Assembly of the *Trypanosoma brucei* 60S Ribosomal Subunit Nuclear Export Complex Requires Trypanosome-Specific Proteins P34 and P37

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We previously identified two *Trypanosoma brucei* RNA binding proteins, P34 and P37, and determined that they are essential for proper ribosomal assembly in this organism. Loss of these proteins via RNA interference is lethal and causes a decrease in both 5S rRNA levels and formation of 80S ribosomes, concomitant with a decrease in total cellular protein synthesis. These data suggest that these proteins are involved at some point in the ribosomal biogenesis pathway. In the current study, we have performed subcellular fractionation in conjunction with immune capture experiments specific for 60S ribosomal proteins and accessory factors in order to determine when and where P34 and P37 are involved in the ribosomal biogenesis pathway. These studies demonstrate that P34 and P37 associate with the 60S ribosomal subunit at the stage of the nucleolar 90S particle and remain associated subsequent to nuclear export. In addition, P34 and P37 associate with conserved 60S ribosomal subunit nuclear export factors exportin 1 and Nmd3, suggesting that they are components of the 60S ribosomal subunit nuclear export complex in *T. brucei*. Most significantly, the pre-60S complex does not associate with exportin 1 or Nmd3 in the absence of P34 and P37. These results demonstrate that, although *T. brucei* 60S ribosomal subunits utilize a nuclear export complex similar to that described for other organisms, trypanosome-specific factors are essential to the process.

*Trypanosoma brucei* is a parasitic protozoan and the causative agent of African sleeping sickness in humans and nagana in cattle (26). It is an organism that, while extensively studied, continues to pose a serious threat to human health and economic development in sub-Saharan Africa (29). It is one of only a few diseases that is 100% fatal if left untreated and, due to the toxicity of current treatments and increasing incidence of parasite drug resistance, new chemotherapeutic agents are a necessity (1, 8). Of particular interest for drug development is the necessity of parasite drug resistance, new chemotherapeutic agents are a necessity (1, 8). Of particular interest for drug development in addition to the toxicity of current treatments and increasing incidence of parasite drug resistance, new chemotherapeutic agents are a necessity (1, 8). Of particular interest for drug development in this organism, trypanosome-specific factors are essential to the process.

Ribosomal biogenesis is an essential process in all organisms ranging from bacteria to humans, and the framework of this process is highly conserved among the eukaryotic kingdoms (39). The ribosomal biogenesis pathway of the yeast *Saccharomyces cerevisiae* has been extensively studied and often serves as a paradigm for the eukaryotic pathway (Fig. 1). In this organism, the 60S ribosomal subunit consists of 46 ribosomal proteins and three RNA species (5S, 5.8S, and 25S), whereas the 40S ribosomal subunit is comprised of 32 ribosomal proteins and 18S rRNA (12, 13). In addition to the ribosomal proteins that comprise the 60S and 40S ribosomal subunits, approximately 140 *trans*-acting proteins have been identified in yeast as being required for proper ribosomal assembly to occur, most of which function in 60S biogenesis (10). Among these factors are several proteins that are required for the nuclear export of yeast pre-60S ribosomal subunits, including exportin 1 (Xpo1, also known as CRM1) and Nmd3 (14, 20), Mex67-Mtr2 (44), and Arx1 (3, 21).

Following export from the nucleus, yeast pre-60S particles undergo their final maturation steps in the cytoplasm, including addition of the final large ribosomal proteins, release and recycling of nuclear export factors and, ultimately, joining with a 40S subunit/mRNA complex to form the mature 80S ribosome (9, 17, 27, 28, 32). Thus, the work that has been performed in *S. cerevisiae* has demonstrated that, while ribosome biogenesis is very rapid (42), it is a highly complex and multifactorial process.

The ribosomal biogenesis pathway has not been extensively studied in *T. brucei*, but this organism does contain putative homologues to both 40S and 60S ribosomal proteins as annotated in the *T. brucei* gene database (http://www.genedb.org/genedb/tryp/index.jsp). It is also known that some aspects of the 60S pathway contain characteristics similar to other organisms, such as the existence of an L5-5S preribosomal particle (30) and the involvement of a conserved nucleolar protein, NOG1, in 60S subunit biogenesis (23, 31). However, some differences exist as well, including the processing of the 60S rRNA species (28S rRNA) into six smaller rRNA molecules (5, 43) and the involvement of the trypanosome-specific family of proteins, NOPP44/46, in 60S biogenesis (22). In addition, ribosome biogenesis and protein synthesis are developmentally regulated throughout the life cycle of the parasite (2, 4, 38), indicating that factors involved in this process may be similarly regulated themselves.

