

Trypanosome MTR4 is involved in rRNA processing

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ABSTRACT

The yeast putative RNA helicase Mtr4p is implicated in exosome-mediated RNA quality control in the nucleus, interacts with the exosome, and is found in the 'TRAMP' complex with a yeast nuclear poly(A) polymerase (Trf4p/Pap2p or Trf5p) and a putative RNA-binding protein, Air1p or Air2p. Depletion of the *Trypanosoma brucei* MTR4-like protein TbmMTR4 caused growth arrest and defects in 5.8S rRNA processing similar to those seen after depletion of the exosome. TbnNPAPL, a nuclear protein which is a putative homolog of Trf4p/Pap2p, was required for normal cell growth. Depletion of MTR4 resulted in the accumulation of polyadenylated rRNA precursors, while depletion of TbnNPAPL had little effect. These results suggest that polyadenylation-dependent nuclear rRNA quality control is conserved in eukaryotic evolution. In contrast, there was no evidence for a trypanosome TRAMP complex since no stable interactions between TbmMTR4 and the exosome, TbnNPAPL or RNA-binding proteins were detected.

INTRODUCTION

The exosome is an exoribonucleolytic complex formed by 10–11 proven or predicted RNases, and a variety of other accessory proteins (1). The exosome digests many different substrates in the 3'-to-5' direction. In the nucleus, it is responsible for trimming rRNAs and other stable RNAs in order to create the mature forms, and also for degrading aberrant RNAs of all types (2). In the cytoplasm, a main function seems to be the degradation of mRNAs (3). Isolated complexes have very low activity, so it is likely that accessory factors are required to stimulate the exosome to act appropriately on its diverse substrates (2). These accessory factors should assist the exosome in its function but probably also in the recognition and presentation of the substrate. The molecular basis for the specificity of exosome activity, and the nature and mechanism of action of the accessory factors is however in most cases poorly understood.

Candidate factors might include RNA-binding proteins that recruit the exosome to the RNA, activators and regulators of its activity and helicases (2).

RNA helicases are enzymes that unwind folded RNA molecules in an energy-dependent fashion (4). They are able not only to modify RNA–RNA interactions, but also to affect the interaction of RNA with proteins and DNA. Most RNA helicases can be grouped into two different superfamilies: Sfl and Sf2. Sf2 includes the two most predominant families of helicases, DEAD-box and related DEAH/DEXH-box families. The names refer to one of the conserved motifs found in these proteins (5). Helicases belonging to these groups are found throughout evolution and share several features and domains. RNA helicases are associated with every process involving RNA molecules, including transcription, splicing, editing, ribosome biogenesis, RNA export, translation and RNA turnover (4).

In yeast, two putative RNA helicases, Mtr4p and Ski2p, are known cofactors for exosome activity. While Ski2p is found in the cytoplasm and assists the exosome in mRNA degradation (3), Mtr4p is located in the nucleus (6) and is essential for cell viability (7). Mtr4p participates in the biosynthesis of rRNA in the nucleus of yeast. In *Saccharomyces cerevisiae*, as in other eukaryotes, a large polycistronic rRNA precursor is processed to smaller rRNA intermediates by endonucleolytic cleavage. Final trimming of the 3' ends to their mature forms involves the function of the exosome (1,8). After depletion of yeast Mtr4p, 3' trimming of the 7S precursor to the mature 5.8S rRNA was affected, as was degradation of the 5' external transcribed spacer; since mutations in several exosome subunits had the same effect, it seems probable that Mtr4 is an exosome cofactor. Mtr4 depletion also resulted in accumulation of polyadenylated RNA molecules in the nucleolus (6). The reason for this effect was clarified recently upon discovery of the TRAMP complex, which contains Mtr4p, a non-canonical poly(A) polymerase, either Trf4p or Trf5p, and one of two RNA-binding proteins Air1p and Air2p (9–11). *In vitro*, the yeast TRAMP complex (purified via TAP-Mtr4p) polyadenylates substrates, and the reaction is antagonized by the exosome. *In vivo*, yeast lacking the exosome component Rrp6 showed slower growth and accumulation of

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defective stable RNAs, including rRNA, snRNAs and tRNA; in many cases these were shown to be polyadenylated (9–14). Oligoadenylation of rRNA molecules has been observed in a fission yeast (15), *Candida albicans* (16) and human cells (17). Short A tails have also been found on nuclear truncated mRNAs in human cells, and these species again accumulated upon exosome depletion (18). The current model is that after polyadenylation by Trf4p, which is Air2p-dependent, the target RNAs are degraded by the exosome in an Mtr4p-dependent manner (2). This is analogous to the pathway for mRNA degradation in bacteria, where addition of short poly(A) tails to the 3' end of the transcripts targets them for destruction (19). The mechanism by which the TRAMP complex recognizes aberrant RNAs is however unknown.

