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The exosome of *Trypanosoma brucei*

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The yeast exosome is a complex of at least 10 essential 3′–5′ riboexonucleases which is involved in 3′-processing of many RNA species. An exosome-like complex has been found or predicted to exist in other eukaryotes but not in *Escherichia coli*. The unicellular parasite *Trypanosoma brucei* diverged very early in eukaryotic evolution. We show here that *T.brucei* contains at least eight exosome subunit homologs, but only a subset of these associate in a complex. Accordingly, the *T.brucei* exosome is smaller than that of yeast. Both free and complex-associated homologs are essential for cell viability and are involved in 5.8S rRNA maturation. We suggest that the exosome was present in primitive eukaryotes, and became increasingly complex during subsequent evolution.

Keywords: exoribonucleases/exosome/rRNA processing/*Trypanosoma brucei*

Introduction

The African trypanosome *Trypanosoma brucei* causes sleeping sickness in humans and nagana disease in cattle in sub-Saharan Africa. These parasites are transmitted from one mammal to the next by tsetse flies and undergo drastic morphological and biochemical changes in response to the different environments within the insect (procyclic stage) and mammalian (bloodstream stage) hosts (Vickerman, 1985). Trypanosomatid RNA metabolism diverges in several respects from normal eukaryotic paradigms. The two most dramatic examples occur in the editing of mitochondrial RNAs (Estévez and Simpson, 1999), and the processing of cytosolic mRNA. Mature mRNA molecules are generated from long polycistronic precursors via coupled *trans* splicing and polyadenylation (Ullu *et al*., 1996) instead of being individually transcribed. The inability to control transcription of individual mRNAs means that regulation of gene expression, which is essential for survival in different environments, has to be exerted almost exclusively at the post-transcriptional level through control of mRNA degradation and translation (Hotz *et al*., 1997; Di Noia *et al*., 2000).

Ribosomal RNA processing in *T.brucei* is also unusual; the large subunit rRNA is cleaved at multiple sites to yield seven stable RNA fragments, two internal 5′ external transcribed spacer region (5′-ETS) cleavages occur and a single 5.8S rRNA species is produced (White *et al*., 1986; Campbell *et al*., 1987; Hartshorne and Toyofuku, 1999). Until now, nothing was known about the enzymes involved in either mRNA degradation or rRNA processing in trypanosomes.

The turnover and processing of mRNA and rRNA molecules in prokaryotes and eukaryotes involves 3′–5′ exonucleolytic digestion events. In yeast, a 300–400 kDa complex, the exosome, is responsible for many of these reactions (van Hoof and Parker, 1999; Mitchell and Tollervey, 2000), and is present in both the cytosol and the nucleus. The cytoplasmic complex contains at least 10 different components, Rrp4p, Rrp40p–Rrp46p, Csl4p and Mtr3p, while the nuclear complex has an additional subunit, Rrp6p. All these proteins show 3′–5′ exonucleolytic activity or are predicted to be 3′–5′ exonucleases (Allmang *et al*., 1999b). Six of them are related to *Escherichia coli* RNase PH (Rrp41p, Rrp42p, Rrp43p, Rrp45p, Rrp46p and Mtr3p) and three contain an S1 RNA binding domain (Rrp4p, Rrp40p and Csl4p). Rrp44p contains an RNase II-family signature, and Rrp6p is related to RNase D (Allmang *et al*., 1999b). All these components are essential for cell viability, with the exception of Rrp6p where depletion confers a temperature-lethal phenotype (Allmang *et al*., 1999b). Human cells also contain cytosolic and nuclear exosome-like complexes, with sizes ranging between 250 and 700 kDa (Mitchell *et al*., 1997; Brouwer *et al*., 2001). In *Arabidopsis thaliana* the Rrp41p homolog *At*Rrp41p resides in a ~500 kDa complex (Chekanova *et al*., 2000). Despite the conserved features and components, the exosomes of different eukaryotes are clearly not identical. Analysis of the *Caenorhabditis elegans* genome indicates that only three proteins are related to RNase PH, and there is no clear homolog for Rrp4p (van Hoof and Parker, 1999). The six human RNase PH-like genes do not show orthologous pairs with the six yeast ones, and there are no clear homologs for Rrp43p or Mtr3p (Mitchell *et al*., 1997). Analysis of genome organization in the Archaea suggests that there might be an exosome in these organisms (Koonin *et al*., 2001), but no exosome-like complex has been detected in *E.coli* (Deutscher, 1993).

In this paper we present evidence for the existence of a simple exosome complex in *T.brucei* which is involved in the maturation of 5.8S rRNA.

Results

Identification of homologous proteins of the yeast exosome components in T.brucei

A search for exosome components in the unfinished *T.brucei* genome sequencing project revealed that there are homologs for the yeast proteins Csl4p, Rrp4p, Rrp6p,

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Table I. Homologs of the yeast exosome components found in the *T.brucei* partially sequenced genome

T.brucei protein (kDa)	DDBJ/EMBL/GenBank accession No.	*S.cerevisiae* homolog	*C.elegans* homolog	Motif
*Tb*RRP4 (32.7)	AJ308995	Rrp4p, 39%	none	S1 RNA binding
*Tb*RRP6 (78.6)	AJ309000	Rrp6p, 35%	C14A4.4, 32%	RNase D
*Tb*RRP40 (32.0)	AJ308996	Rrp40p, 33%	F59C6.4, 28%	S1 RNA binding
*Tb*RRP41A (27.6)	AJ308997	Rrp41p, 27%	B0564.1, 28%	RNase PH
*Tb*RRP41B (28.0)	AJ309001	Rrp41p, 25%	C14A4.5, 28%	RNase PH
*Tb*RRP44 (109.0)	AJ308998	Rrp44p, 41%	C04G2.6, 40%	RNase II
*Tb*RRP45 (38.9)	AJ308999	Rrp45p, 30%	F37C12.13, 27%	RNase PH
*Tb*CSL4 (32.6)	AJ308994	Csl4p, 28%	K06A9.1B, 26%	S1 RNA binding

The *T.brucei* protein sequences indicated were used to search the *S.cerevisiae* and *C.elegans* protein databases using the BLASTP program. The percentage of identity is presented in each case. The molecular masses of the predicted *T.brucei* peptides are also shown in parentheses.

