Structurally Distinct, Stage-Specific Ribosomes Occur in *Plasmodium*

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Two structurally distinct nuclear genes code for cytoplasmic small subunit ribosomal RNA's in the parasite *Plasmodium berghei*. Stable transcripts from one of the ribosomal RNA genes are found almost exclusively in those stages of the life cycle that develop in the mosquito. When the parasite infects the mammalian host, transcripts from the second gene become the predominant small subunit ribosomal RNA species.

SMALL SUBUNIT RIBOSOMAL RNA'S (18*S* RRNA'S) SERVE pivotal roles in the protein synthesis machinery of prokaryotic and eukaryotic cells. They are generally transcribed from a family of related genes (*1*) which, by the criteria of oligonucleotide cataloguing (*2*) and primer extension techniques (*3*), appear to be nearly identical within a given organism. This conservation of sequence presumably reflects functional constraints on the molecule that are required for optimal translational efficiency. Variations in ribosome structure related to translational control mechanisms are thought to be restricted to modification or absence of certain ribosomal proteins and to altered concentrations of cytoplasmic factors. A coarse control over protein synthesis during an organism's transition between developmental stages could be imagined in which major structural alterations of ribosomes mediate the selection of specific families of messenger RNA's to be translated, although there are no known examples of this. In the case of *Xenopus laevis*, two classes of 5*S* rRNA transcripts that are specific to either somatic or oocyte ribosomes have been reported (*4*), but similar changes for larger ribosomal RNA's have not been documented.

The rRNA's and the genomic organization of rRNA genes of a number of *Plasmodium* species have been studied (*5*). Two different types of ribosomal DNA transcription units have been described from *Plasmodium berghei* (*6*) with two copies of each type of unit being unlinked in the genome. The two classes define similar rRNA's; but the internal transcribed spacer regions, as well as the flanking sequences, display considerable sequence variation (*6, 7*). Heteroduplex analyses have shown that only one of the rDNA gene classes is colinear with rRNA species isolated from asexual bloodstream parasites (*7*). Here we show that the two types of small subunit rRNA genes in *P. berghei* are structurally distinct and their transcription is restricted to different stages of the *P. berghei* life cycle.

Primary sequence of A and C genes. The small subunit rRNA gene transcribed in asexual parasites from the mammalian host's bloodstream (referred to as the A gene) resides on a 5.6-kb Eco RI–Hind III restriction fragment, and its sequence has been reported (*8*). A second *P. berghei* small subunit rRNA gene (the C gene) is contained within an 8.8-kb Eco RI–Hind III restriction fragment. Heteroduplex and S1 nuclease analyses have demonstrated that rRNA's of asexual parasites are colinear with the A gene, but transcripts of the C gene have not been detected. In order to access whether the nontranscribed C gene represents a pseudogene we have subcloned its coding and noncoding strands into the single-stranded phages M13mp18 and M13mp19. Synthetic oligonucleotide primers that are complementary to evolutionarily conserved regions in eukaryotic rRNA's were used to initiate DNA synthesis in the dideoxynucleotide chain termination sequencing protocols (*9, 10*). The sequence of the C gene (determined on both strands) as well as the differences between the A and C genes are shown in Fig. 1. Positions that vary between the two sequences are not randomly dispersed throughout the length of the molecule. There are only four nucleotide differences in the 3′ domain (positions 1237 to 2077), while certain regions in the 5′ and middle domain display considerable variation (there are 18 differences between positions 1123 and 1150). The nonrandom distribution of nucleotide differences between the A and C genes is an indication that the inactive C gene may code for a functional rRNA transcript. If the C gene were a pseudogene, differences between the two rDNA gene classes should not be concentrated in a limited number of regions.

