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Chimeric rRNAs containing the GTPase centers of the developmentally regulated ribosomal rRNAs of *Plasmodium falciparum* are functionally distinct

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ABSTRACT

The human malaria parasite, *Plasmodium falciparum*, maintains at least two distinct types, A and S, of developmentally controlled ribosomal RNAs. To investigate specific functions associated with these rRNAs, we replaced the *Saccharomyces cerevisiae* GTPase domain of the 25S rRNA with GTPase domains corresponding to the *Plasmodium* A- and S-type 28S rRNAs. The A-type rRNA differs in a single nonconserved base pair from the yeast GTPase domain. The S-type rRNA GTPase domain has three additional changes in highly conserved residues, making it unique among all known rRNA sequences. The expression of either A- or S-type chimeric rRNA in yeast increased translational accuracy. Yeast containing only A-type chimeric rRNA and no wild-type yeast rRNA grew at the wild-type level. In contrast, S-type chimeric rRNA severely inhibited growth in the presence of wild-type yeast rRNA, and caused lethality in the absence of the wild-type yeast rRNA. We show what before could only be hypothesized, that the changes in the GTPase center of ribosomes present during different developmental stages of *Plasmodium* species can result in fundamental changes in the biology of the organism.

Keywords: development; malaria parasite; ribosome; *Saccharomyces cerevisiae*; translation

INTRODUCTION

The human malaria parasite *Plasmodium falciparum* is unusual in having a low number of structurally distinct sets of ribosomal RNA genes located on different chromosomes (Wellems et al., 1987), although the arrangement of this rDNA as an 18S–5.8S–25S unit is that of a typical eukaryote. The unique feature of these *Plasmodium* gene sets is their stage-specific expression during the parasite life cycle (Gunderson et al., 1987; Waters et al., 1989; Li et al., 1994a). *P. falciparum* is an obligate intracellular parasite that infects erythrocytes during the asexual stages of development (schizogony), which results in the clinical symptoms of malaria. A-type rRNA is detected in asexual (blood) stages. The parasite is transmitted by the bite of an anopheline mosquito, where a motile form of the parasite (sporozoite) is injected into the peripheral blood supply. S-type rRNA is detected in sporozoites purified from the salivary glands of infected mosquitoes. Sporozoites migrate rapidly to the liver following the bite of an infected mosquito, where development takes place in hepatocytes to complete the cycle. At least two distinct types (A- and S-type) of rRNA are therefore detected, corresponding to distinct stages of the parasite's life cycle. The switch from A- to S-type gene expression can be monitored during development of the parasite in the mosquito (Li et al., 1994b), and the corresponding S- to A-type transition in the liver has been found during development of sporozoites in a cultured hepatocyte cell line (Zhu et al., 1990). This suggests that switches in rRNA gene expression are a critical feature during development in the life cycle of the malaria parasite.

For most *Plasmodium* species, the overall sequence similarity between the A- and S-type rRNA genes is

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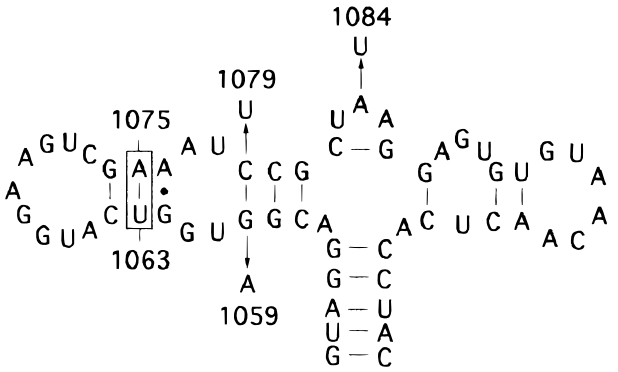
about 80%, and a detailed structural comparison of the 5.8S–28S rRNA genes from *P. falciparum* shows that most of the differences are located in variable regions and in internal transcribed spacer (ITS) regions (Rogers et al., 1995), with a very high degree of sequence conservation (>95%) in conserved regions (Rogers et al., 1996). However, in the GTPase center of the *P. falciparum* S-type rRNA, differences exist that are found in no other eukaryotes (Fig. 1). This suggests a functional difference between the A- and S-type rRNAs, although there has been no direct biological evidence that this is the case. In addition, functional differences between the two types of ribosomes would mean that they are not interchangeable and that switches between the types of rRNA gene expression trigger parasite development. In an attempt to define functional differences, the yeast wild-type GTPase center was replaced with corresponding A- and S-type GTPase centers in a plasmid containing a complete yeast rDNA repeat encoding the 18S, 25S, 5.8S, and 5S rRNAs. The effect of the *Plasmodium* GTPase centers was examined in yeast using a system whereby yeast wild-type rDNA can be replaced entirely with the plasmid-borne chimeric rDNA (Chernoff et al., 1994; Liebman et al., 1995; Liu & Liebman, 1996).