Rrp40p, Rrp44p and Rrp45p and two homologs for Rrp41p (Table I). Since in most of the cases the sequences were not complete, we cloned genomic fragments containing the *T.brucei* genes and then sequenced them to identify the full-length open reading frames (ORFs) (trypanosome genes almost never contain introns). The homologs were defined by several criteria. First, they were the best hits in the trypanosome databases. Secondly, when the complete sequence was re-scanned against DDBJ/EMBL/GenBank and in individual genome databases, the putative homologs again showed the maximal identity and E scores. Thirdly, the typical motifs found in exosome components or putative exosome components in other organisms were identified (Allmang *et al*., 1999b; van Hoof and Parker, 1999). In the case of *Tb*CSL4, the BLAST scores were far too low to allow definitive identification and we only recognized this protein after we had found it in the purified *T.brucei* exosome (see below). The low scores for *Tb*CSL4 are due mainly to the fact that the N-terminal domain is not conserved in Csl4p homologs although the functional motifs are present (van Hoof *et al*., 2000). We found no other RNase PH-like proteins that could be the trypanosome homologs for Rrp42p, Rrp43p, Rrp46p or Mtr3p. It is possible, however, that they are present; the *T.brucei* genome sequence is not yet complete and partial sequences with low conservation (like *Tb*CSL4) could be unidentifiable.

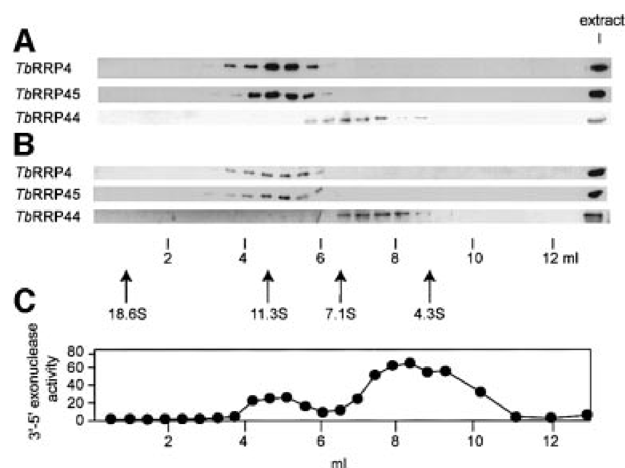

Fig. 1. *Tb*RRP4 and *Tb*RRP45, but not *Tb*RRP44, are found in a complex. (**A**) Cytosolic and (**B**) nuclear extracts from procyclic *T.brucei* were fractionated through 10–30% glycerol gradients. Aliquots of each fraction and original extracts were subjected to SDS–PAGE and immunoblotting analysis using antibodies against *Tb*RRP4, *Tb*RRP45 or *Tb*RRP44 (A and B) or assayed for 3′–5′ exonuclease activity (**C**). The exonuclease activity is expressed as the percentage of substrate digested (see Materials and methods). The sedimentation coefficients of marker proteins processed in parallel are indicated.

*Tb*RRP4 and *Tb*RRP45 are present in an 11S complex

To analyze whether there is an exosome-like complex in *T.brucei*, polyclonal antibodies were raised in rabbits against *Tb*RRP4, *Tb*RRP44 and *Tb*RRP45. Subcellular fractionation experiments showed that *Tb*RRP4, *Tb*RRP44 and *Tb*RRP45 were in both the cytosol and the nucleus. They were present at similar levels in both bloodstream and procyclic cells so were not developmentally regulated (data not shown). We fractionated cytosolic and nuclear extracts from procyclic *T.brucei* by glycerol density gradient centrifugation, and individual fractions were tested by immunoblotting analysis and also for 3′–5′ riboexonuclease activity (Figure 1). *Tb*RRP4 and *Tb*RRP45 co-sedimented in a complex with an estimated sedimentation coefficient of ~11S (240 kDa). In contrast, *Tb*RRP44 sedimented at ~6S (110 kDa), the expected size of a monomeric form of the protein. No free *Tb*RRP4 or *Tb*RRP45 species was detected. When we measured 3′–5′ riboexonuclease activity in the cytosolic fractions, we observed two peaks, the less active of which sedimented with the complex at ~11S. The sedimentation behavior of *Tb*RRP4, *Tb*RRP44 and *Tb*RRP45 after fractionation of cytosolic and nuclear extracts from bloodstream trypanosomes resembled that of procyclic cells (data not shown). These results suggest that there is an exosome-like complex in both the cytosol and the nucleus of *T.brucei*. The *T.brucei* exosome appeared to be considerably smaller than the yeast exosome, indicating that it might contain fewer components.

*Tb*RRP4 is a processive, 3′–5′ riboexonuclease that does not complement the yeast *rrp4-1* allele

A His_6-*Tb*RRP4 fusion protein was expressed in *E.coli* and purified to homogeneity, in order to test whether it had the exonucleolytic activity expected of a component of the

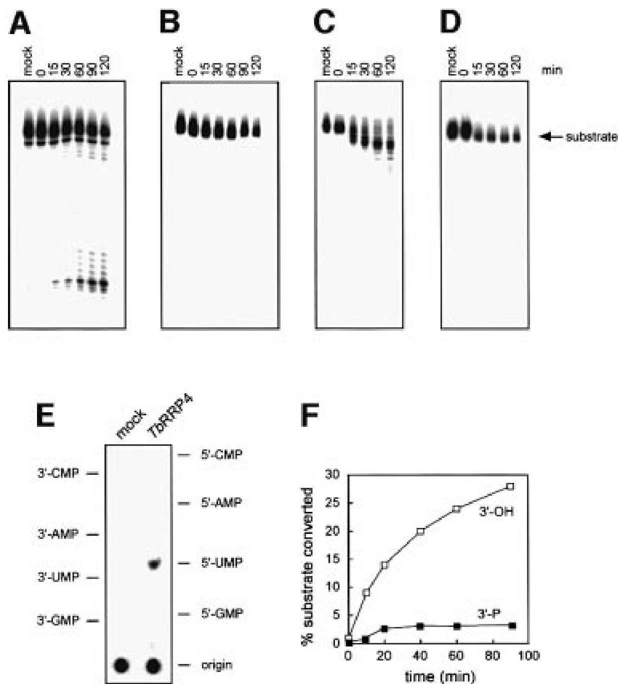

Fig. 2. Recombinant His$_6$-*Tb*RRP4 and the purified *T.brucei* exosome show 3′–5′ exoribonuclease activity *in vitro*. Purified His$_6$-*Tb*RRP4 was incubated in the presence of a 5′-labeled (**A**) or 3′-labeled RNA substrate (**B**) for the times indicated, and the reactions electrophoresed in PAGE–urea gels. In the mock lane the RNA substrate was incubated for 120 min using the same reaction conditions, without recombinant His$_6$-*Tb*RRP4. Exonuclease activity was assayed for the purified exosome complex under the same conditions using the 5′- (**C**) or 3′-labeled (**D**) substrate. (**E**) Separation of the reaction products by TLC. The RNA substrate was incubated in the absence (mock) or in the presence of His$_6$-*Tb*RRP4. The migration of 5′- and 3′-nucleoside monophosphates is also indicated. (**F**) Exonuclease activity of His$_6$-*Tb*RRP4 measured in the presence of the RNA substrate without (open squares) or with (filled squares) a phosphate group at the 3′ end.