There is further evidence supporting the hypothesis that...
developmentally regulated factors are involved in *T. brucei* ribosomal biogenesis. Our laboratory has shown that the developmentally regulated, trypanosome-specific proteins, P34 and P37, associate with 5S rRNA (34), ribosomal protein L5 (K. Hellman, K. Prohaska, and N. Williams, unpublished data), and a family of nucleolar phosphoproteins, NOPP44/46 (33). More recently, our laboratory has discovered that, when P34 and P37 expression is knocked down using RNA interference (RNAi), it is lethal to the cells (18). In addition, there is a 25-fold decrease in the levels of 5S rRNA, and the polysomal profile of P34/P37 RNAi cells shows a decrease in 80S ribosome formation, concomitant with an overall decrease in protein synthesis (18). These results demonstrate that P34 and/or P37 is involved in the ribosomal biogenesis pathway and/or translation initiation.

Because ribosomal biogenesis is a highly complex process, there are several points in the pathway at which P34 and/or P37 may be involved. In Fig. 1, we present a simplified version of the yeast pathway as a model for our system, since it has been extensively studied. Beginning in the nucleolus, the 90S particle is the first complex formed in the pathway (15), which includes 60S ribosomal protein L3 (27, 35). Following cleavage to form separate 66S and 43S particles, 60S ribosomal protein L11 is added to 66S in the nucleolus (16, 27), after which the particle is released into the nucleoplasm (24). Following further nucleoplasmic maturation, the pre-60S particle associates with exportin 1 through the Nmd3 adaptor protein (14, 20) and is then exported to the cytoplasm. Once in the cytoplasm, the final 60S ribosomal proteins are added as one of the last maturation steps (9, 27) prior to subunit joining. Finally, the mature 60S subunit is joined with an mRNA-bound 40S subunit to form the mature 80S ribosome (25) and translation initiation can take place. As indicated in Fig. 1, P34 and P37 may become associated with the maturing 60S complex at any one of these steps in the ribosomal biogenesis pathway. However, the association of P34 and P37 with exportin 1 (19) supports a role(s) in nuclear export of the 60S ribosomal subunit for these proteins.

In order to determine when and where P34 and P37 are involved in ribosomal biogenesis, we have utilized subcellular fractionation together with immune capture experiments specific to various ribosomal proteins and ribosomal biogenesis accessory factors based on the yeast pathway. These experiments demonstrate that *T. brucei* requires trypanosome-specific proteins in the otherwise highly conserved ribosomal biogenesis pathway.

**MATERIALS AND METHODS**

**Cell culture.** Procyclic-form *T. brucei brucei* strain 427 was grown in Cunningham’s medium supplemented with 10% fetal calf serum (6). Clonal P34/P37 RNAi cells have been described previously (19). Induction of double-stranded RNA specific to P34 and P37 was initiated by addition of tetracycline (1 μg/ml; Sigma, St. Louis, MO) to a concentration of 1 × 10⁶ cells/ml. Cells were harvested 4 days postinduction and Western blot analysis was performed, probing for P34 and P37 to confirm the absence of both proteins.

**Antibodies.** The antibodies used for this study were as follows: P34/P37 polyclonal antiserum (47), affinity-purified P34/P37 polyclonal antibody (Bethyl Laboratories, Montgomery, TX), L3 polyclonal antiserum (Affinity Bioreagents, Golden, CO), L11 polyclonal antiserum (Affinity Bioreagents), L10 monoclonal antibody (Novus Biologicals, Littleton, CO), affinity-purified L5 polyclonal antibody (Bethyl Laboratories), S6 polyclonal antiserum (Affinity Bioreagents), affinity-purified Nmd3 polyclonal antiserum (gift from Marilyn Parsons), and TATA binding protein polyclonal antiserum (gift from Vivian Bellofatto).

**Specificity of antibodies.** The ribosomal protein antibodies used in this study were commercially obtained and were raised to recombinant human proteins. Prior to testing the specificity of these antibodies to the trypanosome proteins, we aligned each of the human protein sequences with those from *T. brucei* (see Fig.
S1 to S5 in the supplemental material). We next performed immune capture experiments (described below) with cytoplasmic and nuclear extracts specific for L3, L11, L10, S5, and S6. Subsequent Western blot analyses using the same antibodies used for the immune capture showed that each antibody demonstrated reactivity to the appropriately sized protein and that the majority of each protein was present in the pellet fraction compared to the supernatant (see Fig. S6 in the supplemental material).

We also aligned the yeast Nmd3 protein sequence with the homologous T. brucei protein, which revealed a high degree of identity between the two species (see Fig. S7 in the supplemental material). We utilized an antibody raised to yeast Nmd3 (a generous gift from Arlen Johnson) for Western blot analyses on nuclear and cytoplasmic extracts, the results of which demonstrated that this antibody cross-reacts with a protein of the appropriate molecular weight (data not shown). These results confirmed that T. brucei contains an Nmd3 homologue and that the yeast antibody was useful for further experiments.

**Cellular fractionation.** Cytoplasmic and whole nuclear extracts were prepared as described previously (19). Nucleolar and nucleoplasmic extracts were prepared as described previously (36).

