu I
Dra I
Hae III
Dde I
Ava I
Dra I
Alu I
Dra I
Hae III
Dde I
Ava I
Dra I
Dra I
Eco RI
Hae III
Dde I
Ava I
Dra I
Alu I
370
350
390
350
1000
650
700
670
300
650
960
1900

Figure 6. Copy number analysis. Appropriate quantities of Hpa II digested genomic DNA (G) and duplicates of Pvu II digested plasmid standards corresponding to (1), (3), (6), (9) or (12) 5S gene copies were resolved on a 0.8% agarose gel and examined by Southern blot analysis. Scanning densitometry of this and several other autoradiograms indicated that approximately three 5S rRNA genes are present.



COPY NUMBER

REFERENCES

Bogenhagen, D. F. and Brown, D. D. (1981). Nucleotide sequences in Xenopus 5S DNA required for transcription termination. Cell 24, 261-270.

Brown, D. D. (1984). The role of stable complexes that repress and activate eukaryotic genes. Cell 37, 359-365.

D'Alessio, J. M. (1982). RNA sequencing. In: "Gel Electrophoresis of Nucleic Acids: A Practical Approach." (D. Rickwood and B. D. Hames, eds.), pp 1760-1764. IRL Press, Oxford.

Dame, J. B., and McCutchan, T. F. (1983). The four ribosomal DNA units of the malaria parasite Plasmodium berghei: Identification, restriction map and copy number analysis. J. Biol. Chem. 258, 6984-6991.

Dame, J. B., and McCutchan, T. F. (1984). Identification of 5S and 5.8S ribosomal RNA molecules and their genes in Plasmodium berghei. Mol. Biochem. Parasitol. 11, 301-307.

Dame, J. B., Sullivan, M., and McCutchan, T. F. (1984). Two major sequences classes of ribosomal RNA genes in Plasmodium berghei. Nucl. Acids Res. 12, 5943-5952.

Francoeur, A. M., Gritzmacher, C. A., Peebles, C. L., Reese, R. T., and Tan, E. M. (1985). Synthesis of small nuclear ribonucleoprotein particles by the malarial parasite Plasmodium falciparum. Proc. Natl. Acad. Sci. U.S.A. 82, 3635-3639.

Goman, M., Langsley, G., Hyde, J. E., Yankovsky, N. K., Zolg, J. W., and Scaife, J. G. (1982). The establishment of genomic DNA libraries for the human malaria parasite Plasmodium falciparum and identification of individual clones by hybridisation. Mol. Biochem. Parasitol. 5, 391-400.

Grunstein, M., and Hogness, D. (1975). Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. U.S.A. 72, 3961-3965.

Hadjiolov, A. A. (1985). The Nucleolus and Ribosome Biogenesis. Springer-Verlag/Wein Press, New York.

Kumazaki, T., Hori, H., and Osawa, S. (1982). The nucleotide sequence of 5S ribosomal RNA from a sea anemone, Anthopleura japonica. FEBS Lett. 146, 307-310.

Labhart, P., and Reeder, R. H. (1984). Enhancer-like properties of the 60/81 bp elements in the ribosomal gene spacer of Xenopus laevis. Cell 37, 285-289.

Langsley, G. Hyde, J. E. Goman, M., and Scaife, J. G. (1983). Cloning and characterization of the rRNA genes from the human malaria parasite Plasmodium falciparum. Nucl. Acids Res. 11, 8703-8717.

Langsley, G. Hyde, J. E. Goman, M., and Scaife, J. G. (1983). Cloning and characterization of the rRNA genes from the human malaria parasite Plasmodium falciparum. Nucl. Acids Res. 11, 8703-8717.

Lis, J. T., Prestidge, L., and Hogness, D. S. (1978). A novel arrangement of tandemly repeated genes at a major heat shock site in D. melanogaster. Cell 14, 901-919.

Long, E. O., and Dawid, I. B. (1980). Repeated genes in eucaryotes. Ann. Rev. Biochem. 49, 727-764.

McCutchan, T. F. (1986). The ribosomal RNA genes of Plasmodium. Int. Rev. Cyt. 99, 295-309.

Peattie, D. (1979). Direct chemical method for sequencing RNA. Proc. Natl. Acad. Sci. U.S.A. 76, 1760-1764.

Peterson, R. C., Doening, J. L., and Brown, D. (1980). Characterization of two Xenopus somatic 5S DNAs and one minor oocyte-specific 5S DNA. Cell 20, 131-141.

Piper, P. W., Patel, N., and Lockheart, A. (1984). Processing of the 3' sequence extensions upon the 5S rRNA of a mutant yeast in Xenopus laevis germinal vesicle extract. Eur. J. Biochem. 141, 115-118.

Queen, C., and Korn, C. J. (1984). A comprehensive sequence analysis program for the IBM personal computer. Nucl. Acids Res. 12, 581-599.

Reeder, R. H. (1984). Enhancer and ribosomal gene spacers. Cell 38, 349-351.

Reese, R. T., Langreth, S.C., and Trager, W. (1979). Isolation of stages of the human malaria parasite Plasmodium falciparum from culture and from animal blood. Bull. W.H.O. 57 (suppl. 1), 53-61.

Rinke, J., and Steitz, J. A. (1982). Precursor molecules of both human 5S ribosomal RNA and transfer RNAs are bound by a cellular protein reactive with anti-La lupus antibodies. Cell 29, 149-159.

Selker, E. U., Stevens, J. N., and Metzenberg, R. L. (1985). Heterogeneity of 5S RNA in fungal ribosomes. Science 227, 1340-1343.

Selker, E. U., Yanofsky, C., Driftmier, K., Metzenberg, R. L., Alzner-DeWeerd, B., and RajBhandary, U. (1981). Dispersed 5S RNA genes in N. crassa: Structure, expression and evolution. Cell 24, 819-828.

Shippen, D. E., and Vezza, A. C. (1986). 3' Terminal nucleotide sequence of the 17S ribosomal RNA species from Plasmodium falciparum. J. Parasitol. 72, 178-181.

Shippen-Lentz, D. E., Ray, R., Scaife, J. G., Langsley, G., and Vezza, A. C. (1987). Characterization and complete nucleotide sequence of a 5.8S ribosomal RNA gene from Plasmodium falciparum. Mol. Biochem. Parasitol. 22, 223-231.

Southern, E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-517.

Tabata, S. (1981). Nucleotide sequences of the 5S ribosomal RNA genes and their adjacent regions in Schizosaccharomyces pombe. Nucl. Acids Res. 9, 6429-6437.

Tartof, K. D. (1975). Redundant genes. Ann. Rev. Genet. 9, 355-385.

Tekamp, P. A., Garcea, R. L., and Rutter, W. J. (1980). Transcription and in vitro processing of yeast 5S rRNA. J. Biol. Chem. 255, 9501-9506.

Trager, W., and Jensen, J. B. (1976). Human malaria parasites in continuous culture. Science 193, 673-675.

Unnasch, T. R., and Wirth, D. F. (1983a). The avian malaria Plasmodium lophurae has a small number of heterogeneous rRNA genes. Nucl. Acids Res. 11, 8443-8459.

Unnasch, T. R., and Wirth, D. F. (1983b). The cloned genes of P. lophurae: a novel rDNA structure. Nuc. Acids Res. 11, 8460-8472.

Van der Ploeg, L. H. T., Smits, M., Ponnudurai, T., Vermeulen, A., Meuwissen, J. H. E. T., and Langsley, G. (1985). Chromosome-sized DNA molecules of Plasmodium falciparum. Science 229, 658-661.

Vezza, A. C., and Trager, W. (1981). Preliminary characterization of the major RNA species from the human malaria parasite Plasmodium falciparum. Mol. Biochem. Parasitol. 4, 149-162.

III. CHACTERIZATION AND COMPLETE NUCLEOTIDE SEQUENCE OF A 5.8S RIBOSOMAL RNA GENE FROM PLASMODIUM FALCIPARUM

Dorothy Shippen-Lentz, [1] Ratna Ray, [2] John G. Scaife, [3]
Gordon Langsley, [3] and Anne Carmel Vezza [2]

[1]Department of Biology, University College, University of Alabama at Birmingham, Birmingham, Alabama 35294; [2]Department of Microbiology, Schools of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, Alabama 35294; [3]Department of Molecular Biology, University of Edinburgh, Edinburgh, United Kingdom

Molecular and Biochemical Parasitology 22 223-231 (1987)

SUMMARY

The 5.8S and 5S rRNA components from the FCR-3/The Gambia strain of Plasmodium falciparum have been identified and the complete nucleotide sequence of a 5.8S ribosomal RNA gene determined. Unlike the 5S rRNA species, the 5.8S is a single homogeneous population of molecules of 157 nucleotides. Comparison of its nucleotide sequence with previously reported 5.8S rRNA sequences indicates that it is homologous to these molecules, but distantly related to them. The sequence of the 5.8S rRNA coding region from the pfrib-2 recombinant of the HG13 Gambian isolate of P. falciparum is identical.

INTRODUCTION

Plasmodium falciparum, like other eukaryotic organisms, synthesizes several species of ribosomal RNA (rRNA) molecules. The 25S, 17S and 5.8S RNA species are contained within a single transcription unit (Langsley et al., 1983; Dame and McCutchan, 1983; Dame and McCutchan, 1984). The 25S and 17S rRNA components have been previously characterized and are typically eukaryotic in nature with molecular weight estimates of 1.3-1.5 x 10^6 and 0.72-0.78 x 10^6, respectively (Vezza and Trager, 1981; Hyde et al., 1981; Shippen and Vezza, 1986). However, unlike other eukaryotic rRNA genes which are present in several hundred to thousands of tandemly arranged transcription units, the rRNA genes of plasmodia are low in copy number (4-6 copies per haploid genome) and dispersed throughout the genome (Langsley et al., 1983; Dame and McCutchan, 1983; Unnasch and Wirth, 1983). In addition, restriction endonuclease digestions have shown that the transcription units are heterogeneous in both spacer and coding regions (Langsley et al., 1983; Dame and McCutchan, 1983; Unnasch and Wirth, 1983).

Little is known concerning the 5S and 5.8S rRNA species from malaria parasites. Recent studies with P. berghei and P. falciparum have identified a 5.8S rRNA component which hybridizes to the rRNA transcription units (Langsley et al., 1983; Dame and McCutchan, 1984). A 5S component has been identified in preparations from P. berghei (Dame and McCutchan, 1984). The relationship of these rRNA species to those of other eukaryotes, as well as other species or isolates of plasmodia, is unknown. In this study we present the complete nucleic acid sequence of the P. falciparum 5.8S rRNA species and demonstrate its relationship to previously reported 5.8S rRNA components.

MATERIALS AND METHODS

Cultivation of parasites. The Gambian isolate of P. falciparum (FCR-3) was maintained by serial passage in type A Rh positive human erythrocytes essentially as described by Trager and Jensen with some modifications (Trager and Jensen, 1976). Parasitized erythrocytes were cultured in 32-ounce glass prescription bottles at a 3-4% human erythrocyte cell suspension using RPMI-1640 medium, without HEPES, but supplemented with 0.22% (w/v) sodium bicarbonate and 10% (v/v) human serum. Cultures were incubated at 38°C in a humidified atmosphere containing 5% CO_2 in air. The medium was changed daily. When the parasitemia exceeded 10%, parasitized red cells were subcultured by the addition of fresh erythrocytes and medium so that the final parasitemia was 1-2% and the final red cell suspension 3-4%.

Preparation of parasitized erythrocytes for RNA isolation. Parasitized erythrocytes containing predominantly trophozoites/schizonts were collected by the gelatin flotation method (Reese et al., 1979). The pellet, containing late stage parasitized erythrocytes, was diluted with fresh cells to a final parasitemia of 2-3% in a 3-4% final red cell suspension. The cultures were returned to clean bottles and incubated at 38°C overnight. When the majority of the parasitized erythrocytes were rings, the cultures were gelatin treated to remove any contaminating lymphocytes. Fresh complete medium, filtered to remove any residual lymphocytes from the serum, was added and the cultures were returned to clean bottles. Following an additional 24 hr incubation period, the parasitized erythrocytes were gelatin treated and the trophozoites/schizonts harvested. The parasitized erythrocytes were washed once in low salt buffer (LSB: 150 mM NaCl, 10 mM Tris-HCl, pH 7.4). The nucleic acids were extracted by the addition of sodium dodecyl sulfate (SDS) (final concentration 1% (w/v)) and an equal volume of a phenol mixture as described previously (Vezza and Trager, 1981). The salt concentration was adjusted to 400 mM NaCl and the nucleic acids were ethanol precipitated overnight at -20°C. Following centrifugation, the

pellet was resuspended in 300 µl high salt buffer (HSB: 400 mM NaCl, 10 mM Tris-HCl, ph 7.4) and the nucleic acids precipitated by the addition of 2 to 3 volumes of ethanol. The DNA, which formed a white gelatinous precipitate, was spooled out on a pasteur pipet. The solution, containing the RNA and some contaminating DNA, was then placed at -20°C overnight. Following centrifugation, the sample was redissolved in 100 µl sterile H_2O.

End-labelling and purification of 5.8S RNA. Approximately 15-30 µg of total cellular RNA was either 3' or 5' end-labelled as described previously (Clerx-Van Haaster and Bishop, 1980; D'Alessio, 1982). The sample was diluted with 200 µl HSB, phenol extracted and ethanol precipitated. After centrifugation, the pellet was washed with 80% (v/v) ethanol and redissolved in either Tris-EDTA buffer (TE: 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) or sterile H_2O. The 5.8S RNA component was purified from other cellular RNA species on a 12% (w/v) polyacrylamide gel containing 8 M urea, 100 mM Tris-borate pH 8.2 and 2 mM EDTA. The 5.8S RNA band, visible after autoradiography, was excised from the gel and eluted into 10 mM Tris-HCl, pH 7.6, 500 mM NaCl and 10 mM EDTA (Kumazaki et al., 1982) by first crushing the gel slice through a syringe and then shaking the gel slurry mixture overnight at 37°C. The RNA was recovered by ethanol precipitation of the gel slice supernatant and resuspended in 100 µl of water.

Plasmid and lambda genomic clones. The large Hind III/Ava I restriction fragment of pfrib-1 or pfrib-2, containing the entire 5.8S rRNA coding region (Langsley et al., 1983), was subcloned into the Riboprobe transcription cloning vector pGEM-1 or pGEM-2 (Promega Biotec, Inc., Madison, WI), respectively. EMBL-3 lambda genomic libraries of the African isolate FCR-3/The Gambia were prepared as outlined previously (Maniatis et al., 1982). The libraries were screened with RNA transcripts of the pGEM-1/rib-1 construct using the plaque hybridization procedure of Benton and Davies (Benton and Davies, 1977).

Nucleic acid sequence analyses. Aliquots of approximately 5-10 x 10^4 cpm of purified 5.8S RNA were subjected to either base specific chemical cleavage as described by

Peattie (Peattie, 1979) or enzymatic cleavage (D'Alessio, 1982) using ribonucleases T_1 (G specific), U_2 (A specific), Phy M (A and U) or Bacillus cereus (U and C). The cleavage products were resolved by polyacrylamide gel electrophoresis as described previously (Peattie, 1979). Dideoxy sequence analyses of an EMBL-3 P. falciparum rDNA genomic clone or the pGEM-2/rib-2 subclone were undertaken using the Promega Biotec Gem Seq sequencing system and protocol.

Analyses of nucleotide sequence data. Analyses of the nucleic acid sequence data were performed on an IBM-PC microcomputer using the MicroGenie sequence analysis program (Beckman Instruments, Inc., Palo Alto, CA) originally developed by Queen and Korn (Queen and Korn, 1984). Evolutionary divergence was measured by calculating the dissimilarity (D_{AB}) values for the P. falciparum 5.8S rRNA nucleotide sequence with those reported for other organisms (Erdmann et al., 1985) using the following formula (De Wachter et al., 1985):

$$D_{AB} = -\frac{3}{4} \ln \left[1 - \frac{4}{3} \left(\frac{S}{I+S} \right) \right] \frac{I+S}{N} + \frac{G}{N}$$

I is the number of alignment positions where sequences A and B contain an identical nucleotide. S is equivalent to the number of positions where A is substituted with respect to B. G is the number of places where A contains a gap and B a nucleotide or vice versa and N is the number of positions where at least one of the sequences contains a nucleotide ($N = I + S + G$).

RESULTS

Resolution of small molecular weight rRNA species. Polyacrylamide gel electrophoresis of pCp end-labelled RNA resolves 4 low molecular weight RNA size classes, as shown in Figure 1 lane C. Comparison of these RNA species with 5S rRNA from Escherichia coli (lane A, $M_r = 4 \times 10^4$) and yeast phenylalanine transfer RNA (lane B, $tRNA^{phe}$, $M_r = 2.5 \times 10^4$) indicates that three of these components migrate in positions similar to 5.8S rRNA, 5S rRNA and tRNAs. The molecular weight estimates for the P.

falciparum RNA species are 5.2 x 10^4 (≈157 nucleotides) for the 5.8S, 3.9 x 10^4 (≈117 nucleotides) for the 5S and 2.6-2.9 x 10^4 (≈75-87 nucleotides) for the tRNAs. The tRNA-like species appear in some gels as 2-3 heterogeneous bands; however, the calculated size range of these molecules agrees with those reported previously for other tRNA species (Erdmann, 1985). The high molecular weight 25S and 17S rRNA components, which migrate as a single faint band indicated by the arrow, label poorly in comparison to these small RNA species. The identity of a fourth component, which migrates slightly slower than the tRNA-like species, is unknown. Small nuclear RNAs have been identified in P. falciparum-infected cell extracts (Francoeur et al., 1985); whether this RNA species belongs to that class or comprises another group of tRNAs is not known.

Analysis of the 5.8S rRNA component. It has been reported that the 5.8S rRNA from the crustacean Artemia is composed of a heterogeneous population of molecules which differ by one to several nucleotides at the 5' terminus of the RNA (Ursi et al., 1982). These discrete subclasses of 5.8S rRNA can be resolved by polyacrylamide gel electrophoresis. Comparable results have been reported for the 5S rRNA species from Neurospora crassa (Selker et al., 1985). We, therefore, undertook similar analyses to determine if the 5.8S, as well as the 5S rRNA, component from P. falciparum was composed of subclasses of RNA. High resolution polyacrylamide gel electrophoresis of purified 5.8S rRNA yielded a single discrete species of RNA, as shown in Figure 2, panel A. However, the 5S rRNA, analyzed in an identical manner (Figure 2, panel B), was resolved into three discrete components. These results indicated that the 5.8S rRNA species comprised a homogeneous population of RNA molecules; while the 5S rRNA species, like the N. crassa 5S species, was heterogeneous. Preliminary sequence data indicates that the 5S rRNAs are identical with the exception of 1 to 2 additional nucleotides at the 3' terminus of the RNA (34th Annual Meeting of the American Society of Tropical Medicine and Hygiene, Nov. 3-7, 1985, Miami, FL).

Nucleic acid sequence analyses. Over the past several years sequence analysis of 5.8S and 5S rRNAs has provided a useful tool for phylogenetic comparisons. However, little data are presently available on some protist groups and no sequence data have been reported for the 5.8S RNA from any species of malaria. Chemical and enzymatic nucleic acid sequence analyses of the 5.8S rRNA were undertaken. Aliquots of purified 3' end-labelled 5.8S rRNA subjected to base specific chemical cleavage; while 5' end-labelled 5.8S rRNA was cleaved enzymatically with either ribonucleases T_1, U_2, Phy M or B. cereus. Following resolution of the samples on 20% (w/v) or 10% (w/v) RNA sequencing gels, the majority of the 5.8S rRNA sequence of 157 nucleotides was obtained (data not shown). Difficulties were encountered in the enzymatic sequence analyses of the RNA. Three nucleotides at positions 32, 33 and 48 could not be determined. In order to identify these residues, as well as confirm the RNA sequence, dideoxy sequence analysis of P. falciparum/EMBL-3 lambda recombinant clone (EMBL-3/rib12), containing the entire 5.8S rRNA coding region (A. Vezza, unpublished results), was undertaken using an oligodeoxyribonucleotide primer specific for the 3' or 5' terminus of the 5.8S coding region. Since heterogeneity in the rRNA coding region has been reported (Langsley et al., 1983; Dame and McCutchan, 1983; Unnasch and Wirth, 1983; Vezza and Trager, 1982), we undertook identical analyses with the pGEM-2/rib-2 recombinant of the HG13 Gambian isolate of P. falciparum to determine if its 5.8S coding region was similar to that obtained for the FCR-3 isolate. Comparison of the data obtained from the dideoxy sequence analyses of the EMBL-3/rib12 confirmed the RNA sequence data and identified the ambiguous nucleotides as C residues. In addition, the EMBL-3/rib12 and pGEM-2/rib-2 recombinants contained identical 5.8S coding regions (data not shown). Figure 3 shows the sequence data obtained for the pGEM-2/rib-2 recombinant. The entire sequence from the primer to either the 3' terminus (data not shown) or 5' terminus of the coding region could readily be determined from a single gel. The complete nucleotide sequence is shown in Figure 4.

