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The Importance of the 45 S Ribosomal Small Subunit-related Complex for Mitochondrial Translation in *Trypanosoma brucei*[*]

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Background: Trypanosome mitochondria contain a 45 S ribosomal SSU-related complex with no precedence in other systems.
Results: A selective ablation of the 45 S complex was associated with synthesis inhibition of some mitochondrial polypeptides.
Conclusion: The 45 S complex is a factor required for mitochondrial translation that may have selective effects on different mRNAs.
Significance: The mechanisms of mitochondrial translation in trypanosomes have no counterparts in other eukaryotes.

The mitochondrial 45 S SSU* complex in *Trypanosoma brucei* contains the 9 S SSU ribosomal RNA, a set of SSU ribosomal proteins, several pentatricopeptide repeat (PPR) proteins, and proteins not typically found in ribosomes, including rhodanese domain protein (Rhod) and a 200-kDa coiled-coil protein. To investigate the function of this complex, PPR29, Rhod, 200-kDa protein, and mitochondrial ribosomal protein S17 were knocked down by RNAi in procyclic *T. brucei*. A growth retardation phenotype, a reduction in the amount of the 45 S SSU* complexes, and the preferential inhibition of synthesis of the cytochrome *c* oxidase subunit I over apocytochrome *b* were observed as early as day 2 postinduction of RNAi. On the contrary, the down-regulation of mitochondrial ribosomal protein L3 drastically reduced the amount of the large subunit and indiscriminately inhibited mitochondrial translation. The relative amounts of translation-competent, long poly(AU)-tailed cytochrome *c* oxidase subunit I and edited apocytochrome *b* mRNAs were selectively reduced by ablation of the 45 S SSU* complex. The formation of the 80 S translation complexes, identified by association of the long-tailed mRNAs with the mitoribosomes, was also disrupted. On the other hand, the relative amount of long-tailed edited RPS12 mRNA was not substantially affected, and there was no noticeable effect on the RPS12 translation complexes. In bloodstream trypanosomes, the amount of the 45 S complexes was drastically reduced compared with procyclics. We propose that the 45 S SSU* complex represents a factor required for normal mitochondrial translation that may have selective effects on different mRNAs.

Trypanosomatids belong to a large group of protists known as kinetoplastids, which represent one of the most divergent eukaryotic lineages (1). Among trypanosomatids are the important human pathogens, such as *Trypanosoma brucei*, an agent of African sleeping sickness, and several species of *Leishmania*, causing cutaneous, mucocutaneous, and visceral forms of leishmaniasis, as well as many benign species worldwide (2) (see Ref. 3 for a recent review of the group's biodiversity). The unifying feature of all of these organisms is the presence of the kinetoplast, a mitochondrial compartment enclosing a large mass of highly condensed DNA (kinetoplast DNA) composed of several thousands of interlocked minicircles and a lower number of maxicircle molecules (4–7). The maxicircle genome contains genes for the 9 S small subunit (SSU)[3] ribosomal RNA (rRNA) and the 12 S large subunit (LSU) rRNA, which are the smallest among their counterparts, as well as 18 protein-coding mRNAs but no tRNA genes (8, 9). This mitochondrial system stands out among the others due to the extensive RNA editing required for maturation of the majority of mRNAs (10–13). Twelve of the 18 protein-coding genes contain defects, varying from small (such as a frameshift) to extensive, leaving the entire gene or its large portion virtually unrecognizable. The editing corrects these defects by additions and, less frequently, deletions of uridine (U) residues in the original pre-edited transcripts, resulting in the creation of translatable (edited) mRNAs. In extreme cases, the transcript of a cryptogene, such as ribosomal protein S12 (RPS12), would acquire tens or even hundreds of U residues in order to become a functional mRNA (a pan-edited mRNA). An example of a gene with a relatively small defect is apocytochrome *b* (Cyb); in *T. brucei*, its mRNA acquires only 34 U residues in the 5′-end region, including one residue near the

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[3] The abbreviations used are: SSU, small subunit; PPR, pentatricopeptide repeat; KRIPP, kinetoplast ribosomal PPR protein; RPS12, ribosomal protein S12; LSU, large subunit; LT, long-tailed; ST, short-tailed; COI, cytochrome *c* oxidase subunit I; Cyb, apocytochrome *b*; DM, dodecyl maltoside; qPCR, quantitative PCR; p.i., postinduction; SSPE, saline/sodium phosphate/EDTA.

5′-proximal position to create the initiation codon AUG (a 5′-edited mRNA). Among the genes encoding functional ORFs is cytochrome *c* oxidase subunit I (COI), whose mRNA does not require editing and represents "unedited" transcripts.

Editing is performed by a sophisticated enzymatic machinery (14–18) directed by guide RNAs, short (~30–45-nt) transcripts encoded by kinetoplast DNA minicircles (8, 19–21). Pan-editing involves multiple guide RNAs acting in an orderly fashion until the mRNA becomes fully edited. A pre-edited mRNA undergoes the first round of editing on the 3′-end, with the subsequent editing events progressively spreading toward the 5′-end. The completion of editing at the very 5′-end is a sign that the entire transcript has been edited downstream. It has been hypothesized that the edited 5′-end sequence is recognized by a specific (yet unknown) pentatricopeptide repeat (PPR) protein that directs the polyadenylation/polyuridylation complex to extend the short poly(A) tail, already present in the pre-edited and partially edited mRNA, with the creation of a long poly(A/U) tail (22, 23). This structure is thought to signify achievement of the maturity and translatability of the mRNA and promotes its recognition by mitochondrial ribosomes.

The details of this hypothetical interaction remain unknown. In addition to selecting a fully edited template out of the pool of pre-edited and partially edited molecules (the task facilitated by the long poly(A/U) tails), the ribosome should also select the proper translation initiation codon (24). Kinetoplastid mitochondrial mRNAs usually contain a 35–40-nt untranslated leader sequence on the 5′-end, which lacks any element equivalent to the Shine-Dalgarno sequence. Although in some cases the initiation codon (usually AUG, but sometimes AUA or AUU) is created by editing and is the most 5′-proximal, this is generally not the case, with an encoded AUG sometimes found upstream in frame or out of frame. The translation initiation mechanism, therefore, is expected to differ from both eubacterial and mammalian mitochondrial systems (25). The analogous problem of translation initiation on mRNA with the long 5′ leaders is solved in yeast with the aid of mRNA-specific activators, including members of the PPR protein family (*e.g.* Pet309, Pet111, Aep2, and Cbp1), which stabilize mRNA and tether it to the inner membrane and facilitate recruitment of the ribosome to the correct start codon (26, 27).

Among the non-plant organisms, trypanosomes are unusually rich in the PPR proteins; more than 40 members of this group have been identified so far in *T. brucei* (28–31). For comparison, 15 PPR proteins have been found in baker's yeast and seven in mammals (32, 33). It is plausible that this abundance is related to the higher complexity of the mRNA processing and translation machineries and their increased reliance on sequence-specific RNA recognition, as compared with other organisms. The recent proteomics analyses of the ribosomal complexes isolated by affinity pull-down using tagged S17 and L3 proteins have revealed more than 20 PPR proteins; these proteins were termed kinetoplast r*i*bosomal PPR-containing proteins (KRIPPs) (28). At least six of these KRIPPs had also been found in the unusual 45 S SSU* complex isolated from mitochondria of a related trypanosomatid, *Leishmania tarentolae* (34). As shown by electron microscopy, this complex represented a heterodimer of the ribosomal SSU with a protein mass of approximately equal size. The analysis of ribosomal complexes in mitochondrial lysates of *L. tarentolae* also revealed a relatively large amount of individual ribosomal LSUs sedimenting at 40 S and a much smaller amount of 25–30 S ribosomal SSUs (35). The 50 S ribosomes (monosomes) formed only a minority fraction among these complexes. The monosomes, isolated by sedimentation in sucrose gradients and analyzed with cryoelectron microscopy, were shown to contain the conventional SSU (not the entire SSU*) and the LSU (36). The non-SSU moiety of the SSU* complexes, therefore, is not part of the monosome structure. All of the conserved ribosomal proteins were allocated to specific protein masses in the SSU and LSU, leaving a considerable volume to be occupied by trypanosomatid-specific proteins, including KRIPPs. Detailed protein composition and protein localization within the 50 S monosome still need to be determined.

The composition of the *L. tarentolae* 45 S SSU* complexes was studied after their purification by repeated rounds of gradient sedimentation as well as isolation of dimerized and salt-washed complexes (34). It included at least 40 proteins with a combined molecular mass of 2 MDa (the "core" set). Among these was a set of conserved mitoribosomal proteins, such as S5, S6, S8, S9, S11, S15–S18, and S29, apparently residing within the SSU moiety, as well as proteins that are not commonly found in organellar ribosomes, such as a 200-kDa "coiled-coil" protein or a rhodanese domain protein and others, but conserved LSU proteins were missing. In *T. brucei*, all of the core protein orthologs, plus additional proteins, are found in the S17 affinity pull-down, suggesting that an equivalent complex exists in this organism as well (28, 37). The partitioning of the proteins between the two lobes of the SSU* complex remains to be investigated; however, this can be tentatively inferred from the proteomics data. Thus, a score of proteins from the core list, including KRIPPs, are found exclusively in the S17 affinity pull-down (which contains the free SSU as well as the SSU bound in the monosomes and in the SSU*), as opposed to the L3 affinity pull-down (which contains free LSU and monosome-bound LSU). Due to their inferred absence in the monosomes, these proteins represent likely components of the non-SSU lobe of SSU* complexes. Other KRIPPS can be tentatively attributed to the SSU or LSU complexes (28, 38).

The 45 S SSU* complex is, to the best of our knowledge, unique to trypanosomatids. As such, it may represent an adaptation of the mitochondrial translation apparatus to RNA editing. We had hypothesized earlier that this complex may prevent the SSU from initiating translation of immature templates or may be directly involved in the edited template recognition, probably via interaction of its KRIPPs with mRNA, or play both roles (24, 34). In light of recent findings (28), it is plausible that this complex is involved in recognition of the long-tailed edited form of mRNA. In this work, we seek to verify the functional importance of the 45 S complexes and gain insight into their specific roles. We chose *T. brucei* because this system allows study of the effects of down-regulation of this complex using an RNAi-based reverse genetics approach (39). The specific silencing of the 45 S complex is achievable by targeting its components that are localized in its non-SSU lobe and, therefore, are not shared with mitochondrial ribosomes. Several of these

components, such as a rhodanese-like protein (Rhod), a protein with the highly divergent PPR motif (PPR29), and a large coiled-coil domain protein (200 kDa), were tentatively identified by a recent proteomics analysis (28). We found that down-regulation of these components had a pronounced effect on the stability of the 45 S SSU* complex. The *in vivo* synthesis of the mitochondrial polypeptides COI and Cyb was severely affected, as was stability of the translatable (long-tailed) form of the respective mRNAs and their association with the mitoribosomes. On the contrary, stability of the long-tailed ribosomal protein S12 (RPS12) mRNA was not significantly affected, nor was its association with the ribosomes. The amount of the complex was drastically reduced in bloodstream trypanosomes, which do not synthesize COI and Cyb. We conclude that the 45 S SSU* complex plays an important role in translation of some (*e.g.* COI and Cyb) but not all (*e.g.* RPS12) mRNA.