	1063/1075	1059/1079	1084
Yeast rRNA	C : G	G : C	A
A-type rRNA	U : A	G : C	A
S-type rRNA	U : A	A : U	U

FIGURE 1. GTPase center of *P. falciparum* 28S rRNA. For consistency, numbering refers to the *E. coli* 23S rRNA sequence. The secondary structure of the *P. falciparum* A-type GTPase region is shown. The A-type *P. falciparum* GTPase center differs from the *S. cerevisiae* GTPase domain by a single base pair change, C1063U and G1075A (marked in box) (Waters et al., 1995). The S-type gene has a total of five changes from the yeast sequence in this region, including the 1063–1075 base pair. The three additional changes in the S-type (see arrows) include the G1059A and C1079U base pair change and the A1084U change (Rogers et al., 1996). The S-type A1059–U1079 base pair is unique in eukaryotes and the U1084 is an A in almost all other eukaryotes, Eubacteria, and Archae.

RESULTS

Yeast cells containing a pure population of chimeric ribosomes with A-type but not S-type *P. falciparum* GTPase centers can be obtained readily

To characterize functional differences between developmentally regulated *P. falciparum* A- and S-type rRNAs, we replaced the GTPase domain of plasmid-borne wild-type yeast rDNA sequence with the corresponding domain from A- or S-type rDNA of *P. falciparum* (see Materials and Methods). Control wild-type yeast rDNA *TRP1 LEU2-d* plasmid, pRDN-wt-TL, and four independent constructs of each rDNA chimeric plasmid, pRDN-A-TL and pRDN-S-TL, containing respectively the A- and S-type GTPase regions (Table 1), were transformed into yeast strain L-1521 (Table 2). L-1521 contains a complete deletion of the entire chromosomal rDNA cluster and is kept alive by the high copy plasmid, pRDN-wt-U (*URA3*), which carries a wild-type yeast rDNA repeat (Chernoff et al., 1994). The standard plasmid-shuffle technique (Boeke et al., 1984) was employed in an effort to exchange the wild-type rDNA plasmid in L-1521 for a plasmid with chimeric rDNA. Transformants of L-1521 with pRDN-A-TL, pRDN-S-TL, or control pRDN-wt-TL were selected on −Trp medium and patched on −Leu plates to amplify the chimeric or control pRDN-TL plasmids (see Materials and Methods). Transformants were then replica plated to media containing 5FOA (+FOA medium), which selects for *ura3* cells, and therefore for loss of the *URA3*-plasmid (pRDN-wt-U), thus completing the plasmid exchange. Transformants of L-1521 carrying pRDN-wt-TL or the four independent pRDN-A-TL plasmid constructs all papillated efficiently on +FOA (Fig. 2). The DNA sequence of plasmids isolated from each of four independent A-type FOA-resistant colonies all clearly showed the presence of unique A-type residues and there was no evidence for any wild-type yeast rDNA sequence at these positions (data not shown). Similar results were obtained using primer extension analysis of the rRNA (Fig. 3A), which detected the presence of A-type but not yeast wild-type rRNA. Thus, yeast cells are viable

TABLE 1. pRDN Plasmids.

Name	rDNA	Plasmid markers
pRDN-wt-TL	Wild-type	*TRP1 LEU2-d*
pRDN-A-TL (#1–4)[a]	*P. falciparum* A-type GTPase center	*TRP1 LEU2-d*
pRDN-S-TL (#1–4)[a]	*P. falciparum* S-type GTPase center	*TRP1 LEU2-d*

[a]#1–4 are independently constructed, identical recombinant plasmids.

TABLE 2. Yeast strains.[a]

Name	Genotype
L1521	*MAT**a*** [*psi*$^-$] *ade1-14*$_{UGA}$ *his7-1*$_{UAA}$ *lys2-L864*$_{UAG}$ *trp1*Δ *ura3-52 leu2-3,112 rdn*Δ [pRDN-wt-U]
L1522	Isogenic to L1521 except that it carries plasmid pRDN-2-U instead of pRDN-wt-U
L1524	Isogenic to L1521 except that it has a *sup45* mutation
L1526	Isogenic to L1521 except that it has a *sup35* mutation
L1500	*MAT*α *[psi*$^+$] *ade2-1*$_{UAA}$ *aro7-1*$_{UAG}$ *ils1-2*$_{UAA}$ *leu2-1*$_{UAA}$ *met8-1*$_{UAG}$ *can1-100*$_{UAA}$ *trp1*Δ *ura3-52 sup111-1 rdn*Δ [pRDN-hyg1 ANIS1-UL][b]

[a]Yeast strains were constructed by Y. Chernoff (Chernoff et al., 1994).

[b]*hyg1* and *ANI1* are recessive and semidominant mutations in the 18S and 25S rRNA genes, respectively, causing resistance to antibiotics hygromycin (*hyg*1) and anisomycin (*ANI1*).

and grow well with A-type chimeric rRNA in the absence of yeast wild-type rRNA.

In contrast to the results with A-type transformants shown above, L-1521 transformants with the four pRDN-S-TL independent plasmid constructs usually papillated very poorly on +FOA medium (Fig. 2). However, occasional transformants with each of the four S-type plasmids did show good growth on +FOA at both 20 °C and 30 °C (e.g., the first S2 transformant shown in Fig. 2). DNA and rRNA sequence analyses (data not shown), as well as rRNA primer extension data (Fig. 3B, After FOA), clearly show that yeast wild-type rRNA was present in these FOA-resistant cells, most probably as a result of genetic recombination between S-type and wild-type rDNA plasmids. Next we asked if the rare FOA-resistant colonies arising from S-type

