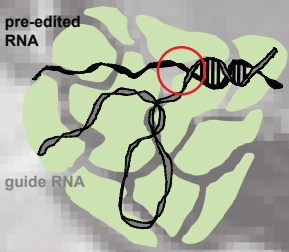
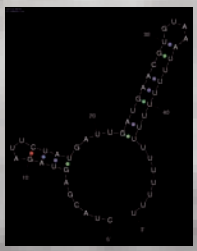
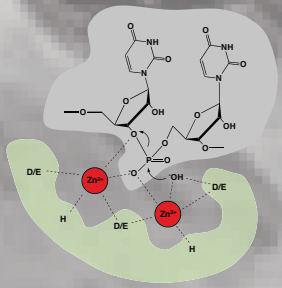
Moritz Niemann

RNA editing in African trypanosomes requires a 3' nucleotidyl phosphatase – the biochemical consequences of the exoUase activity of TbMP42







RNA editing in African trypanosomes requires a 3' nucleotidyl phosphatase – the biochemical consequences of the exoUase activity of TbMP42

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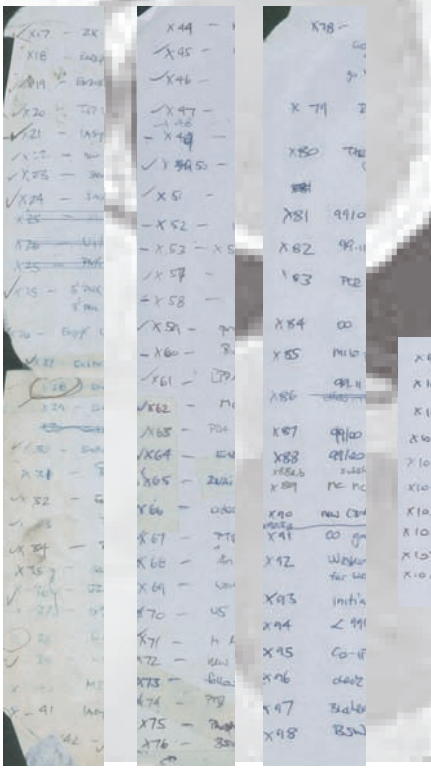
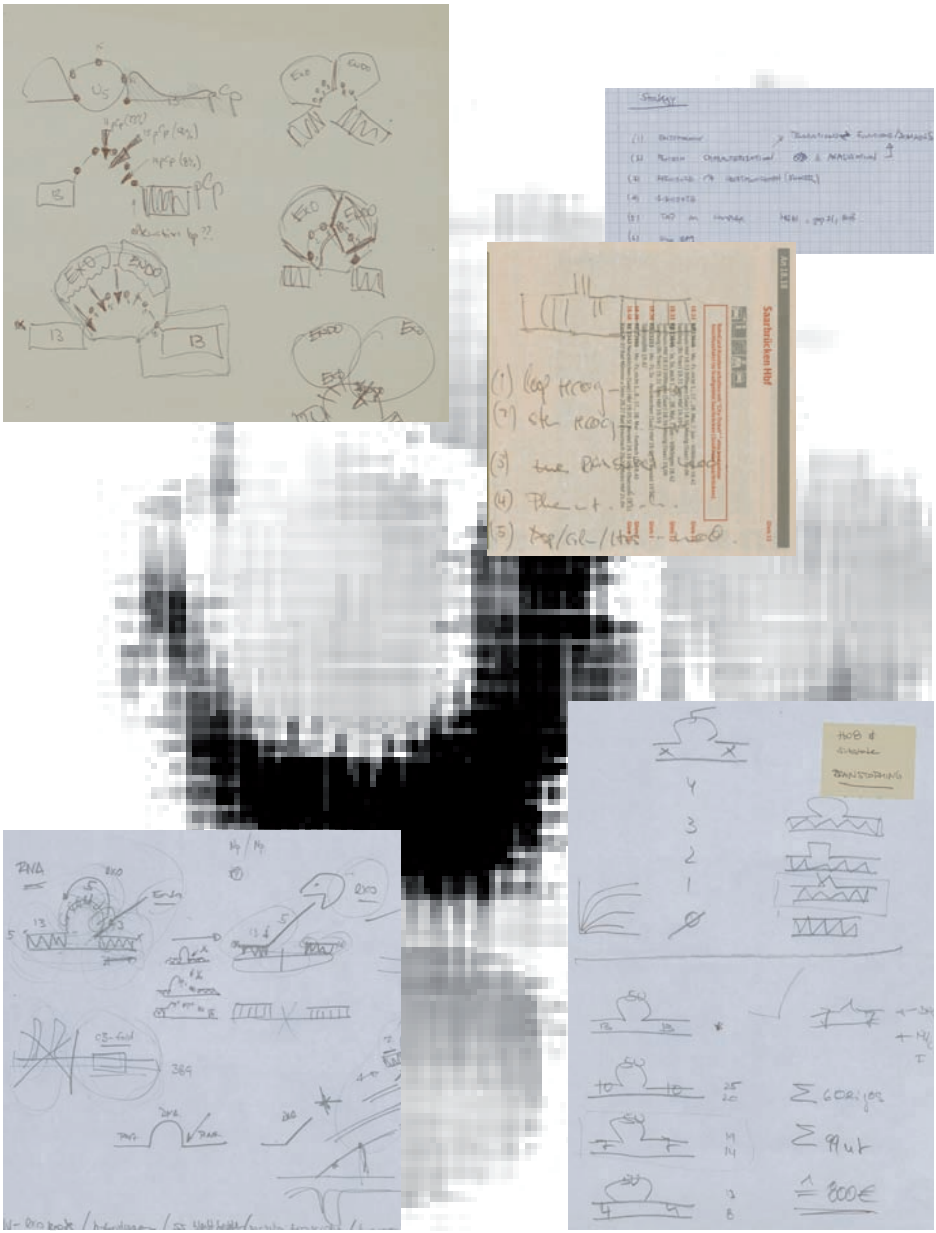


INTRODUCTION



Introduction

"It has not escaped our notice …"

In 1953 Watson and Crick postulated the structure of the DNA double-helix and suggested a copy-mechanism for the imprinted genetic information (Watson and Crick, 1953). In the following years, RNA was assigned the role of delivering that information to and holding together the protein-making machinery as well as collecting the monomeric building blocks needed to construct peptides. Although DNA stores the genetic information, almost all vital processes of the cell revolve around RNA.

RNA structures can function as affinity binding molecules. RNA aptamers are able to adopt complex folded configurations (reviewed in Hermann and Patel, 2000). In procaryotic organisms, riboswitches act as self-regulating circuits and control gene expression co- and post-transcriptionally (reviewed in Schwalbe et al., 2007, and references therein). The binding of a ligand such as guanine (Batey et al., 2004) or thiamine pyrophosphate (TPP) (Winkler et al., 2002) at the RNA's 5' UTR alters the conformation of the RNA and modulates gene expression. The structural change can lead to a transcription termination loop (guanine riboswitch) or influences the accessibility of the Shine-Dalgarno sequence (TPP riboswitch) thereby affecting translation. In eucaryotes, double stranded (ds) RNA serves as an initiator molecule for a variety of gene regulation mechanisms known as gene silencing (Fire et al., 1998). The dsRNA progenitor molecule is processed by the ribonuclease Dicer (Jaskiewicz and Filipowicz, 2008) into small, 21-25nt long dsRNA intermediates: small interfering (si) RNAs and micro (mi) RNAs. These intermediates than associate with protein components to form catalytic ribonucleprotein (RNP) complexes. The incorporated RNA directs the RNP complex to its complementary RNA or DNA sequence. Depending on the recruited RNP, gene silencing can influence either transcription, RNA stability or translation. On the level of transcripton, silencing regulates the mRNA abundance by DNA methylation, chromatin modification or DNA elimination (for a review on RNA interference mechanisms see Filipowicz, 2005; Filipowicz et al., 2005; Sontheimer and Carthew, 2005; Rana, 2007). Post-transcriptional gene silencing (PTGS) mediated by siRNAs is called RNA interference (RNAi). RNAi silences gene expression by sequence specific mRNA degradation in concert with proteins of the so called argonaute family (reviewed in Tolia and Joshua-Tor, 2007). Micro (mi) RNAs are involved in the control of gene expression by translational inhibition (Lee at al., 1993; Doench et al., 2003; Zeng et al., 2003). miRNA expression profiles dictate the level of gene expression by individual translational repression (Lee et al., 2003) and are fully capable of altering the fate of whole cells, tissues and hence whole organisms (summarized in Carrington and Ambros, 2003; Ambros and Chen, 2007; Stadler and Ruohola-Baker, 2008).

RNA is also catalytically active, as discovered in group one intron splicing (see Cech, 1990; and references therein). Although they lack the chemical diversity of amino acid side chains, ribozymes have been proven to be versatile catalysts acting in a wide array of chemical reactions, e.g.: acyl transfer (Lohse and Szostak, 1996), Diels-Alder-cycloaddition (Tarasow et al., 1997; Seelig and Jäschke, 1999), Michael-addition (Sengle et al., 2001), redox reaction (Tsukiji et al., 2003), aldol condensation (Fusz et al., 2005), as well as breaking and joining phosphodiester

bonds (Beaudry and Joyce, 1992; Pan and Uhlenbeck, 1992; Bartel and Szostak, 1993).

The most important ribozyme is the ribosome (Cech, 2000; reviewed in Steitz and Moore, 2003). The reaction center of the protein-making machinery consists of RNA (Ban et al., 2000; Nissen et al., 2000). Hence, RNA alone bestows the catalytic properties that creates proteins from amino acids instead of only providing the structural scaffold for assembling that machinery. The fact, that proteins are assembled from an RNA machine, has lead to the theory, that preceding the time of proteins, life existed only on the basis of RNA: the RNA world hypothesis (Gilbert, 1986). In the current epoch, RNA and proteins act together to orchestrate gene regulation, metabolism and hence life itself. As a matter of fact, two nucleic acid interaction domains, the Zn-finger and the RNA recognition motif (RRM), are present in 5% of the predicted proteins in humans and hence are somehow involved in RNA control, metabolism or stability.

RNA modification and editing

Primary RNA transcripts in eukaryotic cells are subject to extensive modifications in order to mature into functional entities. The unearthing of RNA processing events has unleashed a plethora of RNA modification reactions, e.g.: C to U editing in plant cholorplasts (reviewed in Sugiura, 2008) and mitochondria (reviewed in Takenaka et al., 2008), A to I editing in metazoa (reviewed in Jantsch and Öhman, 2008), tRNA structure editing (reviewed in Alfonzo, 2008) and pre-rRNA editing (reviewed in Reichow et al., 2007) to name only a few. Deletion/insertion type RNA editing in African trypanosomes is a unique post-transcriptional processing

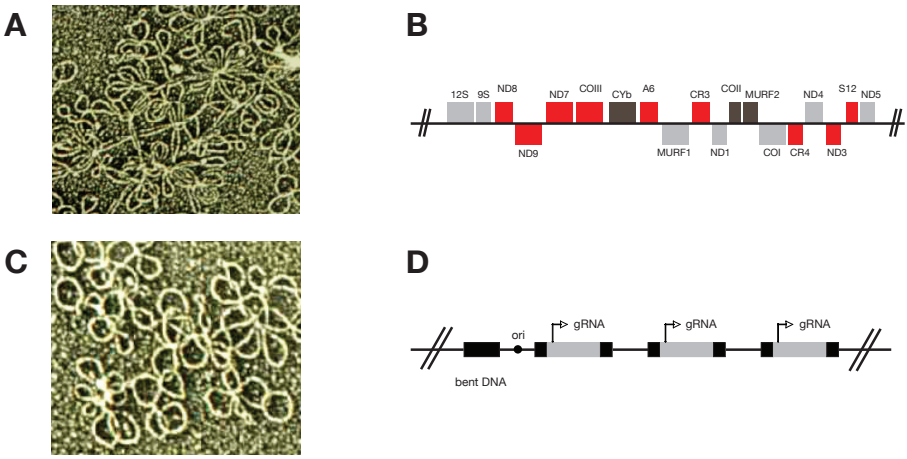


Figure 1: Genomic organization of kinetoplast maxi- and minicircles.
Electron microscopy of the concatenated maxicircle network (**A**) and schematic representation of the maxicircle coding region (**B**). Non-edited transcripts - light grey; limited editing - dark grey; extensive edited sequences (pan editing) – red; 12S, 9S: mitochondrial rRNAs; ND3,4 & 7-9: NADH dehydrogenase subunits; COI-III: cytochromoxidase 1-3; CYb: cytochromoxidase b; CR3,4: C-rich region 3 and 4; MURF1,2: maxicircle unidentified reading frame 1 and 2; A6: ATPase subunit 6; ori: origin of replication. (**C**) Electron microscopy of the interlinked minicircle meshwork and schematic representation of a maxicircle coding region (**D**). gRNA coding regions are represented as grey box.

event that transforms cryptic pre-messenger (pre-m) RNA into functional molecules by the insertion or deletion of exclusively U-residues. The reaction pathway concerns only mitochondria-encoded transcripts of the parasite. In the genus *Trypanosoma brucei* (order kinetoplastida), 18 proteins are encoded on the so called maxicircle genome of the large, singular, complex mitochondrion. The maxicircle genome consists of ~50 identical, circular, concatenated DNA molecules ~20kb in size (Fig. 1A, left) (see Shapiro and Englund, 1995; and references therein). The extent of editing varies from transcript to transcript (Fig. 1A, right). In *Trypanosoma brucei*, MURF1&5, ND 1,4&5 and CO1 are not editited at all, Cyb, CO2 and MURF2 are moderately altered with few insertion and deletion editing events. A6, ND7-9, CO3, CR3-5 and RPS12 are extensively modified with significant sequence alteration (Hajduk et al., 1993): this rearrangement of the coding sequence is refered to as pan-editing (Fig. 2, Simpson and Shaw, 1989). Start and stop-codons are introduced in the process, and without the editing reaction, no functional protein is created. As a consequence, editing is vital to the parasite.