EXPERIMENTAL PROCEDURES

Cell Cultivation, Transfection, and RNAi—Procyclic cells of *T. brucei*, strain 29-13, were cultivated in SDM79 medium (40) at 27 °C supplemented with 10% tetracycline-free fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA), 15 μg/ml G418, and 50 μg/ml hygromycin (39). Bloodstream trypanosomes, strain Lister 427, were cultivated in HMI9 medium (41) at 37 °C, 5% CO_2, with 15% FBS and 2.5 μg/ml G418 (39). The mRNA target regions for RNAi were selected using TrypanoFAN: *RNAit* Web-based service. The following oligonucleotides were used for amplification of the RNAi target regions: M336 (5′-CTCGAGTACCCCAAGTTTAACCAGCG) and M337 (5′-GGATCCGGGTTGCAAGTTCATCCAGT) for S17 mRNA; M338 (5′-CTCGAGCTCCACCGTTTGGTGAGTTT) and M339 (5′-GGATCCAGCCACACATAACCTCCGTC) for L3 mRNA; M453 (5′-AGGATCCCTACAAGGGAAGTGTCCCCA) and M454 (5′-ACTCGAGGAACTCGGGTTCCACACACT) for PPR29 mRNA; M455 (5′-AGGATCCGCGAGTGGTTTAATTGGCAT) and M456 (5′-ACTCGAGTCCTGCATCTCGTACTGACG) for Rhod mRNA; and M457 (5′-AGGATCCCGACACAACACCTCATCCAC) and M458 (5′-ACTCGAGCGGGGAATATAATGCCAATG) for 200-kDa mRNA. DNA manipulations (PCR, purification of plasmids, cloning) were performed using standard procedures. Procyclic *T. brucei* cells were stably transfected with the inducible RNAi constructs based on the p2T7-177 vector (42). Details of the transfection procedure and selection of phleomycin-resistant clones are described elsewhere (43). Effects of the RNAi were investigated by inducing the down-regulation process with 1 μg/ml tetracycline in a logarithmically growing culture at an initial cell density of 5×10^6 cells/ml followed by daily dilutions to the same density over the period of 5–10 days.

Analysis of Mitochondrial Ribosomal Complexes—Procyclic (10^8 cells) or bloodstream trypanosomes (5×10^7 cells) were recovered by brief centrifugation and washed twice with 400 μl of SoTE (20 mM Tris-HCl, pH 7.5, 600 mM sorbitol, 2 mM EDTA). The pelleted cells were lysed in 400 μl of DM buffer (1% dodecyl maltoside, 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 3 mM DTT, 0.1 mM EDTA) supplemented with 400 units of RNasin® Plus RNase inhibitor (Promega, Madison, WI). After 15 min on ice, the lysate was cleared by centrifugation at 17,000 $\times$ *g* for 15 min in a benchtop centrifuge. The 7–30% sucrose gradients made with SGB buffer (50 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 3 mM DTT, 0.1 mM EDTA, 0.05% dodecyl maltoside) were formed using a BioComp gradient station (model 153). Gradients were centrifuged in an SW41 rotor at 17,000 rpm (average relative centrifugal force = 35,670 $\times$ *g*) for 16 h and fractionated into 34 fractions (most experiments) or 17 fractions (Fig. 6). RNA was extracted from gradient fractions using the phenol-chloroform method described earlier (35). RNA from equal size aliquots (25–50% of the gradient fraction material) was transferred onto BrightStar®-Plus hybridization membrane (Ambion) using a Hoefer SlotBlot manifold and immobilized by UV cross-linking. Hybridization probes were prepared by 5′-labeling of the oligonucleotides A304 (5′-TGAACAATCAATCATGGTAATAAGTAGACGATG, specific for 12 S rRNA) and A504 (5′-ACGGCTGGCATCCATTTC, specific for 9 S rRNA) using [γ-^{32}P]ATP (6000 Ci/mmol; PerkinElmer Life Sciences) and T4 polynucleotide kinase. Prehybridizations were performed at 42 °C in 6× SSPE, 5× Denhardt's solution, 0.5% SDS, and 0.2 mg/ml sheared denatured salmon sperm DNA (44), and hybridizations were performed under similar conditions except that SDS was at 0.1%, and yeast tRNA at 20 μg/ml was used instead of salmon DNA. The filters were extensively washed in 2× SSPE, 0.5% SDS at 42 °C and quantified by a Typhoon PhosphorImager. The signals produced by the 9 S and 12 S probes were normalized by hybridization to the tandem 12 S-9 S rRNA immobilized on each hybridization membrane. This transcript was generated *in vitro* by a run-off T7 polymerase transcription of the maxicircle DNA fragment containing the *T. brucei* 12 S and 9 S genes cloned in the pCR® 2.1-TOPO vector (Invitrogen).

Purification of Salt-washed 45 S SSU Complexes*—Kinetoplast-mitochondrial fraction was isolated from *T. brucei* 29-13 cells grown in an 8-liter culture to a density of $20–50 \times 10^6$ cells/ml employing the previously described procedure, which involves hypotonic lysis and purification of the organelles by flotation in Renografin gradients (45). Purified mitochondria were lysed with dodecyl maltoside (DM buffer), and a fraction enriched with the 45 S SSU* complexes was obtained by centrifugation in a 7–30% sucrose gradient as described previously (34). The RNP complexes were recovered from gradient fraction by pelleting at 56,000 $\times$ *g* for 20 h, followed by resuspension in the SGB buffer, which contained 500 mM KCl to destabilize the co-purifying LSU complexes. This was followed by fractionation in another 7–30% sucrose gradient, pelleting, and resuspension in the high salt buffer. This cycle was repeated one more time. The material contained in the final pellet was digested with trypsin and analyzed with liquid chromatography tandem mass spectrometry (LC MS/MS) as described below.

Proteomics Analysis—A Waters system (Waters, Milford, MA) of two-dimensional nano-Acquity ultraperformance liquid chromatography and a Q-TOF Premier tandem mass spectrometer was used for MudPIT (multidimension protein identification technology) analysis to determine protein components of the 45 S SSU* complex. A combination of high pH reverse phase and conventional low pH reverse phase chromatography served as a MudPIT method, following the manufac-

turer's recommendation (Waters, Milford, MA). The solvents for the first dimension LC of high pH reverse phase were 20 mM ammonium formate, pH 10.0, (solvent A) and 100% acetonitrile (solvent B). The online fractionation was carried out with a trap column and percentage cut of solvent B. The peptide sample was loaded onto an XBridge BEH130 C18 trap column (8-μm particle, 300-μm inner diameter, 5 cm long, part no. 186003682, Waters, Milford, MA). The partial loop sample loading method was configured with a 10-μl sample loop in the autosampler for maximal loading with zero sample loss. The fractionation and desalting were carried out using a two-dimensional dilution method configured within the two-dimensional nano-Acquity and controlled with MassLynx 4.1 (Waters, Milford, MA). There were five fractions and one flush based on percentage of solvent B as follows: fraction 1, 13%; fraction 2, 18%; fraction 3, 21.5%; fraction 4, 27%; fraction 5, 50%; fraction 6 (flushing), 70%. Mass spectrometry data were acquired for all of the six fractions. For the second dimension LC, a BEH130 C18 column (1.7-μm particle, 75-μm inner diameter, 20 cm long, part no. 186003544, Waters) was used for peptide separation. A Symmetry C18 (5-μm particle, 180-μm inner diameter, 20 mm long, part no. 186003514, Waters) served as a trap/guard column for desalting and preconcentrating the peptides for each MudPIT fraction. The solvent components for peptide separation were as follows. Mobile phase A was 0.2% formic acid in water, and mobile phase B was 0.2% formic acid in acetonitrile. The separation gradient was as follows: 0–1 min, 3% B; at 30 min, 50% B; at 31 min, 85% B; 31–35 min, 85% B; at 36 min, 3% B; 36–60 min, 3% B. The nanoflow rate was set at 0.3 μl/min without flow splitting. Nano-LC/MS/MS was carried out for each fraction of the MudPIT experiment with a data-dependent acquisition survey method. All acquired raw MS/MS spectra were further processed using ProteinLynx software (Waters, Milford, MA) to generate pkl files, which were uploaded onto a Mascot database search engine (version 2.4, Matrixscience) to search against the most recent *T. brucei* TEU927 database (TriTrypDB Kinetoplastid Genomics Resource, release 5.0, June 2013). The parameters for database searching were as follows: trypsin, 1 missed cleavage; peptide tolerance, 100 ppm; MS/MS tolerance, 0.2 Da; 6 variable modifications, including acetyl (N-term), formyl (N-term), pyro-Glu (N-term Q), pyro-Glu (N-term E), oxidation (HW), and oxidation (M). A random decoy matching was also included during the database searching, which was used to estimate the false discovery rate. Only proteins with at least one significant peptide score of 33 ($p < 0.05$) were considered positive identifications, and with this score cut-off, the false discovery rate was 0% based on the random decoy result.

Northern Blot Hybridization and qPCR—Cells ($2.0–2.5 \times 10^8$) were harvested from the non-induced and RNAi-induced cultures at 24-h intervals over a period of 5 days. Total cell RNA was extracted using TRIzol® reagent (Invitrogen). The RNA (5 μg/lane) was fractionated by electrophoresis in 4% polyacrylamide, 8 M urea TBE gels. The gels were electroblotted onto BrightStar®-Plus nylon hybridization membrane, and the RNA was cross-linked to the membrane by UV irradiation (CL-1000 cross-linker, UVP, Upland, CA). The hybridization probes for COI, edited Cyb, and edited RPS12 mRNAs were prepared by 5′-labeling of the respective antisense oligonucleotides followed by the asymmetric PCR amplification of the antisense DNA strand using a double-stranded template (independently produced by RT-PCR) in the presence of the 5′-labeled oligonucleotide and [α-^{32}P]dATP (6000 Ci/mmol, PerkinElmer Life Sciences), as described in detail elsewhere (46). The antisense oligonucleotides used for 5′-labeling were as follows: A302 (5′-ACTAAGCAACCAAATCCTCCAATAAACATTC) for COI; A208 (5′-CCCATATATTCTATATAAACAACCTGACA) for edited Cyb; and A358 (5′-ACACGTCGGTTACCGGAACT) for edited RPS12. The RT-PCR was done with the same antisense oligonucleotides and the following sense oligonucleotides: A301 (5′-TGCCTATAACTATGGGTGGGTTTACAAAC) for COI; A209 (5′-AAATATGTTTCGTTGTAGATTTTTATTATTT) for edited Cyb; and A357 (5′-CGTATGTGATTTTTGTATGGTTGTTG) for edited RPS12.

The prehybridizations and hybridizations were performed at 56 °C in the same buffer as described above. Three low stringency washes were conducted in 2× SSPE, 0.5% SDS at 56 °C for 0.5 h each and were followed by a high stringency wash in 0.5× SSPE, 0.5% SDS at 65 °C for 0.5 h. Hybridization signals were quantified by a PhosphorImager.

Quantitative PCR was performed as described previously (46). The results were normalized using the cytosolic 18 S rRNA. The following oligonucleotides were used for qPCR: M340 (5′-CGGAATGCGTTCCCTAACCGG) and M341 (5′-TAAGGCCAGGGCATACGACGC) for S17 mRNA; M342 (5′-AGCGCATTCGCACAGACTGGT) and M343 (5′-GTTGCCACTCACGCGGGTTTT) for L3 mRNA; M459 (5′-TGCGTGTCATTCGCTGCCG) and M460 (5′-CGACGCACTGTGGCTGAGCA) for PPR29 mRNA; M461 (5′-TCGTACTTGCGTCTGCAGGCA) and M462 (5′-TGATGCCTTGTGCGCCTGTCT) for Rhod mRNA; and M463 (5′-GGCGGCAGGAGGAGGTGGTA) and M464 (5′-TCAACCAAGGCTGCTCCGCG) for 200-kDa mRNA.