The protozoan parasite *Trypanosoma brucei* causes sleeping sickness in humans and Nagana in cattle. It is transmitted from one mammalian host to another by Tsetse flies. Trypanosomatids diverged very early in evolution (20), and their RNA metabolism differs markedly from that of other eukaryotes. Some examples of this are the high levels of editing that take place in the mitochondria of the parasites (21), transcription of long polycistronic units and generation of mature mRNAs by coupled *trans* splicing and polyadenylation (22). On the other hand, several features of RNA metabolism common to other eukaryotes are found: the parasites possess the exonucleases required for 5'-to-3' mRNA degradation (23) and a functional exosome (24,25). The *T. brucei* exosome has 11 subunits and is found in both the nucleus and the cytoplasm. So far, no difference in the complexes present in the two compartments has been found (26) and no information on trypanosome exosome regulation and specificity is available. The presence of polyadenylated ribosomal RNA in *Leishmania* (27) suggests that nuclear quality control may operate as in other eukaryotes.

Here we report the identification of a putative Mtr4p homolog in trypanosomes, *TbMTR4*. *TbMTR4* localizes to the nucleus and is required for normal 5.8S rRNA maturation. Depletion of *TbMTR4* causes an increase in rRNA polyadenylation. We propose that *TbMTR4* can also act as a cofactor of the nuclear exosome and participate in quality control in the nucleus of trypanosomatids.

MATERIALS AND METHODS

Cell culture

Procyclic form *T. brucei* stably expressing the tetracycline repressor from plasmids pHD449 (28) or pHD1313 (29), with or without T7 polymerase expression (29), were grown in the presence of 0.5 µg/ml phleomycin. They were used to generate all the cell lines described in this work. Cells with RNAi targeting RRP6 were described previously (24).

Plasmids and constructs

To generate the construct for over-expression of a C-terminal TAP-tagged version of *TbMTR4*, the open

Table 1. Oligonucleotides used in this work

CZ1193	5'-ACTTTGCTGCGTTCTTCAAC-3'
CZ1427	5'-GTTTTTATATTCGACACTG-3'
CZ1584	5'-TTGAATCGCATTGAGCACCTGCTTTTTTTTTT TTTTTTTTTVNN-3'
CZ1585	5'-TTGAATCGCATTGAGCACCTGC-3'
CZ2423	5'-TCCGGATCC CAGGTACAGCGACGACG-3'
CZ2424	5'-GAGAAGCTTATGCAGGATGACATACCC-3'
CZ2461	5'-TCCGTTAACCAGGTACAGCGACGACG-3'
CZ2555	5'-AGACTAGAACCGTTGCAGAGACCCC-3'
CZ2556	5'-AGAGCGGCCGACGCGGAAAAGTGGGGTGC-3'
CZ2558	5'-AGACTCGAGATGAAAGATCGGTCGGCC-3'
CZ2559	5'-AGAGGGCCCCCAACTGACGGGGCAGG-3'
CZ2580	5'-GCGTGAGGCTATCTGC-3'
CZ2581	5'-CCTTTGGCACAAACGTCACCACGG-3'
CZ2604	5'-GAGAAGATCTGCATGCATGGATGGTGAGCC GTGTG-3'
CZ2605	5'-CGATCGATGTCGACTCGGCACCACATGGGTAC-3'
CZ2708	5'-CACACTGTGTGGTACTAC-3'
CZ2709	5'-TTTTGTCCCTCTCCAACG-3'
CZ2836	5'-GAGAAGATCTGCATGCGATCGGTCGGCCGCTG-3'
CZ2837	5'-CGGAATTCGTCGACTGTCTCCTGGGGGAC-3'

reading frame (ORF) was amplified using primers CZ2424 and CZ2461. (All primers used are listed in Table 1.) The PCR product was digested with HindIII and HpaI and cloned into pHD924 (24) yielding pHD1680. *Trypanosoma brucei* 449 cells were transfected and hygromycin-resistant clones were checked by western blotting for *TbMTR4* TAP expression after induction with tetracycline.

For myc-tagging of *TbMTR4*, the ORF was amplified using primers CZ2424 and CZ2423. The product was cloned into pHD1484 (30), in frame with a myc-tag. The resulting plasmid (pHD1658) was transfected into cells expressing a TAP-tagged version of the *T. brucei* exosome subunit RRP45 (pHD1337) (24).

The construct used for inducible *TbMTR4* and NPAPL RNAi was based on the 'stuffer' strategy described for *T. brucei* (31). A 630 bp fragment of *TbMTR4* was amplified from genomic DNA (28) using primers CZ2604 and CZ2605, ligated to a stuffer fragment and cloned into pHD1146 (25,32) to yield pHD1763. For *TbNPAPL* primers CZ2836 and 2837 were used, giving a 640 bp fragment and plasmid pHD1903. Procyclic 1313-514 *T. brucei* transfectants were selected with 50 µg/ml hygromycin and cloned by limiting dilution. RNAi induction was achieved by adding tetracycline at a concentration of 100 ng/ml.