Editing events are not dependent on sequence recognition motifs or pre-mRNA structure. The specificity of the U-insertion and deletion reaction is provided by small guiding (g) RNA molecules (Blum et al., 1990). gRNAs are also encoded in the mitochondrial genome. In addition to the ~50 maxicircles, the mitochondrial genome of trypanosomatids consists also of 5.000-10.000 concatentated minicircles (Fig. 1B, left), ~1kb in size (Pollard et al., 1990). Each minicircle encodes for 3-5 gRNA genes (Fig. 1B, right, Pollard et al., 1990). gRNAs are 50-70nts in length and adopt a certain three-dimensional (3D) configuration (Fig. 3 Schmid et al., 1995;

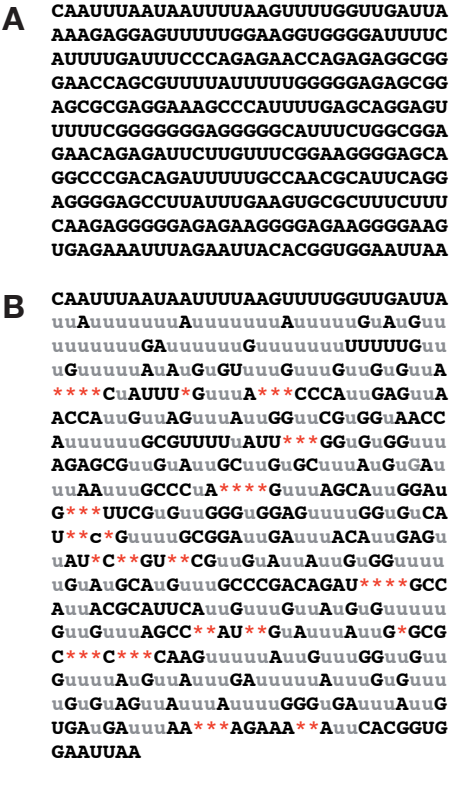


Figure 2: Extensive editing (pan editing) in the ND8 transcript. The pre-edited sequence (**A**) is shown in black letters. Upon editing, 259 U-residues are inserted (grey letters) and 46 are deleted (red stars) from the sequence (**B**).

Herrmann et al., 1997). gRNAs share several other structural characteristics such as a ~15nt long 3' oligo(U)-tail (Blum and Simpson, 1990) and are introduced in more detail in chapter 4. Most important, gRNAs mediate both insertion and deletion editing events (Blum et al., 1990). gRNAs form an imperfect duplex with its cognate pre-mRNA. The sequence information encoded in the gRNA/pre-mRNA hybrid molecule than dictates the editing event (Blum et al., 1990).

At the time of discovering U-insertion/deletion RNA editing, several mechanisms

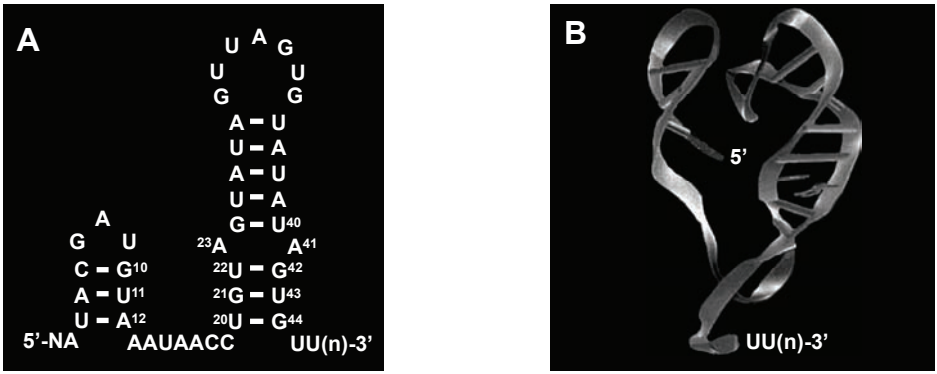


Figure 3: Guide RNA structure.
(A) gRNAs are characterized by two short, imperfect stem-loops and an oligo(U)-tail (Schmid et al., 1995). **(B)** The calculated 3D-structure of the same gRNA as in (A). The two stem-loops fold into a compact arrangement characterized by a triple-nt interaction at the top of the two loops. The model was experimentally verified (Herrmann et al., 1997).

have been proposed for the reaction. In all suggested pathways, gRNA were implicated to direct the editing events. However, the suggested chemical reaction mechanisms leading to insertion and deletion of U-residues differed substantially. One pathway proposed a transesterification event similar to the events in mRNA splicing. This model directly involved the oligo(U)-tail of the gRNA in additon and deletion of U-residues. The 3' OH was proposed to serve as a nucleophile attacking the phosphodiester bond of the pre-mRNA at an editing side (Cech, 1991; Blum et al., 1991). A second transesterification at the inserted or deleted site was considered to re-join the pre-mRNA. The resulting reaction intermediate, a chimeric molecule consisting of a gRNAs 3' end covalently joined to the 3' end of a pre-mRNA, were detected *in vivo* (Blum et al., 1991). gRNA/pre-mRNA chimera were also found *in vitro* (Koslowsky et al., 1992).

A second pathway proposed an enzymatic cascade to facilitate cleavage and ligation reactions, more similar to tRNA splicing. In this model, pre-mRNA is cleaved by an endoribonuclease, U-residues are inserted by a terminal uridylyl transferase (TUTase) or removed by an exoribonuclease and the edited mRNA is re-sealed by an RNA ligase activity (Blum et al., 1990).

Several lines of evidence argued against the transesterification model. For example, stereochemical analysis was inconsistent with the transfer of U-residues directly from the oligo(U)-tail from gRNAs (Frech and Simpson, 1996). Together with the low overall abundance (Riley et al., 1995) and the fact that *in vitro* time course experiments failed to detect chimera before the appearance of edited products (Seiwert et al., 1996), chimera are now considered aberrant byproducts of the editing reaction.

In support of the enzyme cascade model, the biochemical characterization of the editing machinery identified several proteins as key components in the reaction pathway. The discovery of editing specific endonuclease, RNA ligase and TUTase in mitochondrial extracts of trypanosoma or *Leishmania* (reviewed in Madison-Antenucci and Hajduk, 2002; Simpson et al., 2004 and Carnes and Stuart, 2008) lead to the conclusion that

an RNP complex consisting of gRNA, pre-mRNA and proteins provides a reaction platform to facilitate the individual editing reaction editing steps. Glycerol gradient density centrifugation determined the apparent S value of the editosome at around ~20 Svedberg units. This so called editosome contains at least 7 (Rusché et al., 1997), probably 13 (Aphasizhev et al., 2003a) or up to 20 polypeptides (Panigrahi et al., 2001a) depending on the purification protocol. With the development of *in vitro* assays to monitor both insertion and deletion editing of mitochondrial extracts (Seiwert et al., 1994; Kable et al., 1996) and the genetic tool of RNAi at hand, the task of assigning individual peptides to particular reaction steps has progressed considerably (reviewed in Carnes and Stuart, 2008) as has the controversy about these assignments. Although the protein sequences

Table 1. Proteins involved in editing

Name	Motif	Suggested Function
TbMP100	5'/3' exonuclease, EEP-domain	ExoUase
TbMP99	5'/3' exonuclease, EEP-domain	ExoUase
TbMP90	RNaseIII, dsRBM, U1-like	Deletion endonuclease
TbMP81	Zn-finger, OB-fold	Interaction
TbMP67	RNaseIII, dsRBM, U1-like	Endonuclease
TbMP63	Zn-finger, OB-fold	Interaction
TbMP61	RNaseIII, dsRBM, U1-like	Insertion endonuclease
TbMP57	NZ, PAP-core, PAP-assoc	TUTase (editing)
TbMP52	Ligase, tau, K	Ligase
TbMP49	U1-like	Interaction
TbMP48	Ligase, tau, K	Ligase
TbMP47	U1-like	Interaction
TbMP46	RNaseIII?, Pumilio, U1-like	Interaction
TbMP44	RNaseIII?, Pumilio, U1-like	Interaction
TbMP42	Zn-finger, OB-fold	Interaction, Endo/Exonuclease
TbMP41	U1-like	Interaction
TbMP24	OB-fold?	Interaction
TbMP19	OB-fold?	Interaction
TbMP18	OB-fold	Interaction
3' TUTase	NT, PAP-core, PAP-assoc, Zn-finger	TUTase (gRNA)
mHel61	DExH/D-box Helicase	Helicase
TbRGG-1	RGG	Interaction
REAP-1	21-aa repeat	Interaction
RBP16	Cold shock domain, RGG	Interaction
gBP21	R-rich	RNA matchmaking
gBP25	R-rich	RNA matchmaking

List of the editosomal protein inventory. Most peptides are annotaed according to the nomenclature: TbMPxx: Trypanosoma brucei mitochondrial protein, kDa. Sequence motifs are annotated in the middle column. The suggested function is derived from experiments or sequence predictions (see Carnes and Stewart, 2008). „Interaction" means binding to RNA/protein, no catalytic activity has been discovered. EEP: endo-exo-phosphatase; RNaseIII: endoribonuclease motif from RNase III; dsRBM: double-stranded RNA binding motif; U1-like: U1-like Zn-finger motif; Pumilio: Pumilio domain RNA binding motifs; ligase: signature ligase motif; tau and K: putative microtubule associated tau and kinesin light chain domains; NT: nucleotidyl transferase domain; PAP-core and PAP-assoc: poly(A) polymerase core and associated domains; RGG: arginine-glycine-glycine motif; R-rich: arginine-rich domain. DExH/D-box: aspartate-glutamate-x-histidine/aspartate helicase consensus sequence.

of all putative editiosomal components have been known for more than a decade, for most peptides it proves to be difficult to produce correctly folded and active, recombinant (r) proteins. In addition, the analysis and identification of protein function is based on RNAi or classic gene knock-out studies. However, the editosomal reaction platform seems to be a highly redundant machinery. All key activities of the enzymatic cascade are present at least in pairs: two TUTase were found (Aphasishev et al., 2003b), two ligases (McManus et al., 2001), two endoribonucleases (Carnes et al., 2005; Trotter et al., 2005) and at least two exoribonucleases (Brecht et al., 2005; Kang et al., 2005; Rogers et al., 2007). A detailed assessment of the protein inventory is given in in Table 1 and is discussed in the introductions of chapters one, two and three. This redundancy has proven to be an obstacle in RNAi-inspired experiments, since the loss of one enzyme often can be compensated for by its surplus replacement. It was also speculated, that the obvious presence of pairs of key functions is indicative of two separate subcomplexes, one active in insertion and one in deletion editing (Schnaufer et al., 2003).

The association of exoribonuclease activity with a certain protein candidate is especially controverse. From Table 1 it is obvious, that at least 6 proteins possess potential ribonuclease domains (TbMP100, 99, 90, 37, 61 and 42). Obviously, simple gene silencing or gene knock-down studies for these individual components will not be able to resolve the matter. This issue is discussed in detail in chapter three.

Scope of this work

One aim of this study was to assign a function to the editosomal protein TbMP42 (Panigrahi et al., 2001b). Regardless of the purification protocol, the peptide was found to be associated with the editing machinery (Rusché et al. 1997; Aphasizhev et al., 2003a; Panigrahi et al., 2001a). Thus, TbMP42 (42) is considered a core component of the editosome. The peptide contains 389 amino acids and has a molecular mass of ~42kDa. Its pI is 7.6 and it carries a putative mitochondrial import sequence on its N-terminus. (see Figure 4a)

Furthermore, 42 is characterized by two C_2H_2-type Zn-finger consensus sequences at positions 51-79 and 181-209. On its C-terminus, the protein has a predicted oligosaccharide/nucleotide binding site (OB fold) at position 251-389. (Figure 4b) In contrast to the Zn-finger motif, OB-folds are ill defined structural motifs (Theobald et al., 2003) with respect to conserved amino acids. In an OB-fold, antiparallel ß-sheets are arranged in a barrel shaped structure (Theobald et al., 2003). This surface provides an interaction platform for negatively charged ions such as oligosaccharides or oligonucleotides. Specificity is often provided by the loops and turns connecting the ß-sheets, as demonstrated by Cech and co-workers for the protein pot-1. In this example, the connecting turns serve as a clamp that tighten the grip of the protein around its substrate (Lei et al., 2003).

The presence of three potential nucleic acid interaction domains lead to the question, wether 42 interacts with RNA within the context of the editing reaction cycle. Chapter one describes these initial experiments. Although 42 does not contain any classical nuclease motifs, it possess both endo- and exoribonuclease activity *in vitro* and this activity resides in the OB-fold at the C-terminus of the protein (Brecht et al., 2005).

In order to carry out its ribonucleolytic activity, 42 has to bind its substrate. To assess the binding abilities of the protein a set of chemically synthesized RNA substrates were tested in functional assays. Chapter two provides a detailed analysis on the substrate recognition process. In combination with Zn^{2+} depletion studies, the results of chemical modification reactions suggest a metal-ion catalysis mechanism as a possible mode of action for the ribonuclease.

In chapter three, the biochemical consequences of the *in vitro* ribonuclease studies and its implications on the editing reaction are addressed. In summary, the editing reaction cycle requires an additional enzymatic activity: a 3' specific nucleotidyl phosphatase. Two candidate proteins carrying an Endo-Exo-Phosphatase (EEP) domain are implicated to contribute the phosphatase activity to the editing reaction. Again, the above-described redundancy is encountered. Since both candidate proteins were shown to possess exoribonuclease activity *in vitro*, this is discussed.

Considering the complex machinery of the editing reaction cycle, and the necessity for correct RNA editing in order to produce proper proteins, it appears, as if the editing process is highly efficient and optimized to avoid mistakes. The contrary is the case. The steady-state of mRNAs *in vivo* shows a remarkable high degree of mis-edited sequences (Sturm et al., 1992). gRNA molecules grant the specificity for the editing reaction. If that is the case, then why does the machinery produce so many "wrong" sequences? The question arises, whether the protein components make mistakes or if gRNAs exists that "deliberately" direct these "wrong" editing events and if so, why? It has been proposed that these "deliberately wrong" editing events actually serve a purpose. De-

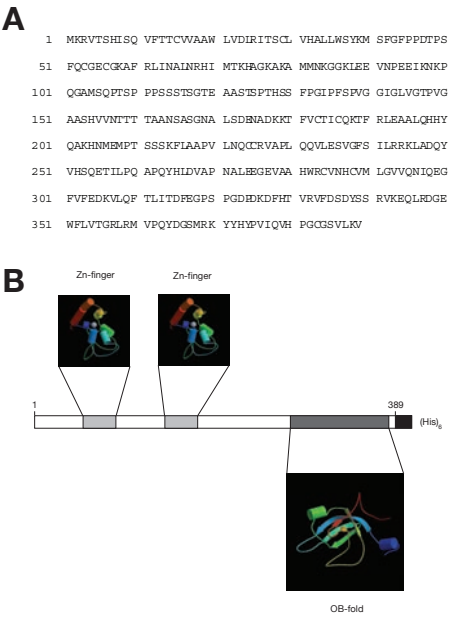


Figure 4: Aminoacid sequence and domain organization of TbMP42.
TbMP42 consists of 389 amino acids **(A)** and has a calculated molecular weight of ~42kDa. The pI of the peptide is 7.6. It has three putative nucleic acid interaction domains: two C_2H_2-type Zn-fingers are located at its N-terminus and an OB-fold is located at its C-terminus **(B)**.

scribed as alternative editing, it was suggested that a "deliberately wrong" editing process could enhance protein diversity (Ochsenreiter and Hajduk, 2006). Chapter four describes an attempt to identify more gRNAs that give rise to alternative edited mRNAs (Madej et al., 2008). However, although mitochondrial RNA was isolated and a size selection was carried out, the amount of gRNA clones is rather small. Intuitively, with the amount of editing and the large amount of "mis"- and semi-edited transcripts at steady-state conditions, one would expect a high abundance of gRNA to be found in such a study. This leads to two conclusions: 1st, an experimental flaw pre-

vents the enrichment of gRNAs or 2nd, gRNA expression and abundance is tightly regulated. As for the protein-orchestrated reaction cycle, RNA editing seems to hold yet some more secrets that await unraveling.

