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Functional Equivalence of Structurally Distinct Ribosomes in the Malaria Parasite, *Plasmodium berghei**

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Unlike most eukaryotes, many apicomplexan parasites contain only a few unlinked copies of ribosomal RNA (rRNA) genes. Based on stage-specific expression of these genes and structural differences among the rRNA molecules it has been suggested that *Plasmodium* spp. produce functionally different ribosomes in different developmental stages. This hypothesis was investigated through comparison of the structure of the large subunit rRNA molecules of the rodent malaria parasite, *Plasmodium berghei*, and by disruption of both of the rRNA gene units that are transcribed exclusively during development of this parasite in the mosquito (S-type rRNA gene units). In contrast to the human parasite, *Plasmodium falciparum*, we did not find evidence of structural differences in core regions of the distinct large subunit rRNAs which are known to be associated with catalytic activity including the GTPase site that varies in *P. falciparum*. Knockout *P. berghei* parasites lacking either of the S-type gene units were able to complete development in both the vertebrate and mosquito hosts. These results formally exclude the hypothesis that two functionally different ribosome types distinct from the predominantly blood stage-expressed A-type ribosomes, are required for development of all *Plasmodium* species in the mosquito. The maintenance of two functionally equivalent rRNA genes might now be explained as a gene dosage phenomenon.

Ribosomes are essential cellular components that play a central role in protein synthesis. It has been demonstrated that the ribosomal RNA (rRNA) has an active role in the assembly, structure, and interaction of the ribosomal subunits (1–3) and a direct role in catalysis and accuracy of protein synthesis (4, 5). The rRNA molecules present in eukaryotic ribosomes are identified by sedimentation properties as 28 S (large subunit (LSU)[1]), 18 S (small subunit (SSU), 5.8 S, and 5 S. The genes encoding the first three rRNA molecules are expressed from a single rRNA gene unit as one polygenic transcript that is subsequently processed. In most eukaryotes, 100–10,000 identical copies of the rRNA gene unit are present per haploid genome, clustered in tandem arrays. Because the rRNA molecule is the catalytic center of the ribosome (1, 5, 6), their sequence conservation presumably reflects functional constraints that are required for optimal translational efficiency.

Unicellular apicomplexan parasites have superficially typical rRNA gene units that are comprised of 18 S, 28 S, and 5.8 S genes, separated by the internal transcribed spacer (ITS) regions and flanked by external transcribed spacer (ETS) regions (7–11). However, in contrast to most eukaryotes, numerous, but not all, apicomplexan species contain a characteristically small number (two to seven) of structurally distinct rRNA gene units that are unlinked in the genome (11–14). This phenomenon has been characterized in the greatest detail in *Plasmodium* spp. parasites that are responsible for malaria. In *Plasmodium*, and in contrast to other apicomplexans that possess dispersed genes, these rRNA gene units are transcribed stage-specifically, thereby reducing the total number of gene units that are simultaneously active (15–19). Based on the differences in expression pattern and nucleotide sequence of the rRNA gene units the existence of three types of structurally different ribosomes in *Plasmodium* has been postulated. The A-type ribosomes are present in the liver and blood stages of the parasite, and the O- and S-type ribosomes are the predominant types produced during development in the mosquito (20). The presence of structural differences among the rRNA molecules has led to the hypothesis that the different ribosome types are also functionally different (15, 18, 19). Two observations support this hypothesis. First, the O- and S-type rRNA genes of the human parasite *Plasmodium vivax* encode SSU rRNA molecules that differ in core regions that are involved in mRNA decoding and translational termination (20). Second, in the human malaria *Plasmodium falciparum*, A-type and S-type LSU rRNA molecules differ in the core regions, and most marked is the distinct GTPase domain in the A- and S-type molecules (18, 21). It is currently a complete mystery why malaria parasites evolved this atypical organization and structure of the rRNA gene units. It has been postulated that these differences reflect the need for the parasite to propagate in two very different environments, the vertebrate host and invertebrate vector (15).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AJ301624, AJ298079, AJ298080, AJ298081, AJ298082. and AJ298083.

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[1] The abbreviations used are: LSU, large subunit; SSU, small subunit; ITS, internal transcribed spacer; ETS, external transcribed spacer; ko, knockout; PCR, polymerase chain reaction; kb, kilobase pair(s); bp, base pair(s); PCRaf, PCR amplified fragment; wt, wild-type.

TABLE I
Oligonucleotides specific for the ETS, SSU, ITS1, ITS2, and LSU regions of the four rRNA units (A/B/C/D) of P. berghei

Specific for	Nucleotide sequence	Name
ETS		
D-ETS	CCACCAACCCAAGCTTATACATTATACATAATAAACCCACAC	L372R
D-ETS	ATACTGTATAACAGGTAAGCTGTTATTGTG	L260R
D-ETS	AAATAGTCAATTAAAATCCTATGG	L392R
C-ETS	GTGTAGTAACATCAGTTATTGTGTG	L270R
SSU		
C/D-SSU	CCCGAATTCAACCTGTTGATCTTGCC	L78R
C/D-SSU	CCCGAATTCACCTACGGAAAACC	332R
C/D-SSU	ATAAAAGCAGTGACAGAAGTC	TM3
A/B-SSU	CATGAAGATATCGAGGCGGAG	TM4
ITS1		
C/D-ITS1	CCCAAGCTTCGCGGATCCACCATGATATGCGTACCTTAG	L427R
C/D-ITS1	CCCAAGCTTTAATTTTTTTATATTTCCCTTGAAC	L412R
C/D-ITS1	CTTAGTGTTTTGTATTAATGACGATTTG	L271R
ITS2		
C-ITS2	TAACGCATATAATTTTACAGGGG	L263R
C-ITS2	CAATTTGCTCACATTGTATATAGG	L264R
D-ITS2	CATTAAACATATATGTTGTTCTCTC	L265R
D-ITS2	CCCAGGTTCCAGTCGCAATAG	L266R
LSU-5′		
C LSU 5′	AACCTACTCATGCAAGTAAGG	L361R
C LSU 5′	AAAATAGAAAATGATGAACCCTC	L648R
D LSU 5′	TGCTCTCCCATCATAAGTTAT	L238R
D LSU 5′	ACATGACTTGCGCCATGAATA	L649R
A/B LSU 5′	CATAGAAATAAATCCATCTTAC	L360R
A/B LSU 5′	GGAAACAGTCCATCTATAATTG	L647R
A/B/C/D-LSU 5′	ATATGCTTAAATTCGGCGG	L87R
LSU-3′		
C LSU 3′	ATTCCGCCACTTAAAAACCTC	L645R
D LSU 3′	TATTCTACGCTTAAAAATCACAC	L646R
A/B LSU 3′	GAACAAATTACTTCATTCATAGC	L644R
A/B/C/D LSU 3′	CGTTAGGAGCATCCCTCAT	L357R
5′ UTR DHFR[a]	GGGAAATCAATGTATTAAAAATAATTATATG	L393
pBS/KS + pUC	AGCGGATAACAATTTCACACAGGA	307A
pBS/KS + pUC	TTTTCCCAGTCACGACGT	328A

[a] UTR, untranslated region; DHFR, dihydrofolate reductase.

To investigate the possible existence of functionally different ribosome types we analyzed the rRNA gene units of the rodent parasite *Plasmodium berghei*, an established model malaria parasite. This parasite contains four distinct copies of the rRNA units (A–D) (22) divided into the blood stage A-type (A- and B-unit) and S-type (C- and D-units), which is transcribed mainly in the proliferative stages in the mosquito (14, 15). It has been possible to distinguish the two S-type SSU genes (C- and D-SSU) on the basis of sequence differences reminiscent of the S- and O-types of *P. vivax* (20). However, we demonstrated that the C-SSU gene is actually a chimera of the A- and D-SSU, and no differences were found in the core regions of the A-, C-, and D-SSU rRNA molecules (23) which might imply functional differences. These results questioned whether two functionally different ribosome types are required for development of *P. berghei* in the mosquito as has been implied for *P. vivax* (20). Here we investigated further the rRNA gene units by comparison of the sequence and structure of the LSU genes of the different units. The sequence information of the LSU and SSU genes allowed us to address possible functional differences between the ribosome types by disruption of the C- and D- (S-type) rRNA gene units. Unexpectedly, neither the structural comparison nor the gene knockout studies revealed evidence for the existence of functionally different ribosome types in *P. berghei*. Only one copy of the S-type genes, either the C- or the D-gene, is sufficient for complete development of the parasite in the mosquito vector. A previously undescribed presence of the A-type rRNA molecules in maturing oocysts may also account for the continued development of gene knockout (ko) parasites in the mosquito. Given the similar phenotype of the C- and D-ko mutants we suggest that the maintenance and transcription of the additional rRNA units in the mosquito represent a gene dosage phenomenon that ensures efficient progression of parasite multiplication in the relatively short lived mosquito vector.