**Immune capture experiments.** Immune capture experiments were done as described previously (19) using antibodies specific to the following proteins: P34 and P37, exportin 1, and L3, L11, L10, S5, and S6. Briefly, 500 μg of the indicated extract was incubated with antibody-cross-linked Dynabeads (Invitrogen, Carlsbad, CA). After incubation, the beads were washed with phosphate-buffered saline and eluted with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. For each immune capture experiment, the protein(s) present in the pellet fractions represents the majority of the protein, compared to that in the unbound fraction (data not shown).

**Western blot analyses.** Following SDS-PAGE, the gels were transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) and blocked in 10% nonfat dry milk. Membranes were incubated with the indicated primary antibody overnight, followed by 10 washes with Tris-buffered saline with Tween and incubation with the appropriate horseradish peroxidase-conjugated secondary antibody. Proteins were detected using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL).

**RT-PCR analyses.** Immune capture experiments were performed as described above, with the modification of incubating the Dynabeads with poly(dI-dC) (Sigma, St. Louis, MO) overnight in order to decrease nonspecific RNA binding. The beads were eluted by resuspension in elution buffer (1% SDS, 50 mM dithiothreitol, and 10% β-mercaptoethanol) and boiling for 5 min. Bound RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) as per the manufacturer’s instructions. Subsequent reverse transcription-PCR (RT-PCR) analyses were performed using primers specific to internal sequences of processed 60S rRNA (28S), 40S rRNA (18S), and the entire processed 5S rRNA sequence (5). A fraction of each reaction mixture was subjected to agarose gel electrophoresis and ethidium bromide staining to visualize the RT-PCR products.

**Sequential immune capture experiments.** Sequential immune capture experiments were performed as described previously (19). Briefly, the primary immune capture against exportin 1 was performed as described for the single immune capture experiments with the modification of starting with 2 mg of nuclear extract. The beads were eluted as described for the RT-PCR analyses, followed by dilution with 1 ml of nonnaturating lysis buffer. Samples were allowed to cool to room temperature and were then used for a secondary immune capture experiment specific for L11. P34/P37 Western blot analyses were performed on the resulting pellet fractions.

**RESULTS**

**Subcellular fractionation** demonstrates that P34 and P37 are present in the nucleolar fraction of procyclic cells. Wild-type procyclic cells were fractionated into whole nuclear, nucleolar, nucleoplasmic, and cytoplasmic extracts. (A) A fraction of each extract was subjected to SDS-PAGE and Western blot analysis probing for P34 and P37. (B) A fraction of each extract was subjected to SDS-PAGE and Western blot analysis probing for specific cellular compartmental markers. NOG1, a nucleolar marker; TBP (TATA binding protein), a nucleoplasmic marker; PGK (phosphoglycerate kinase), a cytoplasmic marker. CE, 50 μg of whole nuclear extract; NO, 100 μg of nucleolar extract; NP, 100 μg of nucleoplasmic extract.

**FIG. 2.** P34 and P37 are present in the nucleolar fraction of procyclic cells. Wild-type procyclic cells were fractionated into whole nuclear, nucleolar, nucleoplasmic, and cytoplasmic extracts. Western blot analyses probing for cellular compartmental markers for each fraction demonstrated that we had isolated distinct subcellular fractions (Fig. 2B), which could be used in subsequent immune capture experiments. In addition, Western blot analysis probing for P34 and P37 demonstrated that both proteins were present in each of our subcellular fractions (Fig. 2A). This figure is representative of three separate experiments. The presence of these proteins in the nucleolar fraction, which had not been demonstrated previously, suggests that they may be involved in earlier nucleolar stages of ribosomal biogenesis. Thus, utilizing subcellular fractionation in conjunction with immune capture experiments, we sought to determine when and where P34 and P37 are involved in the ribosomal biogenesis pathway. Since we had determined previously that P34 and P37 associate with both 5S rRNA (33) and ribosomal protein L5 (Hellman et al., unpublished), two components of the 60S ribosomal subunit, we focused our initial experiments on potential 60S ribosomal protein binding partners.

**P34 and P37 associate with L3-containing 60S ribosomal subunits.** One of the earliest ribosome-associating 60S proteins is L3, which is added to the 90S particle in the nucleolus (27, 35). The association of a protein(s) with L3, particularly in the nucleolus, may be indicative that it functions early in ribosomal biogenesis. Immune capture experiments specific for L3 were performed with whole nuclear, nucleolar, nucleoplasmic, and cytoplasmic extracts from T. brucei, followed by Western blot analyses probing for P34 and P37. These experiments demonstrated that P34 and P37 associated with L3, either directly or indirectly, in all fractions tested (Fig. 3A, P lanes). Although P37 was not detected in the immune capture pellet fraction for all extracts, additional Western blot analyses demonstrated that it is present in each of these fractions (data not shown) but may not be seen in all blots due to its lower abundance relative to P34 (47). In addition, we confirmed that...
our antibody precipitated L3 in each fraction by performing L3 Western blot analyses on each pellet fraction (data not shown). These experiments demonstrated that P34 and P37 are associated with the maturing 60S ribosomal subunit throughout the biogenesis pathway, beginning at an early, nucleolar step.