We *in situ* tagged the *TbNPAPL* ORF by amplifying ~300 bp regions in the 5'UTR of the gene and the 5' region of the ORF. These were then cloned into a suitable vector (33). The 5'UTR region was amplified using primers CZ2556 and CZ2555 while primers CZ2558 and CZ2559 were used for amplification of the 5' region of the ORF. The fragments were cloned upstream and downstream of the BLA/V5 cassette and yielded plasmid pHD1740. Both plasmids were digested with ApaI/NotI and transfected into procyclic 449 *T. brucei* cells bearing plasmid pHD1680 for over-expression of the *TbMTR4* TAP.

Western blotting

Western blotting was done following standard procedures. For detection of V5 tagged proteins, a 1/1000 dilution of the anti-V5 antibody (Invitrogen, Karlsruhe, Germany) was used. PAP antibody (peroxidase anti-peroxidase, Sigma, USA) was used for detection of TAP-tagged *TbMTR4*.

In all the experiments, secondary antibodies for ECL (GE Healthcare) were used and developing was performed with ECLTM western blotting detection system (GE Healthcare, Braunschweig, Germany).

Indirect immunofluorescence

Trypanosomes were washed with phosphate-buffered saline (PBS) and fixed in PBS containing 4% (w/v) paraformaldehyde. After fixation cells were washed and pipetted into each chamber of a four-chambered slide (BD Biosciences, Heidelberg, Germany). Cells were allowed to settle overnight at 4°C, permeabilized with PBS containing 0.2% Triton X-100 and blocked with PBS containing 0.5% gelatine (PBS/G). Fixed-permeabilized cells were incubated for 1 h in an appropriate dilution of the first antibody in PBS/G. Slides were washed twice with PBS and twice with PBS/G, and incubated with the corresponding secondary antibody (Molecular Probes, Invitrogen, Karlsruhe, Germany) diluted 1/500 in PBS/G for 1 h. After washing nucleic acids were stained with DAPI (100 ng/ml). Slides were finally washed, air dried and mounted in 90% glycerol solution in PBS. Images were taken in a Leica DMRXA microscope equipped with a digital camera.

rRNA processing analysis

Total RNA was obtained using peqGOLD TriFast reagent. To study rRNA processing, 2.5 µg of total RNA was denatured in loading buffer for 10 min at 65°C, run on 5% polyacrylamide/urea/TBE gels at 200 V for 3 h and transferred to neutral nylon membranes (24). Hybridizations were done according to standard procedures and the bands detected and quantified using a phosphorimager (Fuji).

Processing of 7S rRNA was analyzed using primer CZ1427, which hybridizes only with the extended form of 5.8S rRNA; primer CZ1193 was used to detect the mature form of 5.8S rRNA. *SRP* was used as a loading control.

Reverse transcription

Total RNA (2 µg) was used as template for reverse transcription reactions using conditions recommended by the manufacturer (Invitrogen, Karlsruhe, Germany). The anchored oligo d(T) primer CZ1584 was used for the cDNA synthesis. The obtained cDNA served as template for the PCR reactions, using oligonucleotide CZ1585, which hybridizes with the anchor region of oligo CZ1584, and specific primers for the ITS7 (CZ2708) and the SR6 (CZ2709) rRNA fragments. For analyzing the levels of tubulin, primers CZ2580 and 2581 were used. The products were analyzed on 2% agarose gels and quantified by densitometry.

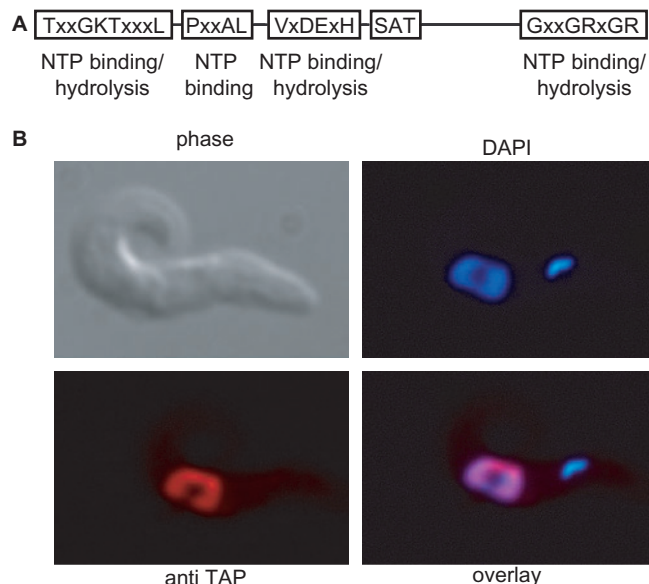


Figure 1. (A) Diagram of the amino-terminal 430 residues of *TbMTR4*, indicating the conserved DEXH helicase motifs and their putative function. (B) Localization of TAP-tagged *TbMTR4*. Over-expressed TAP-tagged *TbMTR4* was detected using anti TAP antibodies. DAPI was used for staining of the nucleus and kinetoplast.