EXPERIMENTAL PROCEDURES

Isolation and Characterization of the LSU Genes of the Different rRNA Gene Units

DNA fragments containing the LSU rRNA genes of the A-, C-, and D-units were isolated for sequencing. The 5′-ends of the A- and C-LSU rRNA genes present in clone pPbSL7.8 (22) and clone pPbSL8.8 (24) respectively, were subcloned by PCR and sequenced completely. The 5′-end of the D-LSU rRNA gene was isolated from a library of size-selected *Kpn*I/*Hin*dIII restriction fragments (range 4–8 kb) derived from genomic DNA (clone 8417 of the ANKA strain) and ligated into the vector pBS/KS. The library was screened by hybridization with oligonucleotide L87R (Table I) specific for a conserved region at the 5′-end of the LSU rRNA genes (position 27–45). The final wash was performed at 42 °C in 3 × SSC, 0.5% SDS. A plasmid (referred to as pPbL4.8), containing the expected 4.8-kb *Kpn*I/*Hin*dIII fragment (11), was isolated, and the insert was completely sequenced. The fragment contained 1.6 kb of the 3′-end of the ITS2 and 3.2 kb of the 5′-end of the D-LSU rRNA gene. We were not able to clone the 5′-end of the second A-type, the B-LSU rRNA gene. The 3′-end of the B-LSU gene (position 3062–3789), present on a 4.1-kb *Hin*dIII fragment (11), was ligated into vector pBR322, resulting in the clone pPbL4.1.[2] This clone was partially sequenced and contained 728 bp of the 3′-end of the B-LSU rRNA gene plus 3.4 kb of downstream sequences. The 3′-ends of the A-, C-, and D-LSU rRNA genes were isolated from a partial *Sau*3A *P. berghei* genomic library in phage λzap-SK (Dr. M. Ponzi, Instituto Superiore di Sanità, Roma, Italy). This library was screened with a 653-bp fragment from the B-LSU rRNA gene, derived from clone pPbL4.1 after digestion with *Hin*dIII and *Ava*I. The final wash was performed at 60 °C in 1 × SSC, 0.5% SDS. Six clones positive for the 3′-end of the LSU gene and

[2] R. M. L. van Spaendonk, A. van Wigcheren, C. J. Janse, and A. P. Waters, unpublished data.

with different insert sizes were obtained. Comparison of the restriction digestion patterns of these clones with the known restriction maps (11) and partial sequence analysis extending into the already sequenced regions of the LSU rRNA genes showed that two clones contained the 3′-end of A-type LSU rRNA genes, three clones the 3′-end of the C-, and one clone the 3′-end of the D-LSU rRNA gene. Subclones made by amplification of the 3′-ends of the A-type, C-, and D-LSU rRNA genes were sequenced completely.

The secondary structure analysis of the *P. berghei* 5.8 S and LSU rRNA molecules was inferred from comparative sequence analysis. The eukaryotic LSU consensus sequence was calculated from an alignment of 23S-like rRNA sequences of 35 representative eukaryotic species.[3] The alignment was made with the Omiga™ 1.0.1 software (Oxford Molecular Group). *P. berghei* positions different from the LSU consensus sequence were checked for compensatory base changes by folding the *P. berghei* rRNA sequence into a three-dimensional structure similar to the secondary structure model for *P. falciparum* (18).

Vectors

The construction of the vectors to disrupt the C- and D-rRNA gene units was as follows.

Disruption Vector—To create vector pMD207 (Fig. 1*B*), we first PCR amplified a 2038-bp fragment from the 5′-end of the C-SSU rRNA gene from plasmid pPbSL8.8 (24), using oligonucleotides L78R and 332R (see Table I; four cycles annealing at 40 °C, 3-min extension followed by 30 cycles annealing at 60 °C, 3-min extension) which introduced unique *Eco*RI sites at either end of the fragment. This fragment was cloned into the unique *Eco*RI site of vector pMD200 (25), resulting in vector pMD207. pMD200 contains the selectable marker cassette with the pyrimethamine-resistant DHFR/TS gene of *P. berghei*. Vector pMD207 has been used for disruption of both the C- and D-rRNA gene units after linearization at the unique *Spe*I restriction site. In pMD207, 21 bp of the C-SSU rRNA gene (nucleotides 2039–2059) are missing, resulting in the introduction of an incomplete copy of the C-SSU rRNA gene after integration of pMD207 in the C- or D-rRNA gene unit (Fig. 1*B*).

Replacement Vectors—Both vectors 387A and 395A (Fig. 1*B*) contain two fragments of the D-rRNA gene unit (24) on either side of the selectable marker cassette present in vector $pD_BD_{Tm\Delta H}D_B$ (26). In both vectors a 723-bp fragment of the D-LSU rRNA gene was cloned downstream of the selection cassette. To obtain this fragment, plasmid pPbL4.8 (see above) containing 1.6 kb of the D-ITS2 and 3.2 kb of the D-LSU rRNA gene was digested with *Taq*I and *Hin*dIII. After size fractionation and purification from gel, the 723-bp *Taq*I/*Hin*dIII fragment was cloned in pBS/KS digested with *Cla*I and *Hin*dIII. The *Hin*dIII site was destroyed by religation after filling out the *Hin*dIII-digested clone. The resulting clone was digested with *Kpn*I and *Eco*RI, and the insert containing nucleotides 2569–3292 of the D-LSU rRNA gene was subsequently cloned into plasmid $pD_BD_{Tm\Delta H}D_B$ digested with *Kpn*I and *Eco*RI, giving rise to plasmid $pD_BD_{Tm\Delta H}D_B$/D-LSU. To create vector 395A, the ITS1 that is 100% identical between the C- and the D-unit,[4] was PCR amplified from plasmid pPbSL8.8 (24) with the oligonucleotides L427R and L412R (Table I; 30 cycles, annealing at 55 °C, 45-s extension), which introduced unique *Hin*dIII sites at either end of the ITS1. The cloning of this 522-bp amplification product (probe C/D-ITS1, Fig. 1*A*) into the unique *Hin*dIII site in plasmid $pD_BD_{Tm\Delta H}D_B$/D-LSU resulted in the formation of plasmid 395A. To create vector 387A, plasmid pPbS5.2 (23) containing the entire D-SSU rRNA gene flanked by a 2.9-kb upstream sequence and by 0.2 kb of the ITS1 was digested with *Nhe*I and *Sac*II. After size fractionation, an 2048-bp *Nhe*I/*Sac*I fragment containing 1480 bp of the upstream sequence and 568 bp of the D-SSU rRNA gene was purified from gel and cloned into pBS/KS digested with *Spe*I and *Sac*II. From this clone a D-unit-specific 868-bp region of the D-ETS (probe D-ETS, Fig. 1*A*), located 612 bp upstream of the D-SSU rRNA gene, was PCR amplified with the oligonucleotides L372R and 307A (Table I). After digestion with *Hin*dIII, the amplification product was cloned into $pD_BD_{Tm\Delta H}D_B$/D-LSU, giving rise to replacement vector 387A.

Transfection of P. berghei with Disruption and Replacement Vectors

Transfection of *P. berghei* and selection of transfected parasites were performed as described previously (26, 27). Briefly, purified schizonts (10^8) of *P. berghei* (ANKA strain, clone 15cy1) were transfected by electroporation with either 40 μg of disruption vector pMD207 (after linearization at the *Spe*I site) or 40 μg of the replacement vectors 395A or 378A (linearized with *Bam*HI and *Eco*RI). Transfected parasites were injected back into rats or mice, and transfected parasites were selected by treatment of the animals with pyrimethamine. Transfected pyrimethamine-resistant parasites were cloned by the method of limiting dilution.