In order to ensure that L3 was being precipitated in the context of the 60S subunit and not simply free protein, we performed RT-PCR analyses on immune capture pellet fractions from experiments performed with cytoplasmic and nuclear extracts. RT-PCR experiments were performed specific to 60S rRNA (28S and 5S) and 40S rRNA (18S) species. The rRNA species found in association with L3, as demonstrated in yeast (27), are shown in Fig. 3B. L3 associates with the 90S particle in yeast (Fig. 1), and if this is also true for *T. brucei*, it should be complexed with not only 28S and 5S rRNA in nuclear extracts but also 18S rRNA. In addition, L3 should associate with 28S and 5S rRNA in cytoplasmic extracts, since it remains associated with mature 60S subunits, and with 18S rRNA subsequent to subunit joining. RT-PCR analyses performed on RNA extracted from L3 immune capture experiments using cytoplasmic and whole nuclear extracts: 1 kb, 1-kb DNA ladder (Invitrogen); -RT, no-RT control; 28S, 28S RT-PCR; 18S, 18S RT-PCR; 5S, 5S RT-PCR; B, immune capture beads alone (control); CE, cytoplasmic extract immune capture; NE, whole nuclear extract immune capture. Results shown are representative of at least three separate experiments.

**FIG. 3.** P34 and P37 associate with 60S protein L3. Immune capture experiments specific to L3 were performed with whole nuclear, nucleolar, nucleoplasmic, and cytoplasmic extracts. (A) Western blot analyses were performed, probing for P34 and P37. B, immune capture beads alone; NE, 50 μg of whole nuclear extract; NO, 50 μg of nucleolar extract; NP, 50 μg of nucleoplasmic extract; CE, 50 μg of cytoplasmic extract; P, immune capture pellet fraction. (B) Diagram depicting the ribosomal complexes and rRNA species with which L3 associates. (C) RT-PCR analyses performed on RNA extracted from L3 immune capture experiments using cytoplasmic and whole nuclear extracts: 1 kb, 1-kb DNA ladder (Invitrogen); -RT, no-RT control; 28S, 28S RT-PCR; 18S, 18S RT-PCR; 5S, 5S RT-PCR; B, immune capture beads alone (control); CE, cytoplasmic extract immune capture; NE, whole nuclear extract immune capture. Results shown are representative of at least three separate experiments.

P34 and P37 associate with 40S ribosomal protein S5-containing ribosomal particles in nucleolar and whole nuclear extracts. An association of P34 and P37 with L3-containing 60S particles in the nucleolar fraction may be interpreted in one of two ways: (i) P34 and P37 are associated with 90S particles or (ii) P34 and P37 associate with the subunit subsequent to the rRNA cleavage event that gives rise to the 66S and 43S subunits. Both 90S and 66S particles are present in the nucleolus and, thus, it is important to distinguish between them to more precisely determine the 60S particle(s) with which P34 and P37 associate. In order to determine whether P34 and P37 are involved in ribosomal biogenesis at the initial 90S particle, we performed immune capture experiments specific to S5, a 40S ribosomal subunit protein that comes into the pathway at the 90S particle stage (Fig. 1) (15). Since P34 and P37 have thus far been shown to associate only with specific 60S subunit factors, an association with S5 in whole nuclear or nucleolar extracts would be indicative of P34 and P37 being present in 90S particles. Immune capture experiments specific to S5 followed by Western blot analyses probing for P34 and P37 demonstrated that both proteins are complexed with S5 in whole nuclear and nucleolar extracts (Fig. 4A, P lanes), which indicates that these proteins become involved in 60S subunit biogenesis at the 90S nucleolar stage prior to subunit separation. However, neither protein associates with S5-containing particles in the nucleoplasm (Fig. 4A, P lane), demonstrating that the association between P34 and P37 and S5 is specific to the precleaved particle and, subsequent to cleavage, they are found specifically with the 60S subunit. In addition, RT-PCR experiments performed on P34/P37 immune captures using whole nuclear extracts demonstrate a complex between these proteins and 18S rRNA, most likely while it is still part of the 35S rRNA precursor (data not shown).

The rRNA species with which S5 associates in yeast are
demonstrated in Fig. 4B. Since S5 is a 40S protein that is added to the 90S particle in the nucleolus and remains associated throughout the 40S subunit maturation pathway (15, 27), it should associate with 18S and 28S rRNA species in nuclear extracts and all three rRNA species in cytoplasmic extracts (Fig. 4B). Our results demonstrate that S5 is complexed with 28S, 18S, and 5S rRNA in both nuclear and cytoplasmic extracts (Fig. 4C). There is some controversy over the stage at which 5S rRNA becomes incorporated into the yeast 60S subunit. Some studies have indicated that it integrates into 90S particles (46), whereas others suggest that it is incorporated into the 66S particle (7). Our results demonstrate that, in *T. brucei*, 5S rRNA incorporates into the 90S ribosomal particle.