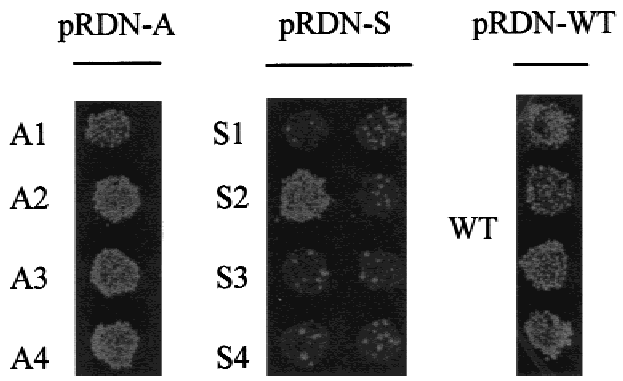


FIGURE 2. Selection for loss of wild-type yeast rRNA in the presence of A- or S-type chimeras. L-1521 containing a complete chromosomal rDNA deletion and pRDN-wt-U was transformed with the chimeric or control *TRP1 LEU2-d* plasmids, pRDN-A-TL, pRDN-S-TL, or pRDN-wt-TL, respectively. Transformants were replica plated on +FOA to select for loss of the yeast *URA3*-plasmid, pRDN-wt-U. Growth on +FOA is indicative of the ability of the transformants to survive the loss of pRDN-wt-U. A-1, 2, 3, 4 and S-1, 2, 3, 4 are independent constructs of pRDN-A-TL and pRDN-S-TL, respectively. Two transformants for each pRDN-S-TL plasmid are shown.

transformants that papillated poorly on +FOA (e.g., both S3 transformants shown in Fig. 2) contained only S-type rRNA. The sequences of rRNA isolated from four rare FOA-resistant colonies all demonstrated the presence of the uniquely wild-type rRNA residues C1063, G1075, G1059, C1079 (data not shown). Although the possibility of small amounts of S-type rRNA could not be eliminated from the sequence data, there was no evidence for the S-type sequence. The rRNA sequence data was further confirmed by an rRNA primer extension experiment (Fig. 3B, After FOA) clearly showing the presence of yeast wild-type rRNA in these FOA-resistant cells.

Yeast cells with a pure population of S-type chimeric ribosomes appear to be lethal

The above plasmid exchange experiment failed to yield cells with a pure population of S-type ribosomes, rather giving rise to FOA-resistant colonies as a result of genetic recombination, converting the S-type to wild-type rRNA sequence. This suggests that the S-type (unlike A-type) chimeric rRNA caused a detrimental effect on cell growth or was unable to support viability of the organism in the absence of functional rRNA. If cells with a pure population of S-type ribosomes were viable but grew poorly, these cells might only arise at a low frequency in a plasmid exchange experiment. Because it is impractical to identify rare events by sequence analysis, we devised a genetic screen. The rationale is based on the expectation that FOA-resistant cells with pure S-type rRNA would be inefficient in allowing loss of a newly introduced URA3-plasmid, which encodes rRNA that is not detrimental to growth, because loss of the URA3-plasmid would leave only S-type rRNA, which reduces cell growth. In contrast, cells that contain a mixture of S-type and wild-type rRNAs would allow the efficient loss of the URA3-plasmid, because wild-type rRNA remains. Thus, the efficiency of such a plasmid loss should allow us to discriminate between the desired FOA-resistant colonies carrying only pRDN-S-TL, and those that resulted from a recombination event, thereby maintaining wild-type yeast rDNA. We used a diploid test to facilitate this screen. Rare FOA-resistant colonies were crossed with strain L-1500 (Table 2) containing a complete rDNA chromosomal deletion and carrying a URA3-rDNA plasmid that efficiently supports cell growth (Liu & Liebman, 1996). We screened 200 diploids for poor papillation on +FOA (inefficient loss of URA3-plasmid), which is the expected phenotype for the desired FOA-resistant parent that only carried S-type rRNA, but no such diploids were found. This indicates that none of the FOA-resistant colonies contained a pure population of S-type rRNA, suggesting that S-type rRNA is probably unable to support cell growth.