Computer and comparative analyses. Analyses of the small rRNA species from various organisms has provided a useful tool in studying phylogenetic relationships. Alignment of the P. falciparum 5.8S rRNA sequence with previously reported sequences (Erdmann et al., 1985), indicated that the P. falciparum sequence was indeed homologous to those obtained from other sources. Several examples are presented in Figure 4. The best alignment was achieved with Thermomyces langinosus; while the worst was with Bombyx mori. The uppermost sequence in each alignment is the P. falciparum sequence. For all sequences analyzed, the range of matches per length was between 48% and 62%.

A more accurate assessment of the relationship between two sequences is the D_{AB} value (Table I). This value takes into account the insertions and deletions that are required in matching two sequences for maximum alignment. The higher the D_{AB} value the more distantly related or divergent the molecules are to one another. It should be mentioned that these values are calculated for the P. falciparum sequence with each of the other sequences and do not represent all possible pairwise comparisons of each sequence with one another. From the data obtained it is evident that there is no close relationship between the P. falciparum 5.8S rRNA with those from other organisms; this is largely due to the unavailability of sufficient data from other protist groups and more importantly other species of malaria. For comparison, the D_{AB} values for the pairs Xenopus laevis and Mus musculus is 0.04, and 0.48 for X. laevis and Tetrahymena paravorax.

Although there appears to be variation in the number of nucleotides, as well as the sequence of the 5.8S rRNA from organism to organism, there is some conservation of nucleotides as indicated by the bold letters in Figure 4. These nucleotides are conserved in all sequences examined thus far including the P. falciparum 5.8S sequence (Erdmann et al., 1985).

DISCUSSION

We have identified 3 to 4 low molecular weight size classes of RNA synthesized during the asexual life cycle of the parasite. On the basis of their electrophoretic mobilities

and molecular weight estimates, three size classes were tentatively identified as the 5.8S rRNA, 5S rRNA and tRNA species of P. falciparum. Unlike the 5S rRNA species which is transcribed from a separate unit, the 25S, 17S and 5.8S rRNA components are processed from a high molecular weight rRNA precursor. In P. falciparum, the mechanisms involved in rRNA transcription and processing are unknown; however, the processing event, which generates the mature 5.8S rRNA species, appears to be precise since a single homogeneous population of 5.8S rRNA molecules can be isolated. It has been shown that there are at least 2 classes of ribosomal RNA genes in P. falciparum which differ in both spacer and coding regions (Langsley et al., 1983; Dame and McCutchan, 1983). The recombinant clones pfrib-2 and EMBL-3/pfrib12 from two Gambian isolates contain identical 5.8S coding regions. We do not know if pfrib-1 (Langsley et al., 1983), which comprises the second class of rRNA genes, also contains an identical 5.8S sequence. This possibility is currently under investigation.

Difficulties were encountered in obtaining the 5.8S rRNA sequence. The 3 C residues, which were subsequently identified by dideoxy sequence analyses, could not be determined using the enzymatic method of RNA sequence analysis. One possible explanation is that these nucleotides are modified in some manner. The 5.8S rRNA from other sources is known to contain pseudouridine and methylated ribose (Walker and Pace, 1983; Nazar et al., 1983). Dictyostelium appears to be the exception since its 5.8S component is unmodified (Walker and Pace, 1983). Although these molecules vary in sequence, as well as length (152 nucleotides for T. paravorax to 170 for Trypanosoma brucei), there are several nucleotides (indicated by the bold letters in Figure 4) that are conserved for all 5.8S rRNA molecules sequenced to date. The reason for such conservation is unknown. Of the 16 conserved nucleotides, the first 13 are postulated to form a major stem-loop structure; while the remaining 3 nucleotides (GAA near the 3' terminus) are located in a short unpaired region (Choi, 1985; Ursi et al., 1983; Vaughn and Sperbeck, 1984). Current data indicate that the 5' and 3' termini of the 5.8S rRNA

molecule interact with the 5' terminal region of the 28S rRNA (Choi, 1985; Ursi et al., 1983; Vaughn and Sperbeck, 1984). Since no sequence data are available on this region from P. falciparum, we are unable to tentatively identify such domains in our 5.8S rRNA species.

Analyses of prokaryotic or eukaryotic rRNA molecules, to establish possible phylogenetic relationships, have received considerable attention (De Wachter et al., 1985; Jacq, 1981; Pace et al., 1986; Marteaux et al., 1985). The RNAs are essential elements in the functioning and structure of the ribosome. Ribosomes and their associated RNAs are conserved in overall structure, and comparative analyses of nucleic acid sequence data have identified homologous regions. Bacteria lack a 5.8S rRNA component, although a homologous region is located at the 5' terminus of the 23S rRNA (Vaughn and Sperbeck, 1984; Jacq, 1981). Similarly, a 5.8S rRNA species is absent in the lower eukaryotic organism Vairimorpha necatrix (Vossbrinck and Woese, 1986). However, a homologous region at the 5' terminus of its 25S rRNA component has been identified. The large and small rRNA species are generally more suitable for phylogenetic analyses owing to their large size and the number of bases available for comparison. The low molecular weight rRNAs seem less than ideal since a limited number of bases are available for analyses (≈120-170); however, the ubiquity, conservation, and ease of isolation, of these molecules make them attractive candidates for such studies. From the D_{AB} values it is evident that additional information on the rRNAs from plasmodia, and the class Sporozoa in general, is needed to determine possible phylogenetic relationships with other organisms and more importantly with other species of malaria.

ACKNOWLEDGMENTS

This study was supported by Public Health Service Grants AI 18767 and AI 20597 from the National Institutes of Health. D.S.-L. is a graduate student supported by a University of Alabama at Birmingham Graduate Fellowship. We wish to thank Dr. Jeff Engler, Director of the Primer Core Facility at UAB, for synthesizing the oligodeoxyribonucleotide primers and Dr. Daniel Jones, Chairman of the Department of Biology, for his encouragement and support.

Figure 1. Polyacrylamide gel electrophoresis of [^{32}P] pCp end-labelled RNA. Total RNA preparations from P. falciparum, E. coli 5S and yeast tRNA^{phe} were end-labelled with [^{32}P] cytidine biphosphate. An aliquot of each RNA sample was resolved by polyacrylamide gel electrophoresis on a 40 cm x 20 cm x 1.5 mm 12% (w/v) polyacrylamide gel containing 8 M urea and TBE (0.1 M Tris-borate, 0.005 M EDTA), pH 8.2. Samples were resolved by electrophoresis at 35 mA for 6 h. Lane 1 contains E. coli 5S RNA, lane 2 yeast tRNA^{phe} and lane 3 total P. falciparum RNA. The arrow indicates the position of the unresolved 25S and 17S rRNA components.

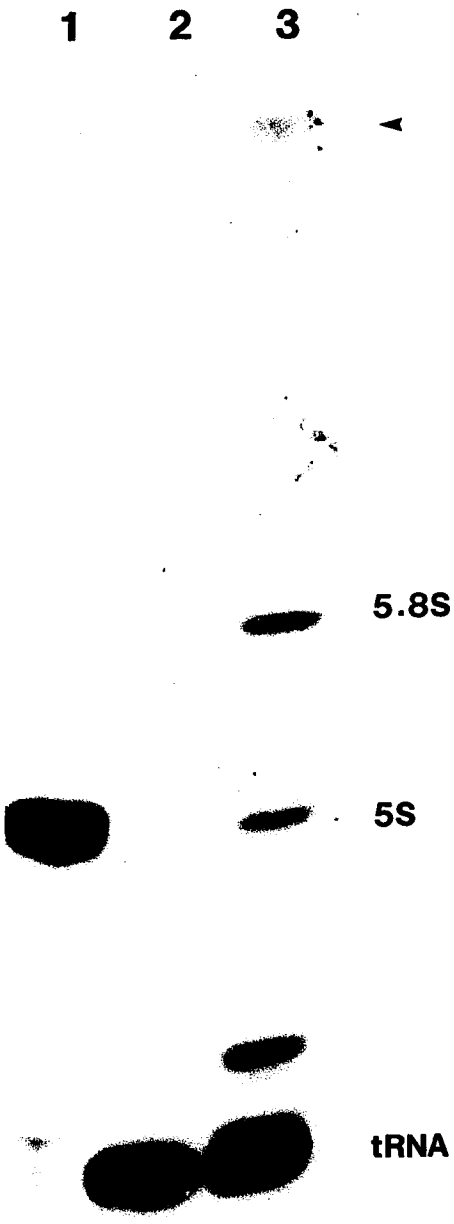

1
2
3
5.8S
5S
tRNA

Figure 2. Polyacrylamide gel electrophoresis of purified 5.8S and 5S rRNAs. Following excision and elution of the 5.8S or 5S rRNA component from a 12% preparative polyacrylamide gel, a small aliquot was analyzed on a 90 cm x 20 cm x 0.38 mm 10% (w/v) polyacrylamide gel with an acrylamide /bis ration of 29:1. The gel contained 3 M urea in TBE. Electrophoresis was performed at 2100 V for 24 h.


A
B

Figure 3. Dideoxy sequence analysis of the pGEM-2/rib-2 construct using an oligodeoxyribonucleotide primer specific for 3' terminal residues 132 to 152.

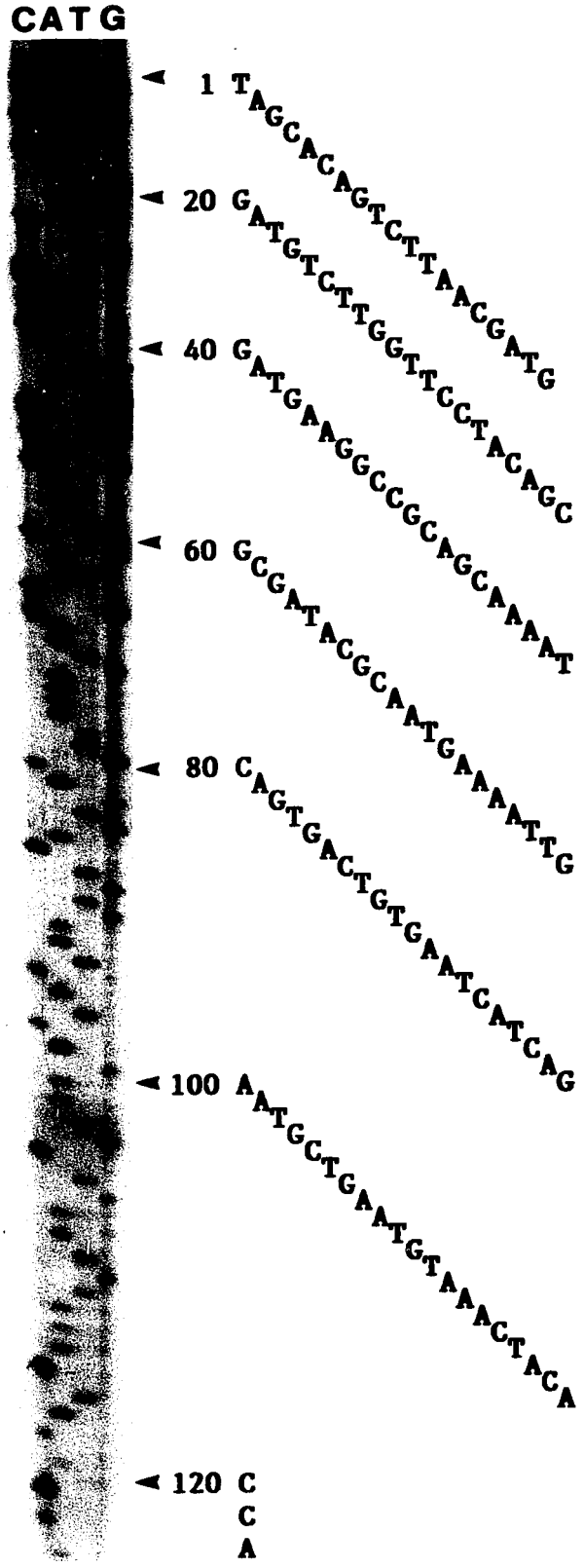

CATG
1 TAGCACAGTCTTACGATG
20 GATGTCTTGGTTCCTACAGC
40 GATGAAGGCCGCAGCAAAAT
60 GCGATACGCAATGAAAATTG
80 CAGTGACTGTGAATCATCAG
100 AATGCTGAATGTAACTACA
120 C
C
A

Figure 4. Nucleotide sequence alignment. The 5.8S rRNA from P. falciparum was compared and aligned to its corresponding sequence in T. lanuginosus, T. pyriformis, T. brucei or B. mori. Dashed lines indicate homologous nucleotides.

Plasmodium falciparum
Thermomyces lanuginosus

```
1          TAGCACAGTCTTAACGATGGATGTCTTGGTTCCTACAGCGATGAAGGCCGCAGCAAAATG
               | | | | ||| |||||  |||||||||||  || ||||||||  ||||||| ||| |
1              AAACUUUCAACAAUGGAPCUCUUGGUUCCGGCAUCGAUGAAGAACGCAGCGAAAPG

61         CGATACGCAATGAAAATTGCAG TGACTGTGAATCATCAGAATGCTGAATGTAAACTACA
           |||||  |  ||||   ||  |||||     |  ||||||||||   |     ||||  |  |  |  |  |
57         CGAUAAGUAAUGUGAAPUGCAGAAUUCCGUGAAUCAUCGAAUCUUUGAACGCACAUUGCG

120        C    CAGCTCTTCGGGGAATAGTGGTACTCCTACAGAAAAT
           |    |  |  |  |||  |||      ||    |||  |   |   | |
117        CCCUCUGGUAUUCCGGGGGGCAUGCCUGUCCGAGCGUCAUU
```

Plasmodium falciparum
Tetrahymena pyriformis b

```
1          TAGCACAGTCTTAACGATGGATGTCTTGGTTCCTACAGCGATGAAGGCCGCAGCAAAATG
            ||  |  |  |  |  ||||  |||||  |||  ||||||      ||||||||  ||||||  |||||
1           AGAAAACUUUCAACGGUGGAUAUCUAGGUUCCCGUGACGAUGAAGAACGCAGCGAAAUG

61         CGATACGCAATGAAAATTGCAGTGACTGTGAATCATCAGAATGCTGAATGTAAACTACAC
           ||||||||||||  ||||||||  ||  |  ||  |||||||||     ||||  |  ||  |
60         CGAUACGCAAUGCGAAUUGCAG AACCGCGAGUCAUCAGAUCUUUGAACG CAAGUGGUG

121        CAGCTCTTCGGGGAATAGTGGTACTCCTACAGAAAAT
            ||  |  |          |  ||  |  |   |  |   |
118        GAGGUGUAAAAACCUUCAUGUUUGUAUCAGUGUGGAA
```

Plasmodium falciparum
Trypanosoma brucei

```
1          TAGCACAGTCTTAACGATGGATGTCTTGG TTCCTACAGCGATGAAGGCCGCAGCAAAAT
            |  ||  ||  |  |||||||||  |||||  ||||||   ||  |||||  |||||||||  |
1           A  AC GT GTCGCGATGGATGACTTGGCTTCCTATTTCGTTGAAGAACGCAGCAAAGT

60         GCGATACGCAATGAAAATTGCAGTGACTGTGAATCATCAGAATGCTGAATGTAAACTACA
           ||||||  |   |   ||||||||   |  |  |  |   ||  |    ||||  |  ||||  |
56         GCGATAAGTGGTATCAATTGCAGAATCATTCATTGCCCA ATCTTTGAACGCAAACGGCG

120        CCAGCTCTTCGGGGAATAGTG GTACTCCTACAGAAAAT
           |  ||  |  ||      |  |  |   |  |  |  ||||  |   |
115        CATGCCCGACGCTCTCTCGAGCATCCCCGTACATACCACATTTCTCAGTGTCGAAT
```

Plasmodium falciparum
Bombyx mori

```
1          TA  GCACAGTCTTAACGATGGATGTCTTGGTTCCTACAGCGATGAAGGCCGCAGCAAAA
            |  |   |  ||  ||| |||||  |||||  ||       ||||||||  |||||  ||
1          AAAUGAUUACCCUGGACGGUGGAUCACUUGGCUCGCGGGUCGAUGAAGAACGCAGUUAAC

59         TGCGATACGCAATGAAAATTGCA GTGACTGTGAATCATCAGAATGCTGAATGTAAACTA
           |||   |  |  ||  ||  ||||  |  ||  |  |  ||||   ||   |||  |  |  |  |
61         PGCGCGUCAUAGUGUGAACUGCAGGACACAUUUGAACAUCGACAUUUCGAACGCACAUUG

118        C ACCAGCTCTCGGGGAAT AGTGGTACTCCT ACAGAAAAT
           |   | |              ||    ||||||  |  ||
121        CGGUCCGUGGAGACACAUCCAGGACCACUCCUGUCUGAGGGCCGGCU
```

TABLE 1. D_{AB} VALUES FOR THE 5.8S RNAs OF P. FALCIPARUM AND OTHER KNOWN ORGANISMS.

Genus/species (Order)	D_{AB} Value
Plasmodium falciparum (Eucoccidiida)	0.00
Neurospora crassa (Pyrenomycetes)	0.52
Saccharomyces cerevisiae (Endomycetidae)	0.53
Chlamydomonas reinhardii (Volvocales)	0.53
Hymeniacidon sanguinea (Porifera)	0.54
Thermomyces langinosus (Plectomycetes)	0.54
Schizosaccharomyces pombe (Endomycetidae)	0.55
Tetrahymena pyriformis (Ciliophora)	0.58
Crypthecodinium cohnii (Dinophyta)	0.59
Lupinus luteus (Angiospermae)	0.61
Acanthamoeba castellanii (Amoebida)	0.62
Dictyostelium discoideum (Acrasea)	0.64
Escherichia coli [rRNB] (Gram-)	0.67
Triticum aestivum (Angiospermae)	0.67
Salmo gairdneri (Osteichthyes)	0.69
Crithidia fasciculata (Kinetoplastida)	0.70
Vicia faba (Angiospermae)	0.70
Drosophila melanogaster (Insecta)	0.70
Gallus gallus (Aves)	0.71
Lytechinus variegatus (Echinoidea)	0.71
Sciara coprophila (Insecta)	0.72
Anacystis nidulans (Gram-, Cyanobacteria)	0.72
pt Nicotiana tabacum (Angiospermae)	0.72
Terrapene carolina (Reptilia)	0.73
Acyrthosiphon magnoliae (Insecta)	0.74
Homo sapiens, Mus musculus (Mammalia)	0.75
pt Chlamydomas reinhardii (Volvocales)	0.75
Bacillus stearothermophilus (Gram+)	0.75
Arion rufus (Gastroda)	0.76
Xenopus borealis (Amphibia)	0.76
Xenopus laevis (Amphibia)	0.77
pt Zea mays (Angiospermae)	0.79
Artemia salina (Crustacea)	0.80
Physarum polycephalum (Myxogastria)	0.81
Philosomia cynthia ricini (Insecta)	0.83
pt Euglena gracilis (Euglenophyta)	0.85
Bombyx mori (Insecta)	0.86

REFERENCES

Benton, W. D., and Davis, R. W. (1977). Screening λgt recombinant clones by hybridization to single plaques in situ. Science 196, 180-182.

Clerx-Van Haaster, C., and Bishop, D. H. L. (1980). Analyses of the 3' terminal sequences of Snowshoe Hare and La Crosse bunyaviruses. Virology 105, 564-574.

Choi, Y. C. (1985). Structural organization of ribosomal RNAs from Novikoff hepatoma. II Characterization of possible binding sites of 5S rRNA and 5.8S rRNA to 28S rRNA. J. Biol. Chem. 260, 12773-12779.

D'Alessio, J. M. (1982). RNA sequencing. In: Gel electrophoresis of nucleic acids: A practical appproach. (Rickwood, D. and Hames, B.D., eds.), 173-197, IRL press, Oxford.

Dame, J. B., and McCutchan, T. F. (1983). The four ribosomal DNA units of the malaria parasite Plasmodium berghei: Identification, restriction map and copy number analysis. J. Biol. Chem. 258, 6984-6991.

Dame, J. B., and McCutchan, T. F. (1984). Identification of 5S and 5.8S ribosomal RNA molecules and their genes in Plasmodium berghei. Mol. Biochem. Parasitol. 11, 301-307.

De Wachter, R., Huysmans, E., and Vandenberghe, A. (1985). 5S ribosomal RNA as a tool for studying evolution. In: Evolution of Procaryotes (Schleifer, K.H. and Stackenbrandt, E., eds.) 115-141, Academic Press, London.

Erdmann, V. A., Wolters, J., Huysmans, E. and De Wachter, R. (1985). Collection of published 5S, 5.8S and 4.5S ribosomal RNA sequences. Nucleic Acids Res. 13 (suppl.), r105-r153.

Francoeur, A. M., Gritzmacher, C. A., Peebles, C. L,. Reese, R. T., and Tan, E. M. (1985). Synthesis of small nuclear ribonucleoprotein particles by the malarial parasite Plasmodium falciparum. Proc. Natl. Acad. Sci. U.S.A. 82, 3635-3639.

Hyde, J. E., Zolg, J. W., and Scaife, J. G. (1981). Isolation and characterization of ribosomal RNA from the human malaria parasite Plasmodium falciparum. Mol. Biochem. Parasitol. 4, 283-290.

Jacq, B. (1981). Sequence homologies between eukaryotic 5.8S rRNA and the 5' end of procaryotic 23S rRNA: evidence for a common evolutionary origin. Nucleic Acids Res. 9, 2913-2929.