Mitochondrial Translation—Cells (10^7) were harvested from the cultures at 24-h intervals. Cytosolic translation was inhibited with cycloheximide, and the products of mitochondrial translation were labeled *in vivo* using [^{35}S] EasyTag Express Protein Labeling Mix (PerkinElmer Life Sciences) as described previously (28). Labeled products were resolved in two-dimensional denaturing gels and revealed by fluorography (47, 48).

RESULTS

45 S SSU Represents an Abundant Class of RNP Complexes*—The proteomics analysis of the S17-TAP affinity pull-down showed that this protein is part of a large ribosomal RNP complex that includes all of the components previously found in the 45 S SSU* complex from *L. tarentolae* (28, 34, 37). In order to verify if the *T. brucei* complex is a direct equivalent of the latter, we have analyzed the mitochondrial ribosomal complexes obtained by lysis of cells with a mild detergent, dodecyl maltoside, followed by fractionation in a sucrose gradient. The ribosomes were detected by slot-blot hybridization of RNA extracted from each fraction with the labeled oligonucleotides specifically hybridizing with the mitochondrial 9 S SSU rRNA or 12 S LSU rRNA. The hybridization signals were normalized by hybridization of each probe with the *in vitro* 9 S-12 S tandem

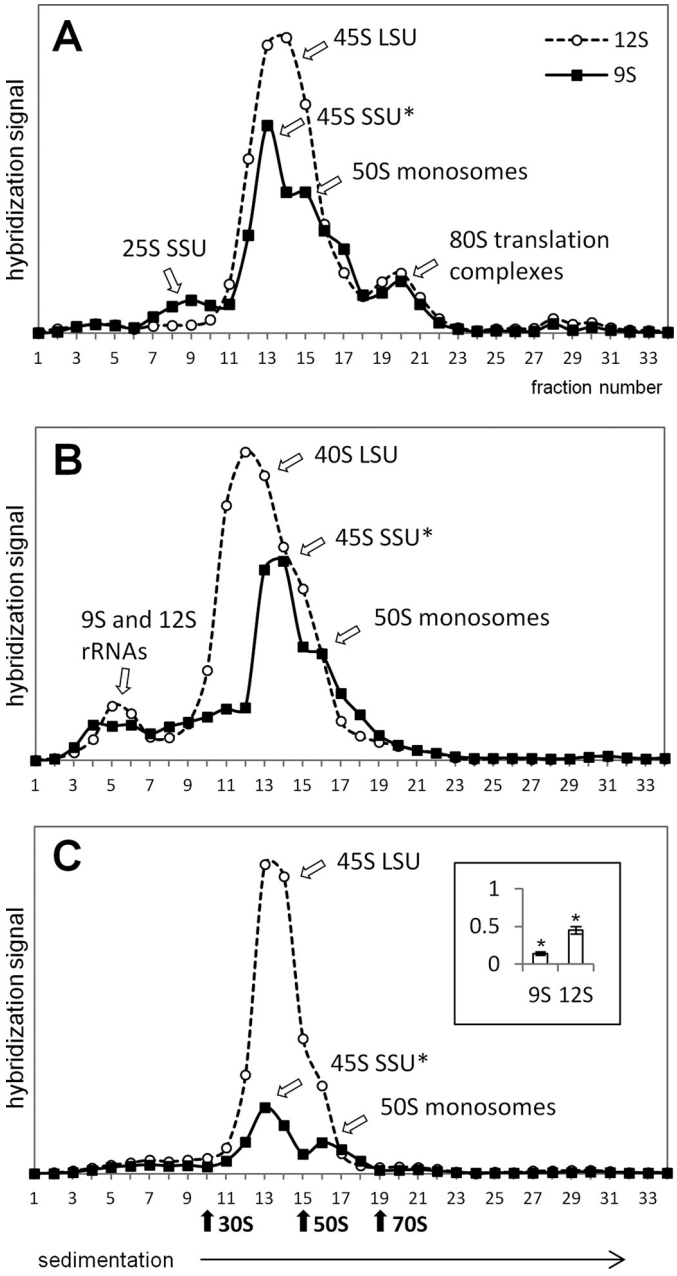


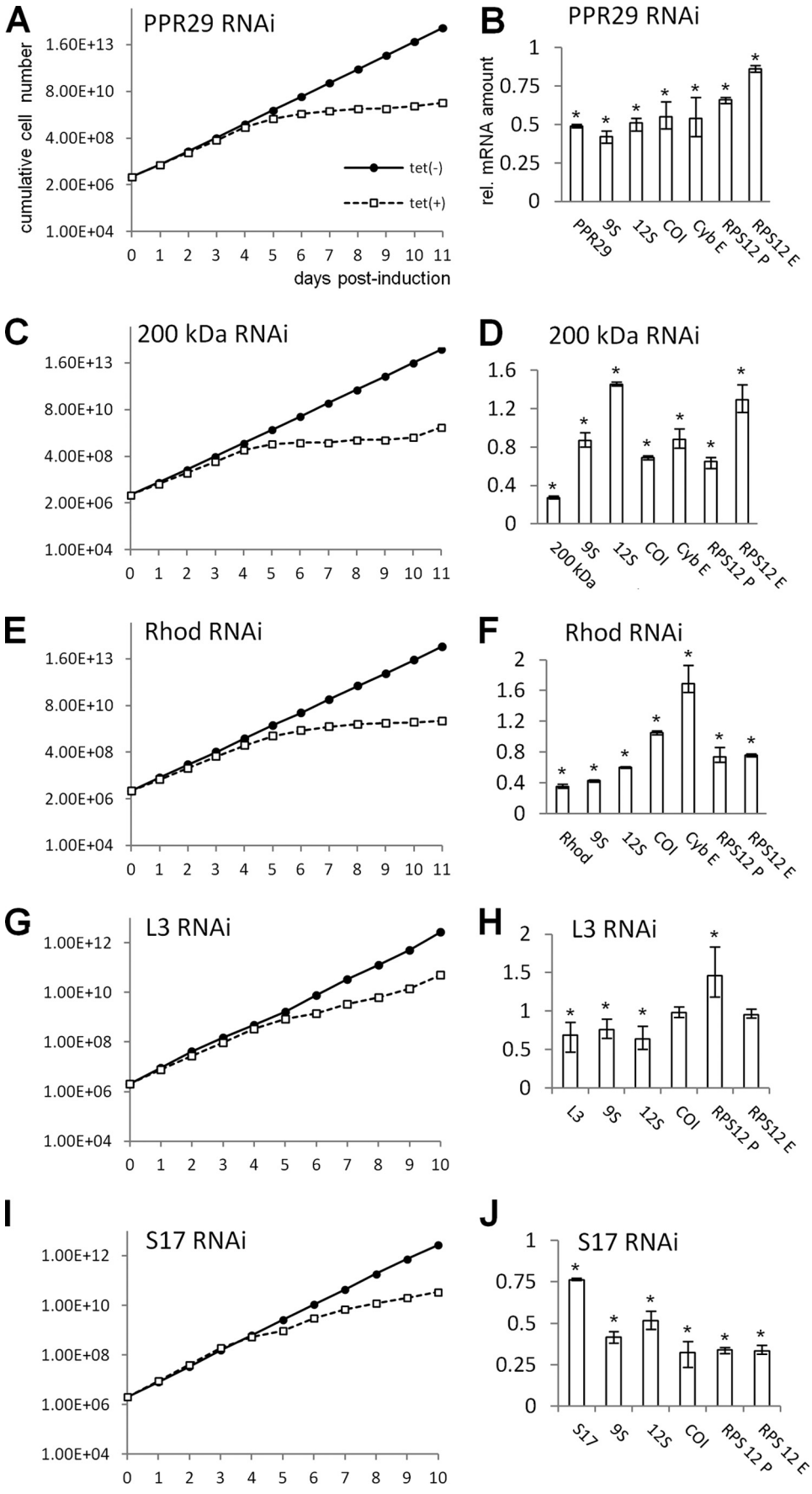
FIGURE 1. **Sedimentation profiles of the 9 and 12 S rRNAs revealing ribosomal ribonucleoprotein complexes contained in total cell lysates of procyclic *T. brucei* 29-13 cells obtained with 1% dodecyl maltoside in 100 mM KCl (*A*) and 500 mM KCl (*B*), and bloodstream *T. brucei* Lister 427 cells lysed with 1% dodecyl maltoside in 100 mM KCl (*C*).** The lysates were fractionated in a 7–30% sucrose gradient (SW41 rotor, 17,000 rpm, 16 h) followed by slot-blot hybridization of the RNA extracted from gradient fractions with ^{32}P-labeled oligonucleotides antisense to the 9 and 12 S rRNAs. The *inset* in *C* shows the relative amount of the 9 and 12 S rRNA in bloodstream trypanosomes in comparison with procyclics as determined by qPCR with respect to the 18 S cytosolic rRNA. The *asterisks* indicate that the observed changes in rRNA levels are statistically significant (Mann-Whitney *U* test, $p < 0.05$). Subunits and ribosomes from *Escherichia coli* were used as sedimentation standards. *Error bars*, S.E.

transcript on the same blot. The sedimentation profile of the mitoribosomal complexes from procyclic *T. brucei* 29-13 cells is shown in Fig. 1*A*. It is rather similar to the profile previously observed in *L. tarentolae* (34, 35). The 9 S RNA shows the main peak at 45 S (termed SSU*, similar to the equivalent complex from *L. tarentolae*, for the reason outlined below) with the 50 S shoulder. A small amount of the 9 S rRNA is also observed in the 25 S region. The bulk of 12 S is observed as an abundant peak also sedimenting at 45–50 S (termed the 45 S LSU peak). The ribosomal RNAs are also seen co-sedimenting as a relatively small peak of 80–85 S.

This sedimentation pattern, and in particular the presence of the two most abundant (and nearly co-sedimenting) peaks formed by the 9 and 12 S rRNA, can be interpreted in two ways: (i) the 45 S peaks may represent individual ribosomal RNP complexes (SSU* and LSU) of nearly the same size, as in *L. tarentolae*; (ii) the cosedimentation of the two ribosomal RNAs in the 45 S region may be due to the existence of 45 S ribosomes (monosome) composed of two smaller size subunits. In order to distinguish between these possibilities, we performed RNAi knockdown of L3 and S17 ribosomal proteins, the components of the LSU-containing and SSU-containing complexes, respectively. As expected, a profound effect in the cells' viability was observed in each case, leading to the growth retardation phenotype (Fig. 2, *G* and *I*). It was noticed that RNAi of L3 preferentially affected the 45 S LSU peak by greatly reducing its relative amount (Fig. 3*D*). The relative abundance of the 45 S SSU* peak did not change as much. And vice versa, when the S17 protein was RNAi-silenced, the relative amount of the 45 S SSU* peak was strongly reduced, whereas the LSU peak was not affected to the same extent (Fig. 3*E*). These results show that the respective particles are not physically connected and, therefore, constitute separate subunits. If the opposite scenario were true (the 45 S material representing the ribosome), a depletion of either subunit would destroy an entire ribosome, leading to equal reduction of both peaks, with the remaining subunit accumulating as individual particles. Obviously, this was not the case. The observed growth retardation phenotype first observed at day 3 or 4 postinduction (p.i.) (Fig. 2, *G* and *I*), apparently followed the effect on the ribosomal particles that was seen already at day 2 p.i. in both cases (data not shown).