Secondary structure models. Small subunit rRNA's can be regarded as mosaics of genetic elements that display varying rates of evolutionary drift. Sequences that are conserved in all organisms are interspersed among regions that display intermediate or very high rates of mutational change (*10, 11*). Comparative sequence analyses have demonstrated that all known small subunit rRNA's can be folded into consensus secondary structures with nearly constant central frameworks or core structures (*12*). Superimposed on this core are primary and secondary structural features that are characteristic of a given phylogenetic grouping. A consensus secondary structure model for the predicted transcripts of the *P. berghei* rRNA genes is shown in Fig. 2. All of the kingdom-specific primary and secondary structural features present in other eukaryotic small subunit rRNA's encoded by nuclear genes are found in the proposed model, and the locations of nonconserved regions are indicated by shaded backgrounds. The differences between the A and C genes are concentrated in three regions, which typically display considerable sequence variation among eukaryotic small subunit rRNA's. A large loop and four helices can be formed in the region between positions

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150 and 300 of *P. berghei* just as in other eukaryotes. There are 26 differences (in the space of 117 positions) between the two genes in this region. The A and C genes also differ in sequence between positions 635 and 914. This region is characteristic of eukaryotic small subunit rRNA's but because convincing phylogenetic proof is unavailable, the secondary structure is not displayed. The region between positions 1123 and 1150 also contains many sequence differences, but the predicted secondary structure in this region appears to be similar in the two genes. Other areas of the molecule that usually display considerable sequence and length variation among eukaryotic organisms are virtually identical between the A and C genes (for example, positions 1420 to 1620 and 1720 to 1780).

We infer from our primary and secondary structure analyses that neither of these are pseudogenes. Differences between them are restricted to regions that are not evolutionarily conserved (*11*), and all of the primary and secondary features typical of eukaryotic small subunit rRNA's are maintained in both genes. The indication that the C gene was not a pseudogene led us to investigate further its expression in other stages of the life cycle.

RNA sequence from sporozoites and blood stage parasites. Partial sequences of the predominant rRNA transcripts from the sporozoite and blood stages of the parasite's life cycle were determined and compared to the corresponding regions of the A and C rRNA genes. We prepared an oligonucleotide primer (TM-73, AACTCGATTGATACACAC), which is complementary to positions 292 to 309 and 280 to 297 of the A and C genes, respectively. The location of TM-73 is enclosed within boxes in the C gene sequence presented in Fig. 1. This sequence is conserved in both rRNA genes and is proximal to a clustering of nucleotide differences between the A and C genes. The TM-73 oligonucleotide was used to prime sequencing reactions for homologous regions in the noncoding DNA strands from the A and C genes cloned into the M13 phages (Fig. 3). The variation between residues 166 and 246 of the

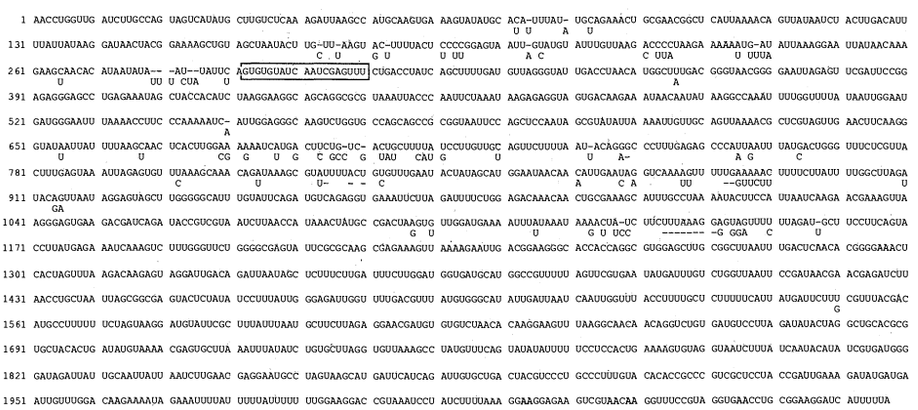

Fig. 1. Sequence of the *Plasmodium berghei* C gene. Synthetic oligonucleotides complementary to evolutionarily conserved regions (*10*) in the coding and noncoding strands of the *P. berghei* small subunit rRNA C gene were used as primers in dideoxynucleotide chain termination sequencing reactions (*9*). The C gene sequence (determined on both strands) is presented, and nucleotide positions that are different in the *P. berghei* A gene are indicated. The boxed region shows the location of the synthetic oligonucleotide primer TM-73, which was used to initiate DNA synthesis in the sequencing ladders shown in Figs. 3 and 4.