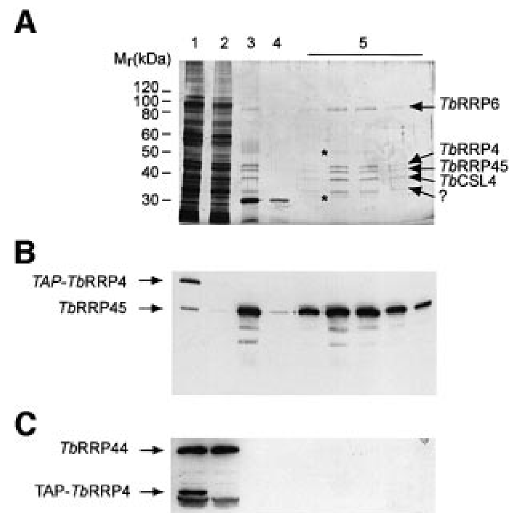

Fig. 3. Purification of the *T.brucei* exosome. The expression of TAP-tagged *Tb*RRP4 was induced by the addition of tetracycline to the culture medium for 48 h, and the associated proteins purified using the TAP method. (**A**) Aliquots from each step (or the whole EGTA-eluate fractions) were analyzed by SDS–PAGE and silver staining. Lane 1, S100 extract; lane 2, IgG chromatography flow-through; lane 3, TEV eluate; lane 4, calmodulin chromatography flow-through; lane 5, EGTA eluate (5 fractions). (**B** and **C**) Immunoblotting analyses of the TAP fractions using *Tb*RRP45 (B) or *Tb*RRP44 (C) antisera. The protein marker is the 10 kDa protein ladder (Gibco BRL).

exosome. Incubation of the purified protein with a 5′-labeled substrate resulted in the accumulation of a shorter oligonucleotide product (Figure 2A), whereas incubation with a 3′-labeled substrate led to a loss of signal of the substrate, with no detectable intermediates (Figure 2B). Thin-layer chromatography (TLC) analysis of the reaction products (Figure 2E) revealed that the enzyme generated nucleoside 5′-monophosphates. The exonuclease was unable to digest a substrate blocked at the 3′ end with a phosphate group (Figure 2F) and was inhibited by EDTA (data not shown). These results are indicative of a hydrolytic, processive 3′–5′ exonuclease that degrades RNA molecules with a 3′ hydroxyl group. Yeast Rrp4p is also a hydrolytic 3′–5′ exonuclease but it has a distributive mode of action.

To test whether *TbRRP4* was able to complement an *rrp4-1* allele, the gene coding for *Tb*RRP4 was cloned and expressed in yeast. The growth of the yeast strain P58 (Mitchell *et al.*, 1997), transformed with plasmids containing either the *TbRRP4* gene, the yeast *RRP4* gene or the cloning vector alone (p415GAL) was compared in Sgal–Leu medium plates at 25 or 37°C (see Materials and methods). The yeast *RRP4* gene was included as a positive control. Neither the cloning vector alone nor the *TbRRP4* gene was able to complement the *rrp4-1* allele (data not shown).

Purification of the T.brucei exosome and identification of the components

To analyze the composition of the *T.brucei* exosome, we purified the complex from procyclic cells using the tandem affinity purification (TAP) method (Rigaut *et al.*, 1999). The TAP tag consists of a calmodulin binding peptide, a tobacco etch virus (TEV) protease cleavage site and two IgG-binding motifs of protein A. We attached the TAP sequence to the C-terminus of *Tb*RRP4, which was known to be present in the 11S complex. TAP-*Tb*RRP4 was expressed in trypanosomes under the control of a tetracycline-inducible *EP1* promoter (see Materials and methods). To check that the TAP-tagged protein was functional, we deleted both of the endogenous (unmodified) *RRP4* genes from the TAP-*Tb*RRP4-expressing cell line (data not shown). The resulting parasites grew normally in the presence of tetracycline (when the inducible TAP-*Tb*RRP4 was expressed), and stopped growing in the absence of TAP-*Tb*RRP4 expression, showing that *Tb*RRP4 is essential for growth and that the tagged protein was functional.

The purification of the *T.brucei* exosome from a cytosolic extract is shown in Figure 3A. The first step involved binding the protein A component of the tag to IgG–Sepharose. The complex was released from the matrix by addition of the TEV protease, then bound to calmodulin beads in the presence of calcium, via the calmodulin binding peptide. After washing, the complex was eluted with EGTA. The eluted complex was analyzed by SDS–PAGE and silver staining (Figure 3A). Five polypeptides were present in apparently equimolar

amounts. Their electrophoretic mobilities corresponded to 90, 45, 42, 38 and 32 kDa (Figure 3A, arrows). In addition, two more, less abundant bands were also seen with apparent molecular weights of 52 and 30 kDa (Figure 3A, asterisks). The 90 kDa band could be resolved as a doublet (90 and 88 kDa) in low-percentage acrylamide gels (data not shown). Both bands correspond to the same protein (see below). Coomassie Blue staining gave similar results except that the 52 kDa band appeared to be more abundant (data not shown). The specificity of the TAP method was monitored by generating a cell line expressing only the TAP tag (see Materials and methods). In this case no bands were detected after purification of the TAP tag peptide (data not shown).

The cell line used for the expression of TAP-*Tb*RRP4 and the purification of the exosome still had one *TbRRP4* allele (see Materials and methods). From western blotting analysis it became clear that the expression of TAP-*Tb*RRP4 led to depletion of the endogenous *Tb*RRP4 (data not shown), suggesting that perhaps free *Tb*RRP4 is unstable. The same complex composition and stoichiometry were obtained upon expression of TAP-*Tb*RRP4 at a concentration of 1 ng/ml of tetracycline, in which both the endogenous and TAP-*Tb*RRP4 proteins are expressed at similar levels, or at 100 ng/ml tetracycline, when only TAP-*Tb*RRP4 could be detected (data not shown).