Genotype Analysis of Knockout Parasites

Infected blood was obtained from mice with a parasitemia between 5 and 40%, and leukocytes were removed using Plasmodipur leukocyte filters (Eurodiagnostica). The parasites were either used for genomic DNA isolation (28), chromosome separation by field inversion gel electrophoresis (29), or parasites were used directly for PCR according to a modified method from Snounou *et al.* (30). 10^5 parasites (obtained from lysed infected erythrocytes) were washed with 250 μl of 1 × PCR buffer (Life Technologies, Inc.), resuspended in 20 μl of 1 × PCR buffer, overlaid with mineral oil, and incubated for 10 min at 100 °C. In each PCR 2 μl of boiled parasite suspension was used as template. To demonstrate correct integration of the vectors we used the oligonucleotides listed in Table I. Disruption of the C-unit with pMD207 was detected with oligonucleotides L270R and 328A (PCR-amplified fragment (PCRaf) of 3.0 kb); disruption of the D-unit was detected with L260R and 328A (PCRaf of 3.0 kb). Wild-type (wt) C- and wt D-units were detected with L271R in combination with L270R and L260R, respectively (both PCRaf sizes of 3.5 kb). Replacement of the C-unit with vector 395A was detected with L270R and L393 (PCRaf of 3.2 kb); D-unit replacement was shown with L260R and L393 (PCRaf of 3.2 kb). In all of these PCRs 40 cycles, annealing at 55 °C, and 4-min extension were used. Wild-type C-unit was detected with L263R and L264R (35 cycles, annealing at 55 °C, 2-min extension, PCRaf of 2.2 kb). For wt D-unit L265R and L266R (35 cycles, annealing at 55 °C, 2-min extension, PCRaf of 1.2 kb) were used. D-unit replacement with vector 387A was detected with L392R and L393 (35 cycles, annealing at 55 °C, 1-min extension, PCRaf of 1.1 kb). Wild-type C- and wt D-unit were detected with L271R in combination with L270R and L260R, respectively (see above).

For Southern analysis of genomic DNA of ko parasites three probes were used. To demonstrate integration of vector pMD207, the C/D-ETS probe, which is specific for the ETS region of the C- and D-units (probe 99S (14)) was used. The C/D-ITS1 probe (see "Vectors"), which is specific for the ITS1 region of the C- as well as the D-unit, was used to show replacement by vector 395A. To visualize replacement of the D-rRNA gene unit by vector 387A, probe D-ETS (see "Vectors") specific for the 5′-ETS region of the D-unit was used. Hybridizations were performed at 60 °C, and their final wash was at a stringency of 0.1 × SSC, 0.5% SDS, twice for 15 min at 60 °C.

Phenotype Analysis of Wild-type and Knockout Parasites

The presence of SSU and LSU rRNA transcripts of the different gene units was analyzed by Northern analysis and RNA dot spot hybridizations. For oocyst RNA 15 midguts were dissected from anopheline mosquitos at day 10 after infection and collected in phosphate-buffered saline. For sporozoite RNA, salivary glands were dissected from 50 mosquitos at day 20 after infection and collected in phosphate-buffered saline. Directly after collection of the parasites, RNA was isolated according to standard methods (28). For Northern blots, RNA was fractionated in guanidine thiocyanate containing agarose gels and blotted to nylon membrane (Schleicher and Schuell) according to the protocol of Goda and Minton (31). For RNA dot spot blots, aliquots of the RNA samples were denaturated and spotted on nylon membrane (Schleicher and Schuell) by a dot slotter apparatus (Bio-Rad). Blots were hybridized with oligonucleotides that are specific for the rRNA genes of the different units (Table I). Hybridizations were performed at 42 °C, and their final wash was at a stringency of 3 × SSC, 0.5% SDS, twice for 10 min at 42 °C. To determine the relative amounts of the different rRNA transcripts, we measured the intensities of hybridization signals using a PhosphorImager (Molecular Dynamics) and the software ImageQuant 3.3 (University of Virginia, ITC-Academic Computing Health Sciences). To correct for differences in specific activity of labeled oligonucleotides, we simultaneously hybridized these probes to plasmids containing the different rRNA genes. The following plasmids were used (Fig. 1*A* for the restriction fragments): pl351, containing the 7.8-kb *Eco*RI fragment of the A/B-unit (pBbSL7.8 (22)); pl316, containing the 8.8-kb *Eco*RI/*Hin*dIII fragment of the C-unit (pBbSL8.8 (11)); pl343, containing the 4.7-kb *Kpn*I/*Hin*dIII fragment of the D-unit

[3] R. R. Gutell, S. Subashchandran, M. Schnare, Y. Du, N. Lin, L. Madabusi, K. Muller, N. Pande, N. Yu, Z. Shang, S. Date, D. Konings, V. Schweiker, B. Weiser, and J. J. Cannone, manuscript in preparation.

[4] R. M. L. van Spaendonk, unpublished data.