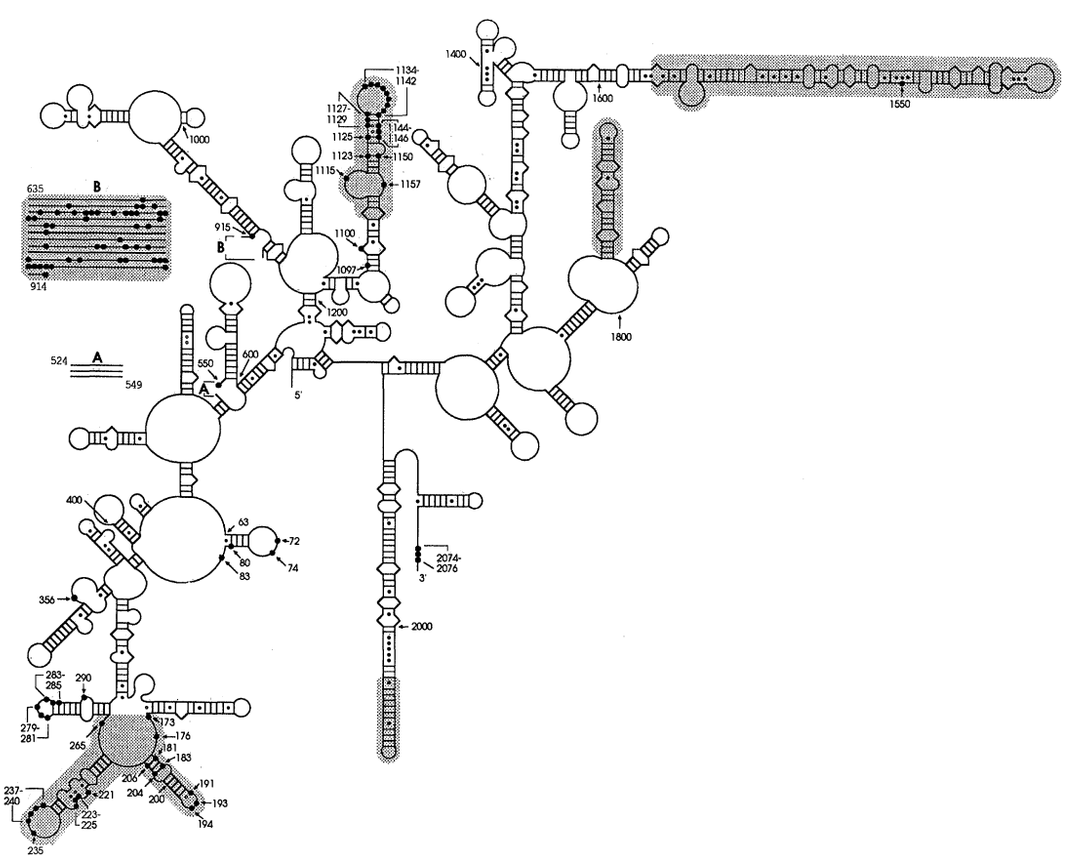

Fig. 2. Consensus secondary structure model for *P. berghei* small subunit rRNA. Transcript from the A and C genes, showing positions that display variation between the two genes (solid circles). Regions that display extreme sequence and length variation in all eukaryotes are indicated by the shaded backgrounds. The numbers refer to the aligned numbering system in the C gene sequence presented in Fig. 1.