Since both *Tb*RRP4 and *Tb*RRP45 co-sediment in an 11S complex (Figure 1), we expected *Tb*RRP45 to copurify with TAP-*Tb*RRP4. Indeed, *Tb*RRP45 was retained in the IgG–Sepharose and calmodulin matrices and was enriched in the EGTA-eluted complex (Figure 3B). TAP-*Tb*RRP4 was also detected with anti-*Tb*RRP45 antibodies (or with anti-*Tb*RRP44 antibodies, see below), because of the presence of the two IgG binding domains within the TAP tag. In contrast, *Tb*RRP44, which is not present in the 11S complex (Figure 1) did not bind to the IgG beads and could not be detected in the purified exosome complex fractions (Figure 3C). When the TAP purification was carried out in the control cell line expressing only the TAP tag, *Tb*RRP45 was detected only in the IgG flow-through, indicating that this protein binds to *Tb*RRP4 and not to the TAP tag (data not shown).

To identify the individual components of the *T.brucei* exosome, the bands were excised from the gel and subjected to trypsin digestion. The generated peptides were analyzed by MALDI mass spectrometry. The observed peptide masses were compared with those obtained from the virtual tryptic digest of the *T.brucei* proteins listed in Table I (see Materials and methods). This approach led to the unambiguous identification of *Tb*RRP6, *Tb*RRP4, *Tb*RRP45 and *Tb*CSL4, as indicated in Figure 3A. Both peptides corresponding to the 90 kDa doublet gave a peptide pattern corresponding to *Tb*RRP6. The doublet migration might be indicative of partial post-translational modification. The MALDI pattern obtained from the 32 kDa band (Figure 3A, question mark) did not match any of the proteins listed in Table I, or any known *T.brucei* protein sequence available in the databases. We are still attempting to identify this and the 53 and 30 kDa bands (Figure 3A, asterisks).

The purified *T.brucei* exosome was incubated with a 5′-labeled RNA substrate in the same conditions as for recombinant His_6-TbRRP4 (Figure 2C). We observed a simultaneous decrease in the lengths of the whole population of substrate molecules, indicative of a distributive riboexonuclease activity. The incubation of the purified exosome with a 3′-labeled substrate led to a loss of signal of the substrate band (Figure 2D). Again, the products of the reaction were 5′-nucleoside monophosphates (data not shown). This indicates that the *T.brucei* exosome shows a distributive and hydrolytic riboexonuclease activity, as in the case of the yeast exosome (Mitchell *et al*., 1997). The exonuclease activity of the *T.brucei* exosome ceased after a 15 min incubation, which may indicate an inactivation of the complex, or that additional factors might be required for proper activity.

The combined molecular weights of the five stoichiometric bands give a value of 217 kDa, which is roughly in agreement with the molecular weight estimated by glycerol gradient analysis, ~240 kDa. The absence of the 110 kDa *Tb*RRP44 from the purified complex was consistent with the previous results from glycerol gradient centrifugation (Figure 1) and with the small size of the *T.brucei* exosome compared with that of yeast. None of the peptide masses obtained by MALDI analysis matched *Tb*RRP40, *Tb*RRP41A, *Tb*RRP41B or *Tb*RRP44. It was notable that *Tb*RRP6 was present in stoichiometric amounts in the purified cytosolic exosome complex. In yeast and in human cells, Rrp6p and the human homolog PM-Scl100 are present only in the nuclear complex (Allmang *et al*., 1999b).

The results so far indicate that the major *T.brucei* cytosolic exosome species is composed of *Tb*RRP4, *Tb*RRP6, *Tb*RRP45, *Tb*CSL4 and an additional, as yet unidentified, protein. Most *Tb*RRP44 is not associated with the exosome.

Effect on cell growth of the inhibition of the expression of individual exosome components by RNA interference

All components of the yeast exosome, except for Rrp6p, are essential for viability (Allmang *et al*., 1999b) and are involved in the processing and degradation of many RNAs (reviewed in van Hoof and Parker, 1999). In order to analyze the functions of the proteins listed in Table I we generated conditional mutants. For each gene, we created a trypanosome line that expressed a double-stranded RNA corresponding to the first 500–800 nucleotides, under the control of the tetracycline-inducible promoter (see Materials and methods). Expression of double-stranded RNA in trypanosomes, also known as RNA interference (RNAi), results in depletion of the corresponding mRNA (Shi *et al*., 2000; Wang *et al*., 2000). The effect of RNAi was checked by northern blotting for every mRNA, and also by immunoblotting for the proteins for which antisera were available. For every cell line illustrated, the target mRNA virtually disappeared after 48 h in the presence of tetracycline (not shown). The proteins analyzed dropped to ~10% of the wild-type levels after 24 or 48 h of induction (data not shown). These reductions are in agreement with studies of several other trypanosome genes (Shi *et al*., 2000; Wang *et al*., 2000).

The effect of depletion of each of the homologs on cell growth is shown in Figure 4. After addition of tetracycline to induce RNAi, cell growth was inhibited. Although minor variations in the kinetics were observed (compare

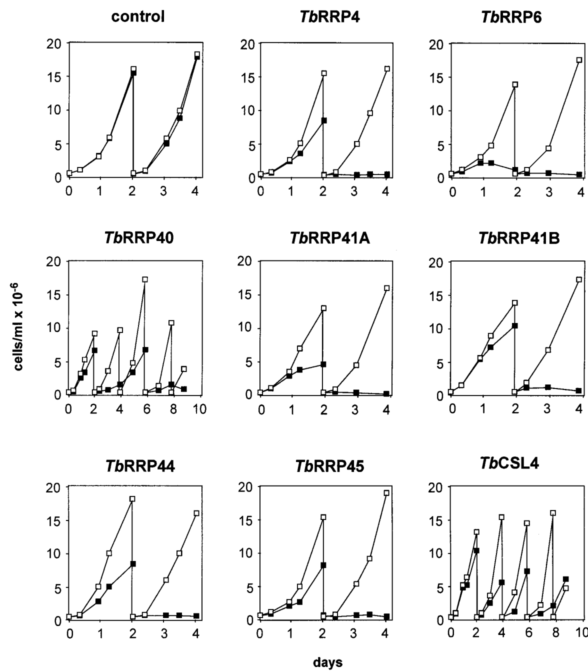

Fig. 4. Depletion of the exosome protein homologs by inducible RNA interference and effects on growth. Trypanosome lines were created that expressed gene-specific dsRNA in a tetracycline-inducible fashion. Each trypanosome line was grown in the absence (open squares) or presence (filled squares) of 100 ng/ml tetracycline to induce RNAi. Cultures were followed for four to nine days and were diluted to 0.4×10^6 cells/ml every 2 days as required.