**P34 and P37 associate with L11-containing 60S ribosomal subunits.** In order to confirm that P34 and P37 remain complexed with the developing 60S ribosomal subunit, we used L11 as a marker for the late-associating proteins that are part of the 66S ribosomal particle (Fig. 1). Immune capture experiments specific for L11 demonstrated an association between P34 and P37 and L11, which is either direct or indirect, in all extracts tested (Fig. 5A, P lanes). Western blot analyses probing for L11 demonstrated that this protein was precipitated from each extract (data not shown).

Figure 5B shows the rRNA species associated with L11 throughout the yeast 60S subunit biogenesis pathway. Because L11 assembles into the maturing 60S particle at the 66S stage (Fig. 1 and 5B) (16), it should be complexed with 28S and 5S rRNA, but not 18S, in *T. brucei* nuclear extracts if this follows the yeast model. In cytoplasmic extracts, L11 should associate with 28S and 5S rRNA through its involvement with the mature 60S subunit and 18S rRNA in the context of the 80S ribosome (Fig. 5B). RT-PCR analyses performed on L11 immune capture experiments from *T. brucei* demonstrate that it associates with 28S and 5S rRNA but not 18S rRNA (Fig. 5C) in the nucleus and with all three rRNA species in cytoplasmic extracts (Fig. 5C).

The results shown in Fig. 5 demonstrate that P34 and P37 associate with the developing 60S ribosomal subunit at multiple steps during its biogenesis, an unusual characteristic for trans-acting factors involved in ribosomal biogenesis. *T. brucei* utilizes a nuclear export complex similar to that described for yeast and mammals.
**T. brucei** 60S subunits associate with exportin 1. In order to determine whether 
*T. brucei* 60S ribosomal subunits associate with exportin 1, we performed immune capture experiments specific for L3 and L11 using whole nuclear extracts. These experiments demonstrated that L3- and L11-containing 60S particles associate with exportin 1, although each does so to a different extent (Fig. 6A, P lanes). We do not currently know if this is due to stoichiometric differences or if it is due to our experimental procedures (e.g., differences in the efficiencies of the antibodies used in the immune capture). These results demonstrate that 
*T. brucei* 60S subunits form an exportin 1-containing nuclear export complex.

**T. brucei** 60S subunits associate with an Nmd3 homologue. In addition to exportin 1, it has been shown that Nmd3 is required for the nuclear export of 60S ribosomal subunits by providing the NES for the 60S-exportin 1 interaction (14, 20, 41). However, it was not known whether 
*T. brucei* contains an Nmd3 protein homologue, so we used the yeast Nmd3 sequence to query the 
*T. brucei* database and found a homologous protein (accession number XP_845721). The two sequences were aligned using ClustalW, which found an approximately 40% identity between the protein sequences from both organisms (see Fig. S7 in the supplemental material). We also determined that an Nmd3 antibody which had been raised to the yeast protein cross-reacted with an appropriately sized protein in 
*T. brucei* extracts (data not shown).

Since we had determined that 
*T. brucei* contains an Nmd3 homologue, we next wished to determine whether exportin 1 and 60S subunits form a nuclear export complex containing this protein. Immune capture experiments specific to exportin 1, L3, and L11 were performed using wild-type procyclic nuclear extracts, followed by Western blot analyses probing for Nmd3. Each protein showed an interaction with Nmd3 (Fig. 6B, P lanes), demonstrating that the 
*T. brucei* 60S ribosomal subunit forms a nuclear export complex containing Nmd3.

**P34 and P37 associate with the 60S nuclear export complex.** Having now demonstrated that 
*T. brucei* 60S ribosomal subunits form a complex with both exportin 1 and Nmd3, we next wished to determine whether P34 and P37 are a part of this complex. We have shown previously that P34 and P37 associate with exportin 1 (19). In order to determine whether this complex also includes pre-60S subunits that are loaded with Nmd3, we performed immune capture experiments specific for P34 and P37 using procyclic whole nuclear extracts. Subsequent Western blot analyses probing for Nmd3 demonstrated that P34 and P37 associate with Nmd3 (Fig. 7A).

In order to confirm that P34 and P37 associate with exportin 1 and the 60S subunit simultaneously, we performed sequential immune capture experiments using nuclear extracts. We have used this technique previously to establish the complex formed between P34, P37, NOPP44/46, and exportin 1 (19). Performing a series of immune captures allows for enrichment of complexes containing specific proteins. We performed the first...
immune capture specific to exportin 1, which was eluted, re-natured, and utilized for a second capture specific to L11. Western blot analyses probing for P34 and P37 demonstrated that these proteins are present in a complex containing both exportin 1 and 60S, although P37 may have been below the level of detection in these experiments due to its lower expression relative to P34 (Fig. 7B). This confirms that P34 and P37 are components of the 60S nuclear export complex and do not simply facilitate the association between 60S and exportin 1.