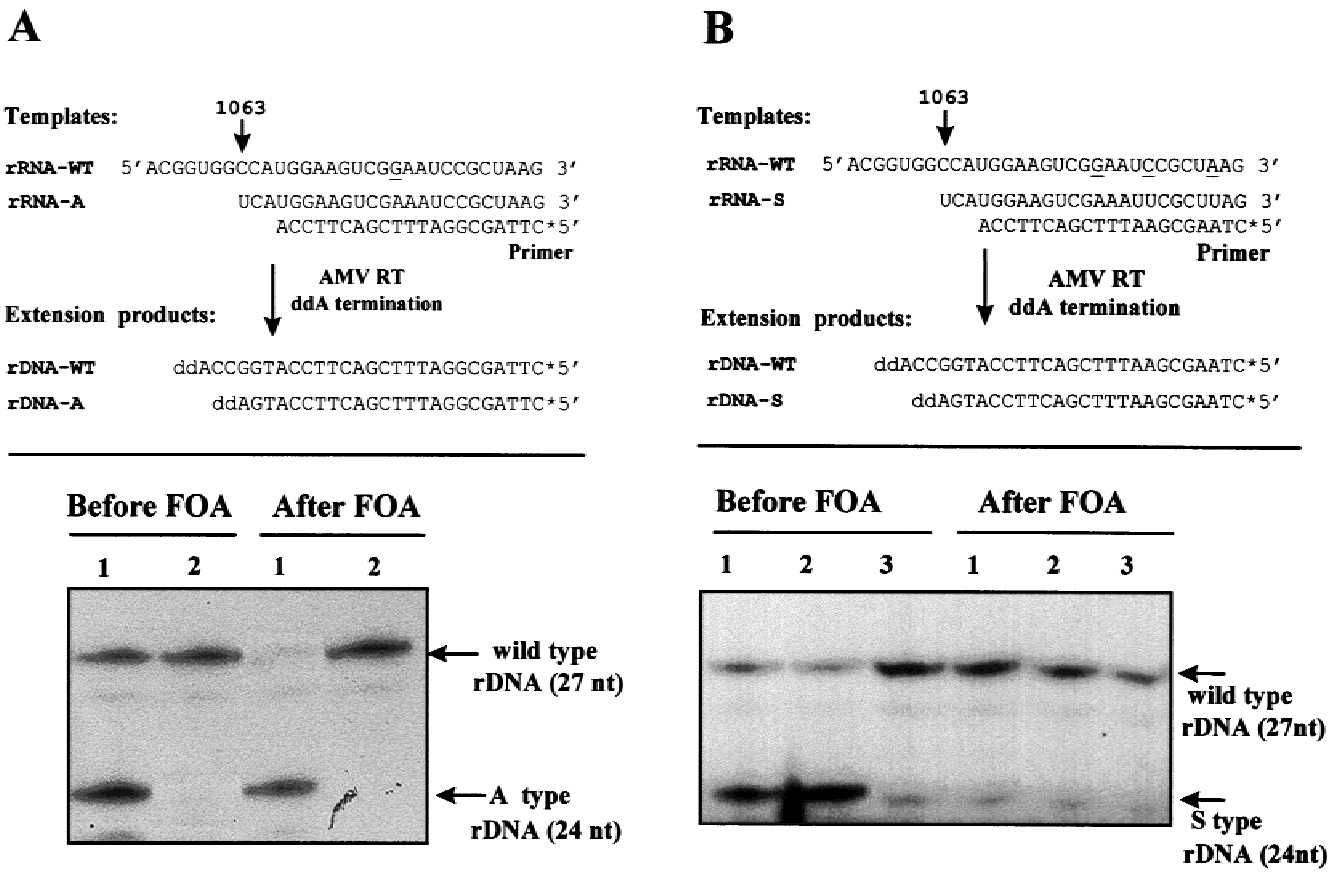


FIGURE 3. Primer extension analysis of rRNA. Total rRNA was isolated from rDNA deletion strain L-1521, which carries pRDN-wt-U, following transformation (**A**) with either pRDN-A-TL (lanes 1) or pRDN-wt-TL (lanes 2) and (**B**) with either pRDN-S-TL (lanes 1 and 2) or pRDN-wt-TL (lanes 3). Analyses are shown before and after selection for loss of pRDN-wt-U plasmid on +FOA medium. Trp^+ transformants that had been grown on −Leu were transferred to both liquid −Trp −Ura medium for total RNA isolation, and to +FOA plates to select for loss of the pRDN-wt-U plasmid. FOA-resistant cells were then grown in YPD for total RNA isolation. Lanes 1 and 2 in B correspond to RNA isolated from transformants that, when placed on +FOA medium, papillated with high and low efficiency, respectively. The DNA sequence of the wild-type and mutant extension products are shown. In A, the primer is completely complementary to the rRNA A-type sequence and has one mismatch with wild-type sequence. In B, the primer is completely complementary to the rRNA S-type sequence and has three mismatches with wild-type rRNA template. Mismatches are indicated by underline.

In another attempt to isolate strains with a pure population of the S-type rRNA, the pRDN-S-TL plasmid was introduced into yeast rDNA-deletion strain L-1522 (Table 2). This strain is isogenic to L-1521 (used above) except that it carries a *URA3*-plasmid containing a mutant rDNA, pRDN-2-U, rather than the wild-type rDNA plasmid, pRDN-wt-U, present in L-1521. The pRDN-2-U plasmid contains a mutation (*rdn-2*) in the small subunit ribosomal RNA that severely reduces growth in the absence of wild-type rRNA (Chernoff et al., 1994). Because cells containing the *rdn-2* mutation exhibit poor growth, it should be easier to exchange pRDN-2-U than pRDN-wt-U for a plasmid containing another deleterious rDNA mutation, e.g., pRDN-S-TL. This method has been used successfully to permit the isolation of yeast cells with a pure population of mutant ribosomes containing the deleterious rDNA mutation, *rdn1-A* (Liebman et al., 1995; Chernoff et al., 1996).

Approximately 300 FOA-resistant colonies isolated from pRDN-S-TL transformants of L-1522 were tested in the above-described diploid plasmid exchange screen. However, almost all cells papillated well, which was indicative of the presence of wild-type rRNA in the FOA-resistant parent. Furthermore, the two colonies that papillated poorly were shown to contain wild-type rDNA. These findings indicate that S-type rRNA is unable to support viability of yeast cells.