RESULTS

Identification of *TbMTR4*

In order to identify putative cofactors of the exosome, a search for DEXD/H-box proteins was performed on the *T. brucei* genomic database using two putative helicases from yeast, Mtr4p and Ski2p. The predicted protein encoded by locus Tb10.6k15.3220 gave the highest scores (e-209 and e-135, respectively) and no other potential homolog was found. The predicted Tb10.6k15.3220 protein also retrieved Mtr4p and Ski2p as the best matches in the yeast genome database. Trypanosome MTR4 has a predicted size of 107.3 kDa and shares 41.5% identity with yeast Mtr4p. All the characteristic residues and motifs of the DEXH-box proteins are conserved (Figure 1A) in the first half of the protein (aa 10–430). Other highly conserved residues are also found in that region, while at the C terminus (aa 430–950) only 29% identity is shared with yeast Mtr4p. The *Leishmania major* homolog has 73.5% identity with *TbMTR4*. *TbMTR4* was TAP tagged and its location in the cell was analyzed by immunofluorescence microscopy (Figure 1B). The TAP-tagged protein was found in the nucleus, similarly to yeast Mtr4p. It was however less abundant in a region with less DAPI staining, possibly the nucleolus.

Function of *TbMTR4* in rRNA processing

One function of yeast Mtr4p is the stimulation of the nuclear exosome. We first wanted to find out whether trypanosome MTR4 has a similar function. Depletion of *TbMTR4* by RNAi caused growth arrest within 48 h, followed by death (Figure 2A). [Addition of tetracycline to trypanosomes without inducible constructs does not affect

growth, the transcriptome or the proteome (34).] We previously showed that in trypanosomes, the nuclear exosome is involved in the processing of 7S to the mature 5.8S rRNA (24). Analysis of rRNA 24 h after depletion of *TbMTR4* showed that there was a 2-fold increase in the ratio of 7S to 5.8S; for the exosome subunit *TbRRP6* the increase was more than 7-fold (Figure 2B).

We next decided to test if *TbMTR4* interacts with the exosome complex. To analyze this we expressed myc-tagged *TbMTR4* in cells bearing TAP-tagged *TbRRP45* (25). After TAP purification, myc-*TbMTR4* could not be detected co-purifying with the exosome (data not shown), suggesting that if any interaction exists, it is very weak and/or transient. The same result was obtained using TAP-tagged *TbRRP6* (26) as bait.

A *T. brucei* Pap2p homolog is in the nucleus

The yeast TRAMP complex adds a short poly(A) tail to defective RNA molecules, and the tail serves as a tag for MTR4-stimulated exosomal degradation. We therefore wanted to explore the possibility that such an activity also exists in trypanosomes. If poly(A) addition is a prerequisite for *TbMTR4*/exosome-dependent degradation of defective rRNAs, a poly(A) polymerase should exist that adds the A tails to the target RNAs. In yeast, Mtr4p interacts with two homologous poly(A) polymerases, Trf4p/Pap2p and Trf5p. They form different complexes which are involved in poly(A) addition and degradation of defective RNAs in the nucleus (10). In order to study whether similar complexes exist in trypanosomes, we searched for homologs of Trf4p in the *T. brucei* genomic database. Locus Tb927.8.1090 encodes a protein that shares 18% identity with the *S. cerevisiae* Trf4p, and we named it *TbNPAPL* (nuclear-poly(A)-polymerase-like). When we searched the yeast genome database using the putative *TbNPAPL* as query we also found Trf5p as best match. Analysis of the *TbNPAPL* sequence with Pfam predicts a nucleotidyl transferase domain between residues 349 and 428 (Figure 3A).

If *TbNPAPL* participates in the degradation of abnormal nuclear rRNAs, it should be present in the nucleus of the trypanosome. However, no nuclear localization signal could be predicted for the putative protein. To study the location of *TbNPAPL* in trypanosomes, we tagged the protein by integrating a sequence encoding a V5 tag at the 5'-end of the *TbNPAPL* gene. By immunofluorescence analysis using anti V5 antibodies we could detect V5-*TbNPAPL* in the nucleus of the parasites (Figure 3B), agreeing with a putative role of the protein in this intracellular compartment.