T. brucei 60S ribosomal subunits fail to assemble with the nuclear export machinery in the absence of P34 and P37. As P34 and P37 are trypanosome-specific proteins associated with the 60S nuclear export complex, which is formed in organisms that lack these proteins, we hypothesized that P34 and P37 may be required for formation of this complex in T. brucei. Utilizing a previously constructed P34/P37 RNAi cell line (18), we examined the 60S-Nmd3 and 60S-exportin 1 interactions in the absence of P34 and P37.

P34/P37 RNAi cells were grown to the appropriate density (1 × 10⁶ cells/ml), at which point tetracycline (1 µg/ml) was used to induce double-stranded RNA expression specific for P34 and P37. The cells were harvested and fractionated into nuclear and cytoplasmic extracts at 4 days postinduction, and Western blot analysis probing for P34 and P37 confirmed the absence of both proteins in each extract (data not shown). It is important to note that the cells were harvested at a point where P34 and P37 were no longer detectable by Western blot analysis but the cells were still viable.

In order to determine whether the association between 60S subunits and Nmd3 was altered in the P34/P37 RNAi cells, we repeated our L3 and L11 immune capture experiments using the nuclear extracts prepared from these cells. These experiments were performed in tandem with the wild-type nuclear extracts in order to make a direct comparison of the 60S-Nmd3 association between the two cell lines. Nmd3 Western blot analyses for the L3 and L11 immune capture experiments as described for Fig. 8, using wild-type and P34/P37 RNAi nuclear extracts. Subsequent Western blot analyses probing for exportin 1 demonstrated that the association between 60S and exportin 1 is lost in the absence of P34 and P37 (Fig. 9, P lanes, compare wild type to RNAi). Together, these results demonstrate that P34 and P37, either directly or indirectly, are required for proper assembly of the 60S ribosomal subunit export complex in T. brucei.

In addition to the experimental results shown, we have performed Western blot analyses specific to L11 using equivalent amounts of wild-type and P34/P37 RNAi nuclear extracts. Results shown are representative of at least three separate experiments.
results from these experiments demonstrate that L11 accumulates in the nucleus when P34 and P37 are not present (approximately 3.5-fold ± 0.01-fold based on three separate experiments [data not shown]), suggesting a defect in 60S ribosomal subunit nuclear export. Also, preliminary results from two-dimensional difference gel electrophoresis performed with P34/P37 RNAi cytoplasmic extracts have shown that L11 protein levels decrease approximately 2.5-fold in the cytoplasm (L. Wang, unpublished data), lending further support to an alteration in the nuclear export of 60S subunits in the absence of P34 and P37.

**P34 and P37 associate with 80S ribosomes.** Since we had established that P34 and P37 associate with 60S subunits in the cytoplasm (Fig. 3 and 5), we next wanted to determine whether they remain so subsequent to 60S-40S subunit joining. We did not detect an association between P34 and P37 and S5 in the nucleoplasm (Fig. 6), suggesting that they act specifically on the 60S biogenesis pathway. A complex containing P34, P37, and a 40S protein in the cytoplasm would indicate that this was due to the subunits being joined to form an 80S ribosome. However, since we determined that P34 and P37 are complexed with S5 in whole nuclear and nucleolar extracts, we chose to use a 40S protein that does not associate with the 90S particle as our marker for 80S ribosomes (40S protein S6). Thus, a complex formed between P34 and P37 and S6 in the cytoplasm could only be due to their association with 80S ribosomes.

Thus, in order to determine whether P34 and P37 are complexed with 80S ribosomes, we performed immune capture experiments specific to the 40S protein S6 using cytoplasmic and nuclear extracts. Western blot analyses of the resulting pellets demonstrated that P34 and P37 associate with S6 in cytoplasmic, but not nuclear, extracts (Fig. 10A, P lanes).

We have illustrated the rRNA species complexed with S6 in yeast in Fig. 10B. S6 incorporates into the maturing yeast 40S subunit in the nucleolus at the 43S particle (Fig. 1 and 10B) (11, 27). If this model is conserved in *T. brucei*, S6 should associate only with 18S rRNA in nuclear extracts, but it should associate with 28S, 18S, and 5S rRNA in cytoplasmic extracts. Our results demonstrate that these are in fact the rRNA species with which *T. brucei* S6 associates in each extract (Fig. 10C), demonstrating that we efficiently precipitated 40S ribosomal subunits.