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CHAPTER ONE

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TbMP42, a Protein Component of the RNA Editing Complex in African Trypanosomes, Has Endo-Exoribonuclease Activity

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Summary

...the formation of an antiparallel RNA/RNA duplex structure between the preedited mRNA and a cognate gRNA molecule. It is assumed that the base pairing interaction is catalyzed by the RNA annealing factors gBP21 and gBP27, which have been identified in *Trypanosoma brucei*, *Leishmania tarentolae*, and *Crithidia fasciculata* (Müller et al., 2001; Blom et al., 2001; Aphasizhev et al., 2003b). The gRNA/pre-mRNA duplex positions an editing site 5′ of the helical element, thereby defining the endoribonucleolytic cleavage site of the preedited mRNA. An endoribonucleolytic enzyme activity has been identified in editing-active mitochondrial fractions (Adler and Hajduk, 1997; Piller et al., 1997; Salavati et al., 2002); however, no candidate protein has yet been characterized. During deletion-type RNA editing, uridy-

TbMP42, a Protein Component of the RNA Editing Complex in African Trypanosomes has Endo-Exoribonuclease Activity

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Summary

RNA editing in trypanosomatids is catalyzed by a high molecular mass RNP complex, which is only partially characterized. TbMP42 is a 42 kDa protein of unknown function that co-purifies with the editing complex. The polypeptide is characterized by 2 Zn-fingers and a potential barrel structure/OB-fold at its C-terminus. Using recombinant TbMP42 we show that the protein can bind to dsRNA and dsDNA but fails to recognize DNA/RNA hybrids. rTbMP42 degrades ssRNA by a 3' to 5' exoribonuclease activity. In addition, rTbMP42 has endoribonuclease activity, which preferentially hydrolyzes non-base-paired uridylate-containing sequences. Gene silencing of *TbMP42* inhibits cell growth and is ultimately lethal to the parasite. Mitochondrial extracts from TbMP42-minus trypanosomes have only residual RNA editing activity and strongly reduced endo-exoribonuclease activity. However, all 3 activities can be restored by the addition of rTbMP42. Together, the data suggest that TbMP42 contributes both, endo- and exoribonuclease activity to the editing reaction cycle.

Introduction

The RNA editing reaction of mitochondrial mRNAs in kinetoplastid protozoa is characterized by an enzymatic reaction cycle that inserts and deletes uridylate nucleotides into otherwise incomplete primary transcripts. The process is catalyzed by a high molecular mass ribonucleoprotein complex, which is composed of pre-edited mRNAs, guide (g) RNAs and an uncertain number of proteins (Madison-Antenucci et al., 2002; Worthey et al., 2003; Simpson et al., 2004). Depending on the enrichment protocol, active RNA editing complexes contain as little as 7 (Rusché et al., 1997), 13 (Aphasizhev et al., 2003a) or up to 20 polypeptides (Panigrahi et al., 2001).

Although not all contributing enzyme activities of a full reaction cycle are currently known, it is generally accepted that the initiation step of the process involves the formation of an antiparallel RNA/RNA duplex structure between the pre-edited mRNA and a cognate gRNA molecule. It is assumed that the basepairing interaction is catalyzed by the RNA annealing factors gBP21 and gBP27 which have been identified in *Trypanosoma brucei*, *Leishmania tarentolae* and *Crithidia fasciculata* (Müller et al., 2001; Blom et al., 2001; Aphasizhev et al., 2003b). The gRNA/pre-mRNA du-

plex positions an editing site 5' of the helical element, thereby defining the endoribonucleolytic cleavage site of the pre-edited mRNA. An endoribonucleolytic enzyme activity has been identified in editing-active mitochondrial fractions (Adler and Hajduk, 1997; Piller et al., 1997; Salavati et al., 2002), however, no candidate protein has yet been characterized. During deletion-type RNA editing uridylate residues are exonucleolytically removed from the 3' end of the 5' mRNA cleavage product and released as UMP. This requires a U-specific 3' to 5' exoribonuclease (exoUase). As for the endoribonuclease, mitochondrial extracts contain exoUase activity (Aphasizhev and Simpson, 2001; Igo et al., 2002) but no candidate protein has been identified to date. Insertion-type editing requires the addition of U-nucleotides to the 3' end of the 5' mRNA cleavage product. This reaction step is catalyzed by a 3' terminal uridylyl transferase (TUTase). The enzyme has recently been cloned from both *Leishmania* and trypanosomes and was characterized as a member of the DNA polymerase β superfamily of nucleotidyltransferases (Aphasizhev et al., 2003c; Ernst et al., 2003).

An editing reaction cycle is completed by the ligation of the processed 5' fragment to the 3' fragment of the pre-mRNA. Two editing-specific RNA ligases (REL1, REL2) have been identified and were biochemically and genetically characterized (McManus et al., 2001; Schnaufer et al., 2001; Huang et al., 2001).

Aside from these core activities evidence exists that several auxiliary factors add to the reaction cycle. Among these factors are proteins which interact and stabilize pre-edited mRNA such as REAP1 (Madison-Antenucci and Hajduk, 2001), polypeptides which can bind to the 3' oligo(U) extensions of gRNAs (TbRGG1) (Vanhamme et al., 1998) or proteins such as mHel61p, a complex-associated putative RNA helicase (Missel et al., 1997; Stuart et al., 2002), which may catalyze the unwinding of fully basepaired gRNAs from edited mRNAs.

Potential candidates for the yet unidentified catalytic components of the editing machinery are proteins that co-purify with the complex (Stuart et al., 2002; Worthey et al., 2003; Simpson et al., 2004). They include polypeptides that have been shown to contain Zn-finger motifs, suggesting direct contact points to nucleic acid ligand molecules (Panigrahi et al., 2001; Huang et al., 2002; Lu et al., 2003). One such protein is TbMP42. The mitochondrial polypeptide has a molecular mass of 42kDa and was first identified in African trypanosomes (Panigrahi et al., 2001). It shares sequence homology to three other *T. brucei* Zn-finger proteins (TbMP81, TbMP63, TbMP18), although the four polypeptides show no sequence homology to other polypeptides. A TbMP42-specific monoclonal antibody was shown to immunoprecipitate deletion and insertion RNA editing activity (Panigrahi et al., 2001) supporting the evidence that the polypeptide is associated with the editing complex.

Here we demonstrate that recombinant TbMP42 binds to dsRNA and dsDNA and has both, endoribonuclease and 3' to 5' exoribonuclease activity. The endoribonuclease activity acts preferentially on looped-out uridylate residues and the exoribonuclease activity terminates on RNA duplex structures. Further we show that gene silencing of *TbMP42* is lethal for the parasite and that TbMP42-minus cells have only residual RNA editing activity. However, the editing deficiency can be rescued by the addition of exogenous rTbMP42, which provides evidence for an involvement of the protein during the editing reaction cycle.

Results

Recombinant TbMP42 Binds ds Nucleic Acids

We cloned *TbMP42* by rapid amplification of cDNA ends (RACE). Using the cDNA sequence information we amplified the genomic copy of *TbMP42* and sequenced the resulting open reading frame (ORF). The ORF is 1179bp in length and codes for a 393 amino acid polypeptide. A Southern blot analysis revealed that *TbMP42* is encoded by a single copy gene and by RT-PCR we determined that the transcript is expressed at equal levels in both major life cycle stages of the parasite (data not shown).

In order to characterize the biochemical properties of TbMP42 we constructed a plasmid-encoded $(his)_6$-tagged version of *TbMP42*. The plasmid was transformed into *E. coli* M15[pREP4] bacteria and upon induction with isopropylthiogalactoside (IPTG) high amounts of recombinant (r) TbMP42 were expressed (Fig. 1A). Since the induction of rTbMP42 caused the formation of inclusion bodies within the bacterial cells, we used inclusion body preparations for the purification of the polypeptide. All purification steps were performed at denaturing conditions (8M urea) and started with a Ni-chelate affinity chromatography step utilizing the $(his)_6$-tag of the recombinant protein. rTbMP42-containing fractions were further purified by anion exchange chromatography, in some cases followed by isoelectric focusing or dye-binding chromatography. The resulting urea-containing protein preparations were dialyzed and characterized in SDS-containing polyacrylamide gels (Fig. 1A). Recombinant $(his)_6$-tagged TbMP42 migrated as a homogenous protein population with the expected electrophoretic mobility of a 43kDa polypeptide. The presence of folded protein domains was mon-

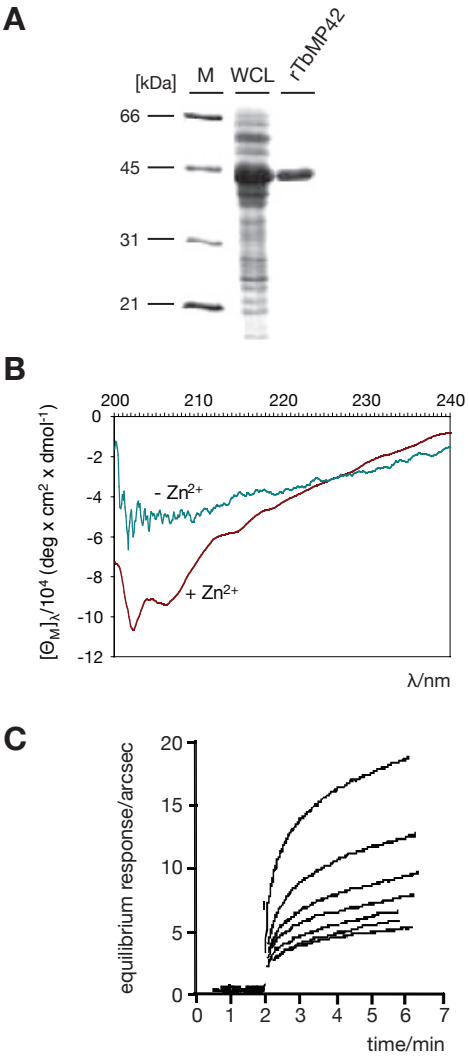

Figure 1: *Structural characterization and RNA binding analysis of rTbMP42*
(A) *SDS-PAGE of a whole cell protein lysate (WCL) from rTbMP42-expressing E. coli in comparison to purified rTbMP42. M - marker proteins.* **(B)** *CD spectra of renatured rTbMP42 (2.6μM) in the presence and absence of Zn^{2+} cations (0.1mM). The spectrum of the folded protein corresponds to 50-60% α-helical content and 10-20% β-sheet structures.* **(C)** *Real time monitoring of the concentration dependent binding of a 15bp dsRNA ligand (bottom to top: 2, 5, 10, 16, 32, 50, 100nM) to rTbMP42.*

itored by circular dichroism (CD) measurements (Fig. 1B) and the spectra indicated that the protein preparations were essentially unstructured. However, the addition of Zn^{2+}-cations induced a folding reaction resulting in protein preparations with 50-60% α-helical content and 10-20% β-sheet structure (Fig. 1B). The critical Zn^{2+} concentration for refolding was determined as ≥0.1mM.

The ability of refolded rTbMP42 to interact with nucleic acid ligands was measured in real time resonant mirror experiments. rTbMP42 was covalently coupled to an amino silane biochip surface and incubated with different nucleic acid ligands. Fig. 1C shows a representative set of binding curves for a 15bp double stranded (ds) RNA ligand at different concentrations. The K_d for the dsRNA/rTbMP42 interaction was calculated as 10nM and binding equilibrium was reached within 2-4min. rTbMP42 was also capable of binding to a 18bp dsDNA ligand to ssDNA (15-18nt) but failed to recognize a 18bp DNA/RNA hybrid (data not shown).

TbMP42-Minus Cells are Not Viable

To identify the mitochondrial function of TbMP42 we performed a gene knockdown experiment by RNA interference (RNAi). A 701bp fragment of the coding region of *TbMP42* was cloned into the RNAi vector pZJM (Wang et al., 2000).The resulting plasmid was linearized and used to transfect insect stage *T. brucei* 29-13 parasites (Wirtz et al., 1999). Ble-resistant transfectants were cloned and the synthesis of *TbMP42*-specific dsRNA was induced by the addition of tetracycline (tet) to the culture medium. Fig. 2A shows a representative growth curve of a clonal *TbMP42* RNAi cell line in the absence and presence of tet. While non-induced parasites grew with a normal doubling time, tet-induced cells showed a severe growth rate phenotype. The parasites stopped multiplying around 120 hours after the addition of tet and eventually died. A molecular analysis of the phenotype revealed that in as little as 48 hours after the induction with tet, both, *TbMP42*-specific mRNA (data not shown) and TbMP42 protein (Fig. 2B) were below the level of detection.

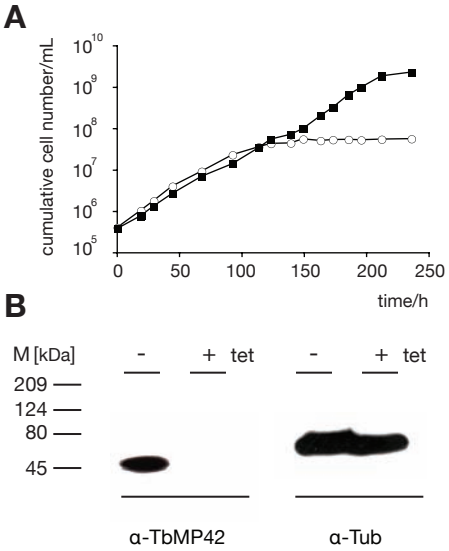

Figure 2: *Phenotypic and molecular analysis of TbMP42-minus trypanosomes*
(A) *Growth behavior of a clonal TbMP42-RNAi trypanosome cell line in the presence (open circles) and absence (filled squares) of tetracycline (tet).* **(B)** *Western blot analysis for TbMP42 and α-tubulin in tet-induced (+tet) and noninduced (-tet) trypanosomes.*

TbMP42-Minus Cells Show Reduced RNA Editing Activity

In order to test whether the described gene knock down phenotype of TbMP42-minus *T. brucei* was correlated with a deficiency of the parasites to perform RNA editing we analyzed the processing reac-

tion directly. For that we used mitochondrial detergent extracts from both, *T. brucei* cells that express TbMP42 and parasites that were treated with tet for 72 hours and therefore lack the protein. The extracts were separated by centrifugation in glycerol density gradients and fractionated. All fractions were assayed for their *in vitro* RNA editing activity using a precleaved U-insertion assay (Igo et al., 2000). The activity sedimented in both samples as a broad peak of approximately 20-35S (Fig. 3A,B). However, the peak fraction derived from the TbMP42-minus cells showed a strongly reduced RNA editing activity of only 10%. This indicated that the absence of TbMP42 severely impacts the editing reaction, though does not totally abolish it. The data further suggest that the absence of the protein does not result in a significant structural rearrangement or even disassembly of the editing machinery. This was further confirmed by analyzing the gradient distribution of the two editing-specific RNA ligases REL1 and REL2 (McManus et al., 2001; Schnaufer et al., 2001; Huang et al., 2001), which was identical in TbMP42-plus and TbMP42-minus cells (data not shown).