Additional support for the conclusion that the 45 S material represents a mixture of two different complexes came from the analysis of cells lysed in the presence of 0.5 M KCl (Fig. 1*B*). In other organisms, this treatment would cause mitoribosomes to dissociate into individual subunits. In *T. brucei*, however, this treatment had no effect on the 45 S SSU* peak. The entire 45 S LSU peak slightly shifted to become 40 S, apparently due to the loss of some loosely bound components. If the original 45 S material were a ribosome, the salt-induced shift of the LSU would also have entailed the respective shift of the SSU peak (if the ribosome did not dissociate) or a release of the individual SSU that would form a large peak in the 25 S area (if the ribosome did dissociate). None of these was observed. The data, therefore, confirm that the particles forming the major SSU* and LSU peaks are physically independent.

These results have shown that the two most abundant types of mitoribosomal RNP complexes in *T. brucei* are the 45 S SSU* complex and 40–45 S LSU complex, as in *L. tarentolae*. In the latter species, the monosomes are relatively low in amount, forming a barely noticeable shoulder at 50 S. They are composed of the 25 S SSU and 40 S LSU (36). Hence, the respective 50 S shoulder of the 45 S SSU* and LSU peaks observed in *T. brucei* may also represent individual monosomes, but this


A
PPR29 RNAi
cumulative cell number
1.60E+13
8.00E+10
4.00E+08
2.00E+06
1.00E+04
tet(-)
tet(+)
0 1 2 3 4 5 6 7 8 9 10 11
days post-induction
B
PPR29 RNAi
rel. mRNA amount
1
0.75
0.5
0.25
0
PPR29
9S
12S
COI
Cyb E
RPS12 P
RPS12 E
C
200 kDa RNAi
1.60E+13
8.00E+10
4.00E+08
2.00E+06
1.00E+04
0 1 2 3 4 5 6 7 8 9 10 11
D
200 kDa RNAi
1.6
1.2
0.8
0.4
0
200 kDa
9S
12S
COI
Cyb E
RPS12 P
RPS12 E
E
Rhod RNAi
1.60E+13
8.00E+10
4.00E+08
2.00E+06
1.00E+04
0 1 2 3 4 5 6 7 8 9 10 11
F
Rhod RNAi
2
1.6
1.2
0.8
0.4
0
Rhod
9S
12S
COI
Cyb E
RPS12 P
RPS12 E
G
L3 RNAi
1.00E+12
1.00E+10
1.00E+08
1.00E+06
1.00E+04
0 1 2 3 4 5 6 7 8 9 10
H
L3 RNAi
2
1.5
1
0.5
0
L3
9S
12S
COI
RPS12 P
RPS12 E
I
S17 RNAi
1.00E+12
1.00E+10
1.00E+08
1.00E+06
1.00E+04
0 1 2 3 4 5 6 7 8 9 10
J
S17 RNAi
1
0.75
0.5
0.25
0
S17
9S
12S
COI
RPS12 P
RPS12 E

has not been verified experimentally. Similarly, the relatively low 25 S peak (Fig. 1*A*) matches with the expected position of individual SSUs in the gradient. As described below, the 80 S peak that contains an approximately equal amount of both rRNAs coincides with (and may represent) the mRNA-ribosome complexes.

*RNAi Targets for Selective Knockdown of 45 S SSU**—Recent proteomics analysis has shown that more than 200 proteins are found in association with the ribosomal protein S17 (28). This abundance reflects the complexity of the translation apparatus as well as the multitude of interactions of the translation and RNA-processing systems. Ablation of many of these components may not, therefore, affect the integrity of the 45 S SSU* particle directly, as opposed to ablation of its structural components. In order to investigate which of these components represent the most tightly bound (integral) components of this complex, we purified the 45 S SSU* complex by three rounds of gradient sedimentation. The co-sedimenting 45 S LSU complexes and loosely bound components were destabilized by resuspension of the pelleted complexes in the 0.5 M KCl buffer solution after each pelleting. This treatment allowed for a significant reduction of the LSU contamination in the final 45 S SSU* preparation (data not shown). Our LC MS/MS analysis of this 45 S SSU* sample revealed, among other components, nearly 30 significant hits representing orthologs of the *L. tarentolae* 45 S SSU* core complex (34), including ribosomal proteins S5, S8, S9, S17, and S18; several KRIPPs (PPR1, -8, -14, -22, and -29); and several atypical ribosomal components (see below) (Table 1). The reduced content of the salt-washed *T. brucei* complexes compared with the *L. tarentolae* complex was probably due to the longer and more extensive salt treatment employed herein. It is reasonable to assume that the protein components that survived this treatment must be firmly embedded in the complex, and their ablation should, therefore, have a strong impact on its stability.

From the above protein list (Table 1), three representative proteins among the atypical components were chosen as the targets for the RNAi assay: the 200-kDa protein (Tb927.8.5200) and Rhod (Tb927.6.4930) as well as PPR29 (Tb927.5.1790), a protein with a rather diverged PPR domain. The selected targets are anticipated to occur within the non-SSU moiety of the SSU complexes, and their ablation would not directly impact the structure of the mitoribosomes. However, due to the remaining uncertainty concerning localization of these proteins inside the SSU* complex and a potential indirect impact of their ablation on the ribosome stability, in comparison, we also investigated the effects of a direct ribosome destabilization by RNAi of the LSU protein L3 as well as the effects of S17 RNAi, which should directly impact both complexes. Above, we have shown that these targets efficiently and selectively eliminate LSU and SSU*, respectively.

Ablation of 45 S SSU by RNAi of PPR29, 200-kDa, and Rhod Proteins*—Upon induction of RNAi, each culture displayed a growth retardation that was first observed at day 4 p.i. and resulted in growth arrest at day 5 p.i. (Fig. 2, *A*, *C*, and *E*). The induced cultures began to recover after day 10 p.i. The analysis of the target mRNA levels by qPCR at days 3 and 4 p.i. showed a decline in the amount of each target (Fig. 2, *B*, *D*, and *F*).

The effects on the 45 S SSU* complexes were investigated daily during the first 5 days p.i. (Fig. 3, *A*, *B*, and *C*) (data not shown). For each of the three RNAi targets, there was a relative decline in the abundance of the 45 S SSU* complex that was first observed at day 2 p.i., and the maximal effect was reached by day 3 p.i. A slight recovery might be observed at later times. No significant effect on the LSU complexes was observed. The decline of the 45 S SSU* complexes was not accompanied by accumulation of the free 9 S rRNA in the gradients, indicating that the unassembled RNA was degraded. This conclusion was corroborated by the qPCR analyses, which showed a decline of the total 9 S rRNA level (Fig. 2, *B*, *D*, and *F*). A similar effect on the relative abundance of the 12 S rRNA was less pronounced (PPR29 and Rhod) or represented an increase (200 kDa).

Inhibition of COI and Cyb Synthesis by Down-regulation of 45 S SSU Complex*—Of the 18 maxicircle-encoded proteins, only two (COI and Cyb) are currently amenable for the direct analysis. These polypeptides can be efficiently separated by a two-dimensional (9 *versus* 14%) Tris-glycine-SDS-PAGE, and their synthesis can be investigated *in vivo* after inhibition of the cytosolic translation with cycloheximide (47, 48). A similarly efficient approach still needs to be designed for analysis of the remaining proteins, which are often even more hydrophobic and prone to loss due to aggregation. We showed previously that the mitochondrial protein synthesis in *T. brucei* (48) was resistant to most inhibitors, including tetracycline, the drug used to induce RNAi. In order to investigate if ablation of the 45 S SSU* complexes directly affects the COI and Cyb synthesis, mitochondrial translation was investigated in *T. brucei* after silencing of the expression of the 200-kDa, Rhod, and PPR29 proteins by RNAi. Each day after the induction, an aliquot (0.5×10^7 cells) was withdrawn from each induced culture and labeled with [^{35}S]methionine and [^{35}S]cysteine. The RNAi experiment was conducted for 5 days, after which time the labeled products from the RNAi-induced and non-induced cells were analyzed in parallel. The COI and Cyb products are visible as two prominently labeled spots, with nearly equal intensity, positioned off the main diagonal of the gel (Fig. 4, *A–C*; *left set* of *panels*). The faint spots, also present off the diagonal (*asterisks*), may represent aggregated forms of COI and Cyb or some of the remaining mitochondrial polypeptides; this has not been investigated. The effect of the RNAi on the COI synthesis was first visible at day 2 p.i.; the impact was well

FIGURE 2. **Growth dynamics (*left*) and relative amounts of the mRNAs and rRNAs (*right*) of the *T. brucei* transfectant clonal cell lines harboring the integrated RNAi-inducible expression constructs: PPR29 (*A* and *B*), 200 kDa (*C* and *D*), Rhod (*E* and *F*), L3 (*G* and *H*), and S17 (*I* and *J*).** RNAi was induced with tetracycline (1 μg/ml) in a logarithmically growing culture at a density of 5×10^6 cells/ml at day 0 (*tet*(+)). Cells were counted daily and diluted to original density. The respective non-induced cultures were used as control (*tet*(−)). The plots show the calculated cumulative cell numbers. Relative amounts of the mRNAs targeted by RNAi (PPR29, 200 kDa, Rhod, L3, and S17); mitochondrial rRNAs (9 and 12 S); and mitochondrial COI, edited Cyb (*Cyb E*), pre-edited RPS12 (*RPS12 P*), and edited RPS12 (*RPS12 E*) mRNAs were determined by qPCR in the RNAi-induced cell lines (day 3) in comparison with the uninduced cells, except that the data for PPR29 mRNA represent day 4. The *asterisks* indicate statistically significant changes in RNA levels (Mann-Whitney *U* test, $p < 0.05$). *Error bars*, S.E.