Kumazaki, T., Hori, H., and Osawa, S. (1982). The nucleotide sequence of 5S ribosomal RNA from a sea anemone, Anthropleura japonica. FEBS Lett. 146, 307-310.

Marteaux, L., Herzog, M., and Soyer-Gobillard, M. -O. (1985). Molecular organization of dinoflagellate ribosomal DNA: Evolutionary implications of the deduced 5.8S rRNA secondary structure. Bio Systems 18, 307-319.

Nazar, R. N., Lo, A. C., and Wilderman, A. G. (1983). Effect of 2'-O-methylation on the structure of mammalian 5.8S rRNAs and the 5.8S-28S rRNA junction. Nucleic Acids Res. 11, 5989-6001.

Pace, N. R., Olsen, G. J., and Woese, C. R. (1986). Ribosomal RNA phylogeny and the primary lines of evolutionary descent. Cell 45, 325-326.

Pace, N. R., Stahl, D. A., Lane, D. J., and Olsen, G. J. (1985). Analyzing natural microbial populations by rRNA sequences. ASM News 51, 4-12.

Peattie, D. (1979). Direct chemical method for sequencing RNA. Proc. Natl. Acad. Sci. U.S.A. 76, 1760-1764.

Queen, C., and Korn, C. J. (1984). A comprehensive sequence analysis program for the IBM personal computer. Nucleic Acids Res. 12, 581-599.

Reese, R. T., Langreth, S. C. and Trager, W. (1979). Isolation of stages of the human malaria parasite Plasmodium falciparum from culture and from animal blood. Bull. W.H.O. 57 (suppl. 1), 53-61.

Selker, E. V., Stevens, J. N., and Metzenberg, R. L. (1985). Heterogeneity of 5S RNA in fungal ribosomes. Science 227, 1340-1343.

Shippen, D. E., and Vezza, A. C. (1986). 3' Terminal nucleotide sequence of the 17S ribosomal RNA species from Plasmodium falciparum. J. Parasitol. 72, 178-181.

Trager, W., and Jensen, J. B. (1976). Human malaria parasites in continuous culture. Science 193, 673-675.

Unnasch, R. R., and Wirth, D. F. (1983). The avian malaria Plasmodium lophurae has a small number of heterogeneous rRNA genes. Nucleic Acids Res. 11, 8443-8459.

Ursi, D., Vandenberghe, A. and De Watcher, R. (1982). The sequence of the 5.8S ribosomal RNA crustacean Artemia salina. With a proposal for a general secondary structure model for 5.8S ribosomal RNA. Nucleic Acids Res. 10, 3517-3530.

Ursi, D., Vandenberghe, A., and De Wachter, R. (1983). Nucleotide sequences of the 5.8S rRNAs of a mollusc and a porifer, and considerations regarding the secondary structure of 5.8S rRNA and its interaction with 28S rRNA. Nucleic Acids Res. 11, 8111-8120.

Vaughn, J. C., and Sperbeck, S. J. (1984). A universal model for the secondary sturcture of 5.8S ribosomal RNA molecules, their contact sites with 28S ribosomal RNAs, and their prokaryotic equivalent. Nucleic Acids Res. 12, 7479-7502.

Vezza, A. C., and Trager, W. (1981). Preliminary characterization of the major RNA species from the human malaria parasite Plasmodium falciparum. Mol. Biochem. Parasitol. 4, 149-162.

Vezza, A. C. and Trager, W. (1982). Comparative analyses of the ribosomal RNA from four isolates of Plasmodium falciparum. Am. J. Trop. Med. Hyg. 314, 718-722.

Vossbrinck, C. R., and Woese, C. R. (1986). Eukaryotic ribosomes that lack a 5.8S RNA. Nature 320, 287-288.

Walker, T. A., and Pace. N. R. (1983). 5.8S ribosomal RNA. Cell 33, 320-322.

Walker, W. F. (1985). 5S and 5.8S ribosomal RNA sequences and protist phylogenetics. Bio Systems 18, 269-278.

VI. ANALYSES OF THE 5.8S rRNA CODING DOMAIN OF PLASMODIUM FALCIPARUM rRNA TRANSCRIPTION UNITS: SEQUENCE HETEROGENEITY AND EVIDENCE FOR GENE INACTIVATION

Dorothy Shippen-Lentz[1] and Anne Carmel Vezza[2]

[1]Department of Biology University College, University of Alabama at Birmingham, Birmingham, Al. USA 35294; [2]Department of Microbiology, Schools of Medicine and Dentistry, University of Alabama at Birmingham, Birmingham, Al. USA 35294

SUMMARY

The 5.8S rRNA coding domains from P. falciparum have been characterized. Southern blot analysis of genomic DNA indicates that these genes form at least 5 different transcriptional classes, a predominant class of approximately 30 members and several minor classes containing only one or two genes. The major class of 5.8S rDNA is identical to the 5.8S RNA present during erythrocytic development while the minor rDNA components contain multiple point mutations, insertions and deletions. These variant genes are transcriptionally inactive and appear to have been generated by an unequal crossing-over event.

INTRODUCTION

The ribosomal RNA genes of most eukaryotic organisms comprise large multigene families, consisting of hundreds to thousands of tandemly repeated units. The 17-18S, 25-28S and 5.8S rRNA components are derived from a single unit and are transcribed as a large precursor molecule which is subsequently processed into the mature RNA. Analyses of the rRNA transcription units from plasmodia suggest that these organisms possess very unusual rRNA genes (reviewed in McCutchan, 1986). Studies with the rodent and avian malaria parasites Plasmodium berghei (Dame and McCutchan, 1983a) and Plasmodium lophurae (Unnasch and Wirth, 1983a) and the human malaria parasite, Plasmodium falciparum (Langsley et al., 1983), indicate that plasmodia contain only four to eight rRNA transcription units per haploid genome. Unlike some protozoa which possess very few rRNA genes, plasmodia do not appear to amplify their rRNA transcription units (Dame and McCutchan, 1983; Cornelissen et al., 1985; McCutchan, 1986). Pulsed-field gradient electrophoresis studies revealed the dispersion of plasmodial rRNA transcription units over several chromosomal loci (Van der Ploeg et al., 1985; Langsley et al., 1987). In addition, restriction endonuclease digestion analyses indicate that the transcription units are heterogeneous in both spacer and coding region (Langsley et al., 1983; Dame and McCutchan, 1983b; Unnasch and Wirth, 1983a) and can be categorized into two to six distinct gene classes. Some plasmodial rRNA transcription units appear to contain introns. Interruptions in both the 17S and 25S rRNA coding regions have been identified in P. lophurae (Unnasch and Wirth, 1983a; Unnasch and Wirth, 1983b). The transcriptional capability of these intron+ rRNA genes has not been determined, but preliminary data suggest that not all of the plasmodial rRNA transcription units are expressed during erythrocyte development (Dame et al., 1984).

The dispersion, diversity and exceptionally low copy number of plasmodial rRNA genes seem to place the parasite at a transcriptional disadvantage for the production of sufficient ribosome components. These units appear to be similar to "orphons" or single-copy dispersed pseudogenes (Childs et al., 1981). However, it is evident that at least one plasmodial rRNA gene class must be transcriptionally active to produce functional ribosomal RNA components. The active rRNA transcription unit(s) have not been identified. Therefore, we have characterized the 5.8S rRNA coding regions from different P. falciparum rRNA transcription units to determine which gene class(es) are expressed during the asexual erythrocytic life cycle. In this report we demonstrate that P. falciparum contains at least 5 distinct classes of rRNA transcription units, one predominant and several minor rDNA components. Only the major rRNA gene class is active during erythrocytic development. The minor rRNA transcription units appear to be pseudogenes.

MATERIALS AND METHODS

Cultivation of parasites. The Gambian isolate of P. falciparum (FCR-3) was maintained by serial passage in type A Rh positive human erythrocytes as described previously (Trager and Jensen, 1976) with a few modifications (Shippen-Lentz et al., 1987). For some studies, the parasites were synchronized using the sorbitol (Lambros and Vanderberg, 1979) and gelatin (Reese et al., 1979) methods. Briefly, when the majority of infected red cells contained trophozoites/schizonts, the cultures were gelatin treated to concentrate late stage parasites and to remove any remaining ring stage infected erythrocytes. The trophozoite/schizonts were then subcultured by the addition of fresh red cells and medium, returned to clean culture bottles and incubated at 37°C for several hours until most of the infected cells contained ring stage parasites. The erythrocytes were then resuspended in 5% sorbitol to rid the sample of trophozoite/schizonts. Following sorbitol treatment, the cultures were returned to clean bottles containing fresh medium and incubated at 37°C until the parasites had matured to the trophozoite/schizont stage.

Nucleic acid isolation. Trophozoite/schizont infected cell pellets were resuspended in 5 volumes of low salt buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4). The nucleic acids were extracted by the addition of 0.1 volume 10% SDS and an equal volume of a phenol/m-cresol/chloroform mixture (Vezza and Trager, 1981). After the addition of 4M NaCl to a final concentration of 400 mM, the nucleic acids were recovered by ethanol precipitation. The majority of the DNA was removed from the sample by spooling onto a pasteur pipet. The remaining nucleic acids, containing the majority of the RNA and some DNA, were precipitated at -20°C overnight. Following centrifugation the pellet was resuspended in sterile water. In some experiments residual DNA was removed from RNA preparations by incubation at 37°C in the presence of RNase-free DNase I.

High molecular weight DNA was extracted from parasitized erythrocytes by disrupting the cell pellet in DNA lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8, 5 mM EDTA) containing 200 µg/ml predigested Pronase and 1% (w/v) SDS. Following incubation at 37°C for 15 to 30 min, the sample was extracted with phenol and chloroform. The DNA was precipitated with ethanol, spooled onto a pasteur pipet and immediately resuspended in 2 ml of 10 mM Tris-HCl (pH 8), 5 mM EDTA. After an overnight incubation at 4°C, the preparation was adjusted to 10 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl (pH 8) and digested at 37°C for 45 min with 20 µg/ml DNase-free RNase A. Following extraction with phenol and chloroform, the DNA was precipitated with ethanol, spooled onto a pasteur pipet, resuspended in 10 mM Tris-HCl (pH 8), 1 mM EDTA and stored at 4°C.

Isolation of λ clones containing the plasmodial rRNA transcription unit. Genomic libraries were constructed using the bacteriophage λ cloning vector EMBL-3 and the cloning protocol outlined by the supplier (Promega Biotec, Inc.). Recombinant phage from unamplified libraries were screened using the plaque hybridization procedure of Benton and Davis (1977) with radiolabelled in vitro generated RNA transcripts from a pGEM-1

recombinant phage containing the previously characterized P. falciparum pfrib-1 rRNA insert (Langsley et al., 1983).

Southern blot analyses. Southern hybridization of restriction endonuclease digested plasmodial genomic DNA was carried out as described previously (Southern, 1975; Shippen-Lentz and Vezza, submitted for publication). The filters were probed with [^{32}P]α-dATP (Amersham) labelled 5.8S cDNA at 65°C O/N, washed and autoradiographed.

Northern blot analyses. Plasmodial RNA was fractionated on a 1.2% agarose gel containing 6% formaldehyde, 12 mM Tris, pH 7.5, 6 mM sodium acetate and 0.3 mM EDTA. Following transfer to GeneScreen Plus (Dupont) membrane filters, as described by the manufacturer, the membranes were probed with [32P] γ-dATP (Amersham) labelled 5.8S oligodeoxyribonucleotides at 42°C for 24 hours. The filters were washed at 42°C for one hour. If deemed necessary, the filters were washed at slightly higher temperatures to eliminate non-specific hybridization of the probes. For dot blot analysis, the RNA was denatured in formaldehyde/formamide prior to application to membrane filters.

5.8S rDNA Sequence Analyses. λ clones were digested with Dra I or Ssp I and the products resolved on a 1% agarose gel. Fragments containing 5.8S rDNA were electroeluted from the gel and recovered by ethanol precipitation. The nucleotide sequence of the 5.8S rRNA coding regions was determined by the primer extension dideoxy sequencing method using the Promega Biotec GemSeq sequencing kit and oligodeoxyribonucleotide primers specific for the 5.8S rRNA sequence.

RESULTS

Five classes of P. falciparum 5.8S rDNA. To determine the total number of rRNA gene classes, P. falciparum genomic DNA was examined by Southern blot analysis and the filters probed with [^{32}P] 5.8S cDNA. In this experiment, plasmodial DNA was digested with Ssp I or Dra I, two restriction endonucleases predicted to cleave outside the 5.8S rRNA coding region based on the 5.8S rRNA sequence (Shippen-Lentz et al., 1987). Digestion with Dra I identified one predominant 5.8S rDNA class and two minor rDNA

components (Figure 1, Lane 2). One major and four minor gene classes were resolved in the Ssp I digestion (Figure 1, Lane 2). The two faint bands in the Ssp I lane denoted by the arrows appear to be the result of a partial endonuclease digestion since they were not present when the analyses were repeated. These date indicate that P. falciparum contains at least four minor and one major 5.8S rDNA classes.

The predominant 5.8S rRNA gene of P. falciparum appears to be present in significantly higher copy number than the remaining 5.8S rDNA classes. Scanning densitometry of the Ssp I digestion suggested a ratio of 1 : 1 : 1 : 1 : 30 for the minor gene classes with respect to the predominant 5.8S rRNA gene class (Figure 2). Similar results were obtained from an analysis of the Dra I lane (data not shown).

Characterization of λ recombinants containing the rRNA transcription units also identified several different classes of 5.8S rDNA. All of the clones analyzed possess a 5.8S rRNA coding region as well as portions of the 17S or 25S rRNA coding regions (data not shown). Digestion with Ssp I or Dra I and subsequent Southern blot analysis indicated that several of the recombinants contain distinct 5.8S rDNA (Figure 3). Of the six clones characterized, 5 contained minor gene classes and only one, λ12, appeared to possess the predominant class of 5.8S rDNA (Figure 3, Lanes 2 and 7). The 5.8S rRNA coding region of this recombinant migrates in the same position as the major 5.8S rRNA gene class previously identified in the genomic blot. From the Dra I analysis, λ12 and λ37 appear to contain the same class of 5.8S rDNA (Figure 3, Lanes 2 and 5); however, the spacer regions surrounding the 5.8S rRNA genes of these clones are clearly distinct as shown by the Ssp I digestion (Figure 3, Lanes 7 and 12). Likewise, the 5.8S rDNA fragment of λ23, which migrates only slightly slower than the λ12 and λ37 fragments in the Dra I digestion (Figure 3, Lane 4), represents a unique class of 5.8S rRNA gene when analyzed by Ssp I (Figure 3, Lane 9). λ9 and λ13, however, may contain the same class of 5.8S rDNA (Figure 3, Lanes 1, 3, 6 and 8). Of particular interest was the λ77 recombinant. Two different classes of 5.8S rDNA were resolved from this clone.

Analysis by digestion with Dra I (data not shown) or Ssp I (Figure 3, Lane 11) suggested that 5.8S rDNA components like the λ9/13 and the λ12 gene classes (Figure 3, Lanes 6-8) were present in the λ77 clone. This finding indicates that two of the P. falciparum rRNA transcription units may be linked. For convenience, the rRNA transcription units have been designated: λ9/13=class I, λ12=class II, λ23=class III, λ37=class IV.

The number of the 5.8S rRNA coding regions in each gene class was determined (Lis et al., 1978; Shippen-Lentz and Vezza, in press). Briefly, genomic DNA digested with Ssp I or Dra I was compared to 5.8S rDNA plasmid standards corresponding to different copy numbers of the 5.8S rRNA genes. The amount of genomic DNA analyzed was calculated to be one genome equivalent based on a P. falciparum genome size of 2.2 x 10^7 base pairs (Goman et al., 1982) and a 5.8S rRNA coding region length of 157 nucleotides (Shippen-Lentz et al., 1987). Comparison of the intensities of the genomic digest to the standards suggested that the minor 5.8S rRNA gene classes are present in only one or two copies while the predominant class II is comprised of approximately 30 rRNA genes.

Heterogeneous 5.8S rRNA coding regions. Southern blot analysis of P. falciparum genomic DNA demonstrated the presence of at least five distinct rRNA gene classes. These results are based on differences in the spacer regions surrounding the 5.8S rRNA genes and not necessarily heterogeneity within the coding domain. DNA sequence analysis of the different cloned 5.8S rRNA genes indicated that the 5.8S rDNA from each rRNA transcription unit is distinct (Figure 4). The nucleotide sequence of the 5.8S rRNA coding region of class II, the predominant class of 5.8S rDNA, is identical to the 5.8S RNA sequence (Shippen-Lentz et al., 1987); however, the 5.8S rRNA coding regions of classes I, III and IV are divergent. Of these variant domains, the 5.8S rRNA coding region of class IV is the most closely related to the predominant gene sequence, differing only by a three base pair insertion at position 126, a single nucleotide deletion at position 130 and a transition at nucleotide 132. The 5.8S rRNA coding regions of class I and III are

characterized by numerous point mutations and insertions, and are only 85% and 79% homologous, respectively (data not shown). The majority of nucleotide changes in the minor gene classes are clustered near the 3' terminus of the coding region, beginning at position 133 and extending to nucleotide 150. The class III 5.8S rDNA domain appears to be truncated near the 3' terminus of the gene and cannot be realigned with the 5.8S rRNA sequence.

Minor 5.8S rRNA gene classes are not expressed during erythrocyte development. Previous studies suggest that the 5.8S rRNA of P. falciparum is a homogeneous population of molecules, 157 nucleotides in length (Shippen-Lentz et al., 1987). Analysis of the plasmodial 5.8S rRNA by high resolution polyacrylamide gel electrophoresis under conditions designed to detect minute sequence or length heterogeneity failed to identify additional 5.8S rRNA species. It is conceivable that these experiments might not detect heterogeneous molecules present in very low quantities or RNAs which are expressed only transiently. Therefore, total cellular RNA was examined by northern and dot blot analyses using probes specific for the divergent regions of the 5.8S rRNA genes to screen for the presence of heterogeneous 5.8S rRNA. The binding sites for these probes are denoted by the underlined nucleotides in Figure 4. These experiments indicate that the variant 5.8S rDNA genes are not transcribed during the asexual life cycle. Only class II rRNA transcripts were detected (Figure 5).

To address the possibility that low copy 5.8S rRNA genes are expressed transiently or at other stages of the asexual life cycle, plasmodial RNA was isolated at different times during erythrocyte development and examined by dot blot analysis. Only transcripts from the class II gene were identified. We cannot rule out the possibility that variant 5.8S rRNA genes are expressed, but their transcripts are degraded too rapidly to be detected. It is also conceivable that the minor gene classes are transcriptionally active during exoerythrocytic development or the sexual life cycle.

DISCUSSION

It has been reported that P. falciparum contains only 4 to 8 rRNA transcription units which are internally heterogeneous and can be categorized into 2 to 3 transcriptional classes (Langsley et al., 1983). Our analyses of the 5.8S rRNA coding regions indicate that P. falciparum contains more than thirty rRNA transcription units which form a predominant and several minor rDNA gene classes. Surprisingly, the minor units were cloned with the same efficiency as the predominant 5.8S rDNA which appears to represent approximately thirty rRNA transcription units. It is conceivable that the rRNA genes of the major gene class lie in regions of the parasite genome which are not easily accessible or amenable for cloning into λ. For example, genes associated with telomeres or relatively small extrachromosomal DNA fragments would not be cloned using the protocol described in the methods section because the restriction endonuclease cleavage of these regions is likely to generate inappropriately sized inserts. Previous studies indicate that the rRNA genes of plasmodia are not extrachromosomal (Dame and McCutchan,1983a; McCutchan,1986); however, the possibility that rRNA transcription units might be located near telomeres bears further investigation. A histidine-rich protein gene of P. falciparum has been mapped to a telomeric region (Wellems et al., 1987) and there is mounting evidence to suggest that this chromosomal location confers a distinct transcriptional advantage (Borst, 1986; Baroin et al., 1987; Wellems et al., 1987). The mechanisms involved in telomeric gene activation are unknown.

Our data are in agreement with previously published reports demonstrating sequence heterogeneity within the plasmodial rRNA transcription units. For P. falciparum, the differences among gene classes are such that the examination of a very small portion of the transcription unit reveals dramatic sequence divergence. Analysis of the 5.8S rRNA coding regions from the minor gene classes indicates that they contain multiple point mutations and short A+T rich insertions when compared to the 5.8S rRNA. Furthermore, the 3' terminus of the 17S rRNA coding region from the class I recombinant which

represents a low copy rRNA transcription unit (data not shown) exhibits a similar degree of divergence when aligned to the 17S rRNA sequence (Shippen and Vezza, 1986).

With such sequence diversity, could functional rRNA molecules be generated from the minor gene classes? To address this possibility, we have screened for the presence of heterogeneous 5.8S rRNA molecules. Our data indicate that the variant rRNA transcription units are not transcribed during the erythrocytic life cycle. This result is reasonable considering the highly conserved nucleotide sequence and secondary structure which characterize rRNA molecules. It has been reported that a single point mutation in one of these highly conserved regions can have drastic consequences for the cell (Spangler and Blackburn, 1985). Therefore, to maintain sequence homogeneity among the rRNA multigene family, a sequence correction mechanism involving gene conversion and/or unequal crossing-over has been postulated (Miller and Brownlee, 1978; reviewed in Hadjiolov, 1985). Both phenomena have been observed in the rRNA multigene family of yeast (Petes, 1980; Klien and Petes, 1981). A major malfunction in this sequence rectification process is likely to be lethal; however, some mistakes or misalignments seem to be tolerated in the large multigene families giving rise to a few non-functional pseudogenes (reviewed in Wilde, 1986). These variant genes usually contain recognizable sequence similarity with their ancestors but are no longer transcriptionally active as a result of mutation accumulation and/or translocation to an unfavorable chromatin environment.