The nuclear results from these experiments revealed that P34 and P37 play a specific role in 60S ribosomal subunit biogenesis, as there is no association with the 40S subunit. The cytoplasmic results demonstrate that P34 and P37 are complexed with 80S ribosomes, suggesting that they remain associated with the translational apparatus, possibly fulfilling a role in subunit joining or translation initiation.

**DISCUSSION**

The work presented here has more precisely defined the role(s) that two trypanosome-specific RNA binding proteins, P34 and P37, play in the process of 60S ribosomal subunit biogenesis. Ribosomal biogenesis has not been extensively studied in *T. brucei*, and the bulk of our current knowledge pertains to rRNA processing events (5, 43). We have shown previously that P34 and P37 associate with several 60S ribosomal subunit components and biogenesis factors, including 5S rRNA (33), 60S ribosomal protein L5 (K. Hellman and K. Prohaska).
Prohaska, unpublished data), and a family of nucleolar phosphoproteins, NOPP44/46 (34). In addition, cells lacking P34 and P37 show phenotypic alterations with respect to SS rRNA levels, formation of 80S ribosomes, and overall cellular translation levels (18). Thus, the goal of the current study was to more specifically determine if, when, and where P34 and P37 are involved in 60S ribosomal subunit biogenesis.

We first demonstrated that we were able to obtain distinct subcellular fractions, including cytoplasmic, whole nuclear, nucleoplasmic, and nucleolar extracts, as determined by Western blot analyses probing for specific compartmental markers (Fig. 2B). We had previously shown that P34 and P37 are nuclear proteins (47) and that they are also present in the cytoplasm (K. Prohaska, unpublished data). However, we had not determined whether they are present in nucleolar preparations. A fraction of each of the isolated extracts was subjected to Western blot analysis probing for P34 and P37, which demonstrated that both proteins are present in the nucleolus (Fig. 2A). Thus, we could perform our interaction studies with each of the extracts in order to determine the extent to which P34 and P37 associate with 60S ribosomal subunits in each subcellular compartment.

We began our studies by examining the interaction(s) between P34 and P37 and L3, as it is one of the first 60S proteins to be added to the developing ribosome (35). Immune capture experiments specific for 60S subunit protein L3 demonstrated an association between P34 and P37 and L3-containing 60S subunits in whole nuclear, nucleolar, nucleoplasmic, and cytoplasmic extracts (Fig. 3A). This was the first indication that these proteins are involved in multiple stages of 60S subunit biogenesis. As L3 is added to the 90S preribosomal particle, an association with P34 and P37 may suggest that they are also present in this early particle. In order to determine whether this is so, we performed immune capture experiments specific to SS, a 40S subunit protein that is also present in 90S particles. P34 and P37 were found in a complex with SS in whole nuclear and nucleolar, but not nucleoplasmic, extracts (Fig. 4A), demonstrating that they come into the 60S biogenesis pathway at the 90S particle stage. Similar immune capture experiments specific for L11, which enters into the pathway at the late, nucleolar 66S particle, demonstrated that P34 and P37 also associate with this protein in all fractions, further confirming that they associate with 60S subunits throughout the biogenesis pathway. For both L3 and L11 immune capture experiments, we determined that 60S complexes, not just free protein, were isolated, as they contained the appropriate interacting rRNA species as demonstrated in yeast (Fig. 3B and 4B). This not only demonstrated that each antibody precipitated ribosomal complexes, but also that the overall framework of the yeast ribosomal biogenesis pathway is conserved in T. brucei.

As P34 and P37 remain complexed with L3 and L11 in both nuclear and cytoplasmic phases, we wanted to determine whether they are components of the 60S subunit nuclear export complex. Although extensively studied in yeast and mammalian cells (14, 20, 40), this complex, which contains exportin 1 and Nmd3, had not yet been demonstrated in T. brucei. We first confirmed that T. brucei contained an Nmd3 homologue via Western blot analysis using an Nmd3 antibody raised to the yeast protein. The antibody cross-reacted with a band of the appropriate molecular weight (data not shown), confirming that this organism contains an Nmd3 homologue. The Campbell laboratory (UCLA) had already demonstrated that T. brucei contains a homologue to the nuclear export factor exportin 1 (45). Thus, we wished to determine whether T. brucei 60S subunits associate with exportin 1 and Nmd3. Immune capture experiments specific for L3 and L11 demonstrated that both proteins associate with exportin 1 and Nmd3 (Fig. 6A and B). These results demonstrated that T. brucei utilizes a nuclear export complex similar to that described for other organisms.