*Tb*RRP6 and *Tb*RRP40 growth curves), all the depleted trypanosomes eventually died with the exception of the *Tb*CSL4 line. The variations could indicate that the different exonucleases have a different importance in cell survival, but might also be due to differences in the turnover of the mRNA, the protein product, or both (Wang *et al*., 2000). These results indicate that all the proteins listed in Table I, with the possible exception of *Tb*CSL4, are essential for the viability of *T.brucei*.

Roles of individual exosome component homologs in 5.8S rRNA processing

The yeast exosome catalyzes the 3′ trimming of a 7S pre-rRNA precursor to yield mature 5.8S rRNA, and the depletion of any of the yeast exosome components results in the accumulation of both the 7S precursor and incompletely processed pre-rRNA species (Allmang *et al*., 1999a). A 5.8S rRNA species with short and heterogeneous 3′ extensions, the 6S pre-rRNA, is also detected in wild-type yeast strains; this rRNA precursor is also processed by the exosome (Allmang *et al*., 1999a). *Trypanosoma brucei* has a 7S species of 0.6 kb, which contains a 3′ extension of ~400 nucleotides (Hartshorne and Toyofuku, 1999) and is therefore a likely substrate for the exosome. The effect of RNAi on the maturation of 5.8S rRNA was tested for all the proteins (Figure 5). Total RNA was obtained from uninduced or induced cells, separated on denaturing polyacrylamide gels, and probed with oligonucleotides specific to 3′-extended 5.8S rRNA (Figure 5A), mature 5.8S rRNA (Figure 5B) or (as a

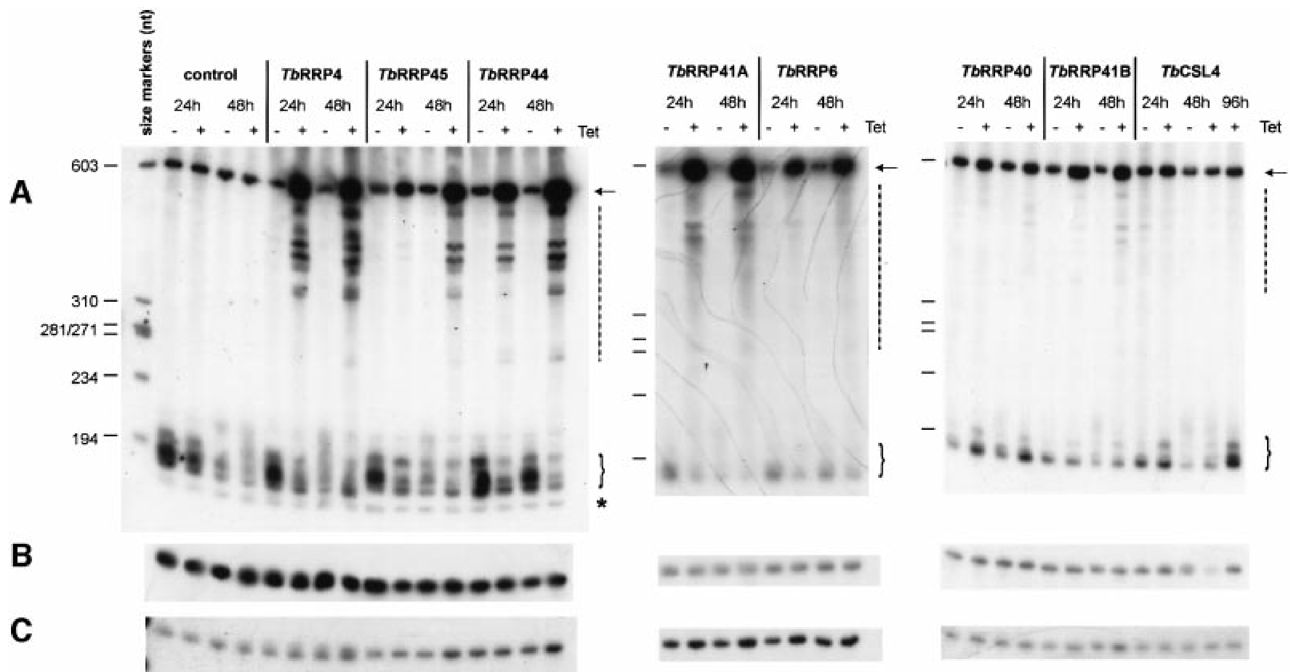

Fig. 5. Effect of depletion of each exosome component homolog on 5.8S rRNA maturation *in vivo*. Total RNA was extracted from parasites grown in the absence (–) or in the presence (+) of tetracycline for 24 or 48 h, and separated in PAGE–urea gels. In the case of *Tb*CSL4, an additional sample taken at 96 h after induction was also included. After electrophoresis the gels were transferred and hybridized to detect (**A**) extended 5.8S rRNA species, (**B**) mature 5.8S rRNA or (**C**) the *T.brucei* signal recognition particle RNA (loading control). The arrow indicates full-length 7S rRNA and the vertical dashed line shows incompletely processed 7S rRNA species. 6S-like species are indicated with a bracket. The asterisk (A, left panel) indicates the mature 5.8S rRNA, which in this particular blot was not completely stripped from a previous hybridization. The size marker is ØX174 DNA digested with *Bsu*R I (MBI fermentas) that was dephosphorylated and labeled with [γ-^{32}P]ATP and polynucleotide kinase.

Table II. Summary table

Protein	Present in the TAP-purified exosome?	Growth phenotype	5.8S rRNA processing phenotype
*Tb*RRP4	yes	essential	1,2
*Tb*RRP6	yes	essential	1
*Tb*RRP40	no	essential	1,3
*Tb*RRP41A	no	essential	1,2
*Tb*RRP41B	no	essential	1,2
*Tb*RRP44	no	essential	1,2,4
*Tb*RRP45	yes	essential	1,2
*Tb*CSL4	yes	slow growth	3

The 5.8S rRNA phenotypes observed were: 1, accumulation of full-length 7S pre-rRNA; 2, accumulation of incompletely processed 7S pre-rRNA species; 3, accumulation of 6S pre-rRNA; 4, reduction of 6S pre-rRNA levels.

loading control) the signal recognition particle RNA (Figure 5C). A 2- to 10-fold accumulation of both full-length and incompletely processed 7S pre-RNA species could be observed upon depletion of *Tb*RRP4, *Tb*RRP45, *Tb*RRP44, *Tb*RRP41A, *Tb*RRP6 and *Tb*RRP41B. Defects were not so evident in the cases of *Tb*RRP40 and *Tb*CSL4, even after prolonged RNAi induction times (Figure 5 and data not shown). Nevertheless, these mutants also showed an accumulation of 6S pre-rRNA and the *TbRRP40* mutant also accumulated 7S pre-RNA. These results are summarized in Table II.