Lastly, we analyzed the capacity of TbMP42-minus cells to perform the editing reaction *in vivo*. This was done by poisoned primer extension experiments testing the abundance of edited apocytochrome b (Cyb) and NADH dehydrogenase subunit 7 (ND7) transcripts in steady state RNA preparations from TbMP42-minus and TbMP42-plus cells (Fig. 3C). In agreement with the above described *in vitro* data, the downregulation of *TbMP42* (for 72 hours) had a negative effect on the abundance of the 2 edited mRNAs, although the cells were still capable of performing the processing reaction to some degree (5-15%).

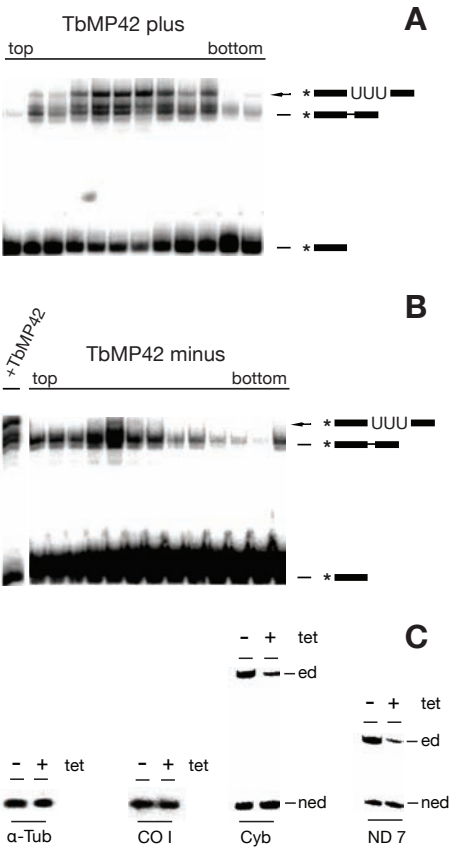

Figure 3: *In vitro RNA editing analysis of TbMP42-minus trypanosomes*
*Mitochondrial detergent extracts were separated in isokinetic glycerol gradients and fractionated. Each fraction was tested for its RNA editing activity. The electrophoretic mobilities of the editing product, the ligation product and the pre-mRNA 5' fragment are given on the right (top to bottom). A * represents the position of the radioactive label.* **(A)** *In vitro RNA editing activity of mitochondrial extracts from TbMP42-plus trypanosomes and* **(B)** *from TbMP42-minus parasites. Top and bottom of the gradients are marked accordingly.* **(C)** *Poisoned primer extension analysis of four mRNAs from the TbMP42-RNAi cell line 72 hours after tet induction: α-Tub - α-tubulin, COI - cytochrome oxidase I, Cyb - apocytochrome b and ND7 - NADH dehydrogenase subunit 7. Extension products representing the edited (ed) and non-edited (ned) versions of the CYb and ND7 mRNAs are indicated. α-Tub is a nuclear transcript and COI a never edited mitochondrial transcript.*

Exogenous TbMP42 Rescues the Editing Deficiency of TbMP42-Minus Cells

Based on the described result we asked whether the addition of rTbMP42 might be able to rescue the reduced editing activity of TbMP42-minus cells. This was experimentally addressed by performing *in vitro* U-insertion editing reactions with a mitochondrial fraction from TbMP42-minus cells. As shown above, the fraction had a reduced editing activity of only 10% (Fig. 4A). Individual samples were supplemented with increasing concentrations of rTbMP42 (0.05-6ng/µL) and as shown in Fig. 4A rTbMP42 was capable of rescuing the editing deficiency in a concentration dependent fashion. At a concentration of 6ng/µL the reaction reached its maximal level, which was in the range of 90% of the value of a fraction that contains endogenous TbMP42 (Fig. 4B). The amount of editing complex-associated rTbMP42 was determined by re-isolating rTbMP42-supplemented complexes by density centrifugation followed by Western blotting. The data showed that 80% of rTbMP42 was complex-associated (data not shown).

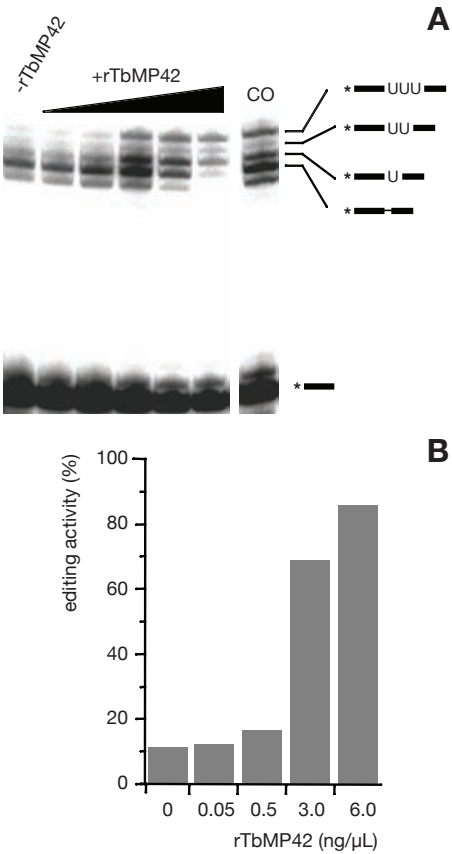

Figure 4: *rTbMP42 is capable of rescuing the editing deficiency of mitochondrial extracts from TbMP42-minus trypanosomes*
(A) *Autoradiogram of a pre-cleaved RNA editing in vitro assay. –TbMP42 represents a sample from TbMP42-minus cells and CO represents a control reaction using a mitochondrial extract from TbMP42-plus cells. The electrophoretic mobilities of the editing product, of two editing intermediates, the non-productive ligation product and the pre-mRNA 5' fragment are given on the right (top to bottom). A * represents the position of the radioactive label.* **(B)** *Quantitative analysis of the signal of the editing product shown in (A).*

Recombinant TbMP42 has 3' to 5' Exoribonuclease and Endo-ribonuclease Activity

Based on the above described finding that rTbMP42 was able to interact with ds nucleic acids we investigated the fate of a synthetic RNA editing substrate upon incubation with rTbMP42. The molecule was termed U5-hybrid RNA (Fig. 5A) and represents a gRNA/pre-edited mRNA hybrid molecule specific for a deletion-type RNA editing reaction. The editing domain is defined by five looped-out uridylate (U) residues, which are flanked by two 13bp helices formed between the gRNA (bottom strand) and the pre-edited mRNA (top strand). Fig. 5B shows a representative result using a U5-hybrid RNA preparation in which the pre-mRNA was radioactively labeled at its 5' end. While the mock treated sample was stable over the entire incu-

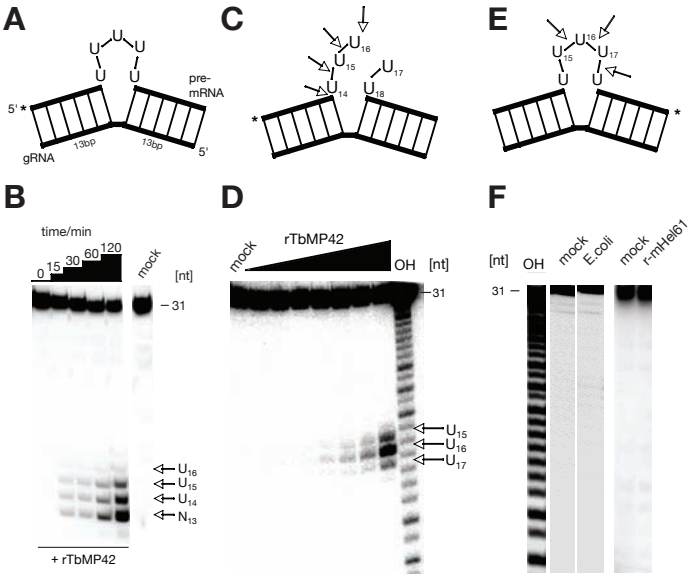

Figure 5: *Exo- and endoribonucleolytic hydrolysis of an RNA editing model substrate by rTbMP42*
(A) *Graphical representation of a pre-mRNA/gRNA hybrid molecule with 5 single-stranded uridylate residues flanked by two 13bp stem structures (U5 hybrid RNA). A * represents the position of the radioactive label.* **(B)** *Time-dependent hydrolysis of U5-hybrid RNA by rTbMP42. Representative autoradiograph of a separation of the exoribonucleolytic hydrolysis products in a denaturing polyacrylamide gel. Hydrolysis positions (U16 – N13) are marked by arrows and are graphically represented in* **(C)**. **(D)** *Endoribonucleolytic hydrolysis of U5-hybrid RNA (the radioactive label (*) is located at the 3'-end of the pre-mRNA). Hydrolysis products are separated by denaturing PAGE and are marked by arrows (U17 – U15). A graphical representation is shown in* **(E)**. *OH represents an alkaline hydrolysis ladder of 5' radioactively labeled input RNA.* **(F)** *Ribonucleolytic hydrolysis of U5-hybrid RNA with unrelated proteins or protein extracts. E.coli – incubation of U5-hybrid RNA with a protein extract fromE.coli cells. r-mHel61 - incubation of U5-hybrid RNA with his-tagged T. brucei mHel61. Both protein samples were treated identical to the purification protocol for rTbMP42.*

bation period (120min), the addition of 7.5ng/µL rTbMP42 resulted in the appearance of 4 pre-mRNA cleavage products varying in length from 16nt to 13nt (Fig. 5B). Thus, rTbMP42 induced a partial ribonucleolytic degradation of the pre-mRNA of U5-hybrid RNA which was suggestive of a 2 step scenario: First, an endoribonucleolytic cleavage at position U16 of the pre-mRNA and second, a 3' to 5' exoribonucleolytic trimming reaction of the 5' cleavage product (Fig. 5C). A comparison of the signal intensities of the different hydrolysis fragments at early time points *versus* late time points suggested a distributive reaction type which is terminated at position 13, the next basepaired nucleotide within the pre-mRNA sequence (Fig. 5C). Although we identified in all of our experiments some minor degradation (<1%) into the second helix of U5-hybrid RNA (position 12 in Fig. 5B), this can be attributed to a breathing reaction and/or alternative secondary structure at the helical end.

In order to experimentally confirm the initial endoribonucleolytic reaction step we used a U5-hybrid RNA preparation that contained a radioactively 3' end labeled pre-mRNA molecule. Upon incubation with rTbMP42 the RNA was predominantly hydrolyzed at the anticipated position (U16, >90%), in addition to some minor cleavage at the two surrounding nucleotides U15 and U17 (Fig. 5D/E). Thus, rTbMP42 shows characteristics of a structure-specific endonuclease, which specifically recognizes looped-out nucleotides.

To exclude the possibility that the two identified ribonucleolytic activities stem

from co-purifying *E. coli* ribonucleases we tested an identical column fraction derived from the parental *E. coli* M15[pREP4] strain that did not contain the *rTbMP42* expression plasmid. This fraction was free of any nucleolytic activity (Fig. 5F). Furthermore, we analyzed another recombinant $(his)_6$-tagged protein preparation to eliminate the possibility of co-purifying *E. coli* ribonucleases through protein/protein interaction (despite the presence of 8M urea throughout the purification). For this we chose mHel61p, which represents another editing complex-associated protein (Missel et al., 1997; Stuart et al., 2002). As above, his-tagged recombinant mHel61p preparations showed no ribonucleolytic cleavage activity (Fig. 5F). Lastly, we tested whether the gRNA molecule in U5-hybrid RNA was hydrolyzed by rTbMP42. However, even a 3 hour incubation with 7.5ng/µL rTbMP42 did not result in any detectable nucleolytic degradation (data not shown). Thus, the rTbMP42-mediated cleavage of U5-hybrid RNA is specific for the pre-mRNA of the gRNA/pre-mRNA hybrid and starts with an endoribonuclease reaction step followed by a 3' to 5' exoribonucleolytic degradation of the 5' cleavage product.

TbMP42-Minus Cells Show Reduced Endo-Exoribonuclease activity

In order to test whether the 2 identified nucleolytic activities of rTbMP42 can be correlated to activities of the RNA editing reaction cycle we analyzed the cleavage pattern of U5-hybrid RNA by an editing-active mitochondrial fraction (EAF) from wildtype trypanosomes. As before, by using complementary radioactive labeling strategies for U5-hybrid RNA, we were able to analyze both ribonucleolytic activities. Fig. 6A shows that wildtype EAF contained endoribonuclease activity which cleaved U5-hybrid RNA at the same position as rTbMP42 (U16>U15>U17). Importantly, a mitochondrial extract from the *TbMP42*-RNAi knockdown strain showed only residual endoribonuclease activity (<5%), suggesting that the majority of the wildtype activity is due to the presence of TbMP42 (Fig. 6A). Adding back rTbMP42 to the RNAi extract fully restored the activity (Fig. 6A).

Identical experiments were performed to analyze the exoribonucleolytic activity with essentially the same result (Fig. 6B). Editing-active fractions from wildtype trypanosomes showed 3' to 5' exoribonucleolytic activity and cleaved U5-hybrid RNA at the same nucleotides as rTbMP42 (U16, U15, U14, N13). The exoribonucleolytic activity was strongly reduced (to app. 5%) in the TbMP42-minus RNAi strain but was completely rescued by the addition of rTbMP42 (Fig. 6B).