Large scale genomic rearrangements are known to occur in P. falciparum. Analyses of the plasmodial chromosomes by pulsed-field gradient (PFG) gel electrophoresis reveal dramatic size polymorphisms of both in vivo and in vitro preparations (Van der Ploeg et al., 1985; Kemp et al., 1985; Corcoran et al., 1986). These genomic rearrangements occur frequently and may involve the duplication or deletion of repetitive DNA as well as coding sequences (Kemp et al., 1985; Corcoran et al., 1986; Wellems et al., 1987). Furthermore, Wellems and coworkers have recently reported that the two histidine-rich protein genes which have similar but different nucleotide sequences

appear to have been generated by duplication and interchromosomal translocation from a common ancestral gene (Wellems and Howard, 1986; Wellems et al., 1987).

The variant rRNA transcription units we have identified may also have arisen by duplication and translocation. A comparison of the 5.8S coding region from the minor gene classes with the transcriptionally active 5.8S gene indicates that the class IV transcription unit was probably the most recent to evolve; its sequence diverges only slightly from that of class II. Moreover, the recombinants containing class IV and the predominant class II transcription units both appear to have tRNA-like sequences associated with them (Figure 5), which suggests that the class IV gene may have been generated by a duplication of the class II rDNA. The class I and III rRNA genes probably appeared much earlier than the class I transcription unit, since these differ dramatically from the functional gene. The apparent 3' truncation of the class III 5.8S rRNA coding domain suggests that this rDNA variant may have originated from a misaligned crossing-over event.

It is not known whether the transcriptionally active units are clustered as has been shown for the P. falciparum 5S rRNA genes (Shippen-Lentz and Vezza, in press) or are scattered throughout the genome. This possibility is currently under investigation. Previous studies indicate that the rRNA transcription units of plasmodia are dispersed over several different chromosomes (Van der Ploeg et al., 1985; Langsley et al., 1987); however, the functional rRNA genes may be associated with each other through a higher order chromatin structure and thus remain subject to sequence rectification mechanisms. The inactive rRNA transcription units appear to have been translocated to regions of the P. falciparum genome which are somehow inaccessible to this correction process. Analysis of the rRNA pseudogenes may help to define the effect of chromosome rearrangement on transcription regulation in P. falciparum.

ACKNOWLEDGMENTS

This study was supported by Public Health Service Grants AI 18767 and AI 20597 from the National Institutes of Health. D.S.-L. is a graduate student supported by a University of Alabama at Birmingham Graduate Fellowship. We thank Dr. Jeff Engler, Director of the Primer Core Facility at UAB, for synthesizing the oligodeoxyribonucleotide primers and Brian Gaupp for his assistance with the scanning densitometry analyses.

Figure 1. Southern blot analysis of Dra I or Ssp I digested genomic DNA. Approximately 10 μg of genomic DNA was digested to completion with either Dra I or Ssp I. Southern blot analysis using [^{32}P] labelled 5.8S cDNA identified several distinct gene classes. The positions of the gene classes identified in the λ recombinants are indicated. The UNK designation indicates an uncharacterized rRNA gene class. The arrows indicate the positions of two restriction fragments that were subsequently identified as partial digestion products.

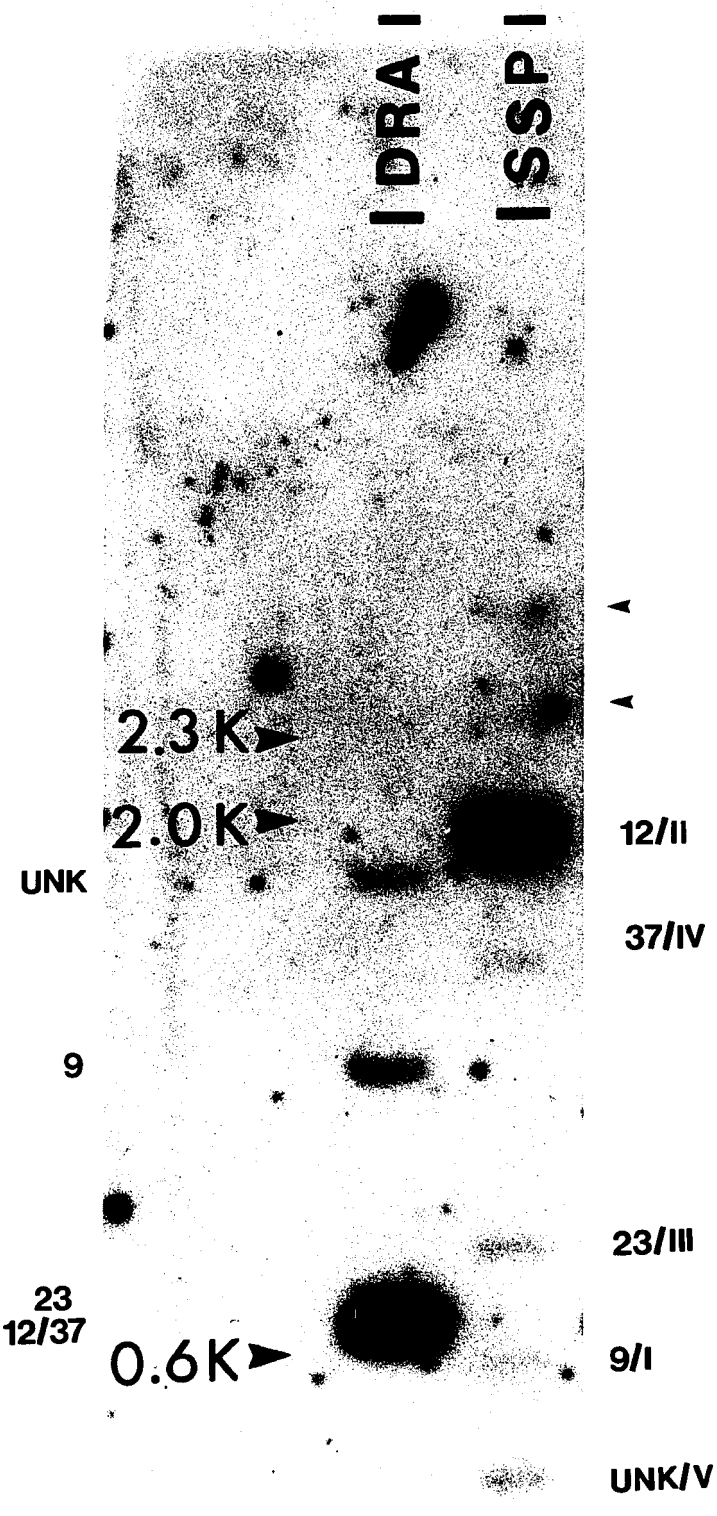

DRA I
SSP I
2.3K
2.0K
0.6K
UNK
9
23
12/37
12/II
37/IV
23/III
9/I
UNK/V

Figure 2. Scanning densitometry of genomic Ssp I cleavage products. Scanning densitometry analysis of the Southern blot profile shown in Figure 1 was undertaken. The positions of the 5.8S rRNA gene fragments corresponding to either the λ or genomic gene classes are indicated. The proportion of each gene class was estimated from the computer output which integrated the area under each peak. The peak at position 85 represents the origin.

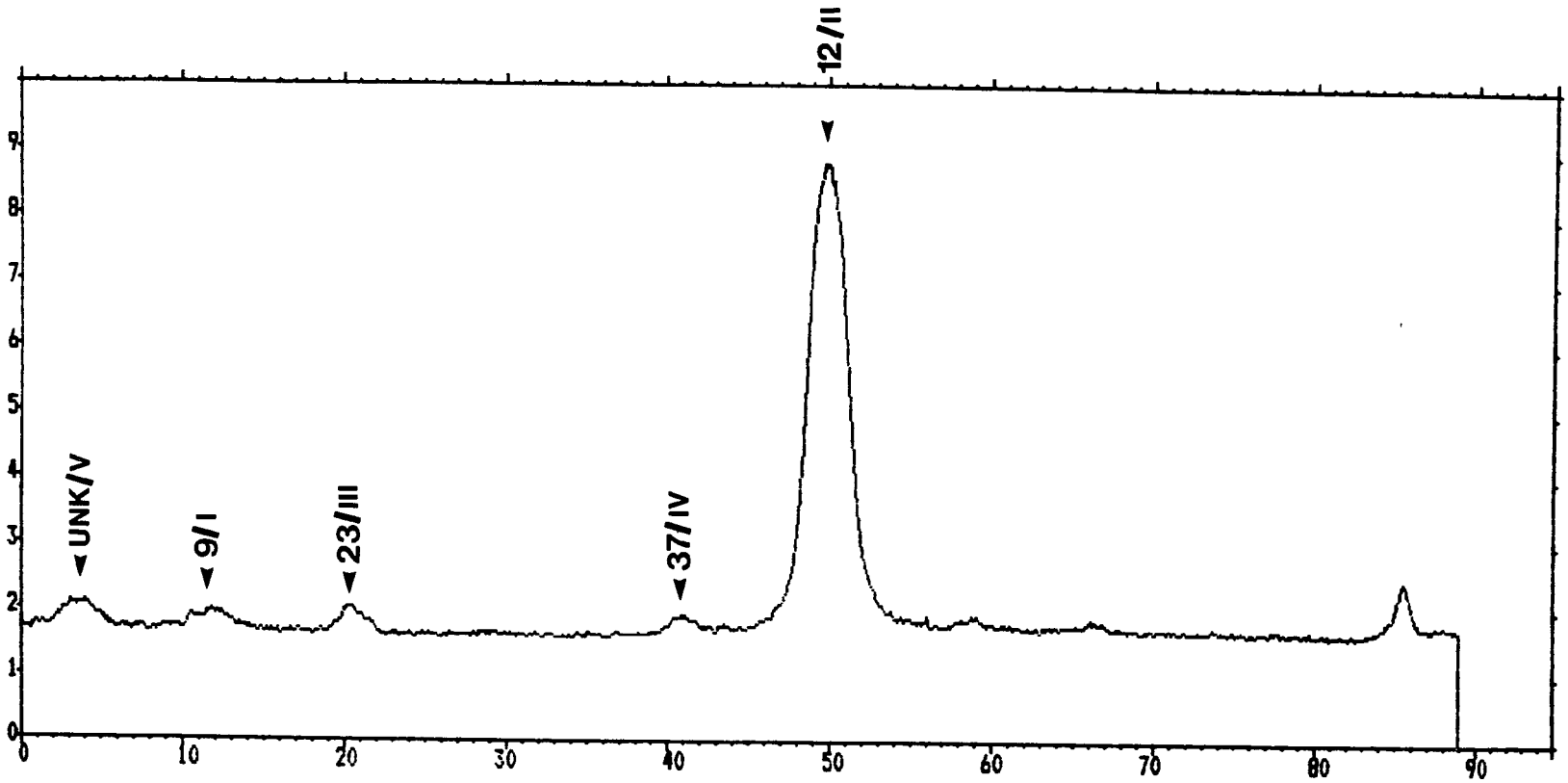
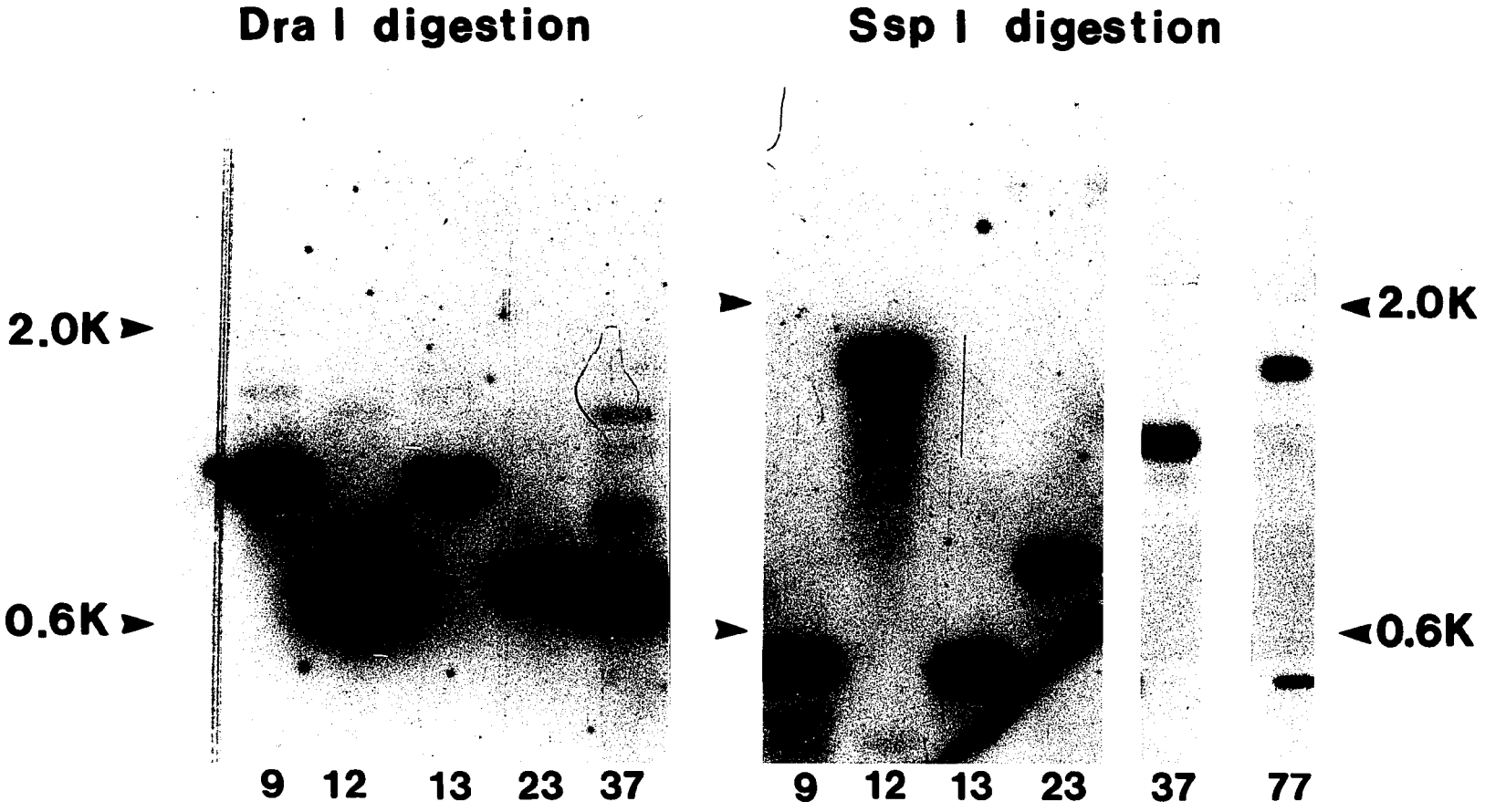

12/II
UNK/V
9/I
23/III
37/IV
0
1
2
3
4
5
6
7
8
9
0
10
20
30
40
50
60
70
80
90

Figure 3. Southern blot analysis of Dra I or Ssp I digestions of the λ recombinants. The λ recombinants were digested to completion with either Dra I or Ssp I. The restriction fragments resolved by agarose gel electrophoresis were analyzed by Southern hybridization using [^{32}P] labelled 5.8S cDNA. Arrows indicate the position of the 2.0 kb and 0.6 kb Hind III λ markers.


Dra I digestion
Ssp I digestion
2.0K
0.6K
2.0K
0.6K
9
12
13
23
37
9
12
13
23
37
77

5.8S rDNA alignment

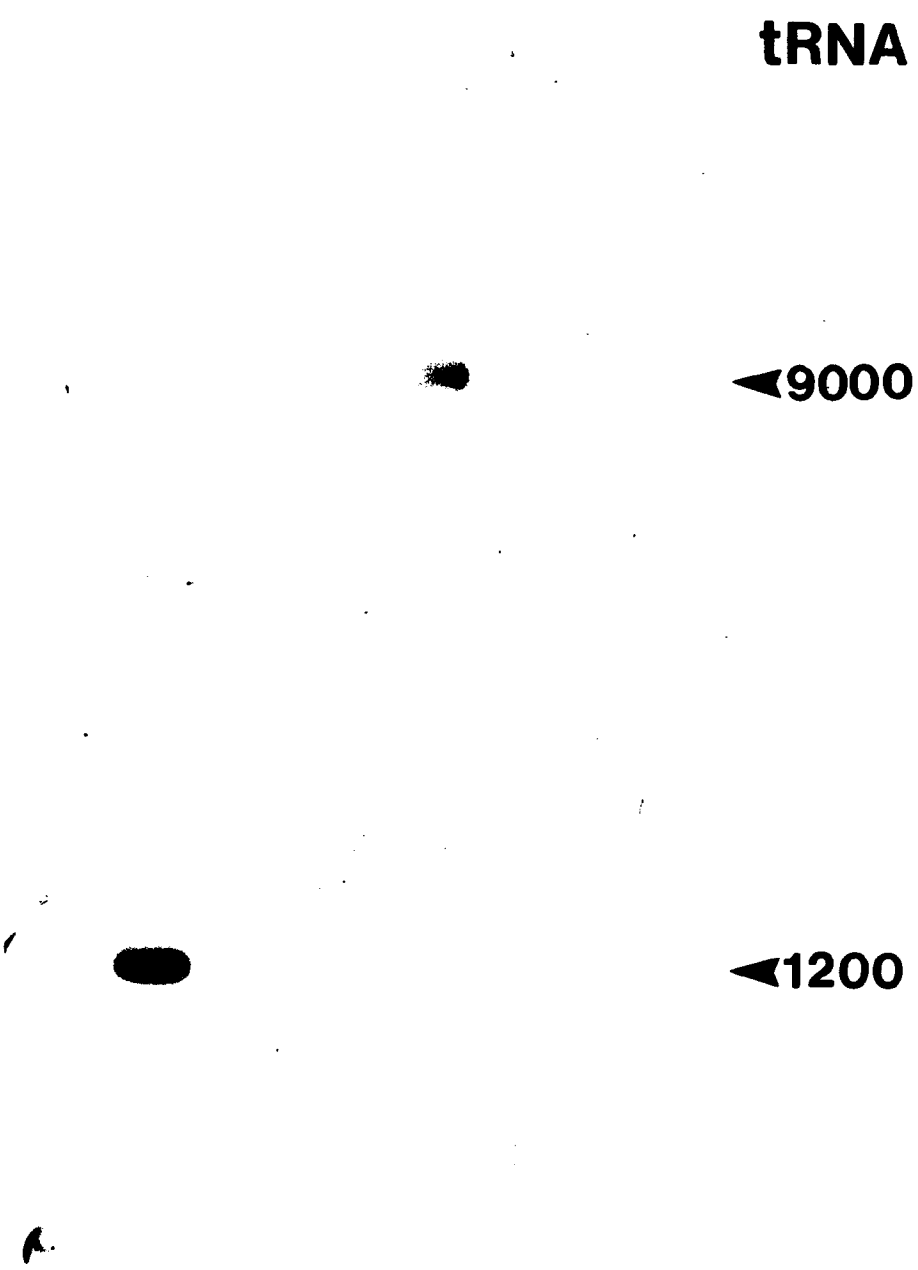
```
                10          20          30          40          50          60
RNA     UAG CACAGU  CUUAACGAUG  GAUGUCUUGG  UUCCUACAGC  GAUGAAGGCC  GCAGCAAAAU
II      TAG CACAGT  CTTAACGATG  GATGTCTTGG  TTCCTACAGC  GATGAAGGCC  GCAGCAAAAT
IV      TAG CACAGT  CTTAACGATG  GATGTCTTGG  TTCCTACAGC  GATGAAGGCC  GCAGCAAAAT
I       TAGCCACAAT  CTTAACGATG  GATGTCTTGG  TTCCTATAGC  GATGAAGGCC  GCAGCAAAGT
III     TAG CACAAT  CTTAACGATG  GATCTCTTGG  CTCTTGTAGC  GATGAAGGCC  GCACGAAAAC
           *    *                  *        *  * **                  **   **

                70          80          90         100         110         120
RNA     GCGAUACGCA  AUGAAAAUUG  CAGUGACUGU  GAAUCAUCAG  AAUGCUGAAU  GUAAACUACA
II      GCGATACGCA  ATGAAAATTG  CAGTGACTGT  GAATCATCAG  AATGCTGAAT  GTAAACTACA
IV      GCGATACGCA  ATGAAAATTG  CAGTGACTGT  GAATCATCAG  AATGCTGAAT  GTAAACTACA
I       GCGCTATGCA  ATGAAAAATG  CAGTGACTGT  GAATCATCAG  AATGCTGAAT  GTAAACTATA
III     GCGATAAGCA  ATGAAAATTG  CAATTACTGT  AAATGATCAG  AATGCTGAAT  GTAAATTACA
           *  *           *       * *       *   *                      *  *

               130         140         150         160
RNA     CC A     G  CUCUU  CGG  GGAA UA  G  UGGUACUCCU  ACAGAAAAU
II      CC A     G  CTCTT  CGG  GGAA TA  G  TGGTACTCCT  ACAGAAAAT
IV      CC A ATT    CCCTT  CGG  GAA  TA  G  TGGTACTCCT  ACAGAAAAT
I       CC ATATTTA  CCCTTATGGG  CAAATTAACG  TGGTATTCCT  ACAGAAAAT
III     CCGATGACTA  TTTTTTTAGG  TATT    CC  CGGTACTCCT  ATTTTTTAT
          * ******  ***  ***    **********  *    *       ******
```

Figure 4. Alignment of the 5.8S rRNA coding regions of the λ recombinants. DNA sequences were determined by the dideoxy sequencing method using primers which hybridized specifically to the 3' or 5' terminal domains of the coding region. The underlined nucleotides indicate the sequence to which the primers hybridized on either the coding or non-coding strand. The asterisks indicate nucleotide changes when compared to the 5.8S rRNA sequence.