In yeast mutants, some reduction in the levels of mature 5.8S rRNA was seen after 12–24 h of exosome component depletion (Mitchell *et al*., 1997; Allmang *et al*., 1999a,b). No depletion of mature 5.8S rRNA was apparent in *T.brucei* (Figure 5B). After 48 h of RNAi induction, *T.brucei* in the presence of tetracycline had divided one to four times, depending on the protein being depleted (Figure 4). RNAi effects on mRNA levels are detected after one cell division (Shi *et al*., 2000). Around 10% of the normal levels of *Tb*RRP4, *Tb*RRP44 and *Tb*RRP45 persisted after 48 h (data not shown) and this is probably also true of the other exonucleases. This might have been sufficient to maintain normal 5.8S rRNA levels under conditions of no or very slow growth, as insufficient division had occurred to dilute it.

We concluded from these experiments that all the *T.brucei* exosome component homologs were involved in rRNA processing, although some of them appeared not to be associated with the exosome.

Discussion

We have identified eight *T.brucei* genes coding for proteins similar to components of the yeast exosome. All but one of the corresponding proteins were essential for cell growth and all were involved in processing of 5.8S rRNA. Our results indicated, however, that the major *T.brucei* cytosolic exosome species was composed of only five proteins: *Tb*RRP4, *Tb*RRP6, *Tb*RRP45, *Tb*CSL4 and an additional, as yet unknown, protein. *Tb*RRP40, *Tb*RRP41A, *Tb*RRP41B and *Tb*RRP44 were not found in the cytosolic exosome complex.

The simplicity of the trypanosome exosome suggests that this complex has become larger and more elaborate during eukaryotic evolution. Six RNase PH-like proteins are present in the yeast exosome and six human ESTs encode RNase PH homologs. The *C.elegans* genome, like that of trypanosomes (so far) contains just three RNase PH-like proteins (Allmang *et al*., 1999b; van Hoof and Parker, 1999). In *C.elegans*, these could be the homologs of Rrp41p, Rrp45p and Rrp46p, whereas the sequences found in trypanosomes seem to most resemble Rrp45p and Rrp41p (two sequences: *Tb*RRP41A and *Tb*RRP41B) (Table I). The *T.brucei* Rrp41p homolog *Tb*RRP41B also resembles Rrp46p, but to a lesser extent. A similar situation exists in *A.thaliana*, which in addition to the genuine Rrp41p homolog *At*Rrp41p (Chekanova *et al*., 2000), possesses another RNase PH-like protein, F12M12.180, which gives maximal similarity to Rrp41p when compared with the yeast protein database. Further RNase PH homologs may be discovered as the *T.brucei* genome is completed; the present level of coverage is probably between 60 and 80%. It is nevertheless striking that, as for *C.elegans*, no possible homologs have so far been found for Rrp42p, Rrp43p or Mtr3p. In contrast to *T.brucei*, *C.elegans* apparently lacks Rrp4p (van Hoof and Parker, 1999; see also Table I).

Using antisera against *Tb*RRP4 and *Tb*RRP45 we found that the cytosolic and nuclear exosomes of *T.brucei* migrated in glycerol gradients at ~240 kDa (Figure 1), which was consistent with the small number of subunits seen. *Tb*RRP40, *Tb*RRP41A and *Tb*RRP41B were absent from the cytosolic exosome, but we can not exclude the possibility that they are present in the nuclear exosome in addition to (or instead of) other components, or are involved in exosome assembly. *Tb*RRP44 migrated as a monomer in both cytosolic and nuclear extracts (Figures 1 and 3C), but it is still conceivable that a very small proportion of *Tb*RRP44 is exosome associated.

Overall, these results indicate that the only components that have been conserved and remained exosome associated throughout evolution are RRP6, RRP45 and CSL4, and that exosome association and composition can by no means be predicted on the basis of sequence homology.

In yeast there are additional, non-exosomal RNase II-like proteins which are essential for a whole variety of processes (including cell division, signal transduction, RNA splicing and mitochondrial biogenesis) (Mian, 1997; Luukkonen and Séraphin, 1999). The *T.brucei* genome also contains at least one other Rnase II-like protein (DDBJ/EMBL/GenBank, No. AJ309002), with a predicted molecular mass of 102 kDa. Trypanosomes expressing RNAi against this protein showed no effects on either cell growth or 5.8 rRNA maturation (data not shown).

Despite the difference in subunit composition, the overall mode of action of the *T.brucei* and yeast exosomes seems to be similar, i.e. distributive and hydrolytic 3′–5′ riboexonuclease activity. Some of the components of the *T.brucei* exosome nevertheless exhibit distinct functional properties. The *T.brucei* Rrp4p homolog, *Tb*RRP4, showed a processive exonucleolytic activity (Figure 2), whereas yeast Rrp4p has a distributive mode of action (Mitchell *et al*., 1997). The human Rrp4p homolog (hRrp4p) can restore the growth of the *rrp4-1* yeast strain at the non-permissive temperature (Mitchell *et al*., 1997), whereas the *TbRRP4* gene could not. This failure could be due to the different exonucleolytic mechanism of *Tb*RRP4

or to an inability of the trypanosome protein to assemble into a functional complex in yeast. All components of the yeast exosome, except Rrp6p, are essential for viability. In *T.brucei*, in contrast, the Rrp6p homolog *Tb*RRP6 was essential, as were the other exosome component homologs, with the possible exception of *Tb*CSL4 (Figure 4). *Tb*RRP6 was present in stoichiometric amounts in the purified cytosolic exosome, whereas in yeast and in human cells Rrp6p and PM-Scl100 can be detected only in the nuclear complex. We do not yet know whether *Tb*RRP6 has specific cytosolic functions.