The Ribonucleolytic Activity of rTbMP42 has a Preference for U Nucleotides

Experiments with partially purified mitochondrial extracts from *T. brucei* have shown that the exoribonucleolytic reaction step of the editing process is a U nucleotide-specific process (Cruz-Reyes and Sollner-Webb, 1996; Lawson et al., 2001; Aphasizhev and Simpson, 2001; Igo et al., 2002). Therefore, we analyzed whether the ribonucleolytic cleavage reactions of rTbMP42 have U-specific characteristics. This was tested by comparing the rTbMP42-induced cleavage of U5-hybrid RNA to another synthetic RNA that contained 5 looped out A nucleotides instead of the 5 Us (Fig. 6C). The appearance of the characteristic hydrolysis products indicated that both pre-mRNAs were endoribonucleolytically cleaved and that the resulting 5' fragment was subsequently trimmed by the 3' – 5' exoribonuclease activity. However, a quantitative comparison demonstrated that the A-substrate

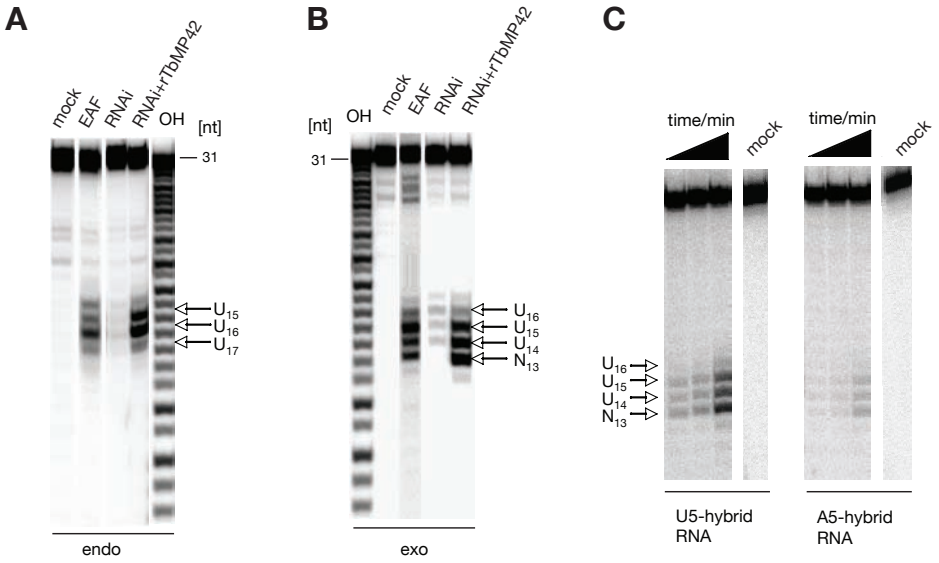

Figure 6: *Endo- and exoribonucleolytic activity of mitochondrial extracts fromTbMP42-plus andTbMP42-minus parasites*

To assay for the 2 ribonucleolytic activities, the pre-mRNA of U5-hybrid RNA was radioactively labeled either at its 3'-end **(A)** *or the 5'-end* **(B)***. The RNA preparations were incubated with glycerol gradient fractions from TbMP42-plus cells (EAF) or from a TbMP42 knocked down RNAi cell line (RNAi). RNAi + rTbMP42 represents a sample where rTbMP42 was added back to the RNAi fraction. Hydrolysis products were separated in denaturing polyacrylamide gels and are marked by arrows. OH represents an alkaline hydrolysis ladder of 5' radioactively labeled input RNA.* **(C)** *Uridylate preference of the endo/exoribonucleolytic activity of rTbMP42. Kinetic (10, 15, 30min) of the rTbMP42-mediated hydrolysis of U5-hybrid RNA in comparison to A5-hybrid RNA. Hydrolysis positions are marked by arrows (U16 – N13).*

was hydrolyzed to a significantly lesser degree in the range of only 5-10% of the U-RNA. Thus, while rTbMP42 can act on U- and A-nucleotides, the U-nucleotide containing pre-mRNA is the preferred substrate.

The Ribonucleolytic Activities of rTbMP42 Reside Within its C-Terminal Half

TbMP42 contains, with its 2 Zn-fingers and a potential C-terminal barrel structure/OB-fold, 3 protein domains known to interact with nucleic acid ligands (Lu et al., 2003; Theobald et al., 2003). This led us to test whether all 3 motifs are required for the ribonucleolytic activities of the protein. We constructed 2 truncated rTbMP42 mutants: First, a N-terminal (NT) variant (amino acids (aa) 1-250) which contains both zinc finger motifs but lacks the potential barrel/OB-fold. Second, a C-terminal (CT) protein variant (aa 251-393) which lacks the 2 zinc-fingers but contains the barrel/OB-fold (Fig. 7A). Both mutant polypeptides were expressed as $(his)_6$-tagged proteins and were purified following the same procedure as outlined for full length rTbMP42 (Fig. 7B). The 2 polypeptides were tested for their endo/exoribonuclease activity using U5-hybrid RNA as a substrate and the results are shown in Fig. 7C. While the Zn-finger-containing N-terminal polypeptide showed no ribonucleolytic activity the C-terminal fragment still contained both activities. This sug-

gests that the two Zn-fingers do not contribute to RNA substrate binding and indicates a role for the C-terminal barrel/OB-fold in both, RNA recognition and hydrolysis.

Discussion

In this study we describe experiments aimed at identifying a molecular function for TbMP42, a protein component of the RNA editing complex in African trypanosomes and *Leishmania tarentolae* (Panigrahi et al., 2001; Aphasizhev et al., 2003a). We characterized the protein as an endo-exoribonuclease, which degrades ssRNA with a 3' to 5' directionality and cleaves RNA molecules endonucleolytically at looped-out nucleotides. The exoribonuclease activity is distributive, it stops at RNA duplex structures and has a preference for U nucleotide-containing RNAs over A nucleotide-containing RNAs. Together, these data suggest that TbMP42 contributes to endo- and exoribonucleolytic reaction steps of the RNA editing cycle.

In support of this hypothesis we were able to show that a recombinant, $(his)_6$-tagged preparation of TbMP42 can rescue the reduced editing efficiency of mitochondrial fractions from an epigenetic *TbMP42* knockdown *T. brucei* strain. The restoration was concentration dependent and reached a maximal level of 90% of the *in vitro* editing activity of a wildtype mitochondrial extract. Thus, rTbMP42 is necessary and sufficient for the restitution of the editing activity in TbMP42-minus mitochondrial extracts. However, it should be noted that due to the pre-cleaved nature of the pre-mRNA substrate the *in vitro* assay only monitores the exoribonucleolytic activity of TbMP42 (Igo et al., 2000). Therefore, full round *in vitro* editing assays have to be performed to verify the result for both activities at the same time.

Further support for the ribonucleolytic activities of TbMP42 comes from the finding that editosome-containing mitochondrial fractions from TbMP42-minus trypanosomes have only residual endo-exoribonuclease activity. As before, the addition of rTbMP42 was able to complement this deficiency, which verified that the two ribonucleolytic activities of editosome-containing protein fractions are by and large due to TbMP42. Unfortunately, since the concentration of RNA editing complexes within these fractions is unknown one cannot deduce any stoichiometric values. Whether one or more rTbMP42 molecules bind to the editing complex remains unclear. However, since editing complexes from TbMP42-minus trypanosomes apparently do not disassemble and are characterized by an apparent S-value similar to complexes enriched from TbMP42-plus cells, it is unlikely that many TbMP42 molecules are part of an active RNA editing complex. Rather, the data are suggestive of a structural situation where only one or a few TbMP42 molecules are localized close to the surface of the editing complex. This is supported by the observation that editing complex-associated TbMP42 is accessible for TbMP42-specific antibodies which have been used to immunoprecipitate the entire complex (Panigrahi et al., 2001). It is further supported by the fact that recombinant TbMP42 assembles into TbMP42-minus editing complexes without any activation or pre-assembly step.

Whether the protein binds to the editing machinery by protein/protein interactions, by RNA/protein interactions or a combination of both cannot be deduced from the presented data. However, since TbMP42 contains two zinc-finger domains, which are not required for the ribonucleolytic activities of the protein, it is tempting to speculate that the Zn-fingers

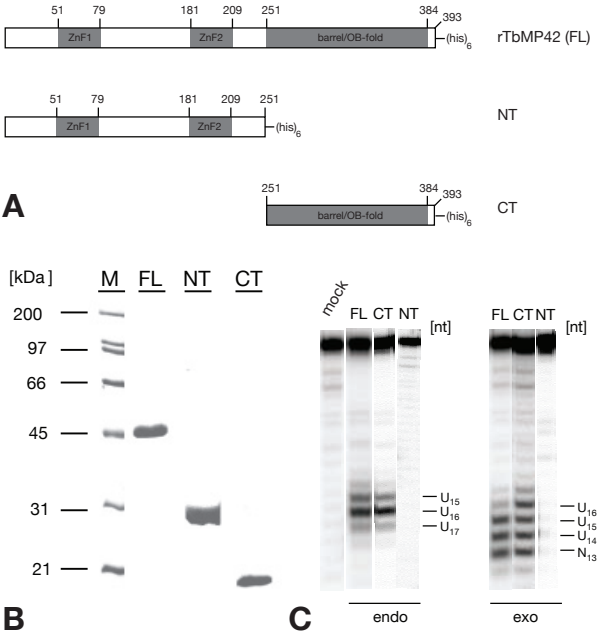

Figure 7: *The ribonucleolytic activities are located within the C-terminal fragment of rTbMP42*
(A) *Schematic representations of the 3 recombinant proteins: FL – full length rTbMP42, NT – N-terminal fragment (27kDa), CT – C-terminal fragment (16kDa). Each polypeptide carries a C-terminal extension of six histidines (his)$_6$ for affinity purification. The positions of the two zinc finger domains are marked as ZnF1 and ZnF2.* **(B)** *SDS-PAGE of the recombinant proteins after affinity purification. M – marker proteins.* **(C)** *Incubation of U5-hybrid RNA with FL, CT and NT. Hydrolysis products were separated in denaturing polyacrylamide gels and are marked by arrows. endo - endoribonuclease assay. exo - exoribonuclease assay*

function as protein/protein interaction sites. This has been shown for Zn-finger proteins in other systems (Rodgers et al., 1996; Kuroda et al., 1996) and has recently been experimentally demonstrated for TbMP63, another Zn-finger protein of the RNA editing complex (Kang et al., 2003).

Based on the position of the identified endonucleolytic cleavage sites of the tested RNA editing model substrate and the fact that rTbMP42 is capable of binding to dsRNA, the most plausible RNA binding motif for TbMP42 seems to be the anchor helix of the pre-mRNA/gRNA hybrid. On the protein level the RNA interaction domain lies within the C-terminal half of rTbMP42. Different structure prediction algorithms calculate a barrel structure for this part of the protein, possibly an oligonucleotide/oligosaccharide binding (OB)-fold (Murzin, 1993; Theobald et al., 2003). OB-folds are characterized by a five-stranded β-sheet coiled to form a closed β-barrel which is capped by an α-helix. The motif has been shown to provide a non-sequence-specific binding platform for single stranded and double stranded nucleic acids through stacking interactions between aromatic amino acid side chains and heterocyclic bases of the bound ligand. This is consistent with our experimental data, which identified binding to different nucleic acid ligands.

Binding of rTbMP42 to dsRNA and dsDNA was dependent on the presence of Zn^{2+} cations. This was, at least in part, due to a refolding reaction of the recombinant protein which was visualized in real time resonant mirror experiments and further experimentally confirmed by CD measurements. Since the two Zn-fingers are dispensable for the ribonucleolytic activities of rTbMP42 this suggests that defined Zn^{2+}-binding sites outside of the 2 Zn-fingers likely act as folding nuclei for the proper folding of the entire protein. Within

this context it is important to note, that a search for known endo- and exoribonucleolytic protein motives within TbMP42 was unsuccessful. Thus, the polypeptide might rely on so far uncharacterized protein domains for its ribonucleolytic activities. On the other hand, there is very limited sequence homology among the exoribonuclease superfamilies (Zuo and Deutscher, 2001) and some exoribonucleases as well as DNA endo-exonucleases have been shown to contain multiple invariant acidic residues, which are involved in metal ion-binding (Sayers and Artymiuk, 1998; Zuo and Deutscher, 2001; Feng et al., 2004). This is consistent with the fact that all known exoribonucleases require divalent cations for their activity and two metal ion catalysis is probably a common feature of exonucleases (Steitz and Steitz, 1993; Zuo and Deutscher, 2001). As a consequence, it seems feasible that the rTbMP42-bound Zn^{2+} cations are not only required for the proper folding of the protein but also for the nucleolytic hydrolysis reactions especially in the context that DNA-specific endo-exonucleases have been found to be Zn-dependent enzymes (Frazer, 1994). TbMP42 contains 34 acidic amino acids and we determined a critical Zn^{2+} concentration of 0.1mM in order to convert unfolded rTbMP42 into active protein. Lastly, we cannot exclude that TbMP42, within the context of the assembled editing complex, has a preference for only one of its ribonucleolytic activities. A similar scenario has been described for mutants of bovine pancreatic ribonuclease A (Cuchillo et al., 2002).

Gene silencing of *TbMP42* stops the parasites from multiplying and leads to cell death after a few days. Therefore, TbMP42 must be considered a required component for cell survival. However, mitochondrial extract in which both, TbMP42 protein and the transcript for the polypeptide are below the level of detection, still show about 10% *in vitro* editing activity and also show edited mRNAs (although at a significantly reduced level). This indicates that TbMP42 is an important component of the editing reaction cycle but it is not essential. One can speculate that in the absence of TbMP42 other ribonucleases of the editing complex can substitute for the lack of the protein especially since molecular redundancy has been shown for several other editing components (for an overview see Simpson et al., 2004). Candidate proteins might be the above-described TbMP42-related Zn-finger proteins TbMP81 and TbMP63 (Panigrahi et al., 2001) or TbMP99 and TbMP100, which have been shown to contain endo-exonuclease phosphatase motifs (Simpson et al., 2004). In addition, experimental evidence exists, which suggests that the molecular architecture of the editing complex involves two different subcomplexes, which physically separate the U-deletion and the U-insertion reaction. This might provide a rational why more than one nuclease activity is required (Schnaufer et al., 2003).

In summary, our analysis identified rTbMP42 as a 3' to 5' exoribonuclease as well as an endoribonucleolytic enzyme. The protein functions as part of the RNA editing complex which suggests that the endonuclease activity contributes to the nucleolytic cleavage of the pre-edited mRNA around an editing site and that the exoribonucleolytic activity is used for the trimming reaction of the U extensions of the 5' cleavage product. Thus, TbMP42 represents a candidate polypeptide for two important activities of the editing reaction cycle.

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Experimental Procedures

Trypanosome Cell Growth

The insect life cycle stage of *Trypanosoma brucei* 427 (Cross, 1975)) and strain 29-13 (Wirtz et al., 1999) was grown at 27°C in SDM-79 medium supplemented with 10% (v/v) heat inactivated bovine fetal calf serum (FCS) (Brun and Schöneberger, 1979). Parasite cell densities were determined by automated cell counting.