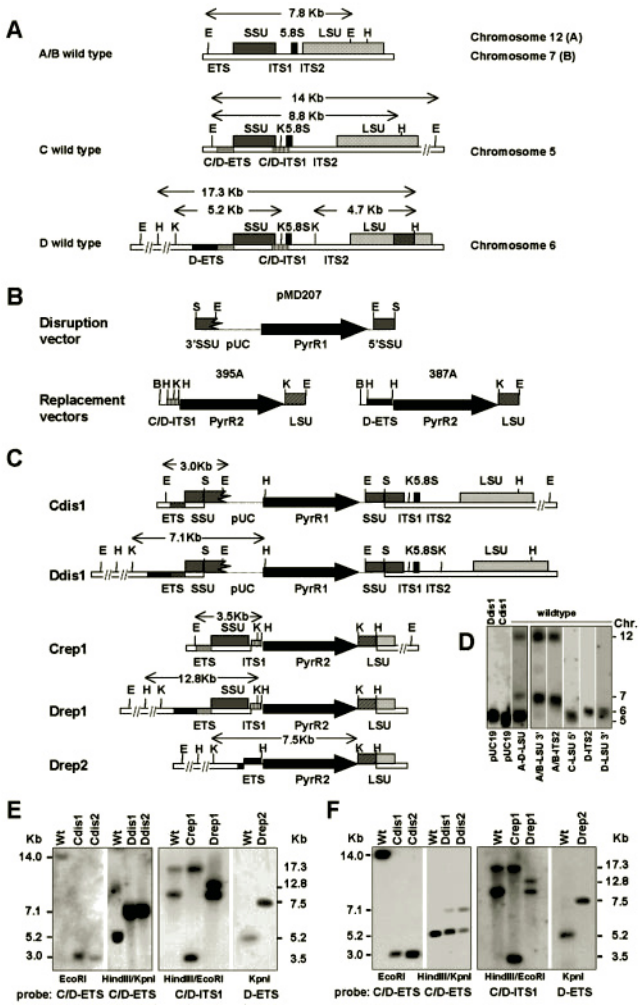

FIG. 1. **Generation of ko parasites containing a disrupted or replaced C- or D-rRNA gene unit.** *Panel A*, schematic representation of the rRNA gene units of *P. berghei* (A/B-, C-, and D-units) and their chromosomal location. The SSU rRNA gene, the 5.8 S, and the LSU rRNA genes are shown in addition to the 5′-ETS and the ITS1 and ITS2. *Arrows* indicate restrictions fragments that have been cloned (see "Experimental Procedures") or that are used for identification of the different units by Southern analysis (see *panels E* and *F*). Fragments that are used as probes for Southern analysis of genomic DNA of ko parasites are *boxed* (C/D ETS, C/D-ITS1, D-ETS). *Panel B*, vectors used to knock out the C- or D-rRNA gene unit. To disrupt the wt C- or D-unit parasites were transfected with vector pMD207, linearized at an unique *Spe*I site. Vector pMD207 contains an incomplete copy of the C-SSU rRNA gene as a target sequence and the pyrimethamine-resistant *P. berghei* DHFR/TS gene (PyrR1). To replace the wt C- or D-unit, the replacement vector 395A or 387A cut with *Bam*HI and *Eco*RI was used. Vectors 395A and 387A both contain the pyrimethamine-resistant *Toxoplasma gondii* DHFR/TS gene (PyrR2). Vector 395A contains the complete S-type ITS1 region and a central part of the D-LSU as target sequences, whereas vector 387A contains the same part of the D-LSU and a fragment of the D-ETS as target sequences. *Panel C*, schematic representation of the disrupted or replaced C- and D-rRNA gene units. Integration of vector pMD207 results in a disruption of the rRNA unit by an incomplete and a complete copy of the SSU gene separated by pUC19 plasmid sequences containing the selectable marker PyrR1 (Cdis and Ddis). In addition, two *Eco*RI restriction sites are introduced by the integrated pMD207 vector (at the 3′-end of the incomplete copy and at the 5′-end of the complete copy of the SSU gene). Integration of vector 395A into the wt C- or D-rRNA unit results in the replacement of the complete 5.8 S gene, the ITS2 region, and the 5′-end of the LSU rRNA gene by pUC19 plasmid sequences containing the selectable marker PyrR2 (Crep1 and Drep1). Integration of vector 387A into the wt D-rRNA unit results in the replacement of the 3′-end of the D-ETS, the SSU gene, the ITS1 region, the 5.8 S gene, the ITS2 region, and the 5′-end of the LSU gene by pUC19 plasmid sequences containing the selectable marker PyrR2 (Drep2). Integration of vector 395A as well as 387A results in the introduction of an *Hin*dIII restriction site at the 3′-end of the ITS1 and a *Kpn*I restriction site at the 5′-end of the remaining LSU sequences. (pPbS5.2 (23)); pl344, containing the 5.2-kb *Kpn*I/*Kpn*I fragment of the D-unit (SSU to ITS1 region).

The phenotypes of the ko parasites were analyzed further using standard technologies for determination of growth and development characteristics of *P. berghei* (32). Asexual blood stage development and gametocyte production were determined in synchronized infections in mice under standardized conditions (33). The gametocyte conversion rate is the percentage of ring forms that develop into gametocytes as determined by counting parasites in Giemsa-stained slides. Ookinete production was determined in *in vitro* cultures (34). The ookinete conversion is the percentage of female gametocytes that develop into mature ookinetes in culture. In each experiment the gametocyte and ookinete conversion rates have been determined in two independent mice/cultures. Oocyst and sporozoite development of ko parasites was analyzed in *Anopheles stephensi* mosquitos after feeding on infected C57BL/10 mice. Mosquitos were fed on days 3 and 4 on mice with a parasitemia of 1–7% resulting from infection on day 0 with 10^7 parasites. In each experiment mosquitos were fed on two mice, and 50–175 engorged females were collected from each mouse. Mosquitos were kept for 20–32 days at a constant temperature of 21 ± 0.5 °C. At different time points after infection, midguts of mosquitos were dissected for monitoring oocyst development and RNA isolation. The mean number of oocysts/mosquito was determined by counting the number of oocysts at day 10 after infection in 30–50 mosquitos. The mean size of oocysts was determined at day 10 as follows. Oocysts from three mosquitos (80–120 oocysts/midgut) were photographed using a light microscope at a magnification of ×400. From these images the size (diameter) of 50–160 oocysts was measured. The infectivity of sporozoites was tested by feeding 25–50 infected mosquitos on naïve mice at day 20 after the infectious blood meal. In each experiment two mice were infected, and

Arrows indicate restriction fragments that confirmed correct disruption or replacement of the rRNA units by Southern analysis (see *panels E* and *F*). *Panel D*, hybridization of oligonucleotides specific for the different rRNA gene units to chromosomes that are separated by field inversion gel electrophoresis. All A/B unit-specific probes hybridized with equal intensity to chromosome 12 and to 7. Hybridization of pUC19 shows the integration of the disruption vector in chromosome 5 and 6 in C-ko and D-ko parasites, respectively. *Panel E*, the genotype of ko parasites before mosquito transmission analyzed by Southern blot hybridizations. To demonstrate correct integration of vector pMD207 into the C-SSU rRNA gene, DNA was digested with *Eco*RI and hybridized to the C/D-ETS probe. In wt parasites, a 14-kb fragment of the C-unit (*panel A*) and a 44-kb fragment (not shown) of the D-unit hybridize to this probe. In the ko parasites, the introduction of an *Eco*RI restriction site at the 3′-end of the incomplete copy of the SSU gene results in a 3-kb fragment (*panel C*) in Cdis1 and Cdis2 and a 20-kb in the Ddis parasites (not shown) which hybridizes to the C/D-ETS probe. To show integration of pMD207 in the D-SSU rRNA gene, DNA was digested with *Hin*dIII and *Kpn*I and hybridized to the C/D-ETS probe. In wt parasites an 18.4-kb *Hin*dIII/*Kpn*I fragment (not shown) of the C-rRNA gene unit and a 5.2-kb *Kpn*I fragment of the D-rRNA gene unit (Fig. 1*A*) hybridize. In the ko parasites an additional *Hin*dIII is introduced at the 3′-end of the ITS1, resulting in a 20.4-kb *Hin*dIII in Cdis parasites (not shown) and a 7.1-kb fragment (Fig. 1*C*) in Ddis1 and Ddis2. To demonstrate integration of vector 395A, DNA was digested with *Hin*dIII and *Eco*RI and hybridized to the C/D-ITS1 probe. In wt parasites, an 8.8-kb *Hin*dIII/*Eco*RI fragment of the C-rRNA gene unit and a 17.3-kb *Hin*dIII fragment specific for the D-rRNA gene unit (*panel A*) hybridize to this probe. In the ko parasites an additional *Hin*dIII site is introduced at the 3′-end of the ITS1 resulting in a 3.5-kb *Eco*RI/*Hin*dIII fragment in Crep1 and a 12.8-kb *Hin*dIII fragment in Drep1 parasites (*panel C*). To show integration of vector 387A, DNA was digested with *Kpn*I and hybridized to the D-ETS probe. In wt parasites a 5.2-kb *Kpn*I fragment of the D-rRNA gene unit (*panel A*) hybridizes, whereas in ko parasites a 7.5-kb *Kpn*I fragment (*panel C*) is present. *Panel F*, the genotype of ko parasites after mosquito transmission analyzed by Southern blot hybridizations. As before mosquito transmission, in Cdis1 and Cdis2 a 3-kb *Eco*RI fragment specific for a disrupted C-unit, in Crep1 a 3.5-kb *Eco*RI/*Hin*dIII fragment specific for a replaced C-unit, in Drep1 a 12.8-kb *Hin*dIII fragment specific for a replaced D-unit, and in Drep2 a 7.5-kb *Kpn*I fragment specific for D-unit replacement are visible. However, in addition to the 7.1-kb *Kpn*I/*Hin*dIII fragment specific for a disrupted D-unit (*panel B*), in Ddis1 and Ddis2 after mosquito transmission a 5.2-kb *Kpn*I fragment specific for wt parasites (*panel A*) is visible as well. These wt parasites arise as the result of reversion events caused by reversal of the integration mechanism leading to excision of the insertion vector. *E*, *Eco*RI; *K*, *Kpn*I; *H*, *Hin*dIII; *S*, *Spe*I; and *B*, *Bam*HI).

TABLE II
Size and identities of the SSU, 5.8 S, and LSU genes of the four rRNA units

The complete sequence is only available for the A-unit of the two A-type units. All evidence indicates that the B-unit is similar to the A-unit. They have identical restriction maps (Fig. 1*A*), comparable sizes of the ITS1 and ITS2, identical sequence of the 5′-ETS region and of the 3′-LSU gene, and comparable hybridization of A-type-specific oligonucleotides to chromosome 7 (B-unit) and 12 (A-unit). The nucleotide sequence data are available under accession numbers AJ301624 (A-LSU), AJ298079 (C-LSU), AJ298080 (D-LSU), AJ298081 (A-5.8 S), AJ298082 (C-5.8 S), and AJ298083 (D-5.8 S).