The pathways of 5.8S rRNA maturation seem to be quite similar in trypanosomes and yeast; in particular, the presence of the 6S rRNA species may indicate a site within the precursor molecule at which fast processive degradation is replaced by a slow distributive processing (Allmang *et al*., 1999a). It has been proposed that different components of the yeast exosome play distinct roles in the maturation of 5.8S rRNA (Allmang *et al*., 1999a). This might also be the case in *T.brucei*, since different patterns were observed after depletion of different exonucleases. For example, a reduction of 6S pre-rRNA was observed after depletion of *Tb*RRP44, while 6S species accumulated after depletion of *Tb*RRP40 and *Tb*CSL4. Depletion of *Tb*RRP6 resulted in accumulation of 7S pre-rRNA, but incompletely processed 7S species were not readily visible.

It is interesting that similar defects in 5.8S rRNA maturation were observed upon depletion of the proteins examined, irrespective of exosome association. It has been suggested that each exosome subunit in yeast is essential because its absence may cause an exosome assembly failure (van Hoof and Parker, 1999). This is most unlikely to be true for *Tb*RRP44 in *T.brucei*, and is improbable for *Tb*RRP40, *Tb*RRP41A and *Tb*RRP41B. An alternative scenario is that maturation of 5.8S rRNA is an orchestrated process involving all these proteins, whether or not they are in a complex, and that a defect in any one disrupts the whole pathway. The availability of several of the higher eukaryotic exosome components as separate entities in trypanosomes is an opportunity to determine their functions, independently of effects on exosome complex stability.

Trypanosoma brucei is one of the earliest diverging eukaryotes containing mitochondria (Fernandes *et al*., 1993). All available data indicate that there is no exosome complex in eubacteria (Deutscher, 1993), but genetic evidence suggests that there may be an exosome precursor in Archaea (Koonin *et al*., 2001). We suggest that individual exonucleases may have begun to associate in a larger complex at or before the onset of the eukaryotic lineage. As organism complexity grew and RNA processing and its regulation became increasingly more complicated, more subunits were added. The simple exosome of *T.brucei* provides a glimpse of these early events.

Materials and methods

Cell culture

Bloodstream and procyclic form *T.brucei* 449 cell lines (Biebinger *et al*., 1997), stably expressing the tetracycline repressor, were used in all experiments. Cells were grown in the presence of 0.2 µg/ml (bloodstream forms) or 0.5 µg/ml (procyclic forms) phleomycin.

Cloning of T.brucei exosome component homologs

Trypanosoma brucei sequences similar to yeast exosome components were identified by comparing the yeast protein sequences against the unfinished *T.brucei* genome using the TBLASTN program (Altschul *et al*., 1990). Oligonucleotides were designed to PCR amplify specific probes, which were used to isolate genomic DNA fragments containing the entire ORFs from partial genomic libraries (Estévez *et al*., 1997) constructed in pGEM4 or pGEM5 (Promega) vectors. The genomic DNA inserts were sequenced by primer-walking and the ORFs identified using the DNAStar and the Gene Construction Kit programs. Searches for Pfam and Prosite motifs were carried out using the ISREC ProfileScan Server (http://www.isrec.isb-sib.ch/software/PFSCAN_form.html), and by comparison of the *T.brucei* proteins with their yeast counterparts. The sequence corresponding to the entire *Tb*RRP41B ORF was taken from the unfinished *T.brucei* chromosome II sequence available at the TIGR *Trypanosoma brucei* Genome Project.

Overexpression of TbRRP4 in E.coli

A His_6-*Tb*RRP4 ORF was PCR amplified from *T.brucei* genomic DNA, cloned into pET-3a (Stratagene; plasmid pHD1191) and transformed into the *E.coli* strain BL21(DE3)pLysS (Stratagene). Bacteria were grown at 22°C to an OD_{600} of 0.2, induced with 1 mM isopropyl-β-D-thiogalactopyranoside and incubated at the same temperature to an OD_{600} of 1.0. Cell lysis was performed in 10 mM HEPES, 5 mM $MgCl_2$, 0.1% NP-40, 300 mM NaCl, 10% glycerol, 20 mM β-mercaptoethanol, EDTA-free protease inhibitor cocktail (Roche) and 20 mM imidazole pH 7.6. The purification of His_6-*Tb*RRP4 using Ni-NTA agarose (Qiagen) was performed according to the manufacturer's instructions [in the same buffer, lacking the protein inhibitor cocktail but containing 1 mM phenylmethylsulfonyl fluoride (PMSF)], and the protein was eluted at 60 mM imidazol. The enzyme was further purified by AffiGel Blue (Bio-Rad) chromatography and eluted with 1.3 M NaCl. This protein preparation was homogeneous by SDS–PAGE and silver staining. The enzyme was dialyzed against 10 mM HEPES, 5 mM $MgCl_2$, 0.1% NP-40, 10 mM NaCl, 10% glycerol, 1 mM dithiothreitol and 1 mM PMSF pH 7.6, and stored in aliquots at –80°C.

Complementation studies in yeast

The yeast expression vector p415GAL (Mumberg *et al*., 1994) was used for complementation of the yeast strain P58 (*MAT*α *ade2 ade3 leu2 ura3 rrp4-1*) (Mitchell *et al*., 1997). The *Tb*RRP4 and the *S.cerevisiae* RRP4 ORFs were PCR amplified from genomic DNA and cloned in p415GAL. Complementation of the *rrp4-1* allele was assayed in plates containing SGal–Leu medium at 37°C (Mitchell *et al*., 1997).

Generation of antisera

Polyclonal antibodies against *Tb*RRP4, *Tb*RRP44 and *Tb*RRP45 were raised in rabbits. For *Tb*RRP4, the protein overexpressed in *E.coli* (see above) was used as an immunogen; ~750 µg of protein were purified by Coomassie Blue SDS–PAGE gels (Schägger *et al*., 1988) and processed for immunization (Harlow and Lane, 1988). Antibodies against *Tb*RRP44 and *Tb*RRP45 were raised using the following synthetic peptides as immunogens: NDTGAGGDDHENSGREGIGEESE(C) (*Tb*RRP44), and HHRRPELTVRGSSVIVHPPHERE(C) (*Tb*RRP45). The cysteine residues were added in order to couple the peptides to maleimide-activated keyhole limpet hemocyanin according to the manufacturer's instructions (Pierce). Immunizations and immunoblotting were performed according to standard procedures (Harlow and Lane, 1988). Antibodies were purified by affinity chromatography using the corresponding peptide coupled to AffiGel-15 (Bio-Rad).