A- and S-type chimeric rRNAs can be detected in cells that also contain wild-type rRNA

We looked for the accumulation of the S- and A-type rRNA transcripts in cells where both chimeric and pRDN-wt-U plasmids were maintained (before plasmid exchange). L-1521 was transformed with pRDN-S-TL, pRDN-A-TL, or pRDN-wt-TL plasmids and primer extension analysis was performed to visualize the rRNA types present. The data in Figure 3 (see "Before FOA") indicate that both the A- and S-type transformants synthesize the corresponding chimeric rRNA in addition to

wild-type yeast rRNA. Note that in Figure 3 the relative amounts of mutant and wild-type rRNA cannot be determined because the primer has mismatches with wild-type rRNA.

Accumulation of rRNA with the *Plasmodium* S-type GTPase center inhibits cell growth in yeast

To determine whether the expression of S-type rRNA impairs cell functioning in vivo, we looked for growth differences in cells harboring a mixed population of A- or S-type and wild-type ribosomes (before selection against pRDN-wt-U). The pRDN-A-TL and pRDN-S-TL plasmids as well as control pDNA-wt-TL were introduced into L-1521 and transformants were selected on −Trp medium. Trp^+ cells were spread on −Leu plates to amplify the chimeric pRDN plasmid and on YPD plates as a control. Differences in cell growth were estimated by colony size (Fig. 4). Transformants carrying a mixed population of A-type and wild-type ribosomes grew as well as wild-type cells on both −Leu and YPD medium. In contrast, cells with the S-type/wild-type ribosome mixture exhibited growth inhibition on −Leu medium, although cell size was unchanged. Doubling time was 4.3 h for pRDN-wt-TL and pRDN-A-TL transformants versus 5.2 h for pRDN-S-TL transformants. This effect was not observed on YPD medium, where the pRDN-TL plasmids were not amplified. Similar results were obtained at 20 °C and 37 °C. The S-type-associated growth inhibition demonstrates that S-type chimeric rRNA adversely affects cell function even in the presence of yeast wild-type rRNA.

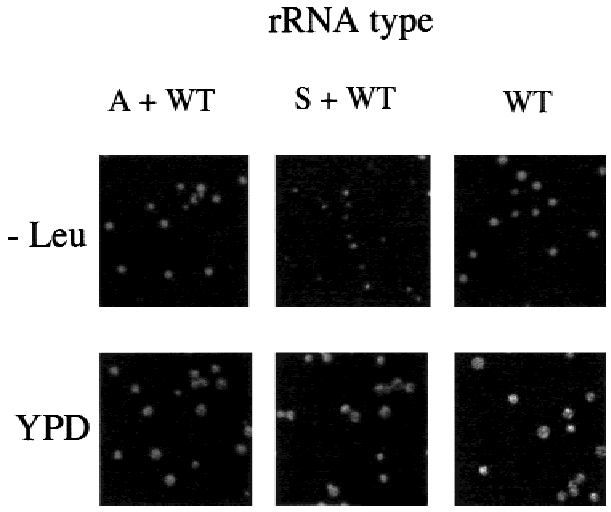


FIGURE 4. Growth phenotype of cells harboring a mixed population of A- or S-type and wild-type ribosomes (A+WT and S+WT, respectively). pRDN-A-TL, pRDN-S-TL, and pRDN-wt-TL plasmids were introduced into rDNA deletion L-1521 strain carrying pRDN-wt-U. Growth was estimated by examining colony size on −Leu medium where the chimeric pRDN-A-TL, pRDN-S-TL, and yeast pRDN-wt-TL plasmids are amplified and on YPD medium, which is nonselective for the plasmids.

Replacement of the *Saccharomyces cerevisiae* 25S rRNA GTPase center with A- or S-type from *P. falciparum* increases translational accuracy

To determine whether the S- or A-type of rRNA alters translational accuracy, we scored for readthrough (suppression) of termination codons in L-1521 transformed with chimeric plasmids. Suppression of the *ade1-14*$_{UGA}$ and *his7-1*$_{UAA}$ nonsense alleles would allow the transformants to grow on media lacking adenine and histidine, respectively. However, no detectable suppression of *ade1-14*$_{UGA}$ and *his7-1*$_{UAA}$ was observed in the presence of A- or S-type rRNA either before or after plasmid exchange (data not shown).

A similar approach was used to define if A- or S-type rRNA could increase translational accuracy. This was detected by a reduction in ribosomal suppression of nonsense codons. We used isogenic strains L-1526 and L-1524, bearing, respectively, mutations in release factors Sup35p and Sup45p, which cause suppression of the *ade1-14*$_{UGA}$ and *his7-1*$_{UAA}$ nonsense alleles. In addition, each strain contained a stable deletion of the chromosomal rDNA repeats and carried the pRDN-wt-U plasmid. The pRDN-A-TL and pRDN-S-TL plasmids were introduced into these strains and Trp^+ transformants were spotted on −Ade −Trp and −His −Trp plates to score for changes in the efficiency of nonsense suppression. Note, leucine was present in all media used in this experiment to avoid the poor growth caused by amplification of pRDN-S-TL on −Leu medium. The reduced ability to grow on −Ade −Trp and −His −Trp (Fig. 5) clearly shows that both A- and S-type rRNAs cause a strong inhibition of *sup45*-mediated suppression even in the presence of wild-type rRNA. This implies that both A- and S-type rRNAs increase translational accuracy. A similar antisuppressor effect of the chimeric rRNA was observed in the presence of *sup35* (data not shown).