Figure 5. Northern blot analysis of erythrocytic stage RNA. Total RNA was resolved by agarose gel electrophoresis. Following transfer to GeneScreen Plus membranes, the filters were cut into strips and then hybridized with [^{32}P] labelled oligonucleotides specific for the 3' terminal region of the 5.8S coding domains of the class I, II, III and IV recombinants.


I
II
III
IV

Figure 6. Southern blot analysis of a Ssp I digestion of the λ recombinants. Recombinant DNA from each of the six λ clones was digested to completion with Ssp I. Following resolution by agarose gel electrophoresis, the cleavage products were transferred to nitrocellulose membranes. The filters were hybridized to [^{32}P] labelled plasmodial tRNA which had been gel purified. The approximate size of the labelled restriction fragments is indicated in base pairs.


tRNA
9000
1200
9 12 13 23 37 77

REFERENCES

Barion, A., Prat, A., and Francois, C. (1987). Telomeric site position heterogeneity in macronuclear DNA of Paramecium primaurelia. Nucl. Acids Res. 15, 1717-1728.

Benton, W. D., and Davis, R. W. (1977). Screening λ gt recombinant clones by hybridization to single plaques in situ. Science 196, 180-182.

Borst, P. (1986). Discontinuous transcription and antigenic variation in trypanosomes. Ann. Rev. Biochem. 55, 701-732.

Childs, G., Maxson, R., Cohn, R. H., and Kedes, L. (1981). Orphons: Dispersed genetic elements derived from tandem repetitive genes of eucaryotes. Cell 23, 651-663.

Corcoran, L. M., Forsyth, K. P., Bianco, A. E., Brown, G. V., and Kemp, D. J. (1986). Chromosome size polymorphisms in Plasmodium falciparum can involve deletions and are frequent in natural parasite populations. Cell 44, 87-95.

Cornelissen, A. W. C. A., Langsley, G., Walliker, D., and Scaife, J. (1985). Gametocytogenesis and ribosomal rRNA gene organisation in rodent malarias Plasmodium chabaudi and Plasmodium berghei. Mol. Biochem. Parasitol. 14, 165-174.

D'Alessio, J. M. (1982). RNA sequencing. In "Gel Electrophoresis of Nucleic Acids: A Practical Approach." (D. Rickwood and B. D. Hames, eds.) pp.173-197. IRL Press, Oxford.

Dame, J. B., and McCutcham, T. F. (1983a). Cloning and characterization of a ribosomal RNA gene from Plasmodium berghei. Mol. Biochem. Parasitol. 8, 263-279.

Dame, J. B., and McCutchan, T. F. (1983b). The four ribosomal DNA units of the malaria parasite Plasmodium berghei: Identification, restriction map and copy number analysis. J. Biol. Chem. 258, 6984-6991.

Dame, J. B., Sullivan, M. and McCutchan, T. F. (1984). Two major sequence classes of ribosomal RNA genes in Plasmodium berghei. Nucl. Acids Res. 12, 5943-5952.

Dame, J. B., and McCutchan, T. F. (1984). Identification of 5S and 5.8S ribosomal RNA molecules and their genes in Plasmodium berghei. Mol. Biochem. Parasitol. 11, 301-307.

Goman, M., Langsley, G., Hyde, J. E., Yankovsky, N. K., Zolg, J. W., and Scaife, J. G. (1982). The establishment of genomic DNA libraries for the human malaria parasite Plasmodium falciparum and identification of individual clones by hybridisation. Mol. Biochem. Parasitol. 5, 391-400.

Grunstein, M., and Hogness, D. (1975). Colony hybridization: a method for the isolation of cloned DNAs that contain a specific gene. Proc. Natl. Acad. Sci. U.S.A. 72, 3961-3965.

Hadjiolov, A. A. (1985)."The Nucleolus and Ribosome Biogenesis." Springer-Verlag/Wein Press, New York.

Kemp, D. J., Corcoran, L. M., Coppel, R. L., Stahl, H. D., Bianco, A. E., Brown, G. V., and Anders, R. F. (1985). Size variation in chromosomes from independent cultured isolates of Plasmodium falciparum. Nature 315, 347-350.

Klien, H. L., and Petes, T. D. (1981). Intrachromosomal gene conversion in yeast. Nature 289, 144-148.

Lambros, C., and Vanderberg, J. B. (1979). Synchronization of Plasmodium falciparum erythrocytic stages in culture. J. Parasitol. 65, 418-420.

Langsley, G., Hyde, J. E., Goman, M., and Scaife, J. G. (1983). Cloning and characterization of the rRNA genes from the human malaria parasite Plasmodium falciparum. Nucl. Acids Res. 11, 8703-8717.

Langlsey, G., Sibilli, L., Mattei, D., Falanga, P., and Mercereau-Puijalon, O. (1987). Karyotype comparison between P. chabuadi and P. falciparum: analysis of a P. chabuadi cDNA containing sequences highly repetitive in P. falciparum. Nucl. Acids Res. 15, 2203-2211.

Lis, J. T., Prestidge, L., and Hogness, D. S. (1978). A novel arrangement of tandemly repeated genes at a major heat shock site in D. melanogaster. Cell 14, 901-919.

McCutchan, T. F. (1986). The ribosomal RNA genes of Plasmodium. Int. Rev. Cyt. 99, 295-309.

Miller, J. R., and Brownlee, G. G. (1978). Is there a correction mechanism in the 5S multigene system? Nature 275, 556-558.

Petes, T. D. (1980). Unequal meiotic recombination within tandem arrays of yeast ribosomal RNA genes. Cell 19, 765-774.

Reese, R. T., Langreth, S. C., and Trager, W. (1979). Isolation of stages of the human malaria parasite Plasmodium falciparum from culture and from animal blood. Bull. W.H.O. 57 (suppl. 1), 53-61.

Shippen, D. E., and Vezza, A. C. (1986). 3' Terminal nucleotide sequence of the 17S ribosomal RNA species from Plasmodium falciparum. J. Parasitol. 72, 178-181.

Shippen-Lentz, D. E., Ray, R., Scaife, J. G., Langsley, G., and Vezza, A. C. (1987). Characterization and complete nucleotide sequence of a 5.8S ribosomal RNA gene from Plasmodium falciparum. Mol. Biochem. Parasitol. 22, 223-231.

Shippen-Lentz, D. E., and Vezza, A. C. The three 5S rRNA genes from the human malaria parasite Plasmodium falciparum are linked. Mol. Biochem. Parasitol., In press.

Southern, E. (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98, 503-517.

Spangler, E. A., and Blackburn, E. H. (1985). The nucleotide sequence of the 17S ribosomal RNA gene of Tetrahymena thermophila and the identification of point mutations

resulting in resistance to the antibiotics paromomycin and hygromycin. J. Biol. Chem. 260, 6334-6340.

Trager, W., and Jensen, J. B. (1976). Human malaria parasites in continuous culture. Science 193, 673-675.

Unnasch, T. R., and Wirth, D. F. (1983a). The avian malaria Plasmodium lophurae has a small number of heterogeneous rRNA genes. Nucl. Acids Res. 11, 8443-8459.

Unnasch, T. R., and Wirth, D. F. (1983b). The cloned genes of P. lophurae: a novel rDNA structure. Nucl. Acids. Res. 11, 8460-8472.

Van der Ploeg, L. H. T., Smits, M., Ponnudurai, T., Vermeulen, A., Meuwissen, J. H. E. T., and Langsley, G. (1985). Chromosome-sized DNA molecules of Plasmodium falciparum. Science 229, 658-661.

Vezza, A. C., and Trager, W. (1981). Preliminary characterization of the major RNA species from the human malaria parasite Plasmodium falciparum. Mol. Biochem. Parasitol. 4, 149-162.

Wellems, T. E., and Howard, R. J. (1986). Homologous genes encode two distinct histidine-rich proteins in a cloned isolate of Plasmodium falciparum. Proc. Natl. Acad. Sci. USA 83, 6065-6069.

Wellems, T. E., Wallider, D., Smith, C. L., de Rosario, V. E., Maloy, W. L., Howard, J., Carter, R., and McCutchan, T. F. (1987). A histidine-rich protein gene marks a linkage group favored strongly in a genetic cross of Plasmodium falciparum. Cell 49, 633-642.

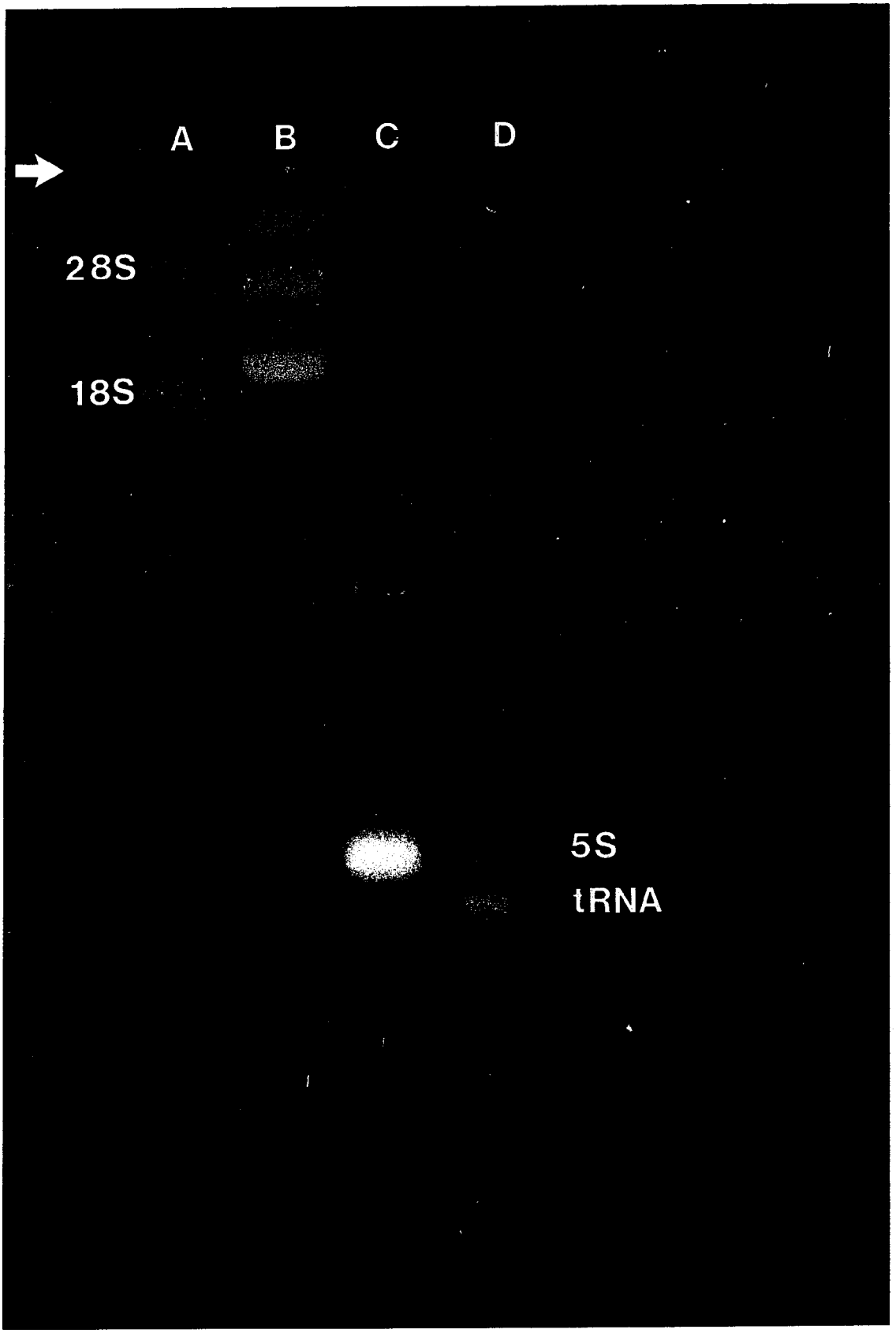
Wilde, D. C. (1986). Pseudogenes. CRC Critical Reviews in Biochemistry. 19, 323-352.

V. DISCUSSION AND SUMMARY

The preceding manuscripts describe the characterization of the ribosomal RNA and ribosomal DNA from the malaria parasite Plasmodium falciparum. In addition to the 25S and 17S rRNA components, P. falciparum contains several small RNA species, including a 5.8S and 5S rRNA (Figure 1). Except for their presence in total RNA preparations, these molecules had not been studied further.

The 5.8S and 5S rRNA molecules from a wide variety of other organisms have been analyzed in great detail. A comparative analysis of these sequences indicates that certain nucleotides are universally conserved for all species studied to date. This conservation also extends to the secondary structure level; numerous models designed to predict the folding of the 5S and 5.8S rRNA have been postulated (Vaughn and Sperbeck, 1984; Erdmann et al., 1985). Therefore, it is not surprising that the 5S and 5.8S rRNA molecules present in a particular organism usually constitute very homogeneous populations. Some variation in length or nucleotide sequence appears to be tolerated, however. Analysis of the 5.8S and 5S rRNA molecules from Xenopus, yeast and several other organisms indicates that these components may be heterogeneous; however, the secondary structure or folding of these molecules is preserved. The divergent molecules can be generated by several different mechanisms, including the transcription of variant rRNA genes (Selker et al., 1985), inefficient transcription termination (Bogenhagen and Brown, 1981) and inaccurate processing of the precursor rRNA species (Tekamp et al., 1980). This finding is particularly interesting in considering the rRNA genes of plasmodia, since these units are known to be heterogeneous in their coding regions. If transcribed simultaneously, these genes might give rise to different RNA populations. To address this possibility, the 5.8S and 5S rRNA species from P. falciparum were analyzed by high resolution polyacrylamide gel electrophoresis (HRPAGE), a technique which detects single

Figure 1. Agarose gel electrophoresis of P. falciparum RNA. A total RNA preparation from P. falciparum was resolved on a 1.5% agarose gel containing ethidium bromide. Lane 1 is BHK rRNA; Lane 2, P. falciparum nucleic acids; Lane 3, E. coli 5S rRNA marker; and Lane 4, yeast $tRNA^{phe}$ marker. The arrow denotes the origin.


A
B
C
D
28S
18S
5S
tRNA

nucleotide differences, and by RNA sequence analysis. These experiments are described in the first two manuscripts.

The 5S rRNA. The 5S rRNA of P. falciparum was resolved into three discrete subclasses by HRPAGE. To determine whether the heterogeneity was due to differences in molecular length or in nucleotide sequence, RNA sequence analyses were undertaken. These studies demonstrated that the three 5S RNA species are identical in sequence and vary only by the presence of one to two additional U residues at the 3' terminus. In vivo and in vitro analyses indicated that these three subclasses are present in similar proportion; therefore, it seems unlikely that non-specific breakdown could account for the length heterogeneity. Moreover, this type of variation was not observed in the analyses of the 17S RNA species (see appendix) or the 5.8S rRNA component which was isolated in an identical manner. To address the possibility that the heterogeneity is the result of inaccurate transcription termination or aberrant RNA processing, the plasmodial 5S rRNA genes were isolated and characterized.

The 5S rRNA sequence from P. falciparum is typically eukaryotic and can be folded into the 5S RNA secondary structure model (Erdmann et al., 1985) as shown in Figure 2. In addition, the plasmodial 5S rRNA molecule contains those nucleotides which appear to be highly conserved in other 5S RNA sequences. Alignment of these sequences with the P. falciparum 5S RNA component indicates extensive homology, especially with the 5S RNA species from some of the ciliated protozoa (Figure 3). However, the plasmodial RNA sequence appears only distantly related to other 5S RNA molecules when additional factors are considered. It is possible to compare two nucleotide sequences by calculating a dissimilarity coefficient or D_{AB} value which reflects the evolutionary relatedness between the different molecules by taking into account the number of matches, mismatches and spaces needed to align the sequences for maximum homology (DeWatcher et al., 1985). Calculation of the dissimilarity coefficients for these alignments does not

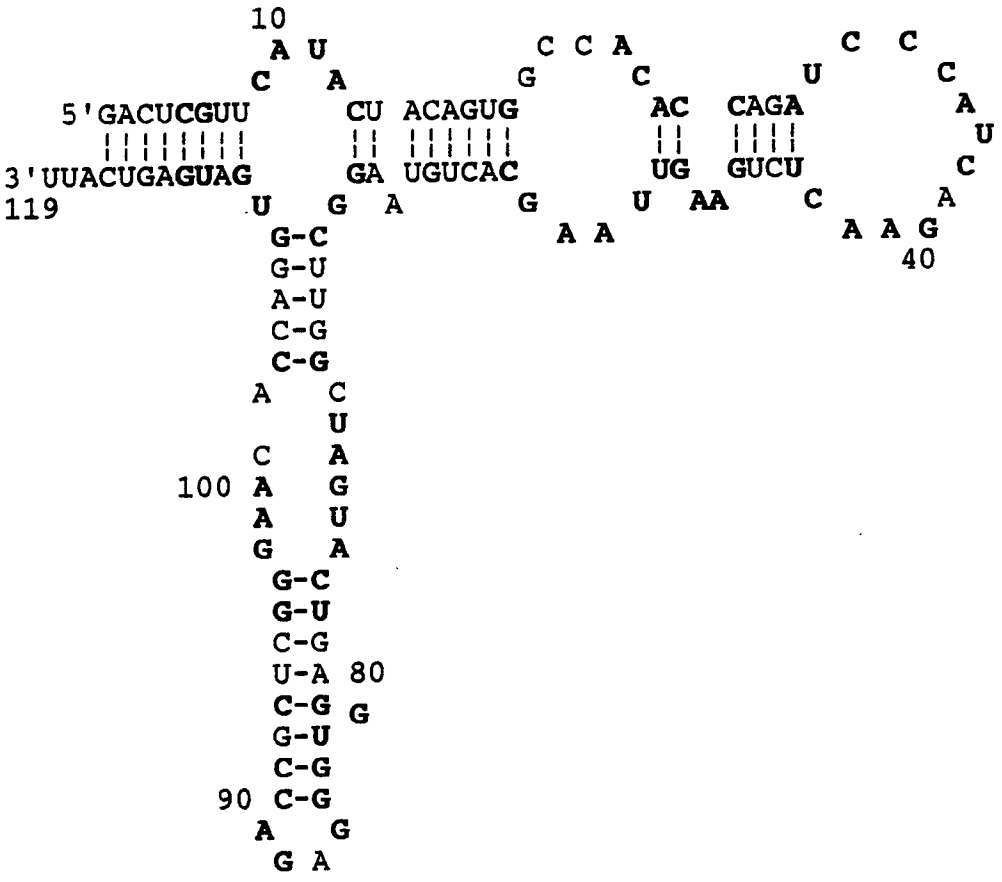


Figure 2. Possible folding of the P. falciparum 5S rRNA based on the Erdmann 5S rRNA secondary structure model (Erdmann et al., 1985). Bold nucleotides indicate bases which are highly conserved among eukaryotic 5S rRNA sequences.

Figure 3. Nucleotide sequence alignment. The P. falciparum 5S rRNA was aligned with the 5S rRNA sequences from four other lower eukaryotic organisms. Dashed lines indicate homologous nucleotides.