We have shown previously that P34 and P37 associate with exportin 1 (19). Due to their association with the 60S subunit in both nuclear and cytoplasmic preparations, we hypothesized that P34 and P37 may associate with exportin 1 in the context of the 60S subunit nuclear export complex. We performed P34/P37 immune capture experiments using whole nuclear extracts, followed by Nmd3 Western blot analysis, which demonstrated that these proteins do form a complex (Fig. 7A). Additionally, we showed that P34 and P37 form a complex with exportin 1 and the 60S subunit simultaneously (Fig. 7B), demonstrating that these proteins are components of the 60S nuclear export complex in T. brucei. These results led us to ask whether P34 and P37 might be required for the formation of the 60S nuclear export complex in T. brucei. Thus, we repeated the L3 and L11 immune capture experiments with wild-type and P34/P37 RNAi nuclear extracts, followed by Nmd3 and exportin 1 Western blot analyses (Fig. 8 and 9). We found that 60S subunits failed to associate with either export factor in the absence of P34 and P37, demonstrating that these proteins are not only part of, but are also required for, formation of the 60S nuclear export complex. These were significant findings, as this complex is highly conserved among other organisms. We have shown that much of the complex is conserved in T. brucei; however, P34 and P37, which are trypanosome specific, are essential to the formation of the complex as well.

In addition to their nuclear role(s) in 60S biogenesis, we have also demonstrated that P34 and P37 remain associated with mature 80S ribosomes in the cytoplasm (Fig. 10A) (18). Taken together, the results presented here demonstrate that P34 and P37 play multiple roles in ribosomal biogenesis, including assembly of the 60S export complex in the nucleus and, possibly, that they fulfill a role(s) with respect to mature 60S subunits and 80S ribosomes in the cytoplasm.

Although we have established here that P34 and P37 are required for assembly of the 60S nuclear export complex in T. brucei, further experiments need to be performed in order to confirm whether they are involved in the transport process. In yeast, multiple factors are required for nuclear export of 60S subunits in addition to exportin 1 and Nmd3, possibly due to the large size and hydrophilic nature of the 60S subunit (3, 21). These factors include the Mex67-Mtr2 heterodimer (44) and Arxl (3, 21). We queried the T. brucei database for the Mex67, Mtr2, and Arxl protein sequences. Mex67 and Arxl BLAST searches returned top hits that were hypothetical proteins which, when aligned with the queried sequences using ClustalW, did not show a significant extent of similarity. The Mtr2 BLAST search returned only three proteins, none of which demonstrated similarity to the Mtr2 protein. It may be that P34 and P37 represent trypanosome-specific proteins that fulfill similar roles as the proteins that have been identified in yeast, although we must consider the possibility that these proteins
play an indirect role in this process. Future experiments will focus on determining whether P34 and P37 accompany the 60S subunit during transit to the cytoplasm.

Another possibility that we must consider is that P34 and P37 also serve a role in 5S rRNA localization and incorporation into the maturing 60S subunit within the nucleus. Our laboratory has previously demonstrated that P34 and P37 associate with 5S rRNA (33) and that, in their absence, 5S rRNA levels decrease drastically (18). It is possible that, in the absence of P34 and P37, 5S rRNA does not incorporate into the maturing 60S subunit, leading to the inability of 60S to be recognized as competent for nuclear export. This might ensure that incorrectly formed subunits are not exported to the cytoplasm. An example of such a surveillance mechanism was found in yeast, where 60S subunits containing mutant 5S rRNA species accumulated in the nucleoplasm (7). Further experiments are being performed in order to more precisely define the role(s) of P34 and P37 in 5S rRNA localization and incorporation into 60S subunits. This will determine whether they function in the classical L5-5S pathway or whether they represent a distinct pathway specific to trypanosomes.

The fact that P34 and P37 remain associated with mature 80S ribosomes suggests that they play a role(s) in the cytoplasmic phase of ribosomal biogenesis and/or translation initiation. Polysomal profiles performed on P34/P37 RNAi cells have demonstrated an increase in free 60S subunits, a decrease in 80S ribosomes, and a decrease in total cellular translation (18). These results showed striking similarity to those obtained with yeast strains containing mutant L10 protein (9). The cause of the yeast phenotype was determined to be a defect in subunit joining, indicating that P34 and P37 may fulfill a similar cytoplasmic role. Current work in our laboratory is focused on determining whether P34 and P37 are involved in subunit joining, which could account for the decrease in translation in their absence.

The requirement for trypanosome-specific proteins in a highly conserved process such as ribosomal biogenesis may be related to the life cycle of *T. brucei* and the requirement of the parasite to adapt to two separate host environments. It has been shown that protein synthesis and ribosomal biogenesis differ between procyclic and bloodstream-form trypanosomes (2, 4, 38). Thus, these developmentally regulated processes likely involve factors that are similarly regulated themselves. The levels of P34 and P37 proteins are differentially regulated throughout the life cycle of *T. brucei* (47), which may, in part, relate to the differences in ribosomal biogenesis and protein synthesis between the two life cycle stages. Further experiments in bloodstream-form cells are required to address this possibility. Finally, the results presented in the current work have created the foundation for studying the role(s) of P34 and P37 in ribosomal biogenesis in *T. brucei*. The trypanosome-specific nature of these proteins and their requirement for an essential process may render them targets for future chemotherapeutic interventions in treating African trypanosomiasis.

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