DISCUSSION

Functionally important domains of ribosomal RNA, for example, the decoding domain and peptidyl transferase domain, are remarkably conserved in evolution (for review see Green & Noller, 1997). The rRNA domain, which together with ribosomal proteins promotes hydrolysis of GTP, appears to be located in a small and highly conserved domain in the 5′ region of the large subunit rRNA (Cundliffe, 1994). In *Escherichia coli*, this domain corresponds to nt 1052–1112, and a direct interaction between this region and the translocation factor EF-G (Moazed et al., 1988), the antibiotic thiostrepton (an inhibitor of ribosome-associated GTP hydrolysis)

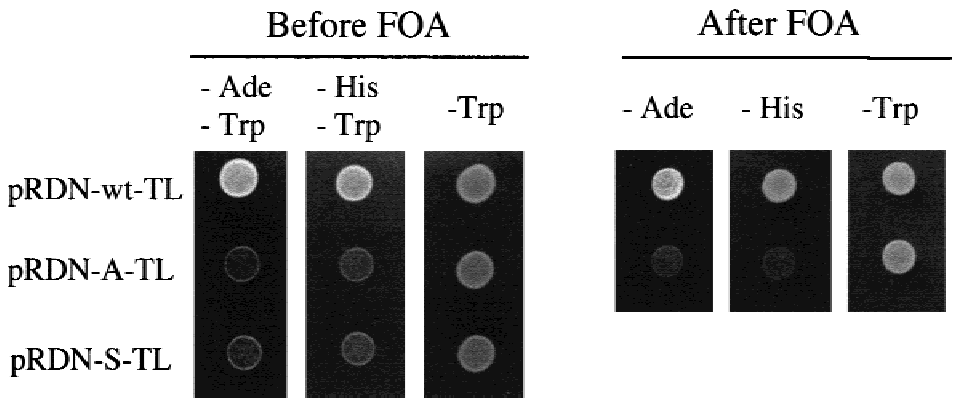


FIGURE 5. Replacement of the *S. cerevisiae* GTPase center in the 25S rRNA with *P. falciparum* A- or S-type alters translational accuracy by decreasing the level of nonsense codon misreading. L-1524 transformants with the indicated plasmids were maintained on −Trp medium. Spots show growth on the indicated media. L-1524 contains a chromosomal rDNA deletion, carries pRDN-wt-U, and has a mutation in the *SUP45* gene that results in growth on −His and −Ade media despite the presence of the *his7-1*$_{\mathbf{UAA}}$ and *ade1-14*$_{\mathbf{UGA}}$ nonsense mutations. This suppression was reduced in L-1524 transformants bearing either pRDN-A-TL or pRDN-S-TL both before (pRDN-A-TL and pRDN-S-TL) and after (pRDN-A-TL) plasmid exchange, relative to L-1524 control transformants with pRDN-wt-TL.

(Rosendahl & Douthwaite, 1994; Rogers et al., 1997), and ribosomal protein L11 (Rosendahl & Douthwaite, 1993), all implicate this region as being functionally important for GTP hydrolysis. This region also probably interacts with the peptidyl transferase site, located in the 3′ region of the large subunit rRNA (Mankin et al., 1994). The extreme conservation in biological function of the GTPase rRNA center was shown by the replacement of the *E. coli* GTPase center with the homologous region from yeast, as well as by the replacement of the yeast sequence with the *E. coli* equivalent (Musters et al., 1991; Thompson et al., 1993). There were no apparent deleterious effects detected in cells with either of these *E. coli*–yeast hybrid rRNAs, although detailed studies were not possible because of the presence of wild-type ribosomes. Also, *E. coli* L11 binds to the large subunit RNA from eukaryotes (Thompson et al., 1993), whereas the yeast homologue (L15) binds the equivalent region in mouse rRNA (el-Baradi et al., 1987). These results suggested that the rRNA GTPase centers are functionally interchangeable between species, and even between kingdoms. Our current data show this is not always the case.

P. falciparum is known to contain two distinct sets of the rDNA genes, A- and S-type, whose nonsynchronous expression is regulated during the life cycle of the organism. The switch in synthesis between the rRNA types may reflect different requirements that vary with the state of parasite development. Indeed, the finding that several residues of the GTPase region of the S-type rRNA differ from the highly conserved expectations found in the A-type suggested that there might be functional differences between the GTPase regions of these two types of rRNA. Because the isolation of necessary quantities of pure A- and S-type ribosomes from *P. falciparum* to test this hypothesis is impractical, we turned to a yeast system where cells containing pure populations of ribosomes with the S- and A-type rRNA GTPase domains could be obtained. Using this system, we showed that sequence variations between the GTPase centers of the A- and S-type genes are sufficient to affect biological change.

Yeast cells containing a deletion of all chromosomally encoded rDNA genes, which were kept alive by the presence of a multicopy wild-type rDNA plasmid, were used. These cells contained a pure population of plasmid-encoded rRNA. To characterize the effect of A- and S-type GTPase rRNA mutations, new rDNA plasmids carrying the mutations of interest were introduced into the cells to replace the original wild-type rDNA plasmid. The successful loss of the wild-type rDNA plasmid was only possible if the mutant rRNA encoded by the new plasmid was able to form functionally active ribosomes. In these experiments, the original plasmids were designed to contain the *URA3* gene because resistance to FOA medium selects for *ura3* cells and therefore for the loss of the *URA3* plasmids. We found that although it was easy to obtain yeast cells with a pure population of ribosomes with A-type GTPase centers, the analogous plasmid exchange experiment with S-type chimeras failed. Although all examined FOA-resistant colonies from the A-type transformants contained uniquely A-type rRNA, the screen for FOA-resistant cells among S-type transformants instead detected low-frequency recombination events, yielding wild-type rRNA sequences in the absence of the *URA3* gene. Thus, although yeast with a pure population of the A-type GTPase center were viable and did not inhibit growth, yeast with a pure population of the S-type changes appeared to be lethal, suggesting that *Plasmodium*-specific S- and A-type GTPase mutations make an essential functional difference, at least in yeast.