To find out whether *TbNPAPL* or other proteins were stably associated with *TbMTR4*, TAP-tagged *TbMTR4* was over-expressed in procyclic *T. brucei*, affinity purified on IgG sepharose and released by TEV protease (see Supplementary Data). All bands apart from the over-expressed *TbMTR4*-TAP were very faint, and the predominant protein identified in all bands was *TbMTR4* (data not shown). Thus it was clear that the over-expressed *TbMTR4* was not stoichiometrically associated in a complex with other proteins. *TbNPAPL* and exosome

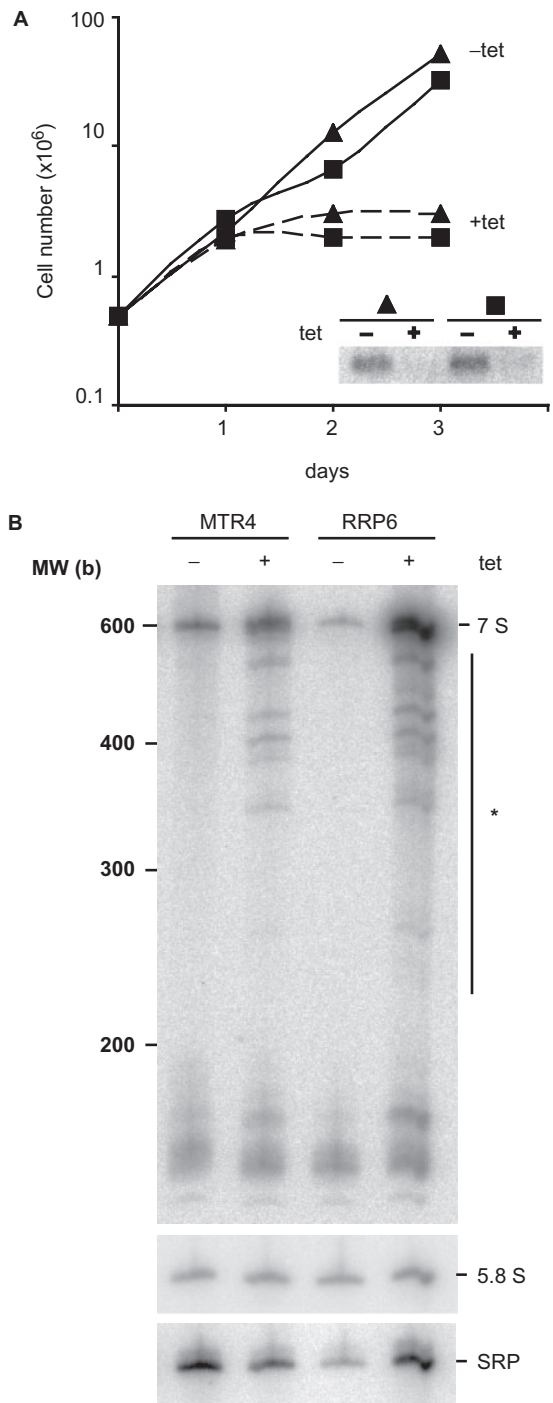


Figure 2. Depletion of *TbMTR4* by inducible RNAi affects cell growth. (A) Trypanosomes expressing a *TbMTR4*-specific dsRNA were grown in the absence (solid line) and presence (dashed line) of 100 ng/ml tetracycline to induce RNAi-mediated depletion. Cultures were followed for 3 days and diluted to 0.5×10^6 cells/ml when required. The inset is a northern blot showing depletion of the *MTR4* mRNA; rRNA staining was similar in all lanes (data not shown). (B) Effect of depletion of *TbMTR4* on 5.8S rRNA maturation *in vivo*. Total RNA was extracted from trypanosomes grown in the absence (-) or presence (+) of tetracycline for 24 h, and separated in polyacrylamide-urea gels. The gels were transferred to nylon membranes and hybridized with probes to detect extended or mature 5.8S rRNA. Mature 5.8S and full-length 7S rRNA are shown, while the incompletely processed species are marked with an asterisk. Cells depleted of the exosome subunit RRP6 were used as the control for incomplete rRNA processing.