Cloning of TbMP42

TbMP42 was cloned by rapid amplification of cDNA ends (RACE). Total RNA was isolated from $3x10^9$ *T. brucei* cells and used for the isolation of poly(A)$^+$ RNA using oligo $(dT)_{25}$ latex beads. Hundred ng of poly(A)$^+$ RNA were incubated with 50µM of an oligo $(dT)_{27}$ primer for 45min at 42°C in the presence of 10U M-MuLV reverse transcriptase (RT) for the synthesis of cDNA. Samples were RNase H digested, phenol/chloroform extracted and after ethanol precipitation dissolved in 20µL TE pH8.0. The cDNA preparation was used as template for the RACE amplification using the following primers: 3'-RACE primers: GAYGGIGARTGRTTYYTIGTIAC IGG and TNGARGARGTIAAYCCIGARGA RATIAA. 5'-RACE primers: GAACAGTTTC TGTACTATATTG, AGAGGGTCCCTCGAA GTCGTG and GTGCTTCGCCTGGTAAT GGTGTTG. The 5' RACE and 3' RACE products were sequenced and the sequence information used to PCR amplify the full length *TbMP42* gene from *T. brucei* genomic DNA using primers TbMP42-5': CGCACCGAGGAGGGTGAAGTGG and TbMP42-3': AGAGGGTCCCTCGAAGTC TGTG. The PCR product was cloned into pBS SK$^-$ (Stratagene) and the nucleotide sequence of both strands of the insert was determined by automated sequencing.

Purification of rTbMP42

Full length *TbMP42* was amplified from genomic DNA using primers CGT CATGAAGCGTGTTACTTCACATATTTC and GAAGATCTCACCCTCAACACTGAC CCACAG. The PCR product was cloned into plasmid pQE60 (Qiagen). Two truncated versions of *TbMP42* were generated by restriction endonuclease hydrolysis of the PCR product. The DNA sequences translate into a N-terminal (aa 1-250) and a C-terminal variant (aa 251-393) of TbMP42. All three constructs were transformed into *E. coli* M15pREP4 (Qiagen). Positive clones were verified by DNA sequencing. Protein expression was performed in 1L bacterial cultures, induced by the addition of 1mM isopropylthiogalactoside (IPTG) at an OD_{600} of 0.5-0.6. Cells were grown for an additional 3h and harvested. The proteins were isolated from inclusion bodies in lysis buffer (10mM Tris/HCl pH8.0, 0.1M NaH_2PO_4, 8M urea). Lysates were loaded onto a Ni^{2+} chelating column and bound proteins were eluted using a pH step gradient (pH6.3; pH5.9; pH3.0). rTbMP42-containing fractions were further purified by anion exchange chromatography at denaturing conditions (8M urea) in some cases followed by isoelectric focussing and dye-binding chro-

matography. Purified rTbMP42 preparations were proteolytically cleaved and the resulting peptide sequences analyzed by mass spectroscopy (MALDI-TOF). No contaminating peptides from *E. coli* ribonucleases were identified. Denatured rTbMP42 was refolded by dialysis at 4°C against 20mM Hepes pH7.5, 30mM KCl, 10mM $Mg(OAc)_2$, 5mM $CaCl_2$, 1mM $ZnSO_4$. The percentage of active rTbMP42 was determined in ligand binding experiments using a resonant mirror system (see below). Typically about 5% of the recombinant protein preparations were capable of binding to ds nucleic acids. CD spectra were recorded at a protein concentration of 0.12mg/mL at 20°C between 260-190nm. Secondary structure contents were calculated according to Provencher and Glockner, 1981.

In Vitro RNA Editing Assay

RNA editing-active protein extracts were prepared from mitochondrial vesicles isolated by nitrogen cavitation (Hauser et al., 1996). The vesicles were lysed as described by Göringer et al., 1994. Cleared extracts (app. 10mg) were fractionated as in Pollard et al., 1992 and tested for their *in vitro* uridylate insertion RNA editing activity using a precleaved editing assay. The three RNA reactants were prepared by solid phase RNA synthesis: 5' mRNA fragment (5'CL18): GGAAGUAUGAGAC GUAGG; 3' mRNA fragment (3'CL13): AUUGGAGUUAUAG-NH_2 (amino-modified at the 3' end); gRNA (gPCA6-2A): CUAUAACUCCGAUAAACCUACGUCU CAUACUUCC. The 5' mRNA fragment was radioactively labeled using $\gamma(^{32}P)$-ATP and T4 polynucleotide kinase. All radioactive RNA preparations were purified in urea-containing polyacrylamide gels and renatured in 6mM Hepes/KOH pH7.5, 50mM KCl, 2.1mM $MgCl_2$, 0.1mM Na_2EDTA, 0.5mM DTT by heating to 70°C (2min) followed by a slow cooling interval down to 30°C before chilling on ice.

Gene Silencing by RNAi

Gene silencing of *TbMP42* by RNAi was performed using the conditional RNAi system of Wang et al., 2000. A 701bp DNA fragment from the 3' end of *TbMP42* was cloned into plasmid pZJM. Ten micrograms of the plasmid were linearized with *Not*I and 10^9 cells of *T. brucei* strain 29-13 (Wirtz et al., 1999) were transfected by electroporation. Samples were transferred into 50mL conditioned SDM 79 medium containing 20% (v/v) FCS, 50µg/mL hygromycine (hyg) and 15µg/mL neomycine (neo). After over night incubation phleomycine (2.5µg/mL) was added and antibiotic-resistant parasites were cultured for an additional 2 weeks. Clonal *TbMP42* RNAi cell lines were established by plating on agarose plates (Carruthers and Cross, 1992). The formation of TbMP42-specific dsRNA was induced by the addition of 1µg/mL tetracyline (tet).

Analysis of the TbMP42 RNAi Strain

Total RNA was isolated from 10^9 trypanosome cells according to Chomczynski and Sacchi, 1987. Cells were harvested 24, 48, 72, and 96h after tet induction. The transcript abundance of *TbMP42* was measured by RT-PCR using the primers: GGGTTTGTATTTGAAGACAAAGTTCTCC and AGAGGGTCCCTCGAAGTCTGTG. The abundance of TbMP42 after tet induction (48h) was verified by Western blotting using a monoclonal anti-TbMP42 antibody and a $(his)_6$-specific antibody. Poisoned primer extension reactions were performed as in Lambert et al., 1999.

Ribonuclease Activity Assays

RNA editing model substrates were prepared by solid phase RNA synthesis:

U5 pre-mRNA: GGGAAAGUUUGUAUU UUUGCGAGUUAAGCC, A5 pre-mRNA: GGGAAAGUUUGUAAAAAAGCGAGUUA UAGCC, gRNA: GGCUAUAACUCGCUC ACAACUUUCCC. 50-250 pmoles of the U5 or A5 pre-mRNAs were radioactively labeled either at their 5' ends with T4 polynucleotide kinase and $\gamma(^{32}P)$-ATP (3000Ci/mmol) or at their 3' ends by T4-RNA ligase and (5'-^{32}P)-pCp (3000Ci/mmol). Gel purification of the labeled pre-mRNAs was followed by annealing to the gRNA oligonucleotide and the dsRNA product was further gel purified in semi-denaturing (1M urea) polyacrylamide gels. Annealed RNAs (50fmoles, specific activity ~ 0.3µCi/ pmole) were incubated at 27°C for 3h with various concentrations of rTbMP42 in 20mM Hepes/KOH pH7.5, 30mM KCl, 10mM $Mg(OAc)_2$, 5mM $CaCl_2$, 1mM $ZnSO_4$, 0.2mM DTT, 0.5mM ATP, 0.04mM UTP. The cleavage products were separated in denaturing polyacrylamide gels and analyzed by phosphorimaging.

Optical Biosensor Measurements

The binding of rTbMP42 to different nucleic acid ligands was measured in real time using a resonant mirror system (Affinity Sensors). rTbMP42 (0.1mg/mL in 20mM $Na_xH_yPO_4$ pH7.4, 130mM NaCl, 5mM KCl, 2mM $MgCl_2$) was immobilized to the surface of a polyglutaraldehyde-activated amino silane micro-cuvette for 30min at 27°C. Remaining activated sites were blocked with bovine serum albumin (BSA, 1mg/mL) and washed. The rTbMP42-coated surface was equilibrated with binding buffer (20mM Hepes, pH7.8, 100mM KCl,1mM $MgCl_2$, 1mM $ZnCl_2$). Equilibrium dissociation constants (K_d) were derived from plots of the equilibrium resonant angle shifts as a function of the ligand concentration and fitted to the binding curve of the Langmuir adsorption isotherm. The following nucleic acid ligands were tested: ssRNA: CGGAUA UCAUACCGUC; dsRNA: GACGGUAU GAUAUCG / CGGAUAUCAUACCGUC; ssDNA: GGATATACTATAACTCCA; dsDNA: TGGAGTTATAGTATATCC / GGATATACTA TAACTCCA; DNA/RNA hybrid: TGGAGT TATAGTATATCC / GGAUAUACUAUAACU CCA.

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CHAPTER TWO

TbMP42 is a structure-sensitive ribonuclease that likely follows a metal-ion catalysis mechanism

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ABSTRACT

RNA editing in African trypanosomes is characterized by a uridylate-specific insertion and/or deletion reaction that generates functional mitochondrial transcripts. The process is catalyzed by a multi-enzyme complex, the editosome, which consists of app. 20 proteins. While for some of the polypeptides a contribution to the editing reaction can be deduced from their domain structure, the involvement of other proteins remains elusive. TbMP42, is a component of the editosome that is characterized by two C_2H_2-type zinc finger domains and a putative oligosaccharide/oligonucleotide-binding (OB) fold. Recombinant TbMP42 has been shown to possess endoribonuclease activity in vitro, however, the protein lacks canonical nuclease motifs. Using a set of synthetic gRNA/pre-mRNA substrate RNAs we demonstrate that TbMP42 acts as a topology dependent ribonuclease that is sensitive to base stacking. We further show that the chelation of Zn^{2+} cations is inhibitory to the enzyme activity and that the chemical modification of amino acids known to coordinate Zn^{2+} inactivates rTbMP42. Together, the data are suggestive of a Zn^{2+}-dependent metal-ion-catalysis mechanism for the ribonucleolytic activity of rTbMP42.

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Abstract

RNA editing in African trypanosomes is characterized by a uridylate-specific insertion and/or deletion reaction that generates functional mitochondrial transcripts. The process is catalyzed by a multi-enzyme complex, the editosome, which consists of app. 20 proteins. While for some of the polypeptides a contribution to the editing reaction can be deduced from their domain structure, the involvement of other proteins remains elusive. TbMP42, is a component of the editosome that is characterized by two C_2H_2-type zinc finger domains and a putative oligosaccharide/oligonucleotide-binding (OB) fold. Recombinant TbMP42 has been shown to possess endo/exoribonuclease activity *in vitro*, however, the protein lacks canonical nuclease motifs. Using a set of synthetic gRNA/pre-mRNA substrate RNAs we demonstrate that TbMP42 acts as an RNA topology-dependent ribonuclease that is sensitive to base stacking. We further show that the chelation of Zn^{2+} cations is inhibitory to the enzyme activity and that the chemical modification of amino acids known to coordinate Zn^{2+} inactivate rTbMP42. Together, the data are suggestive of a Zn^{2+}-dependent metal-ion-catalysis mechanism for the ribonucleolytic activity of rTbMP42.

Introduction

The insertion/deletion-type RNA editing in kinetoplast protozoa such as African trypanosomes is a unique posttranscriptional modification reaction. The process is characterized by the site-specific insertion and/or deletion of exclusively U nucleotides into otherwise incomplete mitochondrial pre-messenger RNA (pre-mRNA). RNA editing relies on small, non-coding RNAs, termed guide RNAs (gRNAs), which act as templates in the process. The reaction is catalyzed by a high molecular mass enzyme complex, the editosome, which represents a reaction platform for the individual steps of the processing cycle (reviewed in Madison-Antenucci and Hajduk, 2002; Simpson et al., 2004 and Stuart et al., 2005). An editing cycle starts with the annealing of a pre-edited mRNA to a cognate gRNA molecule. The hybridization is facilitated by matchmaking-type RNA/RNA annealing factors (Müller et al., 2001; Blom et al., 2001; Müller and Göringer, 2002; Aphasizhev et al., 2003a; Schumacher et al., 2006) that generate a short intermolecular gRNA/pre-mRNA duplex located proximal to an editing site. The pre-mRNA is then endoribonucleolytically cleaved at the first unpaired nucleotide (Seiwert et al., 1996; Kable et al, 1996; Piller et al., 1997) and in insertion editing, a 3' terminal uridylyl transferase (TUTase) adds U nucleotides

to the 3' end of the 5' pre-mRNA cleavage fragment (Aphasizhev et al., 2003b; Ernst et al., 2003). In deletion editing, U's are exonucleolytically (exoUase) removed from the 5' cleavage fragment with a 3'-5' directionality (Aphasizhev and Simpson, 2001; Igo et al., 2002). Lastly, the two pre-mRNA fragments are re-sealed by an RNA ligase activity (McManus et al., 2001; Schnaufer et al., 2001; Huang et al., 2001).

Over the past years our knowledge of the protein inventory of the editosome has significantly increased. Depending on the enrichment protocol, active RNA editing complexes contain as little as 7 (Rusché et al., 1997), 13 (Aphasizhev et al., 2003c), or up to 20 polypeptides (Panigrahi et al., 2001a). Protein candidates for every step of the minimal reaction cycle have been identified, thereby confirming the general features of the above-described enzyme-driven reaction mechanism. However, many of the enzyme activities are present in pairs or in even higher numbers of protein candidates (for a review see Carnes and Stuart, 2008). This redundancy is not understood but has been used to suggest that insertion and deletion editing are executed by separate subcomplexes (Schnaufer et al., 2003). Within this context, several candidate proteins have been suggested to account for the different ribonucleolytic activities of the editosome. TbMP90, TbMP67, TbMP61, TbMP46 and TbMP44 all contain RNase III consensus motives and TbMP100 and TbMP99 possess endo-exo-phosphatase (EEP) domains (Worthey et al., 2003). TbMP90 seems to play a role in deletion editing (Trotter et al., 2005) while TbMP61 was suggested to contribute to insertion editing as demonstrated by gene knock-out studies (Carnes et al., 2005). However, none of the candidate proteins has been shown to execute nuclease activity *in vitro*. TbMP100 and TbMP99 were shown to possess a U nucleotide-specific exoribonuclease activity *in vitro*, but gene knock-out or RNA interference (RNAi)-mediated knock-down studies remained elusive (Kang et al., 2005; Rogers et al., 2007).