It was still possible, however, that yeast with only S-type chimeric rRNA were viable, but infrequent, and were therefore not detected among the background of *ura3* wild-type rDNA recombinants, which also cause resistance to FOA. We therefore devised additional screens to detect pure S-type cells, which might appear in such a low frequency. A total of 500 FOA-resistant colonies were tested in these screens, but all failed the test. Furthermore, 300 of these FOA-resistant colonies were obtained in a plasmid exchange experiment with a *URA3* plasmid carrying the *rdn-2* mutant rDNA. Loss of the *rdn-2* plasmid was expected to be more efficient than loss of a wild-type rDNA plasmid because of the deleterious effect of the *rdn-2* mutation on cell growth. The failure to recover yeast cells inheriting a pure population of the S-type rRNA strongly suggests that, in contrast to the A-type rRNA, the S-type chimeric rRNA is not able to support cell survival when wild-type rDNA genes are deleted.

Although cells with a pure population of the S-type chimeric ribosomes could not be obtained, cells containing a mixture of S-type and wild-type rRNA were viable. Cells with a mixture of wild-type and the A- or S-type chimeras were shown to decrease the nonsense suppression efficiency of mutations in the translational termination factor genes, *SUP35* and *SUP45*. Although this antisuppressor activity may reflect a direct interaction between the GTPase center and the termination factors (Moffat et al., 1991; Murgola et al., 1995), it may also result from interactions with other components of the translational machinery. For instance, if mutations in the GTPase domain reduce the activity of the translation elongation factor EF-1α, the efficiency of stop codon misreading would be decreased, thereby causing antisuppression. Indeed, recent experimental data indicate that a base change at position 1067 of the *E. coli* GTPase center may directly affect the action of the elongation factor EF-Tu (Saarma et al., 1997).

Although a mixture of A- and wild-type ribosomes had no effect on growth rate, cells with a mixed population of S-type and wild-type ribosomes exhibited severe growth inhibition. It is possible that this effect is due to a malfunctioning of ribosomes with the S-type rRNA during protein synthesis. Alternatively, expression of the S-type rRNA may cause a reduction in wild-type rRNA, leaving the total level of rRNA unchanged. If S-type rRNA fails to assemble into ribosomal particles, the shortage of functional ribosomes could inhibit growth. Finally, growth inhibition may also result from titration by nonfunctional S-type rRNA of some limited factors essential for rRNA processing or protein synthesis. Taken together, the S-type specific dominant growth inhibition phenotype, as well as its inability to support cell growth, provide strong evidence that differences in the GTPase domain of the A- and S-type rRNAs are functionally significant. The question of whether this functional difference reflects variations in the GTP hydrolysis rate awaits additional experimental evidence.

MATERIALS AND METHODS

pRDN plasmids

Table 1 lists pRDN plasmids used in this study. All the pRDN plasmids are shuttle vectors containing yeast 2 μm and *E. coli* ColE1 origins of DNA replication and they all carry the 9-kb (yeast wild-type, mutant, or chimeric) rDNA repeat, including coding regions for 18S, 5.8S, 25S, and 5S rRNA, with their original PolI promoter and PolIII promoter sequences. The rDNA plasmids carry combinations of the *URA3*, *TRP1*, or *LEU2-d* genes as transformation selection markers. For example, plasmids of the pRDN-U class carry *URA3*, whereas plasmids of the pRDN-TL class carry *TRP1* and *LEU2-d*. The *LEU2-d* allele contains a defective promoter and is used to select for plasmid amplification (Parent et al., 1985). Yeast cells transformed with pRDN-TL plasmids are originally selected on −Trp followed by transfer to −Leu media. Only those transformants with an elevated *LEU2-d* copy number can compensate for the Leu$^-$ phenotype of the parental strain, allowing cells to grow on medium lacking leucine.

The pRDN-2-U plasmid contains the *rdn-2* mutation in the 18S rRNA gene (Chernoff et al., 1994). The pRDN-A-TL and pRDN-S-TL plasmids are identical to pRDN-wt-TL except that the rDNA sequence of the former plasmids have changes in the GTPase center of the 25S rRNA specific to *P. falciparum* A- and S-type rDNA sequences. Plasmid pRDN-wt-TL was digested with *Mlu* I and *Eag* I (New England Biolabs) and a 587-bp DNA fragment was recloned in the corresponding unique sites in plasmid pSL1190 (Pharmacia). PCR was performed with the recombinant pSL1190 plasmid as template (approximately 1 ng) with oligonucleotide pairs 1142/1140 or 1142/1141. These primers were designed to introduce mutations specific to *P. falciparum* A- and S-type rDNA sequences into the yeast GTPase region of the 28S rRNA gene. Gene S-specific oligonucleotide, (1140), is: 5′-ATA TGG CGG CCG GTG AGT TGT TAC ACA CTC CT**A** AGC G**A**A TT**T** CGA CTT CCA TG**A** CCA **T**CG TCC GGC-3′ (bold underlined nucleotides indicate mutations in the yeast sequence). Gene A-specific oligonucleotide, (1141), is: 5′-ATA TGG CGG CCG GTG AGT TGT TAC ACA CTC CTT AGC GGA TT**T** CGA CTT CCA TG**A** CCA CCG TCC GGC-3′. Primer 1142 is: 5′-CGA CGT ACG CGT AAT GAA AGT GAA CGT AGG-3′.