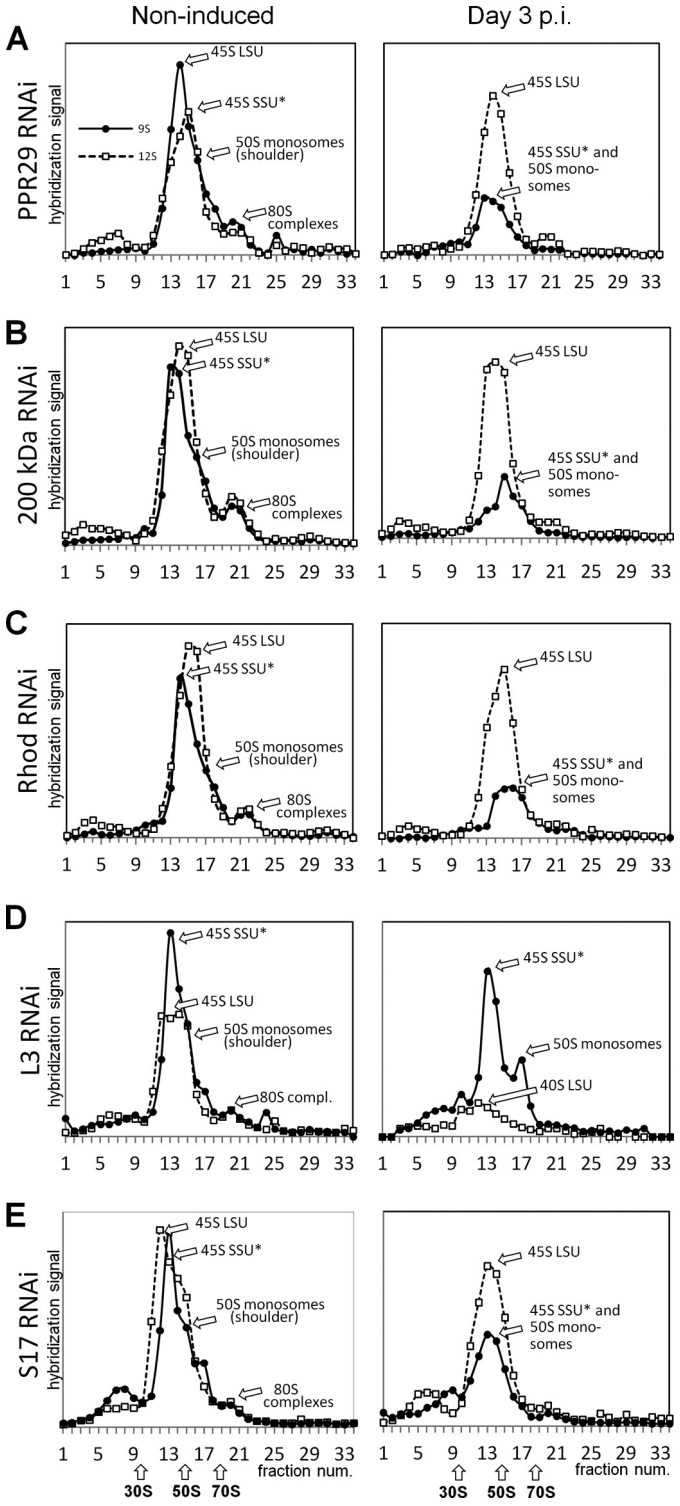


FIGURE 3. **Effects of the RNAi-mediated down-regulation of the PPR29 (*A*), 200-kDa (*B*), Rhod (*C*), L3 (*D*), and S17 (*E*) mRNAs on mitochondrial ribosomal complexes in *T. brucei*.** Cells from the non-induced cultures (*left column* of *panels*) and the cultures at day 3 postinduction (*right column* of *panels*) were lysed with dodecyl maltoside, and the lysates were fractionated in 7–30% sucrose gradients (SW41, 17,000 rpm, 16 h). RNA isolated from the gradient fractions was hybridized with the 9 S rRNA and 12 S rRNA-specific oligonucleotide probes. The plot shows the normalized hybridization signal in arbitrary units.

pronounced at day 3 p.i. (Fig. 4, *A–C*, *right set* of *panels*); this labeled product became undetectable at the end of the experiment at day 5 p.i. in each induced cell culture (data not shown).

TABLE 1

Components of salt-washed 45S SSU* complexes of *T. brucei*

Proteins with scores below the threshold (33) are not shown. Proteins highlighted by boldface type represent the RNAi targets used for down-regulation of the *T. brucei* 45S SSU* complexes in this study.

T. brucei GeneDB ID	Counterpart in the *L. major* complex	No. of peptides detected	Mascot score	Structural feature, gene ID
*Significant hits (**score >33**, $p < 0.05$), these proteins were also found in the Leishmania complex (Maslov et al. 2007)*				
Tb927.2.4400	LmjF27.1895	11	310	armadillo
Tb927.8.5200	**LmjF16.1230**	**11**	**228**	**coiled coil**
Tb927.10.6300	LmjF36.1860	7	222	mitoriboprotein S5
Tb927.5.4040	LmjF33.0010	7	220	coiled coil
Tb927.7.3050	LmjF22.0990	7	198	
Tb927.3.5240	LmjF29.0430	3	176	KRIPP8 (PPR)
Tb927.11.1250	LmjF27.0630	4	151	
Tb927.10.6850	LmjF36.2310	5	140	mitoriboprotein S18
Tb927.8.5280	LmjF26.2270	3	118	
Tb927.6.2080	LmjF30.0650	2	114	KRIPP22 (PPR)
Tb927.9.6510	LmjF15.0780	4	94	
Tb927.6.2180	LmjF30.0740	2	93	
Tb927.6.4560	LmjF30.3220	2	86	
Tb927.9.11280	LmjF35.3850	2	81	mitoriboprotein S17
Tb927.8.3110	LmjF23.1190	1	76	mitoriboprotein S9
Tb927.11.2530	LmjF33.2510	2	75	
Tb927.11.5500	LmjF24.0830	1	73	KRIPP1 (PPR)
Tb927.6.4930	**LmjF30.3530**	**1**	**71**	**Rhodanese**
Tb927.9.11120	LmjF35.3940	1	70	
Tb927.11.13890	LmjF32.0650	1	69	RNA ligase
Tb927.10.10400	LmjF05.0340	1	67	mitoriboprotein S11
Tb927.11.11470	LmjF28.2180	2	64	KRIPP14 (PPR)
Tb927.10.13300	LmjF18.0800	2	57	mitoriboprotein S8
Tb927.5.1790	**LmjF15.0410**	**2**	**54**	**PPR29**
Tb927.10.3250	LmjF03.0220	2	46	
Tb927.7.3240	LmjF22.1220	1	46	
Tb927.10.3580	LmjF03.0630	2	44	
Tb927.5.1510	LmjF15.0080	1	42	
LSU components (likely contaminants):				
Tb927.10.600		1	68	mitoriboprotein L29
Tb927.7.4710		1	66	mitoriboprotein L46
Tb927.11.6000		1	57	mitoriboprotein L4
Tb927.3.5610		1	43	mitoriboprotein L3
Tb927.11.11630		1	42	putative LSU component
Abundant proteins and housekeeping enzymes(likely contaminants)				
Tb927.11.10150		3	100	crotonase
Tb927.4.3690		2	82	SOD
Tb927.1.4100		2	61	cyt *c* oxidase subunit IV
Tb927.5.2080		1	60	IMP dehydrogenase
Tb927.9.13770		1	59	proteasome Rpn13
Tb927.1.2330		1	54	beta tubulin
Tb927.1.2340		1	48	alpha tubulin

The synthesis of Cyb was more refractory to the RNAi; the product was not significantly affected until day 3 p.i. (Fig. 4, *A–C*) or even day 4 p.i. in the case of Rhod RNAi (data not shown), and although the synthesis of this polypeptide was noticeably reduced thereafter, a small amount of the labeled material was detectable even by the end of the experiment at day 5 p.i. (data not shown).

The effects of down-regulating the 45 S SSU* complex on protein synthesis were compared with those caused by the ablation of mitoribosomes with RNAi of L3 (Fig. 4*D*) and S17 (Fig. 4*E*). Down-regulation of L3 would directly affect the LSU and therefore is anticipated to impair the steps beyond formation of the translation initiation complex, namely the formation of monosomes and elongation. In this case, there was an impact on both the COI and Cyb synthesis that was clearly noticeable

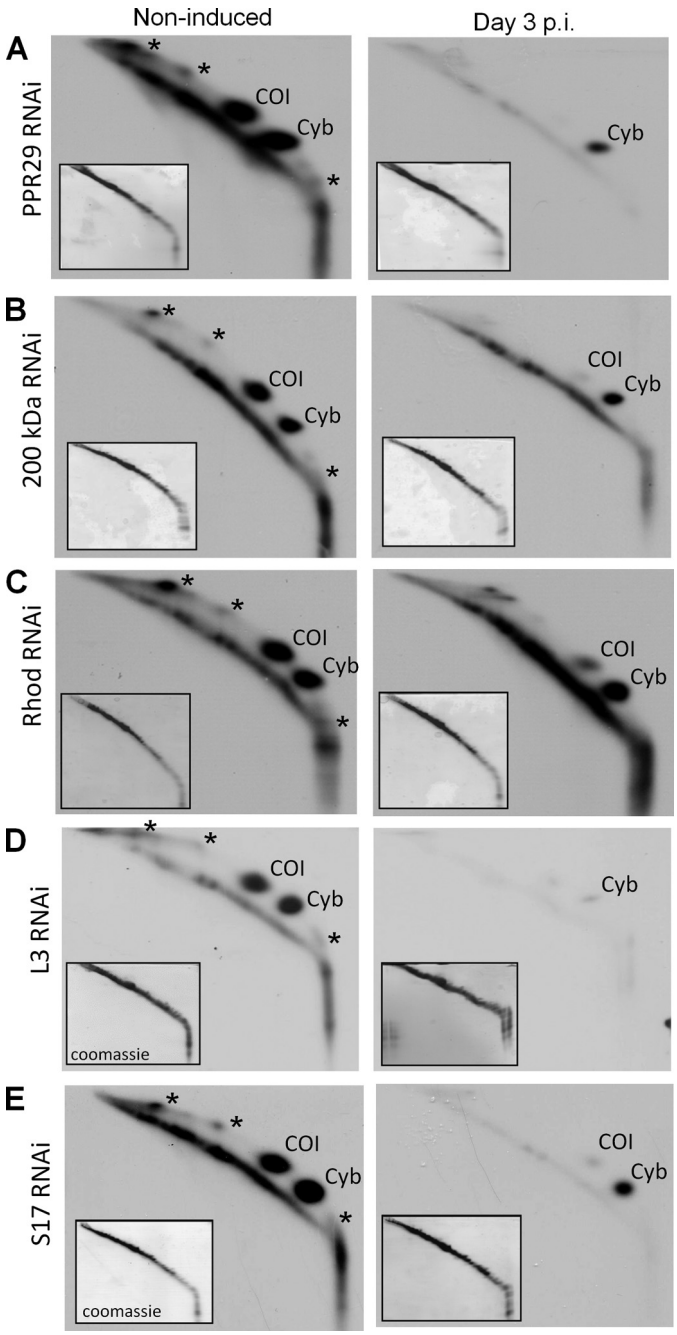


FIGURE 4. **Effects of the RNAi-mediated down-regulation of the PPR29 (*A*), 200-kDa (*B*), Rhod (*C*), L3 (*D*), and S17 (*E*) mRNAs on mitochondrial protein synthesis in *T. brucei*.** Cells in aliquots of the non-induced cultures (*left column* of *panels*) and the cultures at day 3 postinduction (*right column* of *panels*) were labeled with ^{35}S-amino acids in the presence of 100 μg/ml cycloheximide. Proteins were separated by electrophoresis in two-dimensional Tris-glycine-SDS-polyacrylamide (9 *versus* 14%) gels. Labeled proteins were detected by fluorography, and positions of the mitochondrial polypeptides COI and Cyb are indicated. *Asterisks* denote additional mitochondrial translation products that remain unidentified. *Insets* represent the respective Coomassie-stained gels.

at day 2 p.i. (data not shown), and a strong and indiscriminate inhibition of mitochondrial translation was observed as early as day 3 p.i. (Fig. 4*D*). That was clearly different from silencing of the three 45 S SSU* components (Fig. 4, *A*–*C*).

The effect of S17 RNAi (Fig. 4*E*) was similar to those described above for the 45 S SSU* complex but not for the L3 RNAi. As a component of the SSU, S17 is shared by the mitoribosomes with the 45 S SSU* complexes. Down-regulation of S17 would, therefore, impair the functions of both complexes but not necessarily to the same extent. Apparently, its effect on the function of mitoribosomes, which is expected to represent the indiscriminate inhibition of translation, similar to the effect of L3 RNAi, was less pronounced as compared with its effect on the 45 S complexes. These results, and in particular the comparison of depletion of L3 with the effect caused by the RNAi of the PPR29, 200-kDa, and Rhod proteins, indicate that the latter should be attributed to impairment of a special function of the 45 S SSU* complexes and argue against the possibility of these down-regulations causing a disruption of the mitoribosomes.