P. falciparum
T. pyriformis

```
1          G ACUCGUUCAUACUACAGUGGCCACACCAGAUCCCAUCAGAACUCUGAAGUUAAGCACU
           |   |||  |||||||  |||   |||||  |||||||| |||||| |||||||||| |
1          GUUGUCGGCCAUACUAAGGUGAAAACACCGGAUCCCAUUCGAACUCCGAAGUUAAGCGCC

60         GUAAGGCUUGGCUAGUACUGAGGUGGGAGACCGCUCGGGAACACCAGGUGAUGAGUCAUU
            |||||||  ||  |||||||  |||||||  |||||||  |||||  ||  |||  ||     |
61         UUAAGGCUGGGUUAGUACUAAGGUGGGGGACCGCUUGGGAAGUCCCAGUGUCGAUAGCCU
```

P. falciparum
C. fasciculata

```
1          GACU CGUUCAUACUACAGUGGCCACACCAGAUCCCAUCAGAACUCUGAAGUUAAGCACU
           ||  |  ||  ||||||  ||||   |||||| |||||  ||  ||   | ||||||||||||||
1          GAGUACGACCAUACUUGAGUGAAAACACCAUAUCCCGUCCGAUUUGUGAAGUUAAGCACC

60         GUAAGGCUUGGCUAGUACUGAGGUGGGAGACCGCUCGGGAACACCAGGUGAUG AGUCAU
              ||||||  |  ||||||||||||||  |  ||   |||||||||  |   |||  |  |  ||
61         CACAGGCUUAGUUAGUACUGAGGUCAGUGAUGACUCGGGAACCCUGAGUGCCGUACUCCC

119        U
```

P. falciparum
S. pombe

```
1          GACU CGUUCAUACUACAGUGGCCACACCAGAUCCCAUCAGAACUCUGAAGUUAAGCACU
           |  ||  ||  |||||     |  |   ||||||| |||| ||  ||  |  |||  |||||||||
1          GUCUACGGCCAUACCUAGGCGAAAACACCAGUUCCCGUCCGAUCACUGCAGUUAAGCGUC

60         GUAAGGCUUGGCUAGUACUGAGGUGGGAGACCGCUCGGGAACACCAGGUGAUG AGUCAU
             |  |||  |  |  |||||||   |||  ||||||   |   |||||   |   ||||  ||  ||  |
61         UGAGGGCCUCGUUAGUACUAUGGUUGGAGACAACAUGGGAAUCCGGGGUGCUGUAGGCU

119        U
```

P. falciparum
S. cerevisiae

```
1          GACU CGUUCAUA CUACAGUGGCCACACCAGAUCCCAUCAGAAC UCUGAAGUUAAGC
           |   |  ||  ||||  ||||    |     ||||       ||  ||  ||  |  |||  |||||||||
1          GGUUGCGGCCAUAUCUAC CAGAAAGCACCGUUCUCCGUCCGAUCAACUGUAGUUAAGCU

57         ACUGUAAGGCUUGGCUAGUACUGAGGUGGGAGACCGCUCGGGAACACCAGGUGAUG AGU
             |    ||  ||    |  ||||  ||  |||||  ||||    ||  |||    ||||||  ||  |  |
60         GGUAAGAGCCUGACCGAGUAGUGUAGUGGGUGACCAUACGCGAAACUCAGGUGCUGCAAU

116        CAUU
           |
120        CU
```

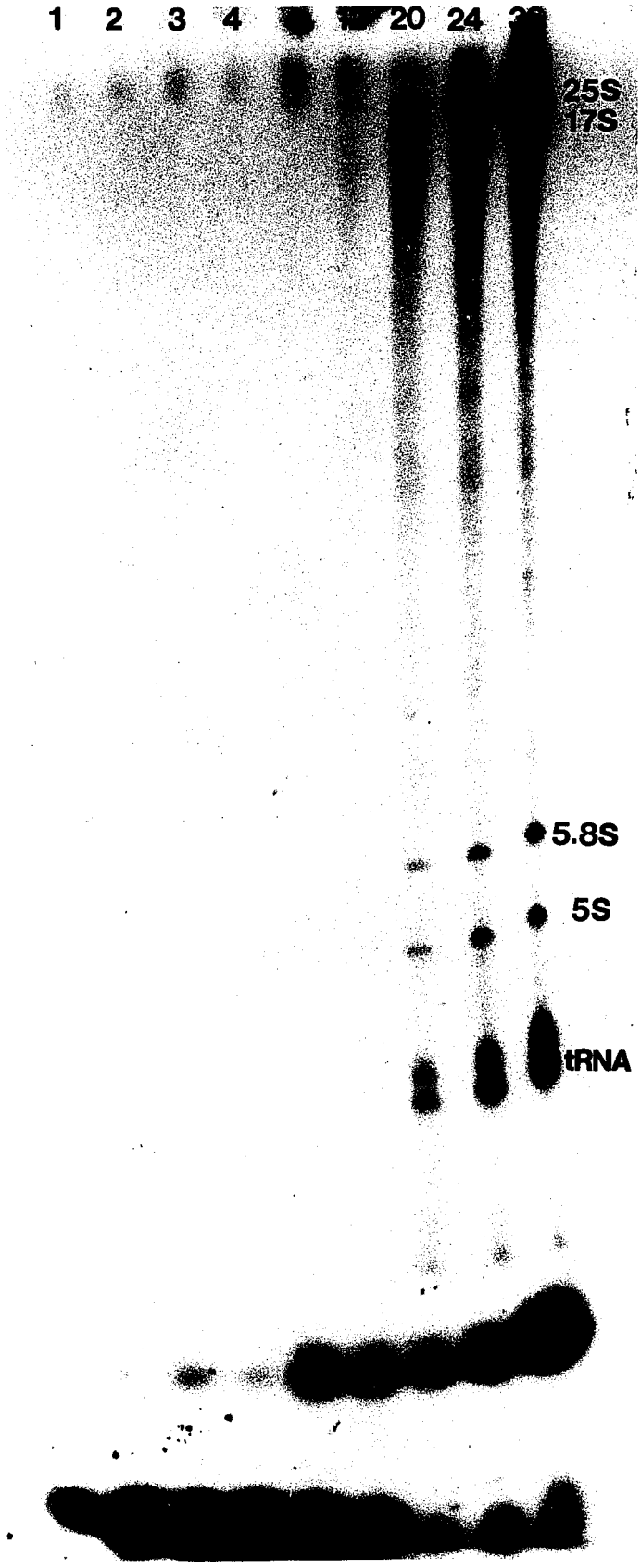
reveal a close relationship between the P. falciparum 5S rRNA and other 5S RNA sequences.

The 5S rRNA genes. The 5S rRNA genes of most organisms are present in very high copy number, are identical in sequence and are arranged as tandemly repeated units. The characterization of the 5S rRNA genes from P. falciparum was of considerable interest since previous studies have demonstrated that the number, structure and organization of the plasmodial large rRNA transcription units are unlike those of other eukaryotic organisms. To determine whether the plasmodial 5S rRNA
genes exhibit similar dispersion, sequence heterogeneity and exceptionally low copy number, further analyses were undertaken.

These studies indicated that P. falciparum contains only three 5S rRNA genes, the lowest number reported for any eukaryotic organism. The three genes are clustered within a 1500 bp fragment of DNA. This is the first example of tandemly arranged genes in plasmodia.

With so few 5S rRNA genes, it is difficult to imagine how the parasite is capable of producing enough RNA for ribosome biogenesis. It has been postulated that during the erythrocytic life cycle, transcription of rRNA is greatest after the genome has replicated and that the combined contribution of these newly synthesized genes is sufficient to meet the demand for rRNA molecules (Dame and McCutchan, 1983b; McCutchan, 1986). In vivo labelling using [^{32}P] orthophosphate indicated that de novo synthesis of P. falciparum rRNA and tRNA is not detectable until 8-12 hours after merozoite invasion (Figure 4). The production of these molecules appears to increase steadily during the remaining 36 hours of the cycle, however. These data demonstrate that the 5S rRNA genes are not expressed constitutively during erythrocytic development. It is not clear whether the increase in rRNA production observed later in the cycle reflects the contribution of newly replicated rRNA genes or if a time lag is required for the synthesis and/or assembly of the rRNA

Figure 4. Ribosomal RNA biosynthesis. Parasitized erythrocytes were synchronized over several 48 hour growth cycles using an alternating gelatin/sorbitol procedure. Nucleic acids were extracted from infected erythrocytes at various times post-invasion, ranging from 1 to 36 hours. The samples were analyzed by PAGE on a 2.5% gel. The migratory positions of the major RNA species are indicated.


1
2
3
4
20
24
25S
17S
5.8S
5S
tRNA

transcription apparatus (polymerase and associated transcription factors). Further studies are needed to address these possibilities.

The presence of only three 5S rRNA genes is even more remarkable when the highly prolific stages of parasite development are considered. During the exoerythrocytic cycle in the liver and sporozoite formation in the mosquito, thousands of parasites are produced in a five to ten day period. Nothing is known about mechanisms employed to meet the tremendous demand for rRNA because it is very difficult to obtain material for study from these developmental stages. It is conceivable that the transcription of 5S rRNA genes is augmented by the presence of enhancer sequences (Labhart and Reeder, 1984; Reeder, 1984) or by the location of 5S rDNA within regions of active unlocked chromatin structure (Hadjiolov, 1985). 5S rRNA enhancer elements have not been identified in other organisms; however, upstream sequences can influence Pol III transcription in vitro (Sprague et al., 1980).

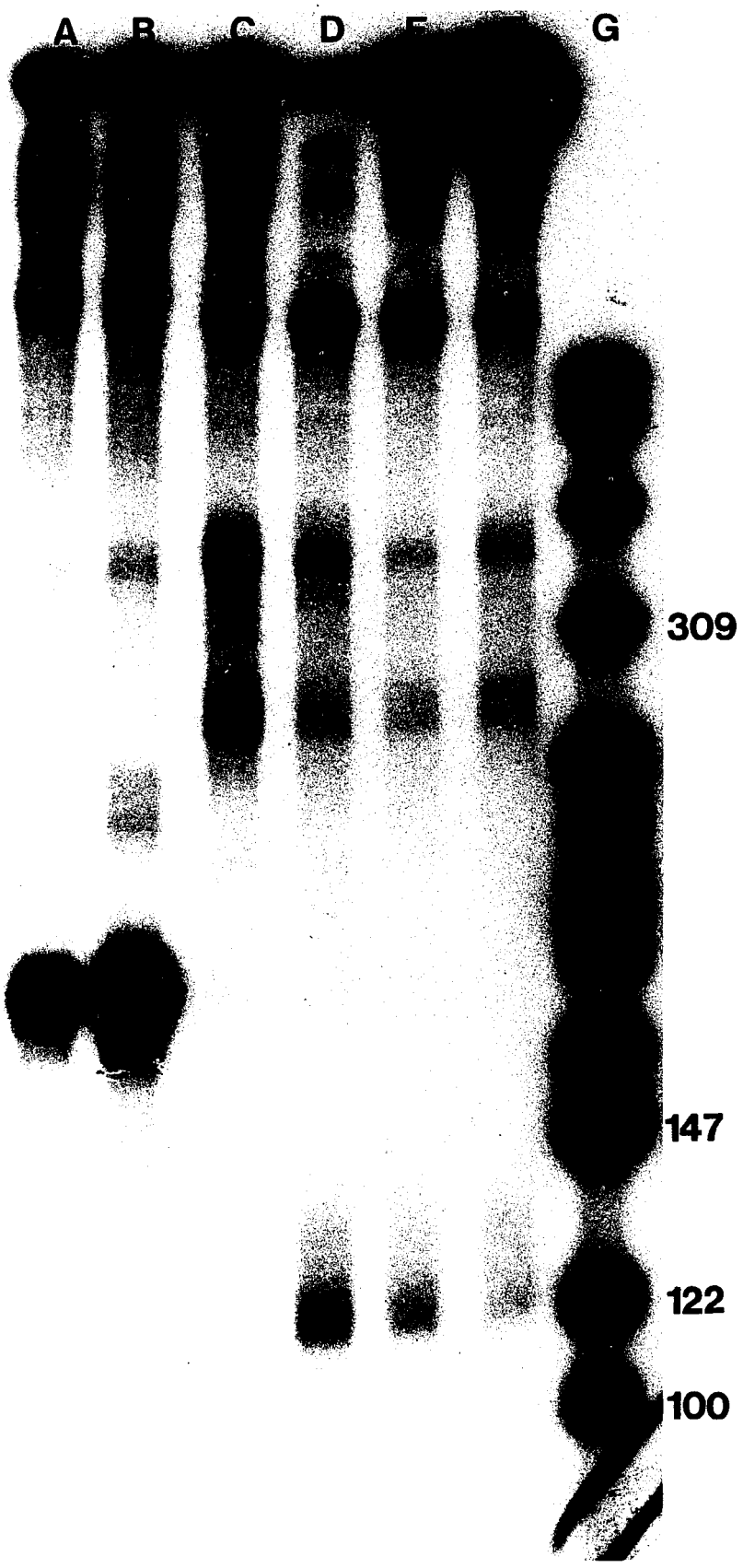
As a first step in studying the expression of plasmodial 5S rRNA genes, DNA sequence analyses were undertaken to define transcription initiation and termination signals. The data indicate that all three 5S rRNA coding regions are identical and are surrounded by divergent A+T rich flanking sequences. Since the 5S rRNA species of P. falciparum exhibit 3' terminal heterogeneity, it was of interest to determine the location of the RNA polymerase III transcription termination signal. The position of this site dictates whether transcription will be halted at the 3' end of the coding region or whether a 5S rRNA precursor molecule will be produced. The Pol III termination signal has been defined as four or more consecutive T residues on the non-coding strand (Bogenhagen and Brown, 1981). In yeast, the termination site lies approximately 20-30 nucleotides downstream from the coding region (Tabata, 1981) which results in the transcription of a precursor molecule. This species is subsequently processed to remove the additional 3' terminal nucleotides; however, the processing event is not always precise and 5S rRNA molecules of slightly different lengths may be produced (Tekamp et al., 1980). For most other

organisms, the transcription termination signal is directly adjacent to the coding region. In this case, 5S rRNA length variation appears to result from a "stuttering" of the polymerase or the transcription complex at the stretch of T residues (Bogenhagen and Brown, 1981).

All of the 5S rRNA coding regions from P. falciparum are directly abutted by six to eight T residues. Therefore, it seems likely that the terminal heterogeneity observed in the 5S RNA molecules is caused by inaccurate transcription termination rather than aberrant rRNA precursor processing. No evidence for a 5S rRNA precursor was encountered in the in vivo or in vitro analyses of total RNA preparations. It is interesting to note that the 3' flanking region of each plasmodial 5S rRNA gene contains a second run of T residues about 20 nucleotides downstream from the first termination signal. Whether these nucleotides can function as a back-up termination site during periods of stress such as heat shock (Rubin and Hogness, 1975) is not known.

One of the most informative approaches for studying transcription regulation in P. falciparum may be the use of in vitro transcription systems which permit the dissection of important regulatory sequences. Genes containing selected mutations can be introduced to define functional domains, influencing the rate or level of transcription. In addition, purification of the active components in the in vitro extract can provide information about the transcription complex and associated factors. As a first step in this direction, the 5S rRNA genes were tested in Pol III in vitro extracts derived from S. cerevisiae or 293 cells, an adenovirus transformed cell line, to determine whether the P. falciparum ICR could be recognized by a heterologous transcription apparatus. The plasmodial 5S rRNA genes were tested with several different yeast extracts and no 5S rRNA transcripts were detected. This result is not surprising since previous studies have shown TFIIIA, one of the Pol III transcription factors, can be species-specific (reviewed in Lassar et al., 1983). However, the ICR of the P. falciparum 5S rRNA gene was recognized by factors within the 293 cell extract and a transcript of approximately 120 nucleotides was generated (Figure 5). The efficiency of the transcription appears to be about 20 fold less for the plasmodial gene than

Figure 5. In vitro transcription analysis of the N2 recombinant. Varying concentrations of the pGEM recombinant containing the N2/Dra I restriction fragment were incubated in S-100 in vitro transcription extracts derived from the adenovirus transformed 293 human cell line. Lanes A and B are expression controls which consist of a plasmid containing the adenovirus VA I gene. Lanes C-F correspond to 0.2, 0.4, 0.8 and 1.6 μg of the pGEM/N2 plasmid. Lane G is Msp I digested pBR322 markers. The size of some marker fragments is indicated. A transcript of approximately 122 nucleotides was identified in lanes D-F.


A
B
C
D
E
G
309
147
122
100

for the VA gene control. Nevertheless, this datum suggests that the regulatory region and possibly the Pol III transcription apparatus of P. falciparum may be similar to those found in other eukaryotic organisms. In addition, these studies have also identified a positive control assay which can be used in developing a homologous in vitro Pol III transcription system for P. falciparum.

The 5.8S rRNA. Unlike the 5S rRNA component, the P. falciparum 5.8S rRNA is a single homogeneous population of molecules 157 nucleotides in length. Since the 5.8S rRNA is generated by the processing of a high molecular weight precursor molecule, it can be concluded from these results that this cleavage event is very precise. Moreover, the presence of a homogeneous population of 5.8S rRNA molecules further suggests either that the 5.8S rRNA coding regions from the different transcription units are identical, or that only a subset of these units are expressed during the erythrocytic life cycle, the stage from which the 5.8S RNA was isolated. The transcriptional activity of the variant rRNA transcription units of P. falciparum is investigated more thoroughly in the third manuscript.

Some difficulty was encountered in determining the 5.8S rRNA sequence; the identity of a few nucleotides could not be ascertained. These unknown bases were later identified as C residues. Previous studies have demonstrated that 5.8S rRNA molecules can contain pseudouridine and methylated ribose (Walker and Pace, 1983; Nazar et al., 1983). The chemical reactions used in the RNA sequence analysis depend on base modification and subsequent cleavage by aniline at the weakened site; therefore, modifications of the base or ribose moiety may render these nucleotides more resistant to cleavage. Such alterations often appear as gaps in the nucleotide sequence ladder. It seems likely that these cytosine residues in the P. falciparum 5.8S rRNA may be modified in some manner; however, the particular alteration is not known.

The 5.8S rRNA from P. falciparum is homologous to the 5.8S rRNA molecules from other organisms; the nucleotides which are thought to be conserved for all organisms studied thus far (Erdmann et al., 1985) are present in the P. falciparum sequence.

However, like the 5S rRNA sequence, the plasmodial 5.8S rRNA species appears to be only distantly related to other 5.8S rRNA molecules when dissimilarity coefficients are calculated (data not shown). These data do not necessarily indicate that the 5S and 5.8S rRNAs of P. falciparum are unusual. It is more likely that these results reflect the lack of sufficient sequence data for protists and, more importantly, other members of the class Sporozoa. It should be noted that phylogenetic comparisons made with small RNA molecules such as the 5S or 5.8S rRNAs are not likely to define precise evolutionary relationships since only 120-160 nucleotides are available for analyses. A more accurate assessment can be determined by comparing the S-rRNA and L-rRNA species; these molecules are several thousand nucleotides in length and provide a much larger sample for comparison. Unfortunately, relatively few S-rRNA and L-rRNA sequences have been published.

The 5.8S rRNA genes. Although the 5.8S rRNA coding region represents less than two percent of the large rRNA transcription unit, characterization of this segment in P. falciparum may provide some insight into the nature of heterogeneity between the rRNA genes and help to identify transcriptionally active units. These studies are presented in the third manuscript. Langsley and co-workers have reported that the rRNA transcription units of P. falciparum are present in only four to eight copies per haploid genome and can be categorized into two to three transcriptional classes by their restriction profiles (Langsley et al., 1983). However, analyses of the P. falciparum 5.8S rRNA coding regions suggest that the rRNA genes identified in the previous study represent only a fraction of the total number of rRNA transcription units present. Southern blot hybridization data indicate that P. falciparum contains approximately 35 rRNA transcription units. These genes are heterogeneous and can be categorized into a predominant class representing about 30 rRNA units and several minor rDNA components which contain only one or two genes. The results obtained in the first study are not easily explained. It is clear that the experimental approaches taken are somewhat different. Assuming the latter estimation is correct and a

significant percentage of these rRNA transcription units are active, the transcriptional analyses of the P. falciparum 5S rRNA genes become even more interesting. Since ribosome biogenesis requires equimolar amounts of L-rRNA, S-rRNA, 5.8S rRNA and 5S rRNA, it is expected that the transcriptional capacity of the three 5S rRNA genes must be exceptionally high to keep pace with the rRNA transcription units.

Sequence heterogeneity has been well documented for the plasmodial rRNA transcription units, although a direct comparison of the nucleotide sequences from the different rRNA genes has not been published. This kind of analysis would not only provide information concerning the extent of divergence, but might also indicate a possible mechanism to explain the emergence of heterogeneous rRNA genes. Since the 5.8S rRNA coding regions have not been previously characterized by restriction endonuclease digestion, it was not known whether these components exhibit sequence variation. To address this possibility, the 5.8S rRNA genes from the predominant class II and the three minor classes were compared by DNA sequence analysis. These studies demonstrated a relatively high degree of heterogeneity between the 5.8S rRNA coding regions. A comparison of the 5.8S rRNA sequence with the 5.8S rDNA indicated that the predominant gene class is complementary to the RNA while the the minor 5.8S rRNA genes contain multiple point mutations, deletions and insertions. The position of these A+T rich insertions is intriguing since the only other organisms known to contain interrupted 5.8S rRNA genes are some species of Dipteran insects (Jordan et al., 1980). In Scaira and Drosophila, the 5.8S rRNA coding regions contain 20-30 bp insertions which lie approximately 30 nucleotides upstream from the 3' terminus (Figure 6). Transcription and subsequent processing of the precursor RNA results in two distinct RNA species, 123 and 30 nucleotides in length, which are hydrogen bonded together. The 5.8S rRNA sequence for the anopheles mosquito has not been published; therefore, it is not known whether the 5.8S rRNA coding regions from P. falciparum are related to the rRNA genes of the vector. Genetic exchange between parasite and host has been postulated (Howell, 1985).