A gene and between 164 and 239 of the C gene include differences in base composition and sequence length. The corresponding regions in stable small subunit rRNA populations from asexual bloodstream parasites of the mammalian host and sporozoites isolated from the salivary glands of the insect were analyzed. The TM-73 oligonucleotide was annealed to RNA templates within bulk RNA populations isolated from different *P. berghei* life cycle stages, and reverse transcriptase–mediated sequencing reactions were performed (*3, 13, 14*). The RNA templates of the asexual parasite produce a "sequencing ladder" that is distinct from that generated when sporozoite RNA is used as a template (Fig. 4). A comparison of Figs. 3 and 4 shows that differences between the genes can be correlated to sequence differences in the stage-specific RNA; the sequence of the RNA from the asexual transcripts is identical to that of the A gene, and the sequence of the RNA from the sporozoites corresponds to the C gene. These results are consistent with similar experimental observations where A gene–specific and C gene–specific oligonucleotides (oligonucleotides that selectively anneal to only the A gene or only the C gene) from several nonconserved regions were used in primer extension analyses. The A gene–specific primers were capable of initiating DNA synthesis in sequencing reactions containing RNA templates from asexual parasites of the mammalian host, and the C gene–specific primers were only capable of priming sequencing reactions using RNA templates isolated from the sporozoite (data not shown).

C gene expression. Using oligonucleotide probes, we have compared the expression of the A and C genes at different stages of the parasite's life cycle. RNA was prepared from blood stage parasites, sporozoites, oocysts, uninfected mosquitoes, and uninfected mice. Samples were taken from mosquito guts that contained *P. berghei* oocysts 8 days after fertilization, and RNA was isolated from mosquito salivary glands that were infected with *P. berghei* sporozoites. Four oligonucleotide probes of differing specificity were used to analyze stable transcripts: (i) a universal small subunit rRNA probe that detects small subunit rRNA from any source; (ii) a *P. berghei* small subunit rRNA probe that detects the small subunit rRNA of *P. berghei* but not that of mouse or mosquito; (iii) a probe specific for the A genes; and (iv) a probe specific for the C gene. We then determined the ability of each probe to hybridize to the different RNA samples. Equivalent amounts of total RNA from each source were applied to each filter. In addition, a 15-fold excess of sporozoite rRNA was applied to one dot on each filter. The relative amount of RNA that bound in each case was determined with the universal probe. Since the RNA from bloodstream parasites contains a low percentage (<1 percent) of gametocytes as well as small quantities of RNA from the host mouse, and the RNA prepared from sporozoite stages contains significant quantities of mosquito RNA, it was necessary to determine the various amounts of parasite small subunit rRNA by hybridization to the *P. berghei* specific probe. The ratio of host to parasite RNA varied to a considerable extent in the individual stage-specific RNA samples.

Data from hybridizations of A and C gene probes to various RNA preparations (Fig. 5) indicate that the C gene transcripts predominate in parasites developing in the mosquito and that the A gene transcripts predominate in bloodstream parasites. The A gene oligonucleotide probe appears to detect A gene transcripts only in RNA from bloodstream stages of the parasite (Fig. 5). Small amounts of the C gene transcripts were detected in RNA from bloodstream stages, but there was more than a 20-fold predominance of the A type RNA. In sporozoites the ratio was reversed. Although there is less sporozoite RNA than bloodstream parasite RNA on the filter (Fig. 4), the C gene probe detected more C gene transcripts in the sporozoite RNA. Ratios were determined by cutting out each spot and counting directly in a scintillation counter. The ratio of the sporozoite RNA to bloodstream parasite RNA on