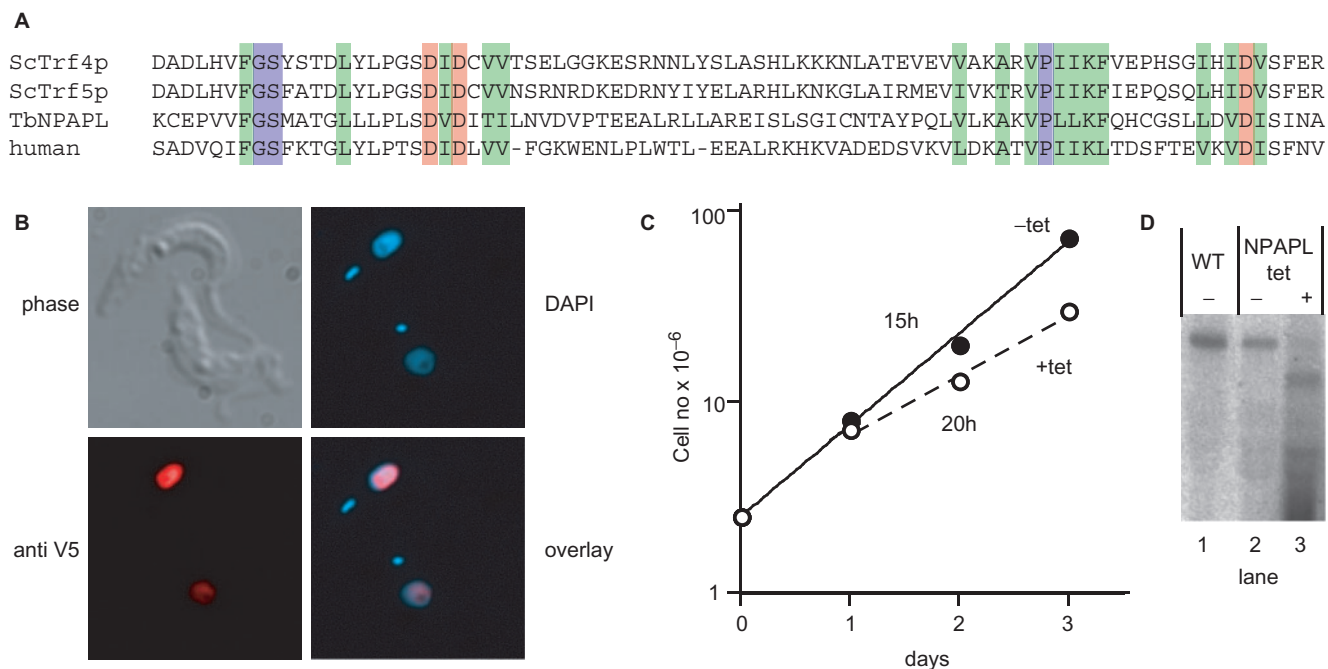


Figure 3. (A) Alignment of the catalytic domains of yeast Trf4p and Trf5p, and the putative human homolog, with the corresponding region of *TbNPAPL*. The green boxes indicate residues conserved among members of the nucleotidyl transferase family (100% similarity), blue and red indicate residues with 100% identity. Catalytic aspartates are shown in red. The human homolog is annotated as DNA polymerase sigma (EC 2.7.7.7) or topoisomerase-related function protein 4-1. (B) Immunofluorescence analysis of cells expressing *TbNPAPL in situ* tagged with the V5 epitope. DAPI: DAPI staining; antiV5: mouse antiV5 antibody with fluorescent anti-mouse secondary antibody. (C) Depletion of *TbNPAPL* mRNA inhibits growth. A cumulative growth curve and a northern blot are shown. Cells containing a tetracycline-inducible *TbNPAPL* RNAi construct were incubated with (open circle, dashed line) or without tetracycline (filled circle, solid line). (D) Northern blot for cells without the RNAi construct (lane 1), and for the *TbNPAPL* RNAi line grown without (lane 2) or with (lane 3) tetracycline.

subunits were not detected. We also expressed a TAP-tagged *TbMTR4*, or the TAP tag alone, in cells expressing V5 *in situ* tagged *TbNPAPL*. V5-*TbNPAPL* was found at similar levels in tandem-affinity purifications of TAP-*TbMTR4* and TAP alone (data not shown), suggesting that it does not form a stable complex with *TbMTR4*. Furthermore, the tandem-affinity purified *TbMTR4* preparation had no detectable polyadenylation activity (see Supplementary Data; data not shown).

We also attempted to generate a trypanosome cell line with inducible RNAi targeting *TbNPAPL*. After several transfections a single line was obtained. These cells grew slower than normal procyclic trypanosomes, which have a doubling time of 10–11 h (compare Figure 3C with Figure 2A) and also had less *TbNPAPL* mRNA than normal cells (Figure 3D), suggesting some leakage of the RNAi construct. After tetracycline addition, the growth rate was further slowed (Figure 3C) but the mRNA was not completely eliminated (Figure 3D).

Roles of *TbMTR4* and *TbNPAPL* in rRNA quality control

In trypanosomes, the rRNA is transcribed as a pre-rRNA which is processed to the individual mature species by endonucleolytic cleavage and 3' trimming (Figure 4A). Unusually, the large subunit RNA is cut into several smaller pieces, which are subsequently trimmed (35). Polyadenylated *Leishmania* rRNAs corresponding to SR6 rRNA and a partially processed precursor including the ITS7 were previously reported (27). To look for

similar polyadenylated rRNAs in *T. brucei*, we made cDNA by priming with oligo d(T) bearing a 5' anchor sequence, then amplified the polyadenylated rRNA species using specific primers (Figure 4B). Both polyadenylated SR6 rRNA and the longer polyadenylated ITS7 intermediate (Figure 4C, lanes 1 and 2) could be detected in trypanosomes. To quantitate the results from different cell lines, we compared the amounts of PCR product—including both the main band and longer species—in serial 10-fold dilutions of the cDNA, using polyadenylated tubulin mRNA as the standard (Figure 4C).