Loss of Long-tailed COI and Edited Cyb mRNAs—In order to gain further insight into the function of the 45 S SSU complexes, we asked if the inhibition of COI and Cyb synthesis is associated with the decline in the mRNA levels. The qPCR analysis has shown that there was a 30% reduction of COI mRNA in the PPR29 and 200-kDa RNAi knockdown cell lines and a smaller change in the Rhod RNAi cell line at day 3 p.i. (Fig. 2, *B*, *D*, and *F*). Similarly, there was some (~30%) decline in the edited Cyb mRNA in the 1790 RNAi cells at day 3 p.i., whereas there was no substantial change in cells depleted for the 200 kDa, and an increase was observed in the Rhod RNAi cells (Fig. 2, *B*, *D*, and *F*). Thus, the changes in mRNA levels, albeit statistically significant according to the Mann-Whitney *U* test, were not of the direction or the scale that would be sufficient to explain the observed inhibition of the COI and Cyb protein synthesis.

In the kinetoplast-mitochondrial system, translatability of an mRNA molecule is conferred by a long poly(A/U) tail (28). Therefore, we investigated if the silencing effect on translation is triggered by the reduction of a relative amount of the long-tailed (LT) form of edited Cyb mRNA and COI mRNA as compared with their short-tailed (ST) forms. To this end, the RNA isolated from non-induced and RNAi-induced cells was investigated by Northern blot hybridization. The results, presented in Figs. 5 and 6, show that upon induction of the RNAi against the PPR29, Rhod, and 200-kDa targets, the edited Cyb (Fig. 5, *A*–*C*) and COI (Fig. 6, *A*–*C*) mRNAs underwent a steady loss of the LT form, exceeding the 2-fold reduction by the end of the experiment at day 5 p.i.; this was paralleled by a proportional gain of the ST form. The decline in the LT form was observed as early as day 1 p.i.

On the contrary, in cells depleted for L3, there was no significant change of the LT edited Cyb mRNA (Fig. 5*D*), and there was actually an increase in the relative amount of the LT COI mRNA (Fig. 6*D*). Therefore, the observed loss of LT edited Cyb and COI mRNAs is specifically linked to the impaired function of the 45 S complexes and not to the ablation of the mitoribosomes.

With the aim to determine whether the loss of the LT forms was general or occurred only in a subset of mRNAs, we have investigated the ratio of the LT and ST forms for edited RPS12 mRNA. In contrast to the COI and Cyb mRNAs, the reduction for the LT RPS12 mRNA was minimal (PPR29 and Rhod RNAi) or even undetectable (200-kDa protein RNAi) (Fig. 7, *A*–*C*). No

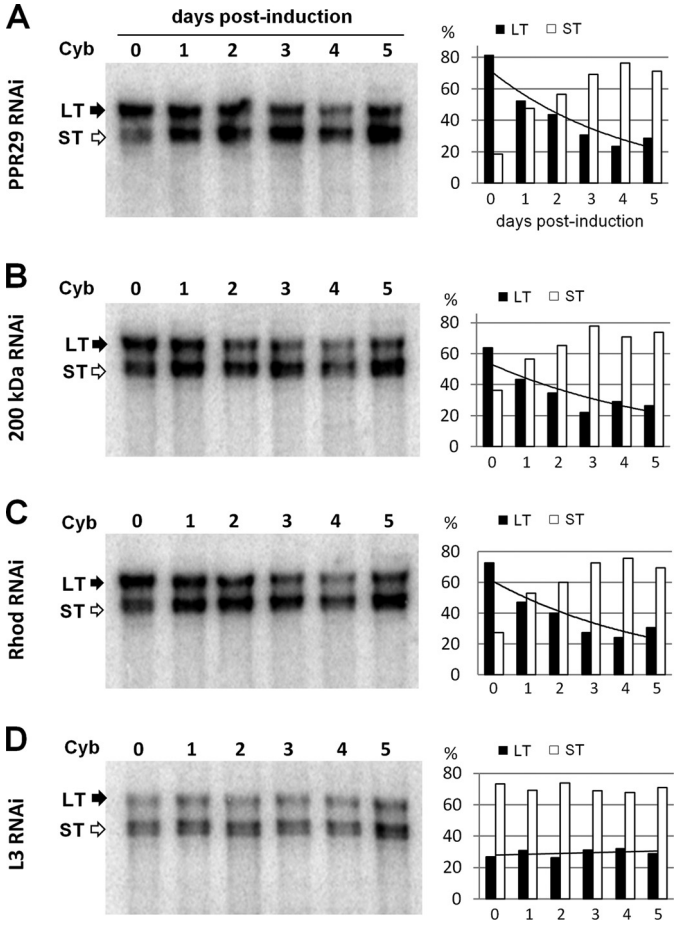


FIGURE 5. **Northern blot hybridization analysis of edited Cyb mRNA in PPR29 (*A*), 200-kDa (*B*), Rhod (*C*), and L3 (*D*) RNAi cells.** Total cell RNA (5 μg) extracted prior to induction of RNAi and during the first 5 days postinduction was fractionated in 4% polyacrylamide-urea gels, electroblotted, and hybridized with respective probes. Positions of the long-tailed (*LT*) and short-tailed (*ST*) forms of the mRNAs are shown in the *left set* of *panels* representing PhosphorImager scans of the hybridization membranes. Percentage ratios of the LT form (*solid bar*) *versus* the ST form (*open bar*) were derived by volume quantification of the respective hybridization bands and are shown to the *right*. Each plot includes a *trend line*.

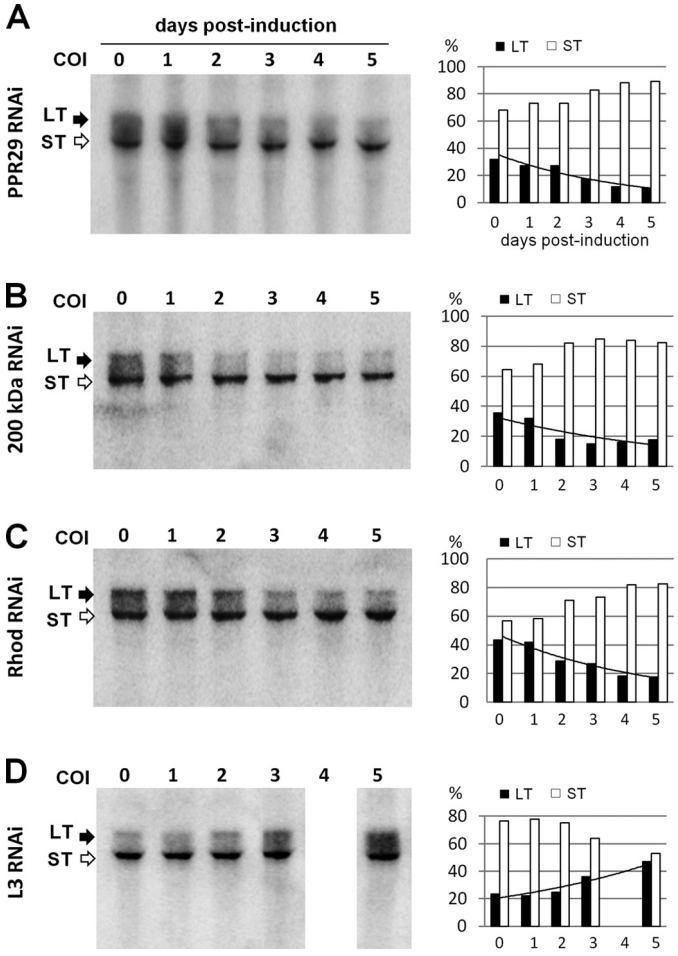


FIGURE 6. **Northern blot hybridization analysis of COI mRNA in PPR29 (*A*), 200-kDa (*B*), Rhod (*C*), and L3 (*D*) RNAi cells.** See the legend to Fig. 5 for experimental details and designations.

effect upon this transcript was imposed by L3 RNAi down-regulation either (Fig. 7*D*).

Effect on Formation of Active Translation Complexes—In order to investigate if the loss of the LT forms in the RNAi-induced cells would affect interactions of the respective mRNAs with mitoribosomes, we investigated the stability of the mRNA-ribosome complexes that had previously been shown to represent translating mitoribosomes (28). Analyzing fractions of a sucrose gradient prepared from the non-induced cells with a probe for the edited RPS12 mRNA, such association can be revealed by the presence of the LT form in the 80 S region of the gradient (Fig. 8*A*, gradient fractions 10 and 11). Northern blot hybridization also shows that the ST form of RPS12 mRNA is mostly found at the top of the gradient (fractions 2–5), apparently as free RNA. These fractions also include the bulk of the LT form, indicating that only a smaller fraction thereof is bound to the mitoribosomes in the 80 S region. These results are consistent with previous findings (28).

Sedimentation analysis of the edited Cyb mRNA revealed a clear segregation of the ST and LT forms (Fig. 8*B*). The ST form of edited Cyb mRNA was found mostly in fractions 3–5, probably as free RNA, whereas its LT form was found almost exclusively in fractions 10–12 with a sedimentation coefficient of 80–90 S. It is noteworthy, however, that the 9 and 12 S rRNA also form a distinct 80–90 S peak, indicating the existence of a particular class of the ribosomal particles (Fig. 1*A*, fractions 19–21). It remains unclear whether these relatively abundant particles actually represent the LT mRNA-ribosome complexes or they if are due to co-sedimenting associations of the 45 S SSU* and 40 S LSU particles. In any case, the LT Cyb mRNA is found almost exclusively in the 80–90 S region, and it is very likely that, as shown previously for the LT RPS12 (28), its presence in this region is due to the association with the mitoribosomes.

The exclusive presence of the LT Cyb mRNA in the 80–90 S region makes it easy to evaluate the relative amount of the putative mRNA-ribosome complexes by slot-blot hybridization. This approach was used to investigate the effects of down-regulation of the PPR29, 200-kDa, and Rhod proteins on the LT Cyb mRNA-ribosome associations. The mRNA hybridization profiles derived from the non-induced and RNAi-induced (day 3 p.i.) cells are shown in Fig. 8*C*. The gradients were normalized using the total hybridization signal, with each fraction showing a percentage thereof. The largest peak, located in fractions 3–5,

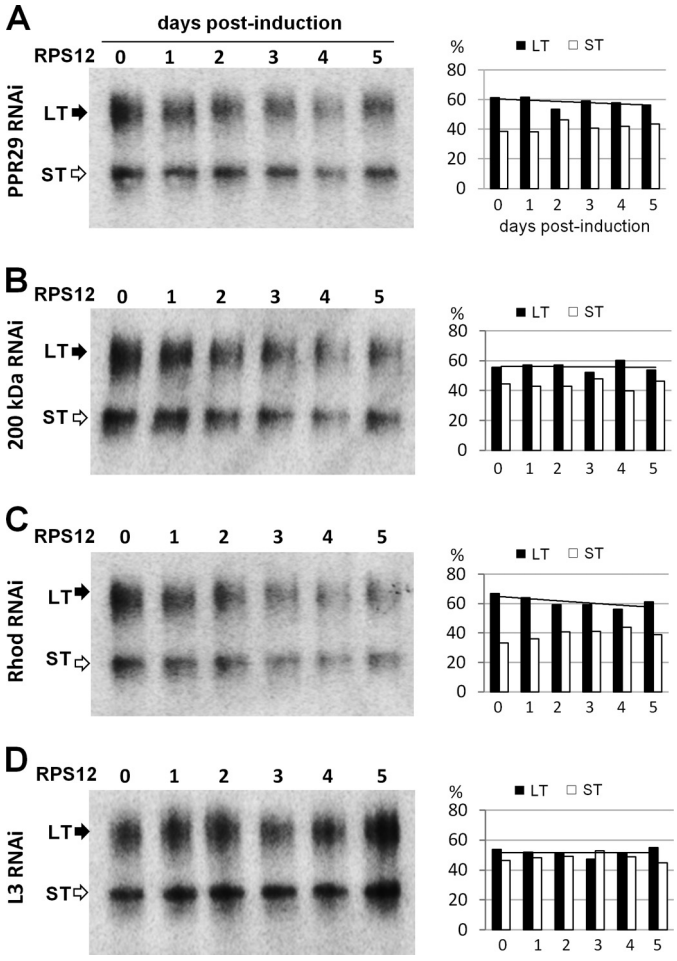


FIGURE 7. **Northern blot hybridization analysis of edited RPS12 mRNA in PPR29 (*A*), 200-kDa (*B*), Rhod (*C*), and L3 (*D*) RNAi cells.** See the legend to Fig. 5 for experimental details and designations.

corresponds to the free ST mRNA and remains unaffected by the RNAi. On the contrary, the putative LT mRNA-ribosome complexes, identifiable as a smaller peak centered at fraction 11, were noticeably affected by each RNAi, with the most severe reduction of the peak observed after RNAi of Rhod (Fig. 8*C*).