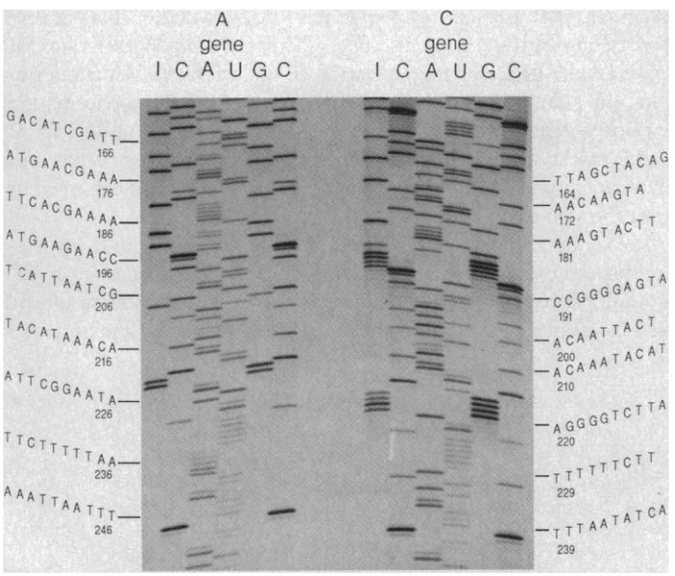

Fig. 3. Sequence analysis of a nonconserved region in the *P. berghei* A and C genes. DNA templates corresponding to the noncoding DNA strands of the two types of *P. berghei* rRNA genes were annealed to the synthetic oligonucleotide TM-73, which is complementary to positions 292 to 309 and 280 to 297 of the A and C genes, respectively. Dideoxynucleotide sequencing protocols (*9, 10*) were used to generate sequencing ladders corresponding to positions 166 to 246 of the A gene and 164 to 239 of the C gene. Numerous transitions and transversions, as well as insertions in the A gene are documented by the autoradiograms.

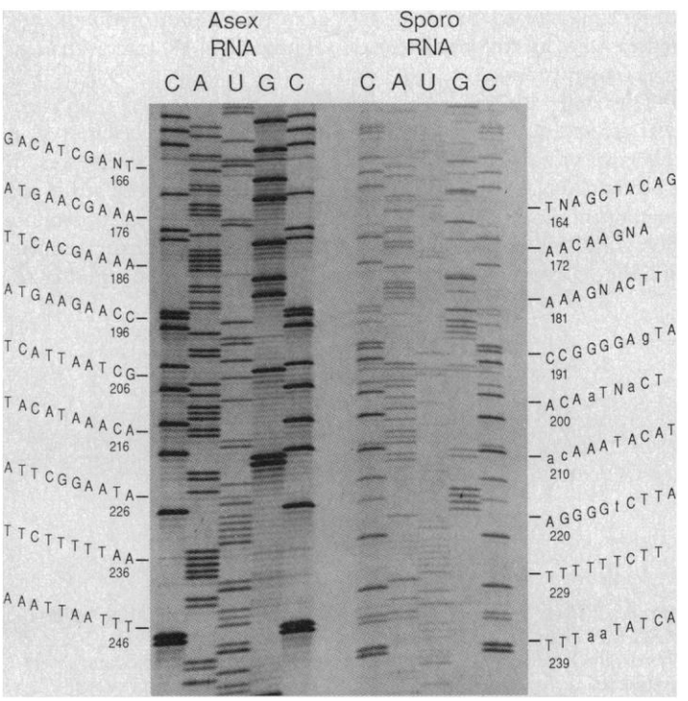

Fig. 4. Direct sequence analysis of small subunit rRNA gene transcripts from asexual (Asex) bloodstream or sporozoite (Sporo) stages of *P. berghei*. Sequencing ladders were produced by reverse transcriptase–mediated primer extension reactions (*14*) that were directed by RNA templates from either *P. berghei* asexual bloodstream parasites or sporozoites isolated from mosquitoes. The synthetic oligonucleotide TM-73 was used to prime the sequencing reactions. The resulting sequences are the complements of the A and C genes presented in Fig. 1. Completely ambiguous nucleotides are indicated by N and uncertain assignments (because of weak or multiple bands) at a given position are represented by lower case letters.

the filter was 1:5 when the *P. berghei*–specific probe was used as a control. The C gene probe detected 4.5 times as much C gene–specific transcript in the sporozoite RNA sample as in the bloodstream parasite RNA samples. We can therefore conclude that the ratio of C gene transcripts to A gene transcripts is greater than 20:1 in sporozoite RNA.