The overall amount of SR6 product was marginally increased in *TbMTR4*-depleted cells (Figure 4C, lanes 3–6), but neither *TbRRP6* RNAi nor *TbNPAPL* RNAi had any effect on the PCR product yield. Similarly, the overall amount of ITS7 product was somewhat decreased in *TbNPAPL* RNAi cells (Figure 4C, lanes 7–10) but unaffected by *TbRRP6* or *TbMTR4* depletion. In no case, however, did the differences exceed 2-fold (a single PCR cycle) so their significance is dubious.

TbMTR4-depletion did, however, have a very clear and reproducible effect on the lengths of both the ITS7 and SR6 PCR products. These were reproducibly longer and more heterogeneous in the *TbMTR4* RNAi cells, especially in the presence of tetracycline (Figure 4C, lane 5). Higher molecular weight species were extended by as much as 100 bp; sequence analysis confirmed that the extensions consisted of poly(A) (data not shown).

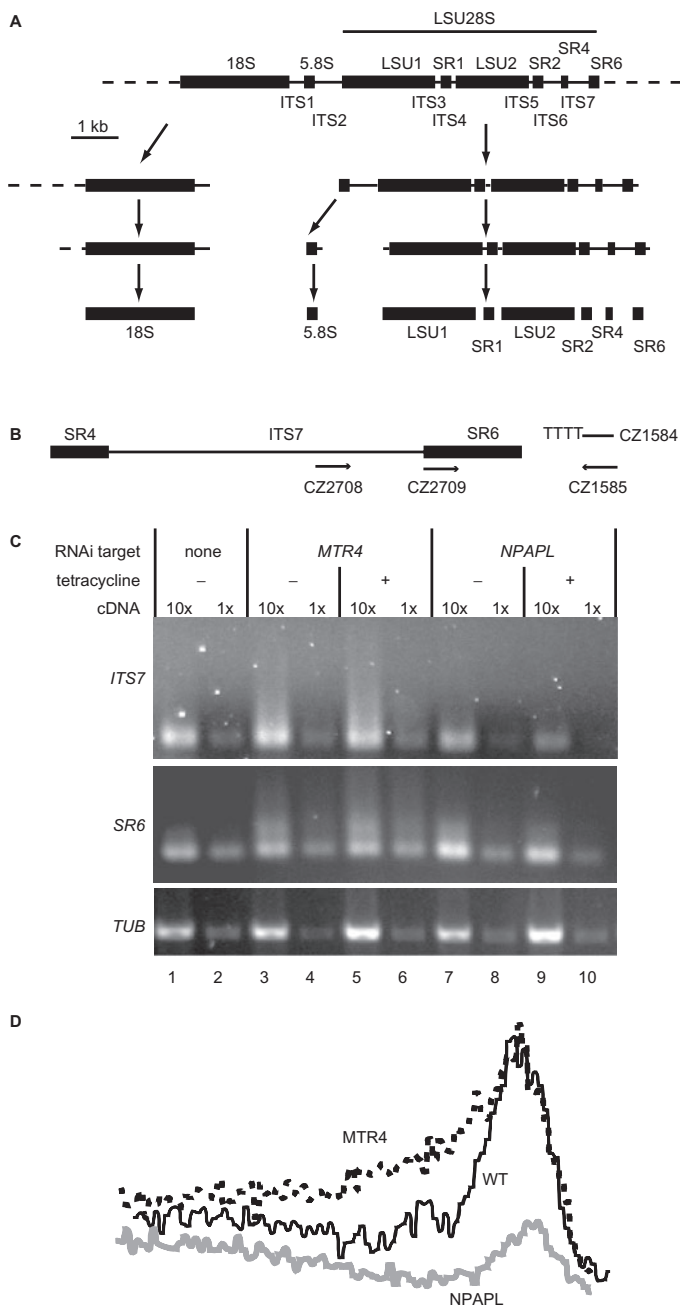


Figure 4. A polyadenylated rRNA precursor accumulates in *TbMTR4* RNAi cells. **(A)** Schematic diagram of the rRNA transcription unit and processing in *T. brucei*. **(B)** Primers used for RT-PCR analysis. **(C)** Total RNA was extracted from trypanosomes growing in the absence (–) or presence (+) of tetracycline and reverse transcribed using the anchored oligo(dT) primer. The cDNA was PCR amplified (25 cycles) with primers specific for ITS7 and SR6 rRNA, and the products separated on a 2% agarose gel. For lanes 1, 3, 5, 7 and 9, undiluted cDNA was used; lanes 2, 4, 6, 8 and 10 are 10-fold dilutions. Quantitation of the 10× lanes revealed that the *TbNPAPL* line had a 30% reduction in signal in the absence of tetracycline (lanes 7 and 8) and a 50% reduction in the presence of tetracycline (lanes 9 and 10). **(D)** The relative signals for ITS7 were quantified by scanning across the gel. The wild-type pattern (lane 1) is shown as a solid line, the *TbMTR4*-depleted pattern (lane 5) by a dashed line and the *TbNPAPL*-depleted pattern (lane 9) by the gray solid line. When the *TbNPAPL* profile was stretched vertically in the graphics program, the overall pattern was indistinguishable from wild type.