TbMP42 is a component of the editosome that does not contain any typical nuclease motif (Panigrahi et al., 2001b). The protein has two C_2H_2-Zn-finger domains at its N-terminus and a putative oligonucleotide/oligosaccharide binding (OB)-fold at its C-terminus. Recombinant (r) TbMP42 has been shown to execute both, endo- and exoribonuclease activity *in vitro*. Gene ablation of TbMP42 using RNAi is lethal to the parasite and mitochondrial extracts lacking the protein have reduced endo/exoribonuclease and diminished RNA editing activity. Adding back recombinant TbMP42 can restore these activities (Brecht et al., 2005). Here we provide experimental evidence that rTbMP42 acts as an RNA structure-sensitive nuclease. Chelation of Zn^{2+} cations inhibits the ribonucleolytic activities of the protein as does the chemical modification of amino acids known to coordinate Zn^{2+}. The results are suggestive of a Zn^{2+}-dependent, metal-ion catalysis mechanism for TbMP42.

Results

The size of the pre-mRNA "U-loop" is a determinant for cleavage activity

U insertion and deletion RNA editing can be reproduced *in vitro* using short, synthetic RNA molecules that mimic the essential structural features of gRNA/pre-mRNA hybrid molecules (Seiwert and Stuart, 1994; Kable et al., 1996; Alatortsev et al., 2008). rTbMP42 has been shown to bind and cleave synthetic gRNA/pre-mRNA pairs (Brecht et al., 2005) and as an

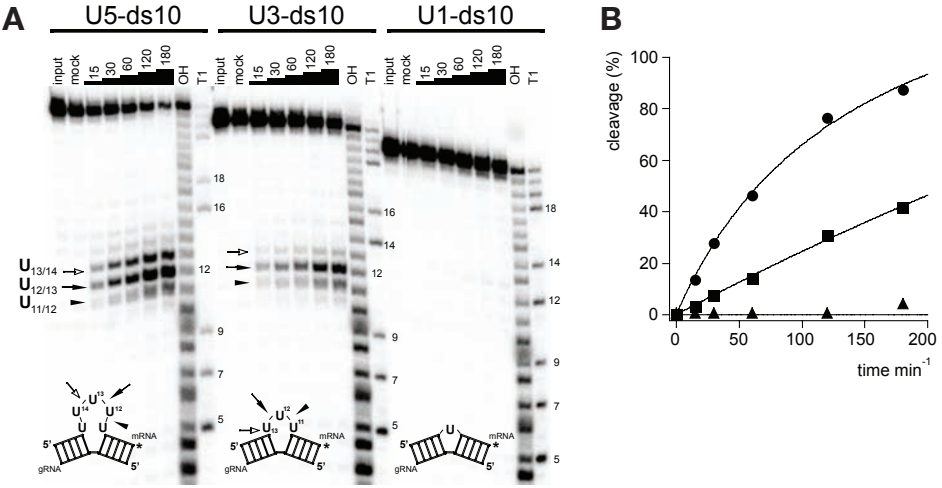

Figure 1: *Loop size is critical for cleavage efficiency.*
(A) *Reaction kinetic of the rTbMP42-mediated cleavage of gRNA/pre-mRNA hybrids U5-ds10, U3-ds10 and U1-ds10. Cartoons of the 3 RNAs are shown below the autoradiograms: top strand - pre-mRNA; bottom strand - gRNA. Radiolabeled (*) RNAs were incubated with rTbMP42 for up to 180min and reaction products were resolved electrophoretically. Cleavage positions and efficiencies are marked by arrows: filled arrow - most efficient cleavage site; open arrow: medium efficiency cleavage site; arrow head: least efficient cleavage site). "input" represents an untreated RNA sample and "mock" a sample that was incubated in the absence of rTbMP42. "T1" represents an RNase T1 hydrolysis ladder and "OH" an alkaline hydrolysis ladder.* **(B)** *Plot of the percentage of cleavage over time (circles: U5-ds10, squares: U3-ds10, triangles: U1-ds10). Data points are fitted to the Langmuir isotherm f(x)=(a*x)/(1+b*x).*

initial step to characterize the ribonucleolytic mechanism of TbMP42 we aimed at determining the substrate recognition specificity of the protein. For that we generated a set of synthetic gRNA/pre-mRNA hybrid molecules. The RNAs differ in the number of single stranded (ss) U's (1U, 3U's, 5U's) within the editing domain of the pre-mRNA sequence, while the two flanking double-stranded (ds) domains were kept constant at 10bp. The RNAs were termed U5-ds10; U3-ds10 and U1-ds10 (Fig. 1A). The three molecules were incubated with rTbMP42 and the generated hydrolysis fragments were separated by gel electrophoresis. Fig. 1A shows a representative time course experiment. After an incubation period of 15min endonucleolytic cleavage of U5-ds10 and U3-ds10 can be detected. U1-ds10 RNA was not cleaved even after 3 hours of incubation. Of the 6 internucleotide bonds that connect the U5 sequence in U5-ds10 only 3 are cleaved: U12/U13, U13/U14 and U11/U12. The same holds true for the 4 phosphodiester bonds in the U3 sequence of U3-ds10. Cleavage only occured at U12/U13, U13/U14 and U11/U12. All other phosphodiester linkages were never cleaved even after prolonged incubation times. Cleavage at the described nucleotides increased over time in the order U12/U13>U13/U14>U11/U12 in U5-ds10 and in the order U12/U13>U11/U12>U13/U14 in U3-ds10. Fig. 1B shows a plot of the percentage of cleavage over time for all three gRNA/pre-mRNA pairs. Cleavage of U5-ds10 follows a saturation function and the reaction is >90% complete after 3 hours. Half-maximal cleavage is achieved after app. 60min. For U3-ds10 a value of 40% is reached after 3 hours.

Lastly, we determined that the 3 hybrid RNAs bind to rTbMP42 with similar affinity. This was done by binding competition experiments and confirmed that the observed cleavage preference of rTbMP42 for the 3 gRNA/pre-mRNA hybrids is a reflection of the structural characteristics of the RNAs and not a consequence of interaction affinities (data not shown).

The helix length of the gRNA/pre-mRNA hybrid affects the cleavage rate

Based on the decribed results, we generated a second, complementary set of gRNA/pre-mRNA hybrid molecules. The 3 RNAs are characterized by 5 single-stranded U nucleotides within the pre-mRNA sequence and are flanked by a variable number of bp in the adjacent stem structures (13bp, 10bp, 7bp). The molecules were termed U5-ds13, U5-ds10 and U5-ds7 (Fig. 2A). In order to analyze the initial endoribonucleolytic cleavage and the subsequent exoribonucleolytic trimming reaction of rTbMP42 simultaneously, the pre-mRNA sequence of the hybrid RNAs was 5' radioactively labeled. For U5-ds13 RNA, initial cleavage was observed at position U16/U17. U5-ds10 and U5-ds7 were cleaved at the same phosphodiester bond, which in these two RNAs corresponds to positions U13/U14 (U5-ds10) and U10/U11 (U5-ds7) (Fig. 2A). Following endoribonucleolytic cleavage, the single stranded 3' U overhangs of all 3 RNAs are degraded in a 3' to 5' direction and the reaction stops at the adjacent double strand irrespective of its length (13bp, 10bp or 7bp). None of the three RNAs was cleaved to completion within 3 hours of incubation. However, the cleavage rates are different for the 3 RNAs. For U5-ds13 50% cleavage was achieved after 75min. For U5-ds10 half maximal cleavage was reached at 120min, for U5-ds7 at 150min (Fig. 2B).

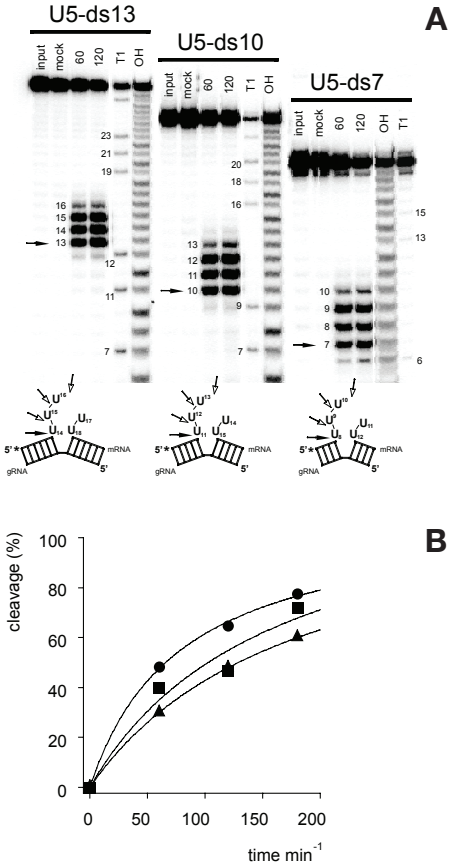

Figure 2: *Stem size influences the cleavage rate.* **(A)** *rTbMP42-mediated cleavage of gRNA/pre-mRNA hybrids U5-ds13, U5-ds10 and U5-ds7. Cartoons of the 3 RNAs are shown below the autoradiograms: top strand - pre-mRNA; bottom strand - gRNA. The position of the radiolabel is shown as a (*). RNAs were incubated with rTbMP42 and reaction products were resolved electrophoretically. Cleavage positions are marked by arrows: filled arrows - cleavage site at the ss/ds RNA junction; open arrows: cleavage sites within the U5-loop sequence. Numbers indicate pre-mRNA nucleotide positions. "input" represents an untreated RNA sample and "mock" a sample that was incubated in the absence of rTbMP42. "T1" represents an RNase T1 hydrolysis ladder and "OH" an alkaline hydrolysis ladder.* **(B)** *Plot of the percentage of cleavage over time (circles: U5-ds13, squares: U5-ds10, triangles: U5-ds7). Data points are fitted to the Langmuir isotherm f(x)=(a*x)/(1+b*x).*

Cleavage requires a 2' OH group

To further analyze the structural and chemical requirements of gRNA/pre-mRNA hybrid molecules for a rTbMP42-driven cleavage reaction, two derivatives of U5-ds10 RNA were synthesized: T5-ds10 and dU5-ds10 (Fig. 3B). While T5-ds10 represents a DNA molecule, dU5-ds10 consits of mainly ribose-moieties except within the "U-loop", which was synthesized from dU-phosphoramidites. As shown in Fig. 3A, rTbMP42 was not able to cleave T5-ds10 even after 3 hours of incubation. By contrast, dU5-ds10 was cleaved, however, at an "unusual" position. Cleavage occurred at the 5' junction of the dU5-loop to the first ribonucleotide within the stem sequence (position A16/dU15). No dU nucleotide within the "U-loop" was cleaved, suggesting a requirement for a nearby 2' hydroxyl group in the cleavage reaction.

Structure probing demonstrates a defined "U5-loop" topology

In order to rationalize the selective cleavage patterns of the different synthetic gRNA/pre-mRNA hybrid molecules by rTbMP42, we analyzed the three-di-

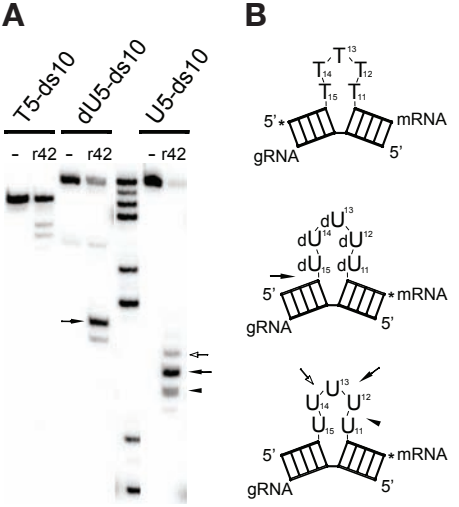

Figure 3: *Cleavage requires a proximal 2'-OH.* **(A)** *rTbMP42 (r42)-mediated cleavage of gRNA/pre-mRNA hybrids T5-ds10, dU5-ds10 and U5-ds10 RNA. The molecules were radiolabeled (*) and incubated with rTbMP42 for 3hrs. Reaction products were resolved in denaturing polyacrylamide gels. "-" represents mock treated samples.* **(B)** *Graphical representations of T5-ds10, dU5-ds10 and U5-ds10 (top to bottom). Cleavage positions/efficiencies are marked by arrows (filled arrows: most efficient cleavage site; open arrow: cleavage site of medium efficiency; arrow heads: least efficient cleavage site). T5-ds10, the "all DNA" molecule, shows an increased electrophoretic mobility.*

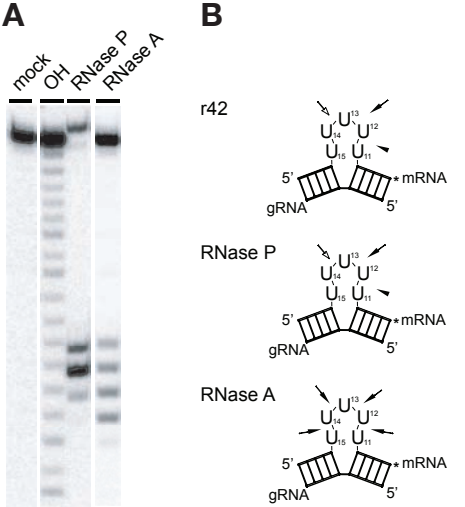

Figure 4: *Structure probing of U5-ds10 RNA. Radiolabeled (*) U5-ds10 RNA was subjected to cleavage reactions with RNase P and RNase A.* **(A)** *Electrophoretic separation of the cleavage products. "mock" - incubation in the absence of rTbMP42; "OH" - alkaline hydrolysis ladder.* **(B)** *Cartoons of U5-ds10 illustrating cleavage positions by the different enzymes. Filled arrows: most efficient cleavage site(s); open arrows: medium efficiency cleavage sites; arrow heads: least efficient cleavage sites. The decreased electrophoretic mobility of full length U5-ds10 RNA in the RNase P lane is due to a loss in charge caused by digestion of the 3'-terminal phosphate group.*

mensional (3D) folding of the "U-loop" sequence by enzymatic structure probing. For that we used the two ribonucleases RNase A and RNase P in conjunction with U5-ds10 as the RNA substrate. While both enzymes are single strand-specific (Raines, 1998; Marquez et al., 2006), RNase P has been shown to be unable to cleave stacked nucleotides (Desai and Shankar, 2003). By contrast, RNase A is known to resolve solvent-exposed as well as stacked nucleotides (Parés et al., 1991). Fig. 4A shows a representative result of the probing data. RNase A cleaves all possible U-loop positions (U11/12, U12/13, U13/14, U14/15) with equal intensity (Fig. 4B). RNase P however, cleaves U5-ds10 at only three positions and with different intensities: U12/U13>U13/U14>U11/U12 (Fig. 4B). This indicates that the U5-sequence is indeed single stranded but 2 of the U's (U12 and U11) display base stacking characteristics (Fig. 5A). rTbMP42 has the same cleavage specificity as RNase P and thus is able to distinguish between solvent-exposed and stacked nucleotides. Fig. 5B shows an energy minimized three-dimensional (3D) model of U5-ds10 derived from a molecular dynamics simulation (Lavery et al., 1995) that integrates the enzyme probing data. The 5-membered "U-loop" is folded back on itself and creates a topology that exposes 3 of the nucleotides to the solvent. The remaining 2 U's are stacked between the two helical elements of the gRNA/pre-mRNA hybrid thereby minimizing entropic costs. Thus, the main scissile phosphodiester bond is mapped to a conformation that resembles a ss/ds-junction rather than a single-stranded, looped-out organization. rTbMP42, similar to RNase P, is unable to resolve stacked nucleotides and cleaves the first unstacked U position within the U-loop of gRNA/pre-mRNA hybrid.