Exact ratios were difficult to determine for the oocyst stage because of technical difficulties in obtaining sufficient quantities of nucleic acids, but a qualitative assessment could be obtained with A and C gene probes of equivalent specific activity. Autoradiography of hybridized RNA dots containing equal amounts of oocyst RNA revealed that most of the *P. berghei* RNA in them was transcribed from the C gene. Long exposures of the film also revealed some A gene transcription.

It is improbable that the dot blot results reflect differential stabilities of A gene versus C gene transcripts during different stages of the *P. berghei* life cycle. Northern blot analyses of sporozoite and asexual RNA probed with A gene– and C gene–specific probes are shown in Fig. 6. Using the A gene–specific probe (Fig. 6A), we were able to detect RNA transcripts in the asexual RNA of *P. berghei* (lane 2) and asexual RNA of *Plasmodium yoelii* (lane 3), a closely related rodent malarial parasite. The predominant RNA species that hybridize with the A gene–specific probe are consistent with chain lengths of 2100 and 2000 nucleotides, as predicted from analysis of the DNA's of *P. berghei* and *P. yoelii*. When identical Northern transfers were probed with a C gene–specific probe (Fig. 6B), a sporozoite transcript approximately 2100 nucleotides long was detected (lane 1). Relatively low levels of the C gene transcripts were detected in the blood stage RNA of *P. berghei* and *P. yoelii*. The proportional differences in signal with the A and C probes are consistent with the results in Figs. 4 and 5. The transcripts complementary to the A and C gene probes are predominantly represented as full-length small subunit rRNA's rather than as breakdown products.

The stage-specific ribosome. Considerable evidence indicates that structurally distinct ribosomal RNA genes are expressed at different stages of the *P. berghei* life cycle. The differences in ribosomal RNA's detected in the various stages cannot be attributed to confusing mitochondrial and nuclear rRNA's, or to contamination with host rRNA's. The A and C genes are 96.5 percent similar, and they contain all of the primary and secondary structure features that are conserved in cytoplasmic small subunit rRNA's defined by nuclear genomes. Mitochondrial small subunit rRNA's are typically eubacterial in character and at least 50 percent of their positions will be different from the nuclear genes. Finally, neither rRNA is like that of previously described mouse rRNA sequences (*13*) or mosquito rRNA.

We have shown that stable transcripts from the *P. berghei* A gene represent the dominant small subunit rRNA species in bloodstream and hepatic-stage parasites while the C gene defines the predominant transcript in the insect stages of the parasite. It is not known whether the switch in predominant rRNA species represents a change in transcription or difference in stabilities between the A gene and C gene transcripts. Heteroduplex analyses indicate that the large subunit rRNA's of the two different gene classes are structurally different (*7*), and their presence in ribosomes may also be stage-specific. Although no stage-specific studies have been done with the rRNA's of other *Plasmodium* species, their ribosomal RNA genes have characteristics similar to those of *P. berghei* (*15–17*). It seems likely that the stage-specific ribosome will be a characteristic of the genus *Plasmodium*.

The rRNA gene organization of *P. berghei* is clearly unusual and may be required for the maintenance of two types of ribosomal RNA transcripts. There are only four gene copies and these are dispersed throughout the genome as opposed to being tandemly repeated. If the genome organization affects the ability to maintain rRNA homogeneity, the unusual organization found in *Plasmodium* may serve to maintain gene differences. The gene differences may be a response to the parasitic life. We have suggested a number of potentially overlapping reasons for such a system (*5–7*). One possibility is that this is a broad mechanism of control over development. Certain populations of messenger RNA may be preferentially translated by the A, C, or A-C hybrid ribosomes. For example, the parasites' transition from asexual to sexual forms could be mediated by such a switch. In fact, some change in the synthesis of rRNA's at this point is indicated by electron microscopy studies showing that the female gametocyte has a higher density of ribosomes than the asexual forms. It is not known whether this putative increase in ribosome content reflects increased transcription rates for active rRNA genes or the activation of additional rRNA transcription units. Another possibility is that the change in transcription is a response to the parasitic life; one area of the genome could be active in the insect stage, while the other is the predominant source of transcripts in the warm-blooded host. This is not essentially different from the two genome theory (*18*).