The 5.8S rDNA of Sciara coprophila and Drosophila melanogaster

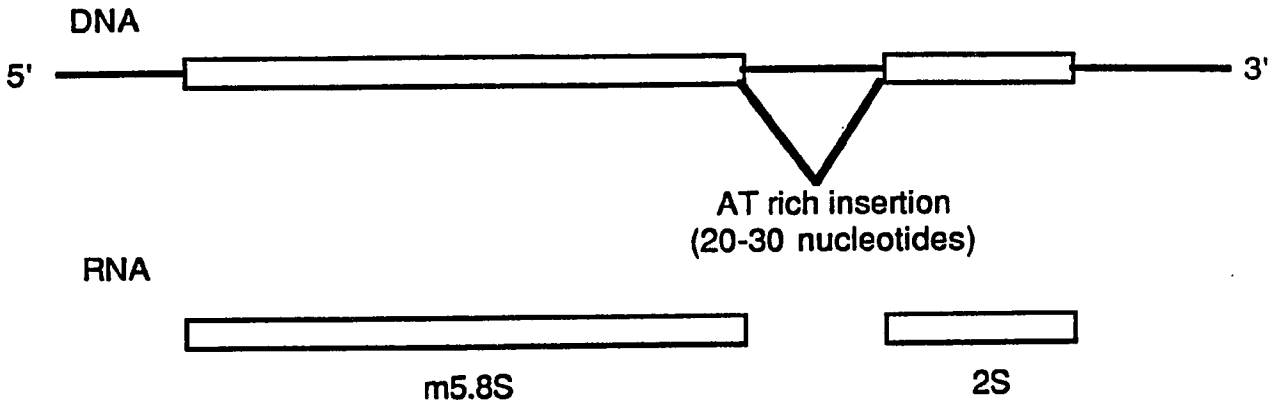


Figure 6. The 5.8S rRNA coding domains of two Dipteran insects. An insertion located approximately 30 nucleotides in from the 3' terminus of the 5.8S rRNA coding region is removed by endonucleolytic cleavage from the large rRNA precursor. Two distinct 5.8S rRNA components are generated.

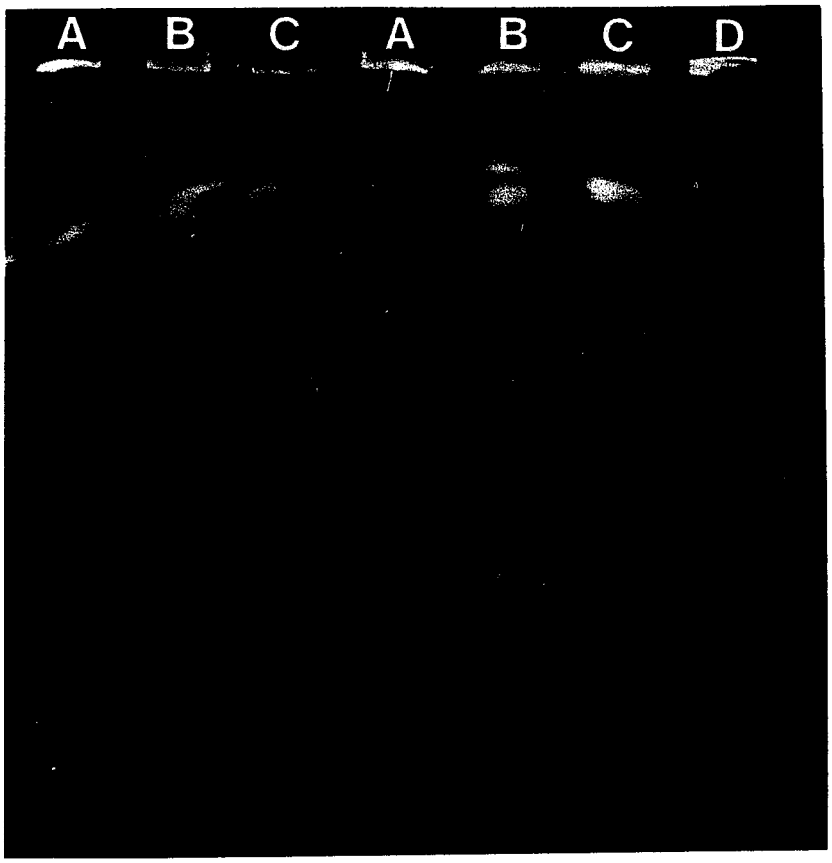
Since the 5.8S rRNA species was shown to consist of a homogeneous population of molecules, it seemed likely that the minor gene classes were transcriptionally inactive, at least during erythrocytic development. Northern blot analysis confirmed this assumption. These data suggest that the minor rDNA variants of P. falciparum are pseudogenes.

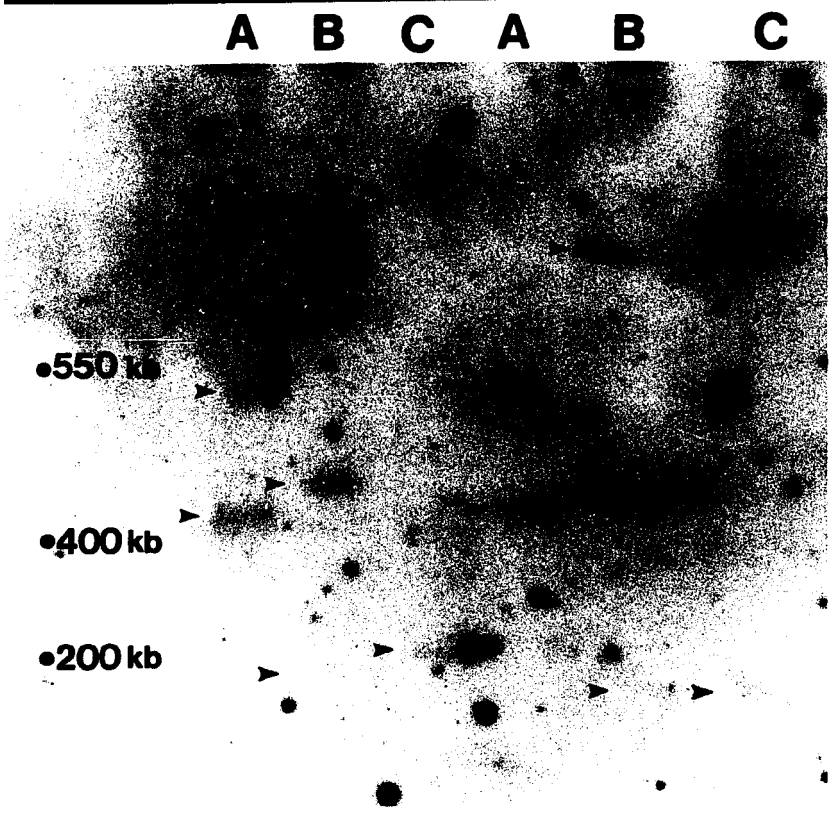
In characterizing the 5.8S rRNA coding regions, several interesting results were obtained which concern the genomic organization of the rRNA transcription units. It has been reported that the rRNA transcription units of plasmodia are not arranged in recognizable tandem repeats (Unnasch and Wirth, 1983b) and are dispersed on several different chromosomes (Van der Ploeg et al., 1985; Langsley et al., 1987; Wellems et al., 1987). Therefore, it was somewhat surprising to find two 5.8S rRNA coding regions, one from a transcriptionally active unit and one from an inactive gene, present within the same λ recombinant. These data indicate that at least two of the P. falciparum rRNA transcription units are linked.

To investigate this possibility further, genomic DNA was digested with restriction endonucleases which recognize statistically rare nucleotide sequences in the plasmodial genome. The DNA fragments generated were separated by PFG electrophoresis and studied by Southern blot analyses. These experiments are particularly informative in gene linkage analyses because very large fragments of DNA can be studied. For the rRNA transcription units, this technique provides a convenient alternative to R-Loop analysis. As shown in Figure 7, the 5.8S cDNA probe hybridizes to several fragments, including several in the 100-150 kb size range. Assuming P. falciparum contains approximately 35 rRNA transcription units, these results suggest that a least some of the rRNA genes may be linked.

Another unusual result was obtained in characterizing the λ recombinants containing the rRNA transcription units. The 5.8S rRNA genes analyzed in these studies were isolated from λ clones selected from a P. falciparum genomic library. Although the transcriptionally active rRNA genes appear to be present in significantly higher copy

Figure 7. Southern hybridization of restriction digests of P. falciparum chromosomes. Agarose blocks containing P. falciparum DNA were digested to completion with either Bgl I (lane A), Bssh II (lane B) or Sma I (lane C). These "infrequent cutters" generate large cleavage products which were resolved by PFG electrophoresis, transferred to nitrocellulose filters and probed with [^{32}P] 5.8S cDNA. The left panel is a photograph of the ethidium bromide stained gel. The right panel is an autoradiogram of the Southern blot analysis. The arrows indicate the positions of several rDNA containing fragments. λ multimer markers are present in lane D.


A B C A B C D


A B C A B C
•550 kb
•400 kb
•200 kb

number than the minor rDNA components, all of the transcriptional classes were cloned with approximately equal efficiency. If each of the 35 rRNA transcription units had an equal probability of selection, the odds of this outcome are extremely small. It seems more reasonable to assume that the transcriptionally active rRNA genes are located in some region of the P. falciparum genome that is not easily accessible or amenable for cloning into λ. One possibility is that these rRNA transcription units exist as extrachromosomal DNA. Several data dispute this idea. Previous attempts to identify extrachromosomal rRNA genes in plasmodia have not been successful (Cornelissen et al., 1985; McCutchan, 1986). Furthermore, none of the PFG studies have indicated the presence of relatively small DNA molecules which hybridize to the rDNA probes ((Van der Ploeg et al., 1985; Langsley et al., 1987; Wellems et al., 1987).

An alternative explanation is that the transcriptionally active rRNA genes of P. falciparum are located near telomeres, the specialized structures at the ends of chromosomes. Telomeres are comprised of repeated sequences of about six to eight nucleotides which are reiterated hundreds to thousands of times (reviewed in Blackburn, 1984). These regions are not amenable to cloning for several reasons. Telomeric sequences show very little variation and, therefore, often do not contain any of the common restriction endonuclease cleavage sites. Moreover, the actual terminus of a chromosome is thought to be a hairpin structure which cannot be ligated to other DNA molecules (Greider and Blackburn, 1985). Perhaps as a consequence of their unusual structure, telomeric regions appear to confer a distinct transcriptional advantage on genes located nearby. The variant-specific surface glycoprotein genes of trypanosomes (reviewed in Borst, 1986) and the A and G surface antigen genes of paramecium (Baroin et al., 1987; E. Blackburn, personal communication) are preferentially activated when they are associated with telomeres. In addition, it has been reported that the telomeric location of a histidine-rich protein gene in P. falciparum may provide a selective advantage for the parasite (Wellems et al., 1987). Based on these data, it seems worthwhile to investigate whether the

transcriptionally active rRNA genes of P. falciparum are somehow associated with telomeres.

The location of the rRNA transcription units may not necessarily remain fixed, however. Several recent studies indicate that large scale chromosomal rearrangements occur frequently in the P. falciparum genome. This finding, in conjunction with the data obtained in the analyses of the 5.8S rRNA genes, suggests a possible origin for the dispersion of the rRNA transcription units and their extensive sequence heterogeneity.

Because of the highly conserved structure and function of the rRNA molecules, it is crucial for cells to maintain sequence homogeneity among the rRNA multigene family and to correct the mutations that normally occur during the course of DNA replication and exposure to various environmental assaults. This is accomplished by at least two recombination mechanisms, gene conversion and unequal crossing-over, which involve either intra- or interchromosomal genetic exchange (Figure 8). Both phenomena have been shown to occur frequently among the rRNA multigene family of S. cerevisiae; however, gene conversion may be the better correction mechanism, since it allows sequence homogenization without altering copy number (Klien and Petes, 1981). Some genetic risk is incurred during unequal crossing-over events. Not only is gene dosage affected, but neighboring sequences may also be deleted or translocated to other areas of the genome (Petes, 1980; Klien and Petes, 1981). In addition, if aberrant sequence alignment occurs prior to recombination, hybrid genes may be created. Unequal crossing-over has been postulated to be a major mechanism for the generation of pseudogenes (Wilde, 1986).

Wellems and coworkers have recently reported that the two P. falciparum histidine-rich protein genes lie on different chromosomes and have related but distinct nucleotide sequences. They suggest that these genes were generated by duplication and interchromosomal translocation from a common ancestral gene (Wellems and Howard, 1986; Wellems et al., 1987).

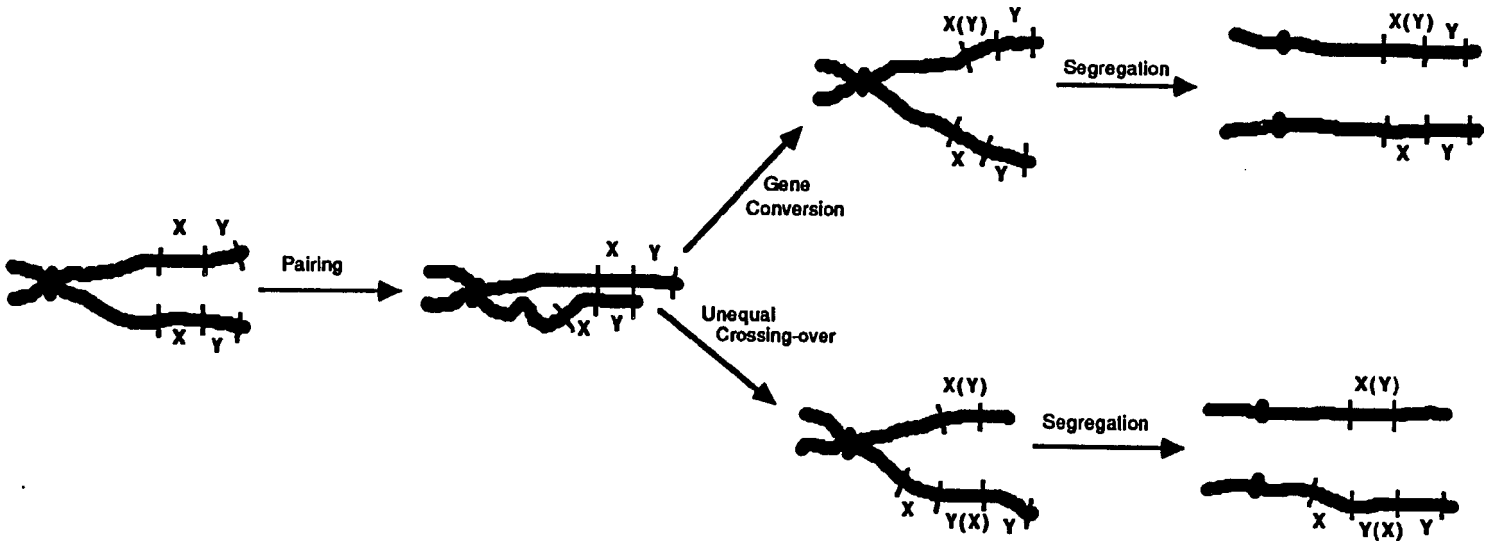


Figure 8. Two correction mechanisms postulated to maintain sequence homogeneity within large multigene families. In gene conversion, the sequence of a recipient gene X is "converted" to the sequence of a donor gene Y. Copy number is not affected. Unequal crossing-over results in two hybrid genes. The number of genes is reduced on one strand and increased on another (adapted from Hadjiolov, 1985).

The dispersion of the rRNA transcription units in P. falciparum is likely to have occurred through a similar process of genomic rearrangement. In this case, the ancestral gene could be any one of the transcriptionally active rRNA transcription units. Not only are the nucleotide sequences of the variant 5.8S rRNA genes very similar to the expressed gene, but hybridization studies reveal that the functional transcription unit and at least one of the nonfunctional rRNA genes are associated with tRNA-like sequences. The inactivation of the rRNA gene variants is not contingent upon their translocation away from other rRNA transcription units. There are several examples of pseudogenes that lie within a cluster of transcriptionally active genes; however, their position in relation to the higher order chromatin structure appears to dictate whether a gene is subject to sequence rectification (reviewed in Wilde, 1986). This may explain the finding of the two linked rRNA transcription units, one active and one inactive. Nevertheless, given the frequency of genomic rearrangements in P. falciparum, it is reasonable to expect that several of the variants are not associated with transcriptionally active rDNA. This possibility can be investigated using PFG analysis and probes specific for the inactive rRNA transcription units. The functional rRNA genes probably remain in this state because they are subject to sequence rectification mechanisms. Since these units are dispersed in the genome, the correction process must somehow be enacted through their chromatin configuration. In contrast, the inactive rRNA transcription units, located near active rRNA genes or translocated to other regions of the genome, appear to have been removed from sequence homogenization constraints and therefore diverge into nonhomology. By comparing the nucleotide sequences of the three variant 5.8S rRNA coding regions with the transcriptionally active rRNA gene, it is clear that the class IV rRNA transcription unit was the most recent to evolve, having only a few mutations. The other pseudogenes, classes I and III, diverged dramatically from the functional gene. The class III variant may be slightly older. The apparent 3' truncation of the class III 5.8S rRNA coding domain

suggests that this rDNA variant may have originated from a misaligned crossing-over event.

Why these rRNA pseudogenes are maintained in the parasite genome, when other functional components such as the knob protein gene can be deleted (Corcoran et al., 1986) is not known. It has been postulated that gene conversion is a mechanism for generating polymorphism as well as maintaining sequence homology. If a variant gene acts as a donor in this process, the mutation is propagated. Pseudogenes may therefore provide a means of genetic diversity (Wilde, 1986). For P. falciparum, these genes may be only one example of the genetic consequences of large scale chromosome rearrangements.

References

Baroin, A., Prat, A., and Caron, F. (1987). Telomeric site position heterogeneity in macronuclear DNA of Paramecium primaurelia. Nucl. Acids Res. 15, 1717-1728.

Blackburn, E. H. (1984). Telomeres: Do the ends justify the means? Cell 37, 7-8.

Bogenhagen, D. F., and Brown, D. D. (1981). Nucleotide sequences in Xenopus 5S DNA required for transcription termination. Cell 24, 261-270.

Borst, P. (1986). Discontinuous transcription and antigenic variation in trypanosomes. Ann. Rev. Biochem. 55, 701-732.

Brown, D. D. (1984). The role of stable complexes that repress and activate eukaryotic genes. Cell 37, 359-365.

Corcoran, L. M., Forsyth, K. P., Bianco, A. E., Brown, G. V., and Kemp, D. J. (1986). Chromosome size polymorphisms in Plasmodium falciparum can involve deletions and are frequent in natural parasite populations. Cell 44, 87-95.

Cornelissen, A. W. C. A., Langsley, G., Walliker, D., and Scaife, J. (1985). Gametocytogenesis and ribosomal rRNA gene organisation in rodent malarias Plasmodium chabaudi and Plasmodium berghei. Mol. Biochem. Parasitol. 14, 165-174.

Dame, J. B., and McCutchan, T. F. (1983a). Cloning and characterization of a ribosomal RNA gene from Plasmodium berghei. Mol. Biochem. Parasitol. 8, 263-279.

Dame, J. B., and McCutchan, T. F. (1983b). The four ribosomal DNA units of the malaria parasite Plasmodium berghei: Identification, restriction map and copy number analysis. J. Biol. Chem. 258, 6984-6991.

Dame, J. B., Sullivan M., and McCutchan, T. F. (1984). Two major sequences classes of ribosomal RNA genes in Plasmodium berghei. Nucl. Acids Res. 12, 5943-5952.

Dame, J. B., and McCutchan, T. F. (1984). Identification of 5S and 5.8S ribosomal RNA molecules and their genes in Plasmodium berghei. Mol. Biochem. Parasitol. 11, 301-307.

DeWatcher, R., Huysmans, E., and Vandenberghe, A. (1985). 5S ribosomal RNA as a tool for studying evolution. In Evolution of Procaryotes (Schleifer, K. H., and Stackenbrandt, E., eds) pp. 115-141. Academic Press, London.

Erdmann, V. A., Wolters, J., Huysmans, E., and DeWatcher, R. (1985). Collection of published 5S, 5.8S and 4.5S ribosomal RNA sequences. Nucl. Acids Res. 13 (suppl.), r105-153.

Francoeur, A. M., Gritzmacher, C. A., Peebles, C. L., Reese, R. T., and Tan, E. M. (1985). Synthesis of small nuclear ribonucleoprotein particles by the malarial parasite Plasmodium falciparum. Proc. Natl. Acad. Sci. U.S.A. 82, 3635-3639.

Goman, M., Langsley, G., Hyde, J. E., Yankovsky, N. K., Zolg, J. W., and Scaife, J. G. (1982). The establishment of genomic DNA libraries for the human malaria parasite Plasmodium falciparum and identification of individual clones by hybridisation. Mol. Biochem. Parasitol. 5, 391-400.

Greider, C. W., and Blackburn, E. H. (1985). Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. Cell 43, 405-413.

Hadjiolov, A. A. (1985)."The Nucleolus and Ribosome Biogenesis." Springer-Verlag/Wein Press, New York.

Howell, M. J. (1985). Gene exchange between hosts and parasites. Int. J. Parasitol. 15, 597-600.

Hyde, J. E., Zolg, J. W., and Scaife, J. G. (1981). Isolation and characterization of ribosomal RNA from the human malaria parasite Plasmodium falciparum. Mol. and Biochem. Parasitol. 4, 283-290.

Ishikawa, H. (1977). Evolution of rRNA. Comp. Biochem. Physiol. 58, 1-7.

Jacq, C., Miller, J. R., and Brownlee, G. G. (1977). A pseudogene structure in 5S DNA of Xenopus laevis. Cell 12, 109-120.