	A/B-unit (A-type)			C-unit (S-type)			D-unit (S-type)		
	SSU	5.8 S	LSU	SSU	5.8 S	LSU	SSU	5.8 S	LSU
Size (bp)	2,059	162	3,789	2,059	167	4,031	2,067	165	3,942
Identity (%)									
A/B-unit					79.0	84.8	90.6	79.6	87.3
C-unit							95.9	95.8	89.0

the parasitemia in these mice was monitored by examination of Giemsa-stained slides.

RESULTS

Comparison of the 5.8 S and LSU Genes of the Different rRNA Gene Units—The nucleotide sequence of the SSU genes of the A-, C-, and D-units of *P. berghei* has already been published (15, 23, 35). The complete nucleotide sequences of the remaining 5.8 S and LSU genes of these units are reported here. The sizes of these genes and the identities between the genes of the different units are shown in Table II. Although we have only sequenced the ETS region (36) and 728 nucleotides of the 3′-region of the LSU of the B-unit, all evidence indicates that the sequences of the A- and B-units are identical. This is based on the identical restriction maps of the A- and B-gene units (Fig. 1*A*), the comparable sizes of the ITS1 and ITS2 as judged by PCR, the identical sequence of the ETS region and of pPbL4.1 encoding the 3′-end of the B-LSU gene (11) with that of the A-LSU gene (this study), and the comparable hybridization intensities of A/B-unit-specific probes/oligonucleotides (Table I) to chromosome 7 and 12 (Fig. 1*D* and data not shown).

A secondary structure analysis of the 5.8 S and LSU rRNA molecules of the 3 units predicted the presence of all six expected domains that are found in the conserved secondary structure model proposed for the 5.8 S/LSU rRNA hybrid of all eukaryotes (37, 38). The 5.8 S RNA molecules of the A- and C-unit differed at the 3′-end in a manner covariant with differences in the 5′-region of the LSU molecules (data not shown) as observed for *P. falciparum* (18). This covariance between these regions of the 5.8 S and LSU genes maximizes the specificity of base pairing between the 5.8 S and LSU molecules of the same unit. Sequence differences between the LSU RNA molecules of the different units were compared with the standard consensus secondary structure model for the LSU molecule of eukaryotes (38). From this comparison it appeared that the majority of sequence differences occurred in the variable regions of the eukaryote-specific LSU molecules (data not shown), whereas in the core regions only 26 out of 1224 positions were different from the eukaryotic consensus sequence (Table III).

If sequence differences between the core regions of different rRNA molecules result in the presence of functionally different ribosome types in *Plasmodium*, it could be expected that those sequence differences would be conserved between different *Plasmodium* species. Therefore we compared differences between the core regions of the A- and S-type LSU genes of *P. berghei* with the published differences between A- and S-type LSU genes of *P. falciparum* (18). *P. falciparum* maintains unique sequence differences in the core region of the LSU gene associated with GTPase activity. The primary structure of this region of the S-type LSU gene of *P. falciparum* more closely resembles that of a bacterial GTPase center. In *P. berghei* we found no differences in the GTPase region between any of the LSU genes. They were identical to that of the A-unit of *P. falciparum* and show a standard eukaryote sequence and structure (position 1051–1108: GTAGGACG**G**UGGUCAUGGAAG-UCGAAAU**C**CGCU**A**AGGAGTGTGTAACAACTCACCTAC; underlined positions are variant in the *P. falciparum* S gene). In the other LSU core regions all variant positions of the *P. berghei* LSU were more similar to the A-unit of *P. falciparum*. In 7 out of 11 positions where *P. falciparum* A- and S-LSU genes differed, all three LSU genes of *P. berghei* units were identical to the A-gene of *P. falciparum*, and 8 of the 11 *P. berghei* positions fit the eukaryotic consensus sequence (Table III, bottom section). The A- and S-type genes of *P. berghei* differ only at five positions in their core regions. Again however, these differences are not conserved in *Plasmodium* because the A- and S-type genes of *P. falciparum* are identical at those positions (Table III). Thus sequence differences between the core regions of the LSU genes of A- and S-type units are a species-specific phenomenon.

19 positions in the core regions which differed from the eukaryotic consensus sequence were conserved among all five *Plasmodium* LSU genes. 13 of these positions were expected to be engaged in the formation of a bp, and these were found to be associated with compensatory changes that maintained the consensus structure (10 of 13) or converted nonstandard Watson-Crick bp to standard ones (3 of 13) (Table III, top section). Therefore, the typical differences from the eukaryotic LSU rRNA core sequence found in *Plasmodium* are supported by compensatory base changes that maintain known secondary structure. The remainder of variant positions is species-specific and, therefore, does not support the notion that stage-specific expression of rRNA is associated with the production of functionally distinct ribosomes.

Generation and Isolation of Knockout Parasites with a Disrupted/Replaced C- or D-rRNA Gene Unit—To study possible functional differences between the C- and D-rRNA molecules, we generated ko parasites with a disrupted or replaced C- or D-gene unit. To knock out the gene units, three different vectors were used (Fig. 1*B*). The disruption vector pMD207 contained an incomplete copy of the C-SSU rRNA gene as target sequence. A single crossover between the SSU rRNA sequences present in pMD207 and in the genome was expected to disrupt the C- and/or D-rRNA gene unit as shown in Fig. 1 (Cdis and Ddis in Fig. 1*C*). In four independent experiments we transfected parasites with vector pMD207 and obtained pyrimethamine-resistant clones. In all experiments we obtained parasites with a disrupted C- or D-rRNA as demonstrated by PCR. We selected two C-disruptant (Cdis1, Cdis2) and two D-disruptant (Ddis1, Ddis2) clones from independent experiments for further analysis. Correct integration of vector pMD207 in the C- or D-rRNA gene unit in the clones was confirmed by PCR (data not shown) and Southern analysis of restricted genomic DNA and of separated chromosomes (Fig. 1, *D* and *E*).

Analysis of both Cdis and Ddis parasites revealed that in a small proportion of the ko parasites the wt genotype was restored. Reversion to the wt genotype occurred through a reversal of the integration mechanism leading to excision of the insertion vector. This reversion phenomenon has been described before in ko parasites of *P. berghei* (39) and can complicate phenotypic analysis (see below). Transfection with replacement vectors results in the specific and irreversible inactivation of rRNA units, preventing restoration of the wt genotype. Therefore, we also constructed the additional re-

TABLE III

Sequence differences in core regions of the LSU rRNA genes between P. berghei, P. falciparum and other eukaryotes

The *P. falciparum* sequence of the LSU rRNA genes is obtained from Rogers et al, (1996). ** Eukaryotic consensus is calculated from an alignment of 35 representative eukaryotic species (Gutell *et al.*, in preparation. Boxed area, GTPase site.)