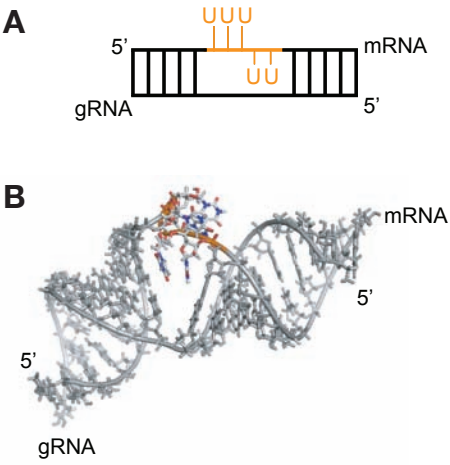

Figure 5: *3D representation of U5-ds10 RNA.* **(A)** *Cartoon of the 2D-structure of the U5-ds10 pre-mRNA/gRNA hybrid illustrating the stacked and looped-out positions of the 5-membered U-loop (orange).* **(B)** *3D model of U5-ds10 RNA derived from MD simulations. Helixes - grey; stacked/looped-out U - color.*

Zn^{2+} chelation and chemical modification of Zn^{2+}-coordinating amino acids

TbMP42 contains no canonical nuclease motif and is only related to 3 other RNA editing proteins of unknown function (TbMP81, TbMP24 and TbMP18) (Panigrahi et al., 2001b). However, TbMP42 is highly homologous to a protein known as LC-7b in Leishmania. The two polypeptides share 51% identity on the amino acid level and based on that, we analyzed whether recombinant LC-7b has ribonucleolytic activity. The protein was expressed in E. coli as a MBP (maltose-binding peptide) fusion protein and was purified to near homogeneity. Although the affinity tag could not be cleaved off after purification, rLC-7b/MBP showed an identical nucleolytic activity and cleavage specificity as rTbMP42 (Fig. 6A).

For rTbMP42 it was shown that Zn^{2+} is required for folding and RNA ligand bind-

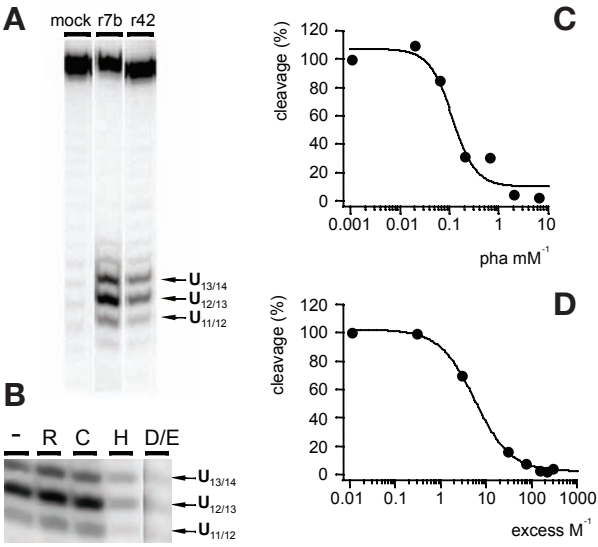

Figure 6: *Zn^{2+} ion chelation and protein modification.*
(A) *Comparison of the rTbMP42 (r42)- and rLC-7b/MBP (r7b)-mediated cleavage reaction using radiolabeled U5-ds10 RNA. "mock" - incubation in the absence of rTbMP42. Cleavage positions are marked by arrows.* **(B)** *Concentration-dependent cleavage inhibition by chelation of Zn^{2+} with 1,10 phenanthroline (pha). Half-maximal inhibition is achieved at a concentration of 0.12mM.* **(C)** *U5-ds10 RNA (3' radiolabeled at the pre-mRNA) cleavage using chemically modified rTbMP42 preparations: R - arginine modified, C - cysteine modified, H - histidine modified, D/E - aspartate/glutamate modified and "-" - unmodified. Cleavage positions are marked by arrows. The inhibition is concentration dependent as shown for the modification of D and E residues* **(D)**. *Half-maximal inhibition is achieved at a 6-fold molar excess of modification reagent over rTbMP42.*

ing (Brecht et al., 2005). Metal ions can serve as catalysts in active sites of nucleases such as in the large superfamily of nucleotidyl-transferases including RNase H, transposase, retroviral integrase and Holliday-junction resolvase (Nowotny et al., 2005; Rice and Baker, 2001; Ariyoshi et al., 1994). In these examples, acidic amino acids and histidines coordinate one or two metal ions, mostly Zn^{2+}, that activate a hydroxyl ion and position the phosphate backbone in close proximity to facilitate the in-line attack of the nucleophile. DNA polymerase and alkaline phosphatase have been suggested to follow a two-metal-ion catalysis mechanism (Beese and Steitz, 1991; Kim and Wyckoff, 1991) and metal ions have also been proposed to be involved in the nucleolytic activity of catalytic RNAs (Steitz and Steitz, 1993).

In order to analyze, whether Zn^{2+} cations play a role in the cleavage reaction of rTbMP42, we tested whether the Zn^{2+}-specific chelator 1,10 phenanthroline is able to inhibit the activity. Fig. 6B shows a representative result. The enzyme activity is inhibited in a concentration-dependent fashion with a half-maximal inhibitory concentration (IC50) of 0.12mM. At concentrations ≥2mM 1,10 phenanthroline completely blocks the cleavage reaction. This indicates a role for Zn^{2+} ions in the catalytic mechanism and further suggests that the blockage of amino acids known to coordinate Zn^{2+} ions (glutamic acid, aspartic acid, histidine) should impact the cleavage reaction as well. To test this hypothesis we covalently modified Asp and Glu residues in rTbMP42 through a carbodiimide-mediated amid bond formation to glycine ethyl esters (Hoare and Koshland, 1966; Hoare and Koshland, 1967). Histidines were modified by carbethoxylation with diethylpyrocarbonate (DEPC) (Miles, 1977) and as controls, we modified arginines and cysteines with phenylglyoxal and N-ethylmaleimide (Takahashi, 1968; Smyth el al., 1960; Smyth et al., 1964). The results are summerized in Fig. 6C/D. As expected, the modification of ar-

ginines and cysteines had no effect on the cleavage activity and specificity of rTbMP42. By contrast, the modification of Asp/Glu and of His blocked the cleavage activity of rTbMP42 in a concentration dependent fashion. At a 100-fold molar excess of modification reagent complete inhibition (≥95%) was achieved.

Discussion

TbMP42 is a component of the editosome that was characterized as an endo/exoribonuclease in vitro (Brecht et al., 2005). The protein contains two zink fingers and a putative OB-fold, but lacks typical nuclease motives. Here we aimed at providing a first picture of the putative reaction mechanism of TbMP42 by studying the RNA recognition and cleavage modality of recombinant TbMP42 using synthetic RNA editing model substrates. Single-stranded, extra-helical U's were identified as determinants for an efficient cleavage reaction, while unpaired, stacked U-nucleotides escape the cleavage reaction. Furthermore, Zn^{2+} ions play a critical role. Zn^{2+}-chelation blocks enzyme function and chemical modification of metal ion-coordinating amino acids abolishes the activity. Thus, we propose that TbMP42 acts as a structure-sensitive ribonuclease that involves Zn^{2+}-ions in its reaction pathway.

Although rTbMP42 has been shown to bind to short ssDNA and dsDNA molecules (Brecht et al., 2005), the "all DNA" substrate (T5-ds10) was not cleaved by the protein. This indicates a general nucleic acid binding capacity for rTbMP42, which likely is mediated by its C-terminal OB-fold. OB-folds are characterized by a barrel-shaped 3D-structure of five (sheets, which provide a non-sequence specific interaction platform for nucleic acids (Theobald et al., 2003). However, the cleavage activity of rTbMP42 is RNA specific. This was confirmed by utilizing a partial RNA/DNA hybrid molecule (dU5-ds10). None of the dU nucleotides within dU5-ds10 was cleaved upon incubation with rTbMP42. Instead, cleavage was directed towards the first phosphodiester linkage that involves a ribose moiety at the 5' RNA/DNA junction. This suggests a general requirement for a 2' hydroxyl group in the cleavage reaction. It further indicates a defined directionality in positioning the OH-group since the corresponding 3' DNA/RNA junction was not cleaved. Lastly, the data point towards a certain structural flexibility within the catalytic pocket. The cleavage site in the RNA/DNA hybrid is some distance away from the cleavage position in the "all RNA" U5-ds10 molecule.

In vivo, TbMP42 must recognize and cleave a wide array of different gRNA/pre-mRNA substrate molecules. They are characterized by varying numbers of looped-out U-nucleotides and based on the described results we propose that rTbMP42 indiscriminately interacts with these RNAs. However, the protein cleaves only solvent-exposed U's located proximal to an RNA helix. Stacked U's on top of helical elements are not cleaved. The 3D-model of U5-ds10 illustrates these characteristics. RNase structure probing and molecular dynamics simulations established that the U-loop in U5-ds10 has defined conformational characteristics: only 3 U nucleotides are solvent-exposed, 2 U's are in a stacked conformation. This is an expected result since bulge regions have been shown to form well-defined structures. The precise topology depends on the chemical nature of the bulged residue(s), the identity of the nucleotides flanking the bulge, and the length of the helical elements (Popenda et al., 2008; Luebke et al., 1997; Zacharias and Sklenar,

1999; Barthel and Zacharias, 2006). Some proteins have been shown to bind and stabilize defined conformers out of an ensemble of different RNA foldings (Valegard et al., 1994; Long and Crothers, 1999; Al-Hashimi, 2005). However, from the presented data we cannot conclude whether that applies to rTbMP42 as well. Furthermore, due to the fact that the length of the adjacent stem regions contributes to the cleavage rate of the reaction, we also cannot exclude that subtle structural differences induced by the accomodation of unpaired U's into the stems propagate through the entire RNA and represent a recognition signal for the protein (Popenda et al., 2008). Importantly, rTbMP42 is not able to resolve stacked positions and thus, the 2 U's in U5-ds10 remain uncleaved. *In vivo*, they likely will be "re-edited" during a subsequent reaction cycle, provided they are in a solvent-exposed i.e. extrahelical conformation. Possibly, this type of structural limitation contributes to the frequent occurence of re- and misediting events that have been observed *in vivo* (Decker and Sollner-Webb, 1990; Sturm et al., 1992). Although U1-ds10 RNA was not cleaved in our assay, depending on the sequence context it is energetically possible that single nucleotides adopt an extra-helical conformation as shown for single-base bulges as part of short model A-form RNAs (Zacharias and Sklenar, 1999). In that case, we would predict that even a single nt bulge will be cleaved by rTbMP42.

Due to the absence of an archetypical nuclease motif in TbMP42, the catalytic reaction center is difficult to trace. However, exoribonuclease super-families in general show very limited sequence homology (Zuo and Deutscher, 2001). For enzymes that rely on metal ion-driven catalysis mechanisms (reviewed in Yang et al., 2006) a large number of structural arrangements of acidic amino acids or electron donating groups can be combined to accommodate the complexation of bivalent cation(s). For TbMP42 we identified Zn^{2+} ions as being crucial for the activity of the protein. Chelation of Zn^{2+} completely abolished nuclease activity. If one or multiple Zn^{2+}-ions are held in place within the catalytic pocket of TbMP42, only certain amino acids are candidates for coordinating the metal ion(s). This includes D and E residues or amino acids capable of donating a free electron pair (H, N, Q). Glutamate and aspartate are the most obvious choices for a direct involvement. Indeed, a conserved Asp residue in conjunction with the scissile phosphate has been identified in all polymerases and nucleases to date to jointly coordinate two metal ions (Yang et al., 2006). Asp seems to be preferred for the coordination of metal ions, probably because it has fewer rotamer conformations than Glu and as a consequence is more rigid. Covalently modifying D and E residues in rTbMP42 resulted in a complete loss of the nuclease activity supporting the above described scenario. The modification of histidines abolished function as well. This could be due to a direct involvement of histidines in the metal ion coordination as shown in the RNase H family (Novotny et al., 2005; Rivas et al., 2005) or because of a proton shuttle function of a His residue (Christianson and Cox, 1999). Since the modification of cysteines did not affect the activity of rTbMP42, this confirms that functional Zn-finger motifs are not required for the activity of the protein as previously suggested by Brecht et al., 2005. Similarly, the modification of arginines did not affect the activity of rTbMP42. This excludes a contribution of the positive side chain of arginines in compensating the polyanionic properties of the bound gRNA/pre-mRNA ligand (Müller and Göringer, 2002). Fig. 7 shows

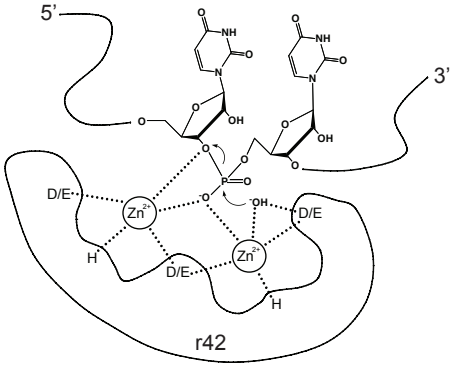

Figure 7: *The putative metal-ion reaction center. Model of the hypothetical catalytic pocket of TbMP42 with 2 Zn^{2+}-ions coordinated (dashed lines) by D, E and H residues in concert with a non-bridging oxygen of the scissile phosphate. (OH-) represents a nucleophilic hydroxyl anion derived from a deprotonated water molecule.*

a model of the putative catalytic pocket of rTbMP42 integrating the above described experimental data.

Taken together, the described data provide a first understanding on how rTbMP42 catalyzes the cleavage of gRNA/pre-mRNA hybrid molecules. The protein interacts with dsRNA domains and recognizes unpaired, looped-out uridylate residues. rTbMP42 acts as structure-sensitive, U-specific endo/exoribonuclease likely following