Jordan, B. R., Latil-Damotte, M., and Jourdan, R. (1980). Coding and spacer sequences in the 5.8S - 2S region of Sciara coprophila ribosomal DNA. Nucl. Acids Res. 8, 3565-3573.

Klien, H. L., and Petes, T. D. (1981). Intrachromosomal gene conversion in yeast. Nature 289, 144-148.

Kumazaki, T., Hori, H., and Osawa, S. (1982). The nucleotide sequence of 5S ribosomal RNA from a sea anemone, Anthopleura japonica. FEBS Lett. 146, 307-310.

Kuntzel, H., Heidrich, M., and Piechulla, B. (1981). Phylogenetic tree derived from bacterial, cytosomal and organelle 5S rRNA sequences. Nucl. Acids Res. 9, 1451-1461.

Labhart, P., and Reeder, R. H. (1984). Enhancer-like properties of the 60/81 bp elements in the ribosomal gene spacer of Xenopus laevis. Cell 37, 285-289.

Langsley, G., Hyde, J. E., Goman, M., and Scaife, J. G. (1983). Cloning and characterization of the rRNA genes from the human malaria parasite Plasmodium falciparum. Nucl. Acids Res. 11, 8703-8717.

Langsley, G., Sibilli, L., Mattei, D., Falanga, P., and Mercereau-Puijalon, O. (1987). Karyotype comparison between P. chabaudi and P. falciparum: analysis of a P. chabuadi cDNA containing sequences highly repetitive in P. falciparum. Nucl. Acids Res. 15, 2203-2211.

Lasser, A. B., Martin, P. L., and Roeder, R. G. (1983). Transcription of class III genes: Formation of preinitiation complexes. Science 222, 740-748.

Long, E. O., and Dawid, I. B. (1980). Repeated genes in eucaryotes. Ann. Rev. Biochem. 49, 727-764.

McCutchan, T. F. (1986). The ribosomal genes of Plasmodium. Int. Rev. Cyt. 99, 295-309.

Miller, J. R., and Melton, D. A. (1981). A transcriptionally active pseudogene in Xenopus laevis oocyte 5S DNA. Cell 24, 829-835.

Nazar, R. N., Lo, A. C., and Wildeman, A. G. (1983). Effect of 2'-O- methylation on the structure of mammalian 5.8S rRNAs and the 5.8S-2S rRNA junction. Nucl. Acids Res. 11, 5989-6001.

Peterson, R. C., Doening, J. L., and Brown, D. (1980). Characterization of two Xenopus somatic 5S DNAs and one minor oocyte-specific 5S DNA. Cell 20, 131-141.

Petes, T. D. (1980). Unequal meiotic recombination within tandem arrays of yeast ribosomal RNA genes. Cell 19, 765-774.

Piper, P. W., Patel, N., and Lockheart, A. (1984). Processing of the 3' sequence extensions upon the 5S rRNA of a mutant yeast in Xenopus laevis germinal vesicle extract. Eur. J. Biochem. 141, 115-118.

Reddy, R., Henning, D., Rothblum, L., and Busch, H. (1986). Some gene variants for 5S RNA are dispersed in the rat genome. J. Biol. Chem. 261, 10618-10623.

Reeder, R. H. (1984). Enhancer and ribosomal gene spacers. Cell 38, 349-351.

Rinke, J., and Steitz, J. A. (1982). Precursor molecules of both human 5S ribosomal RNA and transfer RNAs are bound by a cellular protein reactive with anti-La lupus antibodies. Cell 29, 149-159.

Rosenthal, D. S., and Doering J. L. (1983). The genomic organization of dispersed tRNA and 5S RNA genes in Xenopus laevis. J. Biol. Chem. 258, 7402-7410.

Rubin, G. M., and Hogness, D. S. (1975). Effect of heat shock on the synthesis of low molecular weight RNAs in Drosophila: Accumulation of a novel form of 5S RNA. Cell 6, 207-213.

Selker, E. U., Stevens, J. N., and Metzenberg, R. L. (1985). Heterogeneity of 5S RNA in fungal ribosomes. Science 227, 1340-1343.

Selker, E. U., Yanofsky, C., Driftmier, K., Metzenberg, R. L., Alzner-DeWeerd, B., and RajBhandary, U. (1981). Dispersed 5S RNA genes in N. crassa: Structure, expression and evolution. Cell 24, 819-828.

Spangler, E. A., and Blackburn, E. H. (1985). The nucleotide sequence of the 17S ribosomal RNA gene of Tetrahymena thermophila and the identification of point mutations resulting in resistance to the antibiotics paromomycin and hygromycin. J. Biol. Chem. 260, 6334-6340.

Sprague, K. U., Larson, D., and Morton, D. (1980). 5' Flanking sequence signals are required for activity of silkworm alanine tRNA genes in homologous in vitro transcription systems. Cell 22, 171-178.

Tabata, S. (1981). Nucleotide sequences of the 5S ribosomal RNA genes and their adjacent regions in Schizosaccharomyces pombe. Nucl. Acids Res. 9, 6429-6437.

Tartof, K. D. (1975). Redundant genes. Ann. Rev. Genet. 9, 355-385.

Tekamp, P. A., Garcea, R. L., and Rutter, W. J. (1980). Transcription and in vitro processing of yeast 5S rRNA. J. Biol. Chem. 255, 9501-9506.

Unnasch, T. R., and Wirth, D. F. (1983a). The avian malaria Plasmodium lophurae has a small number of heterogeneous rRNA genes. Nucl. Acids Res. 11, 8443-8459.

Unnasch, T. R., and Wirth, D. F. (1983b). The cloned genes of P. lophurae: a novel rDNA structure. Nucl. Acids Res. 11, 8460-8472.

Van der Ploeg, L. H. T., Smits, M., Ponnudurai, T., Vermeulen, A., Meuwissen, J. H. E. T., and Langsley, G. (1985). Chromosome-sized DNA molecules of Plasmodium falciparum. Science 229, 658-661.

Vaughn, J. C., and Sperbeck, S. J. (1984). A universal model for the secondary structure of 5.8S ribosomal RNA molecules, their contact sites with 28S ribosomal RNAs, and their prokaryotic equivalent. Nucl. Acids Res. 12, 7479-7503.

Walker, T. A., and Pace N. R. (1983). 5.8S ribosomal RNA. Cell 33, 320-322.

Wellems, T. E., Wallider, D., Smith, C. L., do Rosario, V. E., Maloy, W. L., Howard, J., Carter, R., and McCutchan, T. F. (1987). A histidine-rich protein gene marks a linkage group favored strongly in a genetic cross of Plasmodium falciparum. Cell 49, 633-642.

Wellems, T. E., and Howard, J., (1986). Homologous genes encode two distinct histidine-rich proteins in a cloned isolate of Plasmodium falciparum. Proc. Natl. Acad. Sci. USA 83, 6065-6069.

Wilde, D. C. (1986). Pseudogenes. CRC Critical Reviews in Biochemistry 19, 323-352.

VI. APPENDIX. 3' TERMINAL NUCLEOTIDE SEQUENCE OF THE 17S RIBOSOMAL RNA SPECIES FROM PLASMODIUM FALCIPARUM

Dorothy E. Shippen[1] and Anne C. Vezza[2]

[1]Department of Biology, University College;
[2]Department of Microbiology, The Medical Center,
University of Alabama at Birmingham, Birmingham, Alabama 35294

Journal of Parasitology, 72, 178-181.

The human malarial parasite is an obligate intracellular organism which spends the majority of its asexual life cycle within the host erythrocyte. During the course of development the parasite transcribes a variety of parasite specific genes essential for the growth and multiplication of the organism. One such set of genes codes for the parasite specific ribosomal RNA (rRNA) components. It has been reported that these rRNA species with estimated molecular weights of 1.3 x 10^6 and 0.72 x 10^6 are typically eukaryotic (Vezza and Trager, 1981, Molecular and Biochemical Parasitology 4: 149-162). In this report we provide, by nucleic acid sequence analysis, further evidence for the eukaryotic nature of the malarial 17S rRNA species.

Previous studies of the small rRNA component from other eukaryotes have demonstrated a highly conserved sequence of 40 to 50 nucleotides at the 3' terminus. Analyses of these sequences from over 40 different organisms have been useful in studying evolutionary relationships among various species and determining a possible endosymbiotic origin of eukaryotic organelles (Kuntzel and Kochel, 1981, Nature 293: 751-755; Gray and Doolittle, 1982, Microbiological Reviews 46: 1-46; Gray et al., 1984, Nucleic Acids Research 12: 5837-5832; Van Knippenberg et al., 1984, Nucleic Acids Research 12: 2595-2604). Recently, Spangler and Blackburn (1985, Journal of Biological Chemistry 260: 6334-6340) have demonstrated an important functional role for this region in Tetrahymena thermophila. Single base changes in this conserved region of the 17S rRNA molecule confer resistance to the drugs paromomycin and hygromycin. The changes occur at position 43 for hygromycin resistance (U to C) and at position 47 for paromomycin (A to G) (see Table 1). Paromomycin causes mistranslation of mRNA; while hygromycin affects elongation of the polypeptide as well as translation (Palmer and Wilhelm, 1978, Cell 13: 329-334; Wilhelm et al.,1978, Biochemistry 23: 1143-1149; Eustice and Wilhelm, 1984, Biochemistry 23: 1462-1467; Eustice and Wilhelm, 1984, Antimicrobial Agents and Chemotherapy 26: 53-60). It is not known at the present time if drug resistance in malaria can occur by a similar mechanism. Like Tetrahymena the copy

number of the rRNA genes is unlike those associated with other eukaryotic organisms. In most other eukaryotes the rRNA genes are arranged in tandem arrays and are present in high copy number (several hundred to thousands). It has been shown in Plasmodium berghei and Plasmodium falciparum that their rRNA genes are present in low copy number (4 to 8 copies per haploid genome) (Dame and McCutchan, 1983, Journal of Biological Chemistry 258: 6984-6990; Langsley et al., 1983, Nucleic Acids Research 11: 8703-8717; Dame et al., 1984, Nucleic Acids Research 12: 5943-5952). These genes appear to be divided into 2 major transcription units (Langsley et al., 1983, loc. cit.; Dame et al., 1984, loc. cit.). In P. berghei only 1 appears to be transcribed during the erythrocytic life cycle of the parasite (Dame et al., 1984, loc. cit.). Unlike Tetrahymena, malaria rRNA genes do not appear to be amplified.

The data obtained by the Tetrahymena study indicates an important functional role for the highly conserved 3' terminus of the small rRNA molecule. At the present time no data are available on the 3' terminal nucleotide sequence of the 17S rRNA component from any species of malarial parasite. Such data would not only be useful in identifying common phylogenetic relationships but would aid in the analyses of the rRNA transcription units. Therefore, RNA sequence analysis of the 3' terminus of the small rRNA component from P. falciparum was undertaken to elucidate not only its primary structure but to help provide needed information for identification of evolutionary and functionally conserved regions.

The Gambian isolate of P. falciparum was maintained by serial passage in human erythrocytes essentially as described by Trager and Jensen with some modifications (Trager and Jensen, 1976, Science 193: 673-675). Parasitized erythrocytes were cultured in 32-ounce, glass prescription bottles containing RPMI-1640 medium without HEPES but supplemented with 0.22% (w/v) sodium bicarbonate and 10% (v/v) human serum. Cultures were incubated at 38°C in a chamber supplied with a humidified atmosphere containing 5% CO_2. Care was taken during culturing to avoid contamination with host

white cells from either the blood cells or human serum. Parasitized erythrocytes containing predominantly trophozoites/schizonts were collected by the gelatin flotation method (Reese et al., 1979, Bulletin of the World Health Organization 57 (Suppl. 1): 53-61). The nucleic acids were extracted as previously described and the single-stranded RNA was recovered by 2 cycles of LiCl precipitation (Poulson, 1977. In The ribonucleic acids, 2nd ed., Stewart and Letham (eds.). Springer-Verlag, New York, pp. 333-367; Vezza and Trager, 1981, loc. cit.). The RNA pellet, following resuspension in 400 mM NaCl, 10 mM Tris-HCl, pH 7.4, was ethanol precipitated. After washing the pellet in 80% ethanol, the RNA was resuspended in 200 µl of sterile H_2O. The 3' terminus of the RNA was labeled using T_4 RNA ligase (PL/Pharmacia, Piscataway, New Jersey) and cytidine 3', 5'[^{32}P] biphosphate (Amersham Corp., Arlington Heights, Illinois) as previously described (Clerx-van Haaster and Bishop, 1980, Virology 105: 564-574). The RNA species were subsequently resolved by electrophoresis on a 1% (w/v) horizontal agarose gel containing 40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.4 and 0.5 µg/ml ethidium bromide (EtBr). The RNA bands were located by illumination with a longwave (366nm) uv lamp. The separated 17S rRNA component was recovered from the gel by electroelution onto dialysis membranes. After rinsing the membranes in 150 mM NaCl, 10 mM Tris-HCl, pH 7.4, the 17S rRNA preparation was extracted with buffer saturated 1-butanol to rid the sample of EtBr. Following an ethanol precipitation, the samples were subjected to 2 cycles of oligo(dT)-cellulose column chromatography (Vezza and Trager, 1981, loc. cit.). The unbound RNA fraction was collected each time and ethanol precipitated. The final RNA pellet was resuspended in 100 µl of sterile H_2O. Aliquots of approximately 50 to 100 x 10^3 cpm of purified 17S rRNA were subjected to base specific chemical cleavage as described by Peattie (1979, Proceeding of the National Academy of Science 76: 1760-1764). The aniline catalyzed cleavage products were resolved by polyacrylamide gel electrophoresis on either 20% or 10% (w/v) 37.5 cm x 20 cm x 0.38 mm RNA sequencing gels containing 8.3 M urea, 100 mM Tris-HCl, pH 8.2, 2 mM

EDTA. Following electrophoresis the gels were autoradiographed at -70°C using Dupont Cronex film in a film cassette equipped with a Dupont Lightning Plus intensifying screen. The sequence obtained is shown Table 1. The nucleotides at positions 19 and 20 could not be determined. Identical results were obtained from several experiments indicating that the bases in these positions are modified in some manner.

Since it has been previously demonstrated that the 3' terminus of the small rRNA species is highly conserved over the course of evolution, we wished to compare our sequence data with those previously reported for other eukaryotic cytoplasmic rRNA's (Hall and Maden, 1980, Nucleic Acids Research 8: 5993-6005; Rubtsov et al., 1980, Nucleic Acids Research 8: 5779-5794; Salim and Maden, 1981, Nature 291: 205-208; Schnare and Gray, 1981, FEBS Letters 128: 298-304; Lockard et al., 1982, Nucleic Acids Research 10: 3445-3457; Maden et al., 1982, Nucleic Acids Research 10: 2387-2398; Torczynski et al., 1983, Nucleic Acids Research 11: 4879-4890). Such a comparison would ascertain the degree of homology which exists between P. falciparum and other eukaryotic organisms. As summarized in Table 1, there is extensive homology for the first 50 nucleotides with very little sequence variation. Comparison to the trypanosomatid, Crithidia fasciculata, indicates that there are several nucleotides changes (9 out of 50); while other eukaryotes exhibit only 2 to 3 differences. After position 60 the falciparum RNA sequence shows less homology. In the RNA sequences studied thus far from other organisms the nucleotides at positions 19 and 20 have been identified as double methylated adenines $m_2^6Am_2^6A$ (Van Knippenberg et al., 1984, loc. cit.). Since the chemical reactions depend on base modification with subsequent cleavage by aniline at the weakened site, methylation of either the base and/or ribose renders these nucleotides more resistant to modification and/or cleavage. Therefore, these double methylated adenines probably occur in the P. falciparum small rRNA component as well.

For analyses Van Knippenberg et al. (Van Knippenberg et al., 1984, loc. cit.) have divided the 3' terminal 50 nucleotides into 4 regions designated I, II, III and IV as indicated

in Table 1 and illustrated in Figure 1. Region I contains a single-stranded region whose nucleotide sequence appears identical in all small rRNA species examined thus far with fungal, human and mitochondrial RNAs being the exceptions. Region II and II' contain the 18 nucleotides which comprise the stem of a proposed stem-loop structure with region III comprising the loop. Region IV contains a conserved sequence found in a variety of organisms with some mitochondrial and chloroplast sequences varying. Bacterial small rRNA molecules contain the Shine and Dalgarno sequence ...CCUCC... 3' to this region. The hairpin is found in the small rRNA species from bacteria, mitochondria, chloroplasts and cytoplasmic rRNAs and the sequence within this structure is characteristic of the cellular or subcellular origin of the rRNA component (Van Knippenberg et al., 1984, loc. cit.). Eukaryotic cytoplasmic rRNAs have a characteristic UGm$^6_2$Am$^6_2$A loop sequence, while mitochondrial, chloroplast and bacterial RNAs contain a characteristic GGm$^6_2$Am$^6_2$A sequence. The sequence within region II and II' has also been used to categorize various organisms depending on the sequence of nucleotides 17, 18 and 23, 24. Eukaryotic cytoplasmic rRNA's have a characteristic $_{24}$GGUGAACC$_{17}$ sequence. It is not surprising that P. falciparum contains the typically eukaryotic sequence, since previous studies have indicated the eukaryotic nature of the P. falciparum rRNA components. These findings provide not only additional information on the eukaryotic nature of the malarial 17S rRNA species but are useful in analyzing the rRNA transcription unit and its transcribed rRNA precursor molecules. Identification of sequences conserved during transcription and processing would aid in determining the location of spacer regions and potential processing sites and structures within these molecules, as well as leading to a greater understanding of the functional role of the rRNA in the ribosome.

This study was supported by Public Health Service Grants AI 18767 and AI 20597 from the National Institutes of Health. D.S. is a graduate student supported by a University of Alabama at Birmingham Graduate Fellowship. We wish to thank Dr. Daniel Jones, Chairman of the Department of Biology, for his encouragement and support.

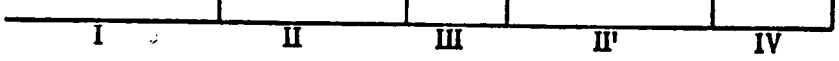
TABLE 1: 3' TERMINAL SEQUENCES OF SOME EUKARYOTIC SMALL rRNAs

AAGACGGUCG AACUXGACUA UCUAGAGGAA GUAAAAGUCG	rabbit
AAGACGGUCG AACUUGACUA UCUAGAGGAA GUAAAAGUCG	rat
AAGACGAUCA AACUUGACUA UCUAGAGGAA GUAAAAGUCG	frog
AAUUUGGACA AACUUGGUCA UUUGGAGGAA CUAAAAGUCG	yeast
AAGUUCACCG AUAUUUCUUC AACAGAGGAA GCAAAAGUCG	crithidia
AAAAACCGUA AAUCCUAUCU UUUAAAGGAA GGAGAAGUCG	falciparum

5' 80 70 60 50

UAAGAAGGUU UCCGUAGGUG AACCUGCGGA AGGAUCAUUA	rabbit[a]
UAACAAGGUU UCCGUAGGUG AACCUGCGGA AGGAUCAUUA	rat[b]
UAACAAGGUU UCCGUAGGUG AACCUGCGGA AGGAUCAUUA	frog[c]
UAACAAGGUU UCCGUAGGUG AACCUGCGGA AGGAUCAUUA	yeast[d]
UAACAAGGUA GCUGUAGGUG AACCUGCAGC UGGAUCAUUUU	crithidia[e]
UAACAAGGUU UCCGUAGGUG **CCUGCGGA AGGAUCAUUA	falciparum

40 30 20 10 3'



Comparison of the 3' terminal nucleotide sequence of the small rRNA component from P. falciparum with other eukaryotic small rRNA's. Nucleotide differences between the malarial sequence and those obtained from other sources are denoted by underlined letters. Roman numerals indicate various regions used by Van Knippenberg et al. (1984, loc. cit.) for comparative analyses of the small rRNA species.

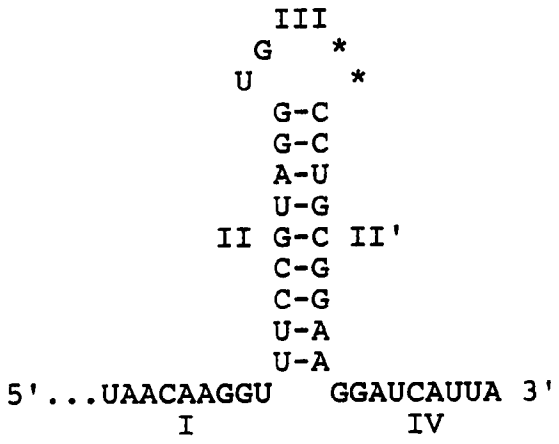


Figure 1. Hairpin structure as proposed by Van Knippenberg et al. (1984, loc. cit.).
* Nucleotide not determined.

GRADUATE SCHOOL
UNIVERSITY OF ALABAMA AT BIRMINGHAM
DISSERTATION APPROVAL FORM

Name of Candidate Dorothy Shippen-Lentz

Major Subject Biology

Title of Dissertation Characterization of the 5S and 5.8S Ribosomal RNA's and Their Genes from the Human Malaria Parasite Plasmodium falciparum

Dissertation Committee:

Chairman

Director of Graduate Program Daniel D. Jones

Dean, UAB Graduate School

Date 12/7/87

PS-1428