Position[a] (*E. coli*)	*P. berghei*			*P. falciparum*		Eukaryotes	Structure[b]	Paired with (nucleotide number)	Structure[c] change
	A	S(C)	S(D)	A	S				
	Different from other eukaryotes conserved within *Plasmodium*								
479	U	U	U	U	U		Loop		
676	G	G	G	G	G	84% A	Loop		
681	U	U	U	U	U	84% G	Helix	796	G–C A–U
682	U	U	U	U	U	84% G	Helix	795	G–C A–U
737	U	U	U	U	U	84% C	Helix	759	G–C A–U
759	A	A	A	A	A	84% G	Helix	737	G–C A–U
768	A	A	A	A	A	81% G	Helix	694	G·U A–U
795	A	A	A	A	A	81% C	Helix	682	G–C A–U
796	A	A	A	A	A	81% C	Helix	681	G–C A–U
802	U	U	U	U	U	84% G	Loop		
821	A	A	A	A	A	84% G	Loop		
822	G	G	G	G	G	84% U	Helix	835	A–U G–C
823	A	A	A	A	A	84% U	Helix	834	U U A–U
835	C	C	C	C	C	84% A	Helix	822	A–U G–C
1859	U	U	U	U	U	48% A	Loop		
2280	A	A	A	A	A	75% G	Helix	2260	U·G A–U
2360	A	A	A	A	A	62% U	Helix	2428	U–A A–U
2428	U	U	U	U	U	83% A	Helix	2360	U–A A–U
2670	G	G	G	G	G	68% A	Loop		
	Different between *P. berghei* A and S Conserved between *P. falciparum* A and S								
240	U	U	A	U	U	32% U; 14% A	Loop		
404	G	A	A	A	A	40% A; 27% G	Loop		
442	G	A	A	G	G	47% G; 28% A	Helix	5.8S	G·U A–U
506	G	A	G	G	G	94% G	Loop		
1789	A	A	U	A	A	82% A	Helix	1775	A–U U U
	Conserved between *P. berghei* A and S Different between *P. falciparum* A and S								
338	G	G	G	A	G	85% G	Loop		
499	U	U	U	A	G	44% G	Loop		
856	G	G	G	C	A	59% C	Helix	921	G–C G·A
1059	G	G	G	G	A	98% G	Helix	1079	G–C A–U
1079	C	C	C	C	U	98% C	Helix	1059	G–C A–U
1084	A	A	A	A	U	94% A	Loop		
1346	G	G	G	G	A	84% G	Helix	1600	G–C A–U
1600	C	C	C	C	U	97% C	Helix	1346	G–C A–U
2271	A	A	A	U	A	75% A	Loop		
2419	G	G	G	G	A	99% G	Helix	2379	U·G A–U
2476	C	C	C	C	U	69% A	Loop		

[a] *E. coli* numbering according to Gutell and Fox (1988) (Ref. 42).

[b] The nucleotides involved in a (non-) standard Watson Crick base pair are indicated as "Helix," the nucleotides that are not involved in base pairs are indicated as "Loop."

[c] For the positions that differ from the eukaryotic consensus sequence and that are involved in base pairs is indicated whether the position "paired with" has changed as well. On the left, the base pair in the eukaryotic consensus sequence; right, the changed base pair in the *Plasmodium* sequence.

placement vectors 395A and 387A to knock out the C- and D-rRNA gene units. Vector 395A would delete the complete S-type 5.8 S gene, the ITS2 region, and the 5′-end of the LSU rRNA gene. Although the vector was based upon sequences of the D-unit it was anticipated that both the C- and D-units could be interrupted because of the high homology between the units in the regions chosen (Drep1 and Crep1 in Fig. 1*C*). We obtained pyrimethamine-resistant parasite clones with a disrupted C- or D-rRNA unit when vector 395A was introduced into the parasite, as demonstrated by PCR. We selected both a C-replacement (Crep1) and a D-replacement (Drep1) clone for further analysis and confirmed correct integration by Southern analysis of restricted genomic DNA (Fig. 1*E*) and of separated chromosomes (data not shown). Vector 387A was D-unit-specific and was designed to recombine with the unique part of the D-ETS region[4] and part of the D-LSU rRNA gene. After integration this vector would interrupt the D-ETS and LSU rRNA gene as well as replace the SSU/ITS1/5.8 S/ITS2 region (Drep2 in Fig. 1*C*). Transfection with vector 387A yielded parasites with a disrupted D-unit as indicated by PCR. One D-replacement clone (Drep2) was selected for further analysis. Correct integration of vector 387A in the D-rRNA gene unit was confirmed by PCR (data not shown) and by Southern analysis of genomic DNA (Fig. 1*E*) and of separated chromosomes (data not shown). In all transfection experiments we never selected ko parasites in which both the C- and D-rRNA gene units were disrupted and/or replaced.

The Presence of Transcripts of the Different rRNA Gene Units in Wild-type and in Knockout Parasites—The availability of the sequence of the SSU and LSU genes of the different units allowed for the design of a number of oligonucleotides specific for the different rRNA molecules (Table I), which were used to

analyze the relative abundance of these molecules in the different developmental stages by Northern analysis and dot spot analysis. As has been shown before, mature S-type transcripts were absent during blood stage development of wt parasites, and all SSU and LSU transcripts in asexual parasites and gametocytes belong to the A-type (Fig. 2, *A* and *E*). S-type transcription in wt parasites starts in the developing ookinete and is up-regulated during oocyst formation (14, 19) (Fig. 2*A*). We note that there is significant A-type RNA present in mature oocysts and in sporozoites (Fig. 2, *A* and *E*). The mature LSU rRNA with a size of about 4 kb is processed, resulting in two fragments: a small 5′-fragment of 800 bp and a large 3′-fragment of about 3 kb (40) (Fig. 2*E*). Because the mature S-type LSU rRNAs are either 153 bp (D) or 242 bp (C) larger than those from the A-type units (Table II), the corresponding 3′-fragments, resulting from the nick at 800 bp from the 5′-end of the mature LSU rRNA molecule, have the same size differences. Based on these size differences the A- and S-type LSU rRNAs could be distinguished on Northern blots (Fig. 2*B*). This allowed for determination of the relative abundance of the A- and S-type transcripts, and we calculated that mature oocysts contain A- and S-type molecules in a ratio of 60% A to 40% S (Fig. 2*B*) This A/S-type ratio was confirmed by the determination of hybridization intensity of different unit-specific oligonucleotides against both SSU and LSU in dot spot experiments. These dot spot experiments revealed also that the S-type transcripts present in both mature oocysts and sporozoites consist of 3–5 times more molecules originating from the D-unit than originating from the C-unit.

To determine whether knock out of the C- and D-rRNA units resulted in the complete absence of rRNA transcripts of these units, RNA from mature oocysts was analyzed (Fig. 2, *D* and *E*, *middle* and *bottom panels*). All C- and D-ko parasites were able to infect mosquitos, and oocyst numbers in mosquitos were comparable to those in mosquitos infected with wt parasites (see below and Fig. 3*A*). In the C-ko parasites no C-unit-specific transcripts were observed, but D-rRNA was present. Conversely, in all D-ko parasites, no D-rRNA was detectable, but the C-transcripts were present. These results demonstrate that knock out of the units completely blocked the synthesis of rRNA transcripts from these units. A-type transcripts were present in oocysts of all ko parasites (Fig. 2*E*, *top panel*). We found no evidence that A-type transcription was up-regulated in oocysts and sporozoites of the ko parasites (results not shown).

Phenotype Analysis of Knockout Parasites with Disrupted or Replaced C- or D-rRNA Gene Unit—Asexual blood stage development of all ko parasites was comparable to wt parasites as determined by the length of the asexual cycle (22–24 h), the number of merozoites/schizont (16 to 24), and the multiplication rate in synchronized infections in mice (results not shown). Also, the development of gametocytes and ookinetes was not affected in all ko parasites (Fig. 3*A*). This lack of an effect on blood stage and ookinete development is consistent with the observations that S-type expression mainly takes place during oocyst development in the mosquito and starts in the (nearly) mature ookinetes. However, oocyst and sporozoite development in the ko parasites was barely affected by the absence of transcripts of the C- or D-rRNA unit. All ko parasites produced normal numbers of oocysts and were able to produce infectious sporozoites (Fig. 3*A*). We only observed a small, but significant and reproducible, difference in the size of the oocysts of these parasites, indicating a slight retardation of the growth of oocyst of the ko parasites (Fig. 3*A*). Only in parasites of Ddis1 did we observe a stronger inhibition of oocyst development (Fig. 3*B*), resulting in the absence of sporozoites in day 10 oocysts in three independent experiments. Surprisingly, the D-ko para-