Following PCR, fragments containing the A- and S-type mutations were purified, digested with *Mlu* I/*Eag* I, and recloned in pSL1190 in the same sites. The sequence of the entire PCR-derived DNA fragment was verified by sequence analysis. The insert was then isolated by digestion with *Mlu* I/*Eag* I and recloned into pRDN-wt-TL, producing recombinant plasmids pRDN-A-TL and pRDN-S-TL. The GTPase domains of four independent isolates of each recombinant plasmid were verified by DNA sequence analysis.

Strains and cultivation conditions

Standard media were used to grow yeast (Rose et al., 1990) and *E. coli* (Sambrook et al., 1989) strains. Yeast transfor-

mation was accomplished by using the lithium acetate procedure (Ito et al., 1983). Synthetic complete medium (Sc) lacking nutrient(s) (e.g., −Trp, −Leu, −Ade, or −His) was used to select for yeast transformants or to score for suppression of nonsense mutations. Incubation on +FOA medium (containing 1 mg/mL 5-fluoro-orotic acid and 12 mg/mL uracil) was used to select for Ura$^-$ yeast cells (see plasmid exchange procedure for details). Yeast strains used in this study are listed in Table 2. L-1521 has a stable deletion of all chromosomal rDNA repeats and carries a wild-type rDNA locus on a multicopy *URA3*-plasmid, pRDN-wt-U. Also, the three nonsense mutations in this strain, *ade1-14*$_{\mathbf{UGA}}$, *his7-1*$_{\mathbf{UAA}}$, and *lys2-L864*$_{\mathbf{UAG}}$ (Chernoff et al., 1994), allow efficient screening for increased levels of misreading (suppression) on −Ade, −His, and −Lys omission media. L-1524 and L-1526 are isogenic to L-1521 except that they contain the recessive omnipotent nonsense suppressors, *sup45* and *sup35*, respectively. L-1522 is isogenic to L-1521, but contains the *rdn-2* mutation in the rDNA locus on the multicopy plasmid. L-1500 has a stable deletion of the entire rDNA cluster in chromosome XII and carries a yeast rDNA plasmid, pRDN-hyg1,ANI1-UL.

E. coli strain DH5α was used for routine cloning. Transformants with rDNA-containing plasmids were grown in LB, supplemented with 100 μg/mL ampicillin at 30 °C. Growth of the *E. coli* transformants at 30 °C rather than at 37 °C was found to reduce rDNA plasmid rearrangement.

Plasmid exchange experiment

Yeast strains bearing a stable chromosomal deletion of rDNA repeats and multicopy rDNA *URA3*-plasmid, pRDN-wt-U, were transformed with either wild-type (pRDN-wt-TL) or chimeric (pRDN-A-TL or pRDN-S-TL) plasmids. Plasmid exchange was accomplished on +FOA medium, selective for Ura$^-$ cells (loss of pRDN-wt-U) (Boeke et al., 1984). Ura$^-$ colonies growing on +FOA are dependent upon the *TRP1 LEU2-d* pRDN-TL plasmid for viability because of the chromosomal rDNA deletion and the loss of the *URA3* pRDN-wt-U plasmid. Primer extension analysis of rRNA as well as RNA and DNA sequence analyses were used to verify the plasmid exchange process.

RNA sequencing and primer extension analysis

Total yeast RNA was isolated as described previously (Schmitt et al., 1990). A modified method of primer extension analysis (Chernoff et al., 1994) was used to detect the presence of chimeric and wild-type 25S rRNAs. The oligonucleotide primers shown in Figure 3 were purchased from Integrated Life Technologies, Inc., gel-purified, and end-labeled with [γ^{32}-P] ATP using T4 polynucleotide kinase. The primers were annealed to total RNA by incubating the annealing mixture (9 μg of total RNA and 1 pmol of primer in 10 μL of water) for 2 min at 90 °C with slow cooling (30–40 min) to 40 °C. Annealed primers (1/10 volume of annealing mixture) were then extended using 1 unit of AMV reverse transcriptase (US Biochemical) in the presence of 150 mM of ddATP and 150 mM of each of dGTP, dTTP, and dCTP for 15–20 min at 37 °C. The presence of a ddATP resulted in termination of DNA synthesis at different positions in the various rRNA species. If S- or A-type rRNA serves as a template, DNA synthesis terminates at position 1063 of the 25S rRNA, producing a DNA fragment of 24 nt. If wild-type rRNA is used as a template, DNA synthesis is extended 3 nt further, producing a DNA fragment of 27 nt. Primer extension products were separated on 20% polyacrylamide gel and visualized by autoradiography.

Ribosomal RNA sequence analysis has been performed as described previously (Powers & Noller, 1993).

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