We then investigated whether the down-regulation of the 45 S SSU* complex has a similar effect on the higher order complex formation by COI mRNA. Although the sedimentation of COI mRNA in the gradients is more difficult to investigate because this long (1700-nt) mRNA is prone to degradation, which occurs during the mild detergent lysis of cells, we were still able to detect two major peaks formed by this mRNA in the sucrose gradients, forming a pattern similar to the one observed with the edited Cyb mRNA (Fig. 8*D*). It still remains to be investigated, however, whether or not the 80–90 S translation complex contains exclusively the LT form of this RNA, especially because this mRNA is unedited and may, therefore, require no special signature for translation. In any case, we have found that the putative COI translation complex was strongly affected by ablation of the 45 S SSU* complexes as was the LT edited Cyb complex.

Finally, in order to verify if the Cyb and COI effects reflected a disruption of specific interactions between these mRNAs and the translation machinery, as opposed to only an indirect impact of the PPR29, 200-kDa, and Rhod RNAi on the ribosomes, we also investigated whether the same set of RNAi would affect the translation complex formed by the edited RPS12 mRNA. We have shown above that these down-regulations do not reduce the relative amount of the LT form of this mRNA. Consistent with this observation, we could not notice any effect caused by these RNAi depletions on the translation complex (investigated at day 3 p.i.) (Fig. 9, *A–C* and *F*). In order to rule out the possibility that this refractory nature of the RPS12 translation complexes is somehow related to the fact that, unlike the LT Cyb mRNA, only a small fraction of the LT mRNA is bound in the translation complex, we also investigated effects of the RNAi of ribosomal proteins S17 and L3, which are expected to directly impact the monosomes. Indeed, in these cases, the RPS12 mRNA translation complex was noticeably affected (Fig. 9, *D–F*). Interestingly, whereas the ablation of S17 led to the disappearance of this complex (Fig. 9*D*), the depletion of L3 resulted in the LT RPS12 mRNA-containing complex, shifting its position to the 45 S region of the gradient (fraction 7) (Fig. 9*E*). The latter observations suggest that within the 80 S complex, the mRNA is associated with the SSU. Overall, these results indicate that formation of the translation complexes by edited Cyb and COI mRNAs is specifically impaired by ablation of the 45 S SSU* complexes.

Reduced Content of 45 S SSU in Bloodstream Trypanosomes*—Ribosomal RNP complexes have also been analyzed in the Lister 427 "single-marker" strain of *T. brucei* (39) grown *in vitro*. The cells were lysed with dodecyl maltoside in the presence of 100 mM KCl, and the ribosomal complexes were analyzed by sedimentation followed by slot-blot hybridization with the 9 and 12 S rRNA-specific probes. The normalized hybridization profiles are shown in Fig. 1*C*. It is noticeable that the relative amount of the 9 S rRNA-containing 45 S complex in the bloodstream stage cells is substantially reduced as compared with the procyclic cells (Fig. 1*A*). This is consistent with the drastic overall reduction of the 9 S rRNA level in bloodstream trypanosome (Fig. 1*C*, *inset*). It still remains to be shown whether this material represents the same complex as in the procyclic trypanosomes or a different complex. The second peak, sedimenting at 50 S, may represent the monosomes, especially because there is a matching shoulder in the 12 S LSU rRNA profile. The plot also shows a relatively large pool of the free 45 S LSU in the bloodstream trypanosomes. The amount of the 12 S rRNA in bloodstream trypanosomes is not as greatly reduced as the 9 S rRNA.

DISCUSSION

The role played by the unusual 45 S SSU* complex in trypanosomatid mitochondrial translation is intriguing. The presence of the PPR proteins in this complex led to the hypothesis that it is involved in sequence-specific mRNA recognition (34). As the first step to address this hypothesis, we have investigated if the SSU* complex plays an indispensable role in the mitochondrial translation.

The *T. brucei* system has been used for this purpose instead of *L. tarentolae* due to the advantages of RNAi in the trypanosomes (39, 49, 50). We have found that the ablation of the three

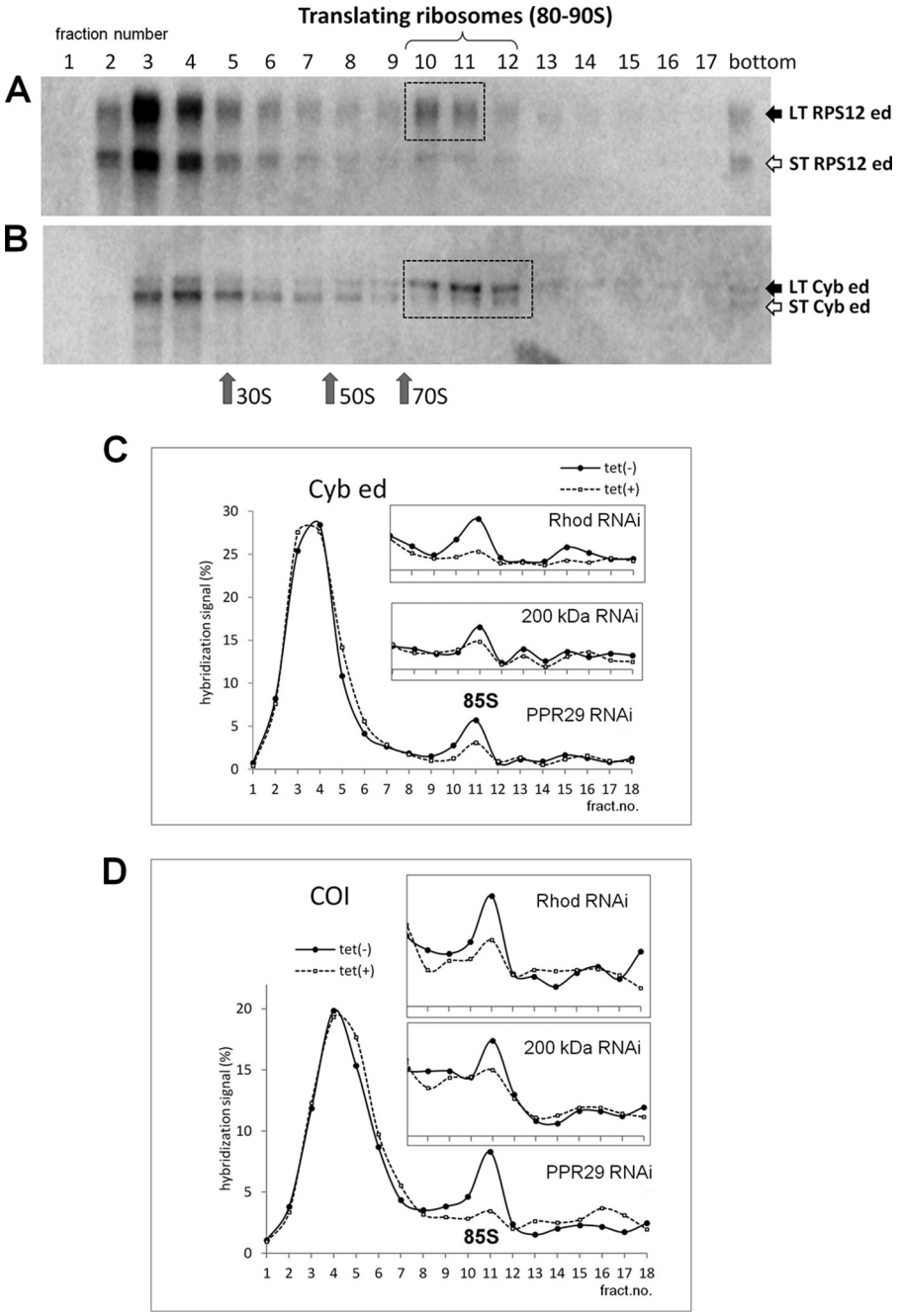


FIGURE 8. **Sedimentation analysis of mRNA-ribosomal complexes.** The parental 29-13 cells were lysed in DM buffer, and the lysate was sedimented through a 7–30% sucrose gradient (SW41, 17,000 rpm, 16 h). RNA extracted from the gradient fractions was fractionated in a 4% polyacrylamide-urea gel, blotted, and probed for edited RPS12 mRNA (*A*) or edited Cyb mRNA (*B*). LT and ST mRNA forms are indicated. *Boxed areas* represent the 80–90 S LT mRNA-ribosome complexes (28). The sedimentation profile of the mitochondrial 9 and 12 S ribosomal RNAs in a similar gradient (except that it was fractionated into 34 fractions instead of 17 fractions) is shown in Fig. 1*A*. Ribosomes and subunits of *E. coli* were used as standards. *C* and *D*, cell lysates were obtained from the cell lines expressing inducible RNAi for PPR29, 200-kDa, and Rhod mRNAs at day 3 postinduction (*tet*(+), *dashed line*) as well as from non-induced cells (*tet*(−), *solid line*) and were gradient-fractionated as described above. RNA from gradient fractions was used for slot-blot hybridizations employing the probes for edited Cyb mRNA (*C*) and for COI mRNA (*D*). The plots show the percentage of the total hybridization signal per fraction.

components of the SSU* complexes have caused a severe growth retardation phenotype in procyclics trypanosomes, similar to the down-regulation of the *bona fide* ribosomal proteins L3 and S17. A strong inhibition of mitochondrial synthesis of COI and Cyb polypeptides was observed in each of those cases, although the effect of down-regulating the SSU* was different from those of down-regulating the ribosome by L3 RNAi as compared with Cyb; the COI synthesis was much more strongly affected by the SSU* RNAi, whereas the indiscriminate inhibition of protein synthesis was seen after the direct L3 RNAi-mediated ablation of ribosomes. The effect of S17 RNAi was similar to that of the other SSU* components, instead of L3 RNAi, indicating that S17 RNAi affected primarily the SSU* complexes instead of the ribosomes. This may occur if the residual amount of the produced S17 in RNAi-induced cells is preferentially directed to the ribosome-bound SSU instead of