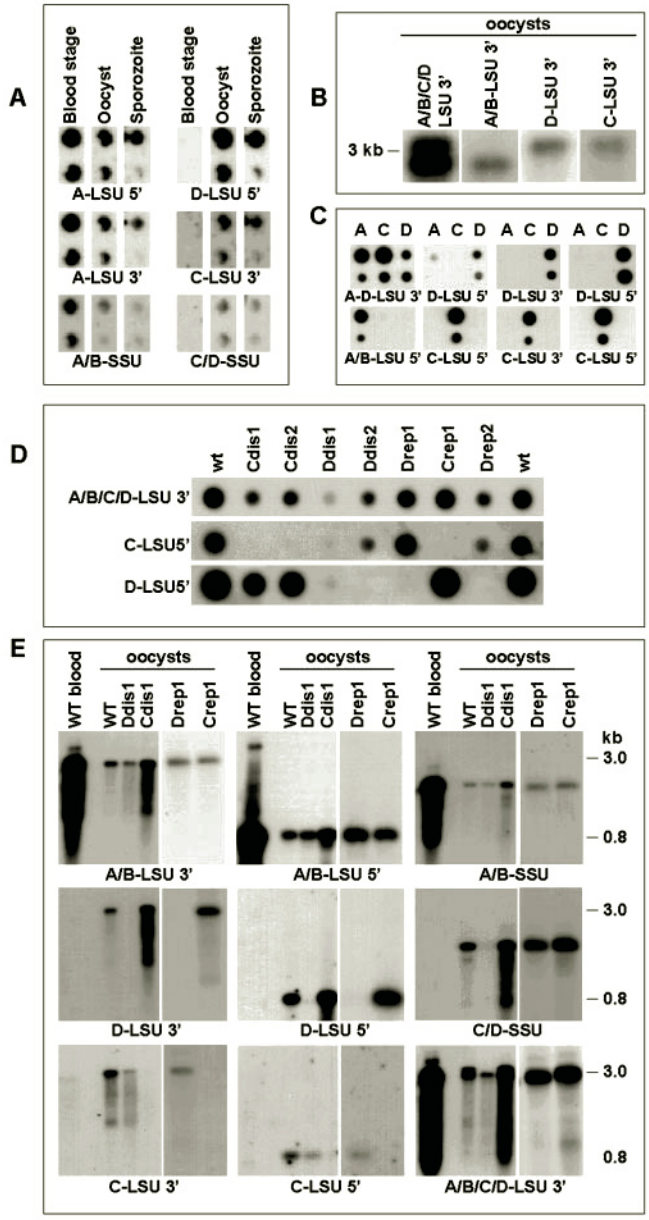

FIG. 2. **The presence of RNA transcripts of the different rRNA units in wt and ko parasites determined by Northern analysis and dot spot experiments.** *Panel A*, the presence of SSU and LSU transcripts of the different rRNA units in blood stages, mature oocysts, and sporozoites of wt parasites, determined by hybridization of RNA to specific oligonucleotides. *Panel B*, the large fragment (~3 kb) of the LSU transcript of the different units in mature oocysts of wt parasites. Small differences (153–242 bp) exist in the size of the LSU genes between the A-type and S-type, resulting in the separation of A- and S-type transcripts. Based on the relative hybridization intensity of these fragments to oligonucleotides (see *panel C*) that recognize all transcripts we estimated by PhosphorImager analysis that the ratio of A- and S-type transcripts is 60%:40%. *Panel C*, hybridization of oligonucleotides specific for the SSU or LSU genes of the different rRNA units (see Table I) to plasmids containing restriction fragments of the individual units. These hybridizations show the specificity of the probes and were used for measurement of the specific activity of labeled oligonucleotides for the determination of the relative abundance of C- and D-transcripts in mature oocysts in dot spot experiments by PhosphorImager analysis (see *panel A*). *Panel D*, the absence of C- and D-LSU transcripts in mature oocysts of C- and D-ko parasites, respectively, as shown by dot spot hybridization with oligonucleotides specific for the different units. *Panel E*, the presence of LSU and SSU transcripts in blood stages and mature oocysts of wt and ko parasites. SSU and LSU transcripts of the A-type units are present in mature oocysts of both wt and ko parasites; transcripts of the C- and D-gene units are absent in C- and D-ko parasites, respectively.

A

	conversion rate (%) asexual/gametocyte	conversion rate (%) gamete/ookinete	oocyst no. Mean (range)	oocyst size (% of wild type)	sporozoites at day 10
wild type	19 (14-23)	61 (50-71)	222 (125-350)	100	++
Cdis1	18 (16-20)	57 (52-61)	312 (289-334)	75* (72-78)	++
Crep1	18	55	158	86*	++
Ddis1	16 (15-17)	54 (51-57)	208 (192-224)	50* (45-54)	--
Ddis2	20	62	207	59*	++
Drep1	21	56	200	78*	++
Drep2	17 (15-19)	67 (57-76)	154 (125 -183)	63* (60-66)	++

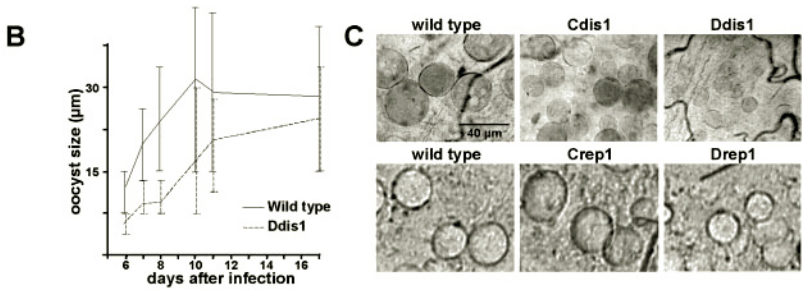

FIG. 3. **Phenotype of wt parasites and of ko parasites containing a disrupted or replaced C- or D-rRNA gene unit, showing a small retardation of the growth of oocysts.** *Panel A*, the development of gametocytes and ookinetes and the numbers of oocysts of ko parasites are comparable to those of wt parasites. A significant difference (* = $p \leq 0.05$; *t* test and Kruskal-Wallis test) is observed in the size of oocysts (mean size with S.D. is shown) at 10 days after mosquito infection. The conversion rate of asexual parasites into gametocytes is the percentage of ringforms that develop into gametocytes. The conversion rate of gametes into ookinetes is the percentage of female gametocytes that develop *in vitro* into ookinetes (28, 30). All ko parasites, except for Ddis1, produced mature oocysts containing sporozoites at day 10 after infection. *Panel B*, the size of oocysts during development of ko parasites (Ddis1) and wt parasites. *Panel C*, 10-day-old oocysts of ko and wt parasites, photographed using a light microscope at a ×400 magnification. Knockout parasites produced normal numbers of oocysts. The only difference between ko and wt parasites is a small one, in the size of mature oocysts.

sites with the same genotype obtained in the second transfection experiment (Ddis2) showed a less pronounced growth inhibition, and these parasites produced infectious sporozoites comparable to the other C- and D-ko clones (Fig. 3*A*). This result suggests that the greater retardation of oocyst growth of Ddis1 parasites resulted from not only the lack of D-unit transcripts but also from an additional defect. The number of sporozoites at day 20 in salivary glands in Ddis2 (11,000–22,000) and Cdis1 (12,000–20,000) was comparable to that of wt parasites (17,000–25,000), and sporozoites of all ko clones were infective to mice. After transmission of the ko parasites through the mosquito, the genotype of all ko parasites transfected with the replacement vectors was the same as before transmission (Fig. 1*F*). In a few experiments of the ko clones transfected with disruption vector pMD207 we observed a contamination of parasites with the wt genotype after mosquito passage (Fig. 1*F*). Those parasites arose as a result of reversion events that can occur using this disruption vector (see above). This population of ko parasites containing parasites with the wt genotype was treated with pyrimethamine during blood stage development in mice, which resulted in the removal of wt parasites from the ko population. This result proved that the wt parasites present in these populations arose as a result of reversion events. The production of infectious sporozoites in the ko clones demonstrates that the presence of two S-type genes is not essential for the complete development of *P. berghei* in the vertebrate host and in the mosquito vector.