Densitometric scanning of the gel (Figure 4D, from Figure 4C, ITS7, lanes 1, 5 and 9) confirmed the visual impression that the poly(A) tails were longer in the *MTR4*-depleted cells. Despite the decrease in yield, no significant effect of *TbNPAPL* depletion on the gel profile was observed.

These results suggest that *TbMTR4* is involved in a nuclear RNA quality-control mechanism that involves polyadenylation of RNA molecules prior to degradation.

DISCUSSION

In this study, we identified in *T. brucei* the putative ATP-dependent RNA helicase *TbMTR4*, a functional homolog of yeast Mtr4p. Our results indicate that although *TbMTR4* is concentrated mainly in the nucleoplasm, rather than the nucleolus, it is involved in the maturation of rRNA. Depletion of *TbMTR4* resulted in accumulation of unprocessed and partially processed 7S rRNA, indicating that *TbMTR4* participates in the maturation of the rRNA precursor to its functional form. In contrast to observations in yeast (7), no decrease in mature 5.8S rRNA was detected. The same phenotype was observed after depletion of exosome subunits in *T. brucei* (24), which would be consistent with a requirement for *TbMTR4* in exosome-mediated rRNA trimming.

Previous studies in yeast have identified Mtr4p as a component of two related complexes, TRAMP4 and TRAMP5 (2), which are located in the nucleus and may have overlapping functions. Both complexes include a poly(A) polymerase (either Trf4p or Trf5p) and an RNA-binding protein (Air2p or Air1p). Trf4p and Trf5p belong to a family of non-canonical poly(A) polymerases which possess the characteristic domains observed in nucleotidyl transferases (as in yeast Pap1p) but lack an RNA-binding domain (36–39). These enzymes need to associate with RNA-binding proteins in order to bind and recognize their substrates, so assembly into larger complexes is expected to be a prerequisite for functionality (2). The possible human homolog of Trf4p (Figure 3) shows 37% identity with conserved functional residues; a role of this protein in quality control has not yet been confirmed. In trypanosomes, we identified *TbNPAPL* as a putative Trf4/5p homolog. Two possible *T. brucei* homologs of Air1p and Air2p (Tb 11.02.4470 and 11.01.1270, respectively) show sequence similarity only within the zinc-knuckle domain, so the status of these candidates is tenuous. Similarly, the best candidate homolog in the human genome (Q8N3Z6) shows only around 20% identity, limited to the Zn-knuckle.

To look for a TRAMP complex in trypanosomes, we used immunoprecipitation of V5-tagged versions and one- or two-step affinity purification. We did not find any stable associations between *TbMTR4*, *TbNPAPL* or the exosome, and the two possible Air1/Air2 homologs were not detected by mass spectrometry in *TbMTR4* preparations. Thus if trypanosomes do contain a TRAMP complex, the interactions are not sufficiently stable to survive purification by the methods we used. Although there is evidence for polyadenylation-dependent nuclear

quality control in human cells (18), we have found no reports of the existence of a TRAMP complex in metazoans. Human Mtr4p (KIAA0052) interacts with the Rrp6p and Rrp47p (*TbEAP3*) homologs via MPP6, a 540-residue protein (40,41). It is not clear whether trypanosomes have MPP6 or not—the best match is a 178-residue protein which consists mainly of a guanylate kinase domain and is 30% identical to the C-terminal 160 residues of MPP6.

We unambiguously detected polyadenylated versions of the small rRNA, SR6 and of an rRNA precursor containing an internal transcribed spacer (ITS7), which should be targeted for destruction. We cannot be certain whether *TbNPAPL* is required for this polyadenylation, since depletion of this putative poly(A) polymerase had no clear effects on levels of two polyadenylated rRNAs—the small SR6 RNA and a precursor, ITS7. Depletion of *TbMTR4*, in contrast, resulted in a clear increase in SR6 and ITS7 rRNA polyadenylation. This shows that addition of poly(A) tails to rRNA does not require *TbMTR4*, but that their removal is stimulated by *TbMTR4*. This supports a model in which *TbMTR4* promotes degradation of the poly(A) rRNAs—perhaps by the exosome, as in yeast (13,42,43). Thus, although there is no evidence for the existence of a TRAMP complex in trypanosomes, our results are consistent with the existence of a polyadenylation-dependent nuclear quality-control pathway involving *TbMTR4*.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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