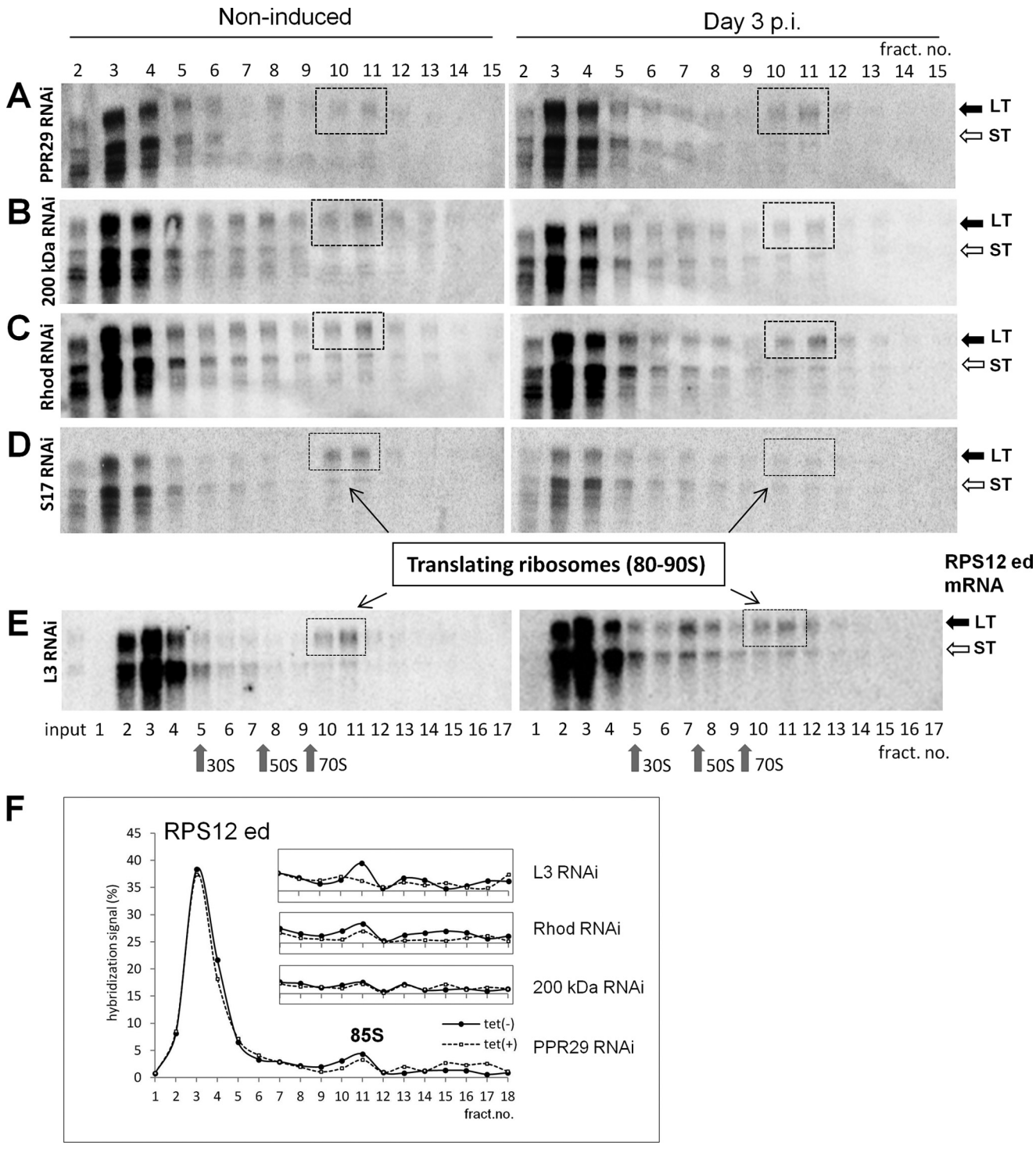


FIGURE 9. **Sedimentation analysis of edited RPS12 mRNA-ribosomal complexes.** The non-induced cells and the cells induced for RNAi targeting the PPR29 (*A*), 200-kDa (*B*), Rhod (*C*), S17 (*D*), and L3 (*E*) mRNAs (at day 3 postinduction) were lysed in DM buffer, followed by sedimentation in a 7–30% sucrose gradient (SW41, 17,000 rpm, 16 h). RNA extracted from the gradient fractions was fractionated in a 4% polyacrylamide-urea gel, blotted, and probed for edited RPS12 mRNA. LT and ST mRNA forms are indicated. *Boxed areas* represent fractions containing the 80–85 S LT mRNA-ribosome complexes. *F*, cell lysates from cell lines induced for L3, Rhod, 200-kDa, and PPR29 RNAi at day 3 postinduction (*tet*(+), *dashed line*) as well as from non-induced cells (*tet*(−), *solid line*) were fractionated in a sucrose gradient as described above. RNA from gradient fractions was used for slot-blot hybridizations employing the probes for edited RPS12. The plots show the percentage of the total hybridization signal per fraction.

the SSU*-bound SSU. Although there was no significant impact on the total mRNA levels, the relative amount of the LT forms of the COI mRNA and the edited Cyb mRNA was reduced after the ablation of the SSU* complexes but not by ablation of the ribosomes by L3 RNAi. This was accompanied by a disruption of the active translation complexes, characterized by the LT form of these mRNAs bound to mitoribosomes (28). These effects were specific for the COI and edited Cyb mRNA; the down-regulation of the SSU* complexes had no significant impact on the LT edited RPS12 mRNA or on its interactions

with the ribosomes. Last but not least, in the bloodstream stage trypanosomes, which possess no cytochrome-based respiratory chain and, therefore, are not expected to synthesize COI and Cyb proteins, the amount of the 45 S SSU* complex was drastically reduced in comparison with the procyclic trypanosomes, which have a fully developed electron-transport chain (18, 51, 52).

These results led us to conclude that the SSU* complex is a factor playing an important role in translation. Moreover, the data suggest that it might be involved in translational regulation of only a subset of mRNAs, specifically those that encode components of the COI and Cyb in procyclic trypanosomes. This is a hypothesis that requires further investigation. It also remains to be investigated if the cytochrome *c* oxidase subunit II and III mRNAs and the respective translation complexes are affected by its down-regulation in a manner similar to COI. Another open question is if the same or different translation factors (complexes) operate with the other mRNAs, in particular those that are expected to be translated in the bloodstream form as well, such as RPS12 and subunit 6 of F_1F_0 ATPase (53). It is noteworthy that a residual amount of the 45 S SSU* material is still observed in the ribosomal sedimentation profile of the bloodstream stage. An intriguing possibility is that there is a family of SSU* complexes with variations in the protein content among its members, which would define their specificity toward different subsets or individual mRNAs.

As shown in this work, the mechanism by which the inactivation of the SSU* complex affects translation of selected mRNAs involves destabilization of the long poly(A/U) tails. This could be achieved through the components of the SSU* complexes shared with the KPAP polyadenylation complex and/or other parts of the mRNA processing machinery. Several such components have been identified by the proteomics analyses in *L. tarentolae* and *T. brucei*, including the PPR29 protein investigated herein and several other PPR (Tb927.11.5500) and non-PPR proteins (Tb927.11.1250, Tb927.11.2530) (17, 28, 43, 46, 54). It is noteworthy that this effect is mRNA-specific, as follows from the observation that the LT of the RPS12 mRNA remains unchanged. This in turn suggests that the down-regulation of the SSU* complex does not impact the polyadenylation/uridylation machinery in general. Moreover, because the short-tailed forms of the COI and edited Cyb mRNAs do not seem to undergo any shortening or degradation, it is likely that the SSU* down-regulation specifically prevents the extension of the existing short poly(A) tail, which would be done by the KPAP1/RET2 complex after its activation by KPAF1 and KPAF2 (28). It was hypothesized previously that a specific recognition of an edited mRNA by a yet unidentified PPR protein on the 5′-end is necessary for the recruitment of the poly(A/U)-tailing machinery to the mRNA 3′-end (23). An attractive scenario is that this recognition may be, in fact, mediated by the SSU* complex; then down-regulation of this complex by RNAi would lead to accumulation of the short-tailed forms. The PPR protein components of the SSU* may be directly involved in the interactions with specific mRNAs. However, this scenario remains purely hypothetical, and no direct interactions of the KPAF proteins with the SSU* complexes have been detected so far.

An alternative possibility is that an interaction of an LT mRNA and the SSU* complex is required for preserving intactness of the long tail, which is synthesized independently. In the absence of this interaction, the long tail is quickly degraded, but the mechanism of such degradation is unknown.

In any case, as shown by the Northern blot analyses, the LT forms of the unedited COI and the edited Cyb mRNAs are noticeably affected as early as day 1 p.i., and that is followed by the inhibition of translation first observed at day 2 p.i. It is obvious that the synthesis of COI was affected more strongly than that of Cyb. The reason for this may be a lower level of the remaining LT COI mRNA, but it may rather be related to the differences in stability of the newly synthesized products. The pulse-chase labeling experiments followed by the analysis of respiratory complexes with blue native gel electrophoresis[4] have shown that all of the newly synthesized COI is found incorporated into the cytochrome *c* oxidase complex. However, most of the labeled Cyb does not get incorporated into the cytochrome bc_1 complex but instead becomes associated with a smaller size protein complex, probably chaperones. This association may protect the nascent Cyb polypeptides from degradation, whereas there is no such protection mechanism for COI. Under the RNAi conditions, the assembly of cytochrome *c* oxidase can be severely impaired, and most of the nascent COI may become quickly degraded.

In the absence of the LT form, inhibition of the synthesis results in impairing the interactions between the mRNAs and the mitoribosomes. This follows from the noticeable reduction of the 85 S translation complexes containing COI and edited Cyb mRNA after the RNAi of the SSU* particles. The exact nature of these complexes still awaits further investigation. Their size suggests that in addition to the 50 S ribosome and the transcripts, these complexes contain additional components.

As shown herein, the 45 S SSU* complexes are indispensable for mitochondrial protein synthesis of the key components of the respiratory chain in procyclic trypanosomes, such as cytochrome *c* oxidase and cytochrome bc_1. It is remarkable that the 45 S complexes seem to be absent or down-regulated in bloodstream trypanosomes in which such a function would not be required. It is, therefore, possible that the down-regulation of the 45 S SSU* complex in the bloodstream stage is involved in developmental regulation of the mitochondrial biogenesis in trypanosomes. The mechanism by which a subset of mitochondrial mRNAs becomes activated for translation may have more than one aspect. On one hand, this activation involves stabilization of the mRNA long poly(A/U) tail (or promotion of its synthesis) with the concomitant formation of the 85 S translation complexes. On the other hand, there may be other potential functions of the 45 S SSU* complex, such as site-specific mRNA recognition, which have not yet been addressed. These include defining its specificity determinants and mapping its binding site(s) on the mRNAs. It would also be of particular interest to investigate its potential for formation of the translation initiation complex, a function that would imply an ability to recognize start codons and interact with translation factors

[4] D. A. Maslov and I. Škodová, unpublished observations.

and the initiator tRNA. In any case, this study shows that the mechanisms of specific translational activation operating in trypanosomes are clearly different from those in yeast (26, 27) or mammals (55). The evolutionary processes responsible for bringing up the puzzling complexity, which marks all aspects of the organization and expression of trypanosomes' mitochondrial genome studied so far, did not bypass its translation apparatus.

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