DISCUSSION

The function of the ribosome appears to be inextricably linked with the conformation of the ribosomal RNA molecules (41). Cells of most organisms contain homogeneous populations of ribosomes with regard to the structure and function of rRNA molecules. Some, but not all, apicomplexan parasites are an exception and produce ribosomes with structurally distinct rRNA molecules encoded by single copy gene units (7–11). This distinction is magnified in *Plasmodium* through the stage-specific expression of the different rRNA genes (14, 18, 20). Based on the structural differences in the core regions of the rRNA molecules, the existence of three different ribosome types in *Plasmodium* has been suggested: A-type ribosomes present during development in the liver and the blood of the host and O- and S-type ribosomes present during development in the mosquito vector. It has been suggested that the maintenance of functionally different ribosome types provides *Plasmodium* with a unique post-transcriptional control mechanism to regulate gene expression (15, 19, 24). The expression of different ribosome forms could be a major step in the commitment of the parasite to a change in life cycle stage by influencing the population of messenger rRNAs (mRNAs) that are translated. Alternatively, through maintenance of functionally different ribosome types *Plasmodium* might be able to optimize protein synthesis during growth in the different environments of the host and the mosquito vector. Finally, structurally distinct ribosomes may simply result from the fact that different segments of the genome are accessible to the transcriptional apparatus during different stages of the life cycle. Direct evidence for the existence of functional differences among the three ribosome types of *Plasmodium* is lacking. The most direct demonstration that structural differences of the rRNA molecules might influence protein synthesis is the observation of the different abilities of the GTPase site of the A- and S-type LSU molecules of *P. falciparum* to influence the growth of transformed *Saccharomyces cerevisiae* expressing chimeric yeast/*Plasmodium* LSU molecules (21).

Through a comparison of the two SSU rRNA genes of *P. berghei* which are expressed during development in the mosquito (S-type rRNA genes), no evidence was found for structural differences in their core regions. This observation and the fact that both genes are expressed simultaneously during the growth of oocysts questioned the existence of two different ribosome types (O- and S-type) in the mosquito stages of this parasite (23). The analysis of the primary structure of the LSU

rRNA genes of the C- and D-unit presented in this paper also demonstrates that there is no structural basis for a distinct classification of ribosomes containing C- or D-rRNA molecules which might be related to known functional differences. The secondary structures of the core regions in both LSU rRNA molecules are comparable, indicating the absence of functional differences between the C- and D-rRNA molecules. In addition to the structural comparisons, we addressed the question of functional differences between the C- and D-rRNA molecules by knocking out the C- and D-rRNA gene units and analyzing the resulting phenotype. These studies strongly support the view that the C- and D-rRNA molecules are not functionally different. The presence of the rRNA of one of the two gene units was sufficient to allow the parasite to undergo full development within the mosquito and for the production of infectious sporozoites. Slight growth retardation of the oocysts of both C- and D-ko parasites might indicate that the existence of two S-type gene units represents a gene dosage effect, comparable to the existence of multiple rRNA genes or genes encoding ribosomal proteins in other organisms (43–45). The fact that the growth retardation of oocysts seems more pronounced when the more highly transcribed D-unit is disrupted rather then the C-unit supports this conclusion. Similarly, the pattern of transcription in other apicomplexan parasites suggests that these parasites maintain the minimum number of rRNA gene units of equivalent function (7–9). A gene dosage effect has also been suggested for the expression of two identical gene copies encoding elongation factor-1a in *Plasmodium* (46), and disruption of one of these copies influenced the growth rate of the parasites.[5]

During development in the liver and blood of the host, *P. berghei* expresses two A-type rRNA gene units, the A- and B-type. All evidence so far indicates that both gene units are identical and expressed simultaneously, which might imply a comparable gene dosage effect for the A-type rRNA gene units. So far we were unable to knock out one of the A-type genes, excluding the collection of supportive evidence for a gene dosage effect. Comparison of the structure of the A- and S-type SSU and LSU rRNA molecules demonstrates also that between the A- and S-type molecules structural evidence is lacking in support of a distinct classification of A- and S-type ribosomes in *P. berghei* which might be related to function. In all core regions**,** the secondary structures of the A- and S-type molecules are essentially identical, and the extensive differences are overwhelmingly confined to the highly variable expansion regions whose structure is well less defined among eukaryotes. The *P. berghei* LSU rRNA molecules match the eukaryotic consensus sequence at all positions known to be associated with catalytic activity. If sequence differences between the core regions of different rRNA molecules result in the presence of functionally different ribosome types in *Plasmodium*, it might be expected that those sequence differences would be conserved between different *Plasmodium* species or that additional species-specific variation at these positions would be observed. However, all positions reported to be variant between the core regions of the *P. falciparum* A- and S-type LSU rRNA molecules are invariant in *P. berghei*, much more similar to the *P. falciparum* A-type, and identical to the eukaryotic consensus sequence. This includes the variant sites in the GTPase center where the *P. berghei* A- and S-type molecules are identical to the *P. falciparum* A-type LSU sequence. The rate of GTPase utilization by the ribosomes of *Plasmodium* was considered as a possible means to control development in the light of different developmental stages containing either S- or A-type ribosomes (21). This control mechanism will thus be absent in *P. berghei* because of the similarity of the GTPase site in all LSU rRNA molecules.

During development of the parasite in the mosquito not only S-type rRNA molecules but also rRNA molecules derived from the A-type rRNA genes are present. *In situ* RNA hybridization revealed that a significant proportion of the rRNA of ookinetes and young oocysts is A-type that originates from the fertilized female gamete (14, 19). In this study we found also that mature oocysts and sporozoites still contain significant amounts of A-type SSU and LSU rRNA molecules, up to 50% of the total amount. The presence of A-type rRNA in sporozoites was an unexpected observation because in other studies it had been reported that A-type rRNA was barely detectable in this stage of development (15, 19, 47). The presence of both A- and S-type ribosomes in mosquito stages, such as immature (19) and mature oocysts (this study), challenges the suggestion that the replacement of different ribosome types has arisen as an adaptive mechanism for efficient protein synthesis during growth in the two very different environments found in the host and the mosquito vector (24). Instead, a mechanism of augmentation of the ribosome population by the introduction of additional ribosome forms can be considered. Moreover, it extends the period during which it might be possible for *P. berghei* to make use of hybrid ribosomes containing both A- and S-type rRNA molecules (19). Unfortunately we were unable to obtain parasites with both S-type rRNA gene units disrupted; therefore we were unable to address the question of whether the transcription of only A-type rRNA gene units would be sufficient for production of infectious sporozoites.

In conclusion, our results do not support the hypothesis that the structural differences between the rRNA molecules result in the presence of functionally different ribosome types. The observations of the lack of structural differences in core regions of all rRNA molecules, the lack of temporal differences in expression of the S-type rRNA gene units, the observation that only one copy of the S-type rRNA genes is sufficient for complete development, and the fact that both A- and S-type ribosomes are active in the mosquito all indicate functional equivalence of the different ribosome types of *P. berghei*. These findings do not exclude that the differences observed in rRNA molecules of other *Plasmodium* species do have functional significance. In contrast to *P. berghei*, the secondary structures of the *P. vivax* O-type SSU rRNA genes and the *P. falciparum* S-type LSU rRNA genes vary in a manner atypical of other eukaryotes. However, based on the result of *P. berghei* one could question whether the presence of functional distinct ribosomes types is a prerequisite for completion of the complicated *Plasmodium* life cycle in two different hosts. The question remains why *Plasmodium* parasites have evolved such an atypical and unique organization of stage-specifically expressed, unlinked rRNA genes that can produce structurally different rRNA molecules. A possibility is that the unlinked organization is a relic of evolution, and the structural differences result from a lack of regular homogenization as in the tandem array organization of rRNA genes of other eukaryotes. These differences might then accumulate but do not significantly alter function. It has been shown that gene conversion does occur among the different rRNA gene units of *Plasmodium* (48), and the evidence reported in *P. berghei* that one of the S-type SSU rRNA genes is a hybrid between an A-type SSU rRNA gene and the other S-type SSU rRNA gene (23) might indicate that significant homogenization can occur among the unlinked gene copies. Another explanation for the atypical organization might lie in the regulation of expression of the rRNA genes. By maintaining unlinked copies under control of different promoters, the parasite may be able to adjust the

[5] M. A. Sperança, H. A. del Portillo, C. J. Janse, and A. P. Waters, unpublished observations.

production of the rRNA molecules depending on the different needs during different phases of development under different environmental conditions. In the relatively short lived mosquito the parasite has to multiply rapidly and efficiently for efficient transmission to a new host. This may require switching on the expression of additional rRNA genes, the S-type genes, to be able to fulfill the need for the production of sufficient numbers of ribosomes in the rapid growing oocyst stage. The significance of the stage-specific expression of structurally distinct ribosomes by *P. berghei* will be illuminated only by gene modification or promoter replacement experiments.

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