In vitro exoribonuclease assays

The RNA substrate was synthesized *in vitro* by T7 RNA polymerase transcription (Cunningham and Ofengand, 1990) of plasmid pET-3a (Stratagene) linearized with *Nde*I, purified in PAGE–urea gels and dephosphorylated using calf-intestine alkaline phosphatase. The purified RNA was labeled either at the 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase or at the 3′ end using [5′-^{32}P]pCp and RNA ligase. To obtain a 3′-labeled RNA lacking a 3′-phosphate group, an aliquot of [5′-^{32}P]pCp-labeled RNA was dephosphorylated using alkaline phosphatase. Internally-labeled RNA was synthesized *in vitro* as above, but [α-^{32}P]UTP was included in the reaction and the concentration of non-labeled UTP was lowered to one-fifth. All labeled RNAs were purified in PAGE–urea gels. Exonuclease assays were performed as described (Mitchell *et al*., 1996) using an excess of substrate over enzyme to ensure that for nearly all substrates, cleavage was the result of the action of only one exonuclease (Bambara *et al*., 1995). The reactions were incubated at

37°C and stopped by the addition of EDTA. To characterize processivity and direction of decay, the reactions were diluted 1:1 with formamide, heated at 65°C for 5 min and loaded in an 8% PAGE–urea gel. Bands were quantified using a phosphoimager (Fuji). For analysis of the released product, the samples were subjected to TLC in PEI-cellulose plates (Ross, 1999). Commercial 5′- and 3′-nucleoside monophosphates (Sigma and Fluka) were resolved in parallel and used as markers. To analyze the 3′–5′ riboexonuclease activity in glycerol gradient fractions, 5 µl of each sample were tested as above and the enzyme activity was measured as the release of acid-soluble counts from a 3′-labeled and dephosphorylated RNA (Ross, 1999), after an incubation of 15 min at 37°C.

Subcellular fractionation and glycerol gradients

To examine the nuclear exosome, nuclear extracts were obtained from ~5×10^9 cells (Field and Field, 1996) and centrifuged for 1 h at 100 000 *g*. The supernatant was allocated and frozen at –80°C. To isolate the cytosolic fraction, the low-speed supernatant obtained after centrifugation of the above cell lysate was further centrifuged for 10 min at 12 000 *g*. The resulting supernatant was ultracentrifuged and the S100 supernatant was stored as above. The separation on glycerol gradients was done essentially as described (Mitchell *et al*., 1997), using as markers bovine serum albumin (4.3S), yeast alcohol dehydrogenase (7.4S), bovine catalase (11.3S) and jack bean urease (18.6S).

Purification of the T.brucei exosome using the TAP method

To create a C-terminal, TAP-tagged version of *Tb*RRP4, the TAP tag (Rigaut *et al*., 1999) was PCR amplified from plasmid pBS1479 (kindly donated by B.Séraphin) and cloned in the *T.brucei* tetracycline-inducible expression vector pHD678 (Biebinger *et al*., 1997) to yield pHD918. The *T.brucei Tb*RRP4 ORF was PCR amplified from genomic DNA and cloned into pHD918, yielding pHD924. *Trypanosoma brucei* procyclic 449 cells in which one *TbRRP4* allele was replaced with a blasticidin resistance gene were transfected with either pHD918 or pHD924. Hygromycin-resistant clones were selected and checked for single integration of the plasmid at the ribosomal rDNA intergenic locus by Southern blotting analysis and also for tetracyline inducibility (Biebinger *et al*., 1997). TAP tag alone (pHD918) or TAP-tagged *Tb*RRP4 (pHD924) expression was induced by adding tetracycline to the medium, and the complex purified as described (Rigaut *et al*., 1999) from S100 cytosolic extracts obtained from 1 l of cells harvested at a density between 1.0 and 1.5×10^7 cells/ml.

Mass spectrometry and protein identification

The components of the *T.brucei* exosome complex were resolved by SDS–PAGE and visualized by Coomassie Blue staining. Individual bands were excised, washed repeatedly with H_2O and H_2O/acetonitrile, shrunk with acetonitrile and digested with 0.25 µg trypsin (sequencing grade modified porcine trypsin from Promega, Madison, WI) in 40 mM ammonium bicarbonate overnight at 37°C. The generated peptides were analyzed by MALDI mass spectrometry. Sample preparation was achieved following the thin film preparation technique (Jensen *et al*., 1996). MALDI mass spectra were recorded in the positive ion mode with delayed extraction on a reflex II time-of-flight instrument (Bruker-Daltonik GmbH, Bremen, Germany). Proteins were unambiguously identified by comparing the peptide mass fingerprint with the theoretical tryptic digestion of homologous proteins of the exosome found in the *T.brucei* genome. Theoretical masses of peptides were calculated using the Sherpa software package (http//:128.95.12.16/Development/Sherpa.html). For every protein analyzed, the experimental peptide masses matched the theoretical ones. In addition, mass patterns due to oxidized methionine or oxidized tryptophan were also confirmatory (Simat *et al*., 1994; Schnölzer and Lehmann, 1997).

RNA techniques

Total RNA was obtained using peqGold Trifast (peqLab, Germany). To study 5.8S rRNA processing, RNA samples were electrophoresed in 5% polyacrylamide–TBE gels, electrotransferred to neutral nylon membranes (1 h at 25 V) and hybridized using standard procedures (Ausubel *et al*., 1997). The oligonucleotides used were CZ1193 (5′-ACTTTGCTGCGT-TCTTCAAC-3′) to detect mature 5.8S rRNA, CZ1427 (5′-GTTTTT-ATATTCGACACTG-3′) to detect 3′-extended 5.8S rRNA species and CZ1478 (5′-CAACACCGACACGCAACC-3′) for the *T.brucei* signal recognition particle RNA (Michaeli *et al*., 1992). Bands were quantified using a phosphoimager (Fuji).

RNA interference

All the constructs used for RNAi were made using the 'stuffer' strategy described for *T.brucei* (Shi *et al*., 2000). Briefly, PCR fragments (500–800 bp) were amplified from genomic DNA, ligated to a stuffer fragment (Shi *et al*., 2000) (kindly donated by Elisabetta Ullu) and cloned into the *T.brucei* expression vector pHD1146 [a pHD678 (Biebinger *et al*., 1997) derivative lacking the T7 promoter]. The resulting plasmids were linearized with *Not*I and transfected into procyclic 449 *T.brucei* cells (Biebinger *et al*., 1997). Transfectants were selected in 50 µg/ml hygromycin and cloned by limiting dilution. RNAi induction was achieved by adding tetracycline to the medium at a concentration of 100 ng/ml.

More information regarding cloning procedures, and oligonucleotide and plasmid sequences can be obtained from the authors.

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