The sequence presented in Fig. 1 can be used to design hybridization probes complementary to stage-specific small subunit rRNA's. In addition to their use as diagnostic tools, these probes could be used to monitor developmental transitions. However, the potential

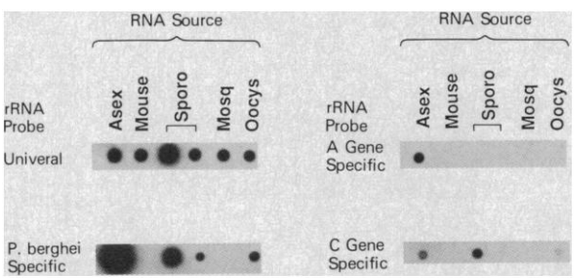

Fig. 5. Analysis of stage-specific transcription. A sample (100 ng) of each RNA was applied to four nitrocellulose filters (*19*). In addition, 1.5 μg of sporozoite RNA was applied to one spot on each filter. The filters were hybridized to four different radioactive probes. The universal probe

TTACCGCG$^{G}_{A}$C$^{T}_{G}$GCTGGC

should be complementary to any small subunit rRNA. The *P. berghei* probe is the sequence ACTCGATTGATACACACT (complementary to positions 291 to 308 and positions 279 to 296 in the A and C genes, respectively). The A gene–specific probe is the sequence CATGAAGATATCGAGGCGGAG (complementary to A gene positions 691 to 711) and the C gene–specific probe is the sequence CTACTCCTTTAAAGAAGATAGTT (complementary to C gene positions 1107 to 1129). Asexual or bloodstream parasites (Asex), sporozoites (Sporo), mosquito (Mosq), and oocysts (Oocys).

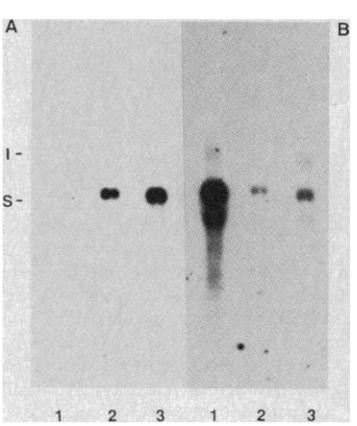

Fig. 6. Northern blot analysis of *Plasmodium* RNA. Bulk RNA was extracted from *P. berghei*–infected mosquitoes (lane 1), blood stage parasites of a *P. berghei* infection (lane 2) and blood stage parasites of a *P. yoelii* infection. After electrophoresis on 1 percent agarose gels, Northern transfers were prepared and challenged with the A gene–specific (**A**) or C gene–specific (**B**) probes described in Fig. 5.

role of structurally distinct ribosomes in the translational control of gene expression has not been explored. If the stage-specific ribosomes selectively translate different messages, it will be important to understand what distinguishes a C type message from an A type message, and how this relates to differences between A and C ribosomes.

Many studies have been directed toward understanding how parasites differ biochemically from their hosts and other free living organisms. We have described a fundamental biochemical difference between *Plasmodium* and all free living organisms that have been studied to date. However, this kind of difference may not be restricted to the *Plasmodium* line of descent. Heterogeneity in the flanking regions of ribosomal RNA genes has also been observed in kinetoplastids and chromophytes (*20*), but differences in the rRNA coding regions have not yet been documented. Further, there are seven ribosomal RNA operons in *Escherichia coli* and microheterogeneity exists in the small subunit rRNA coding regions. It is possible that selective transcription occurs or that transcripts from each operon display different metabolic stabilities in response to varying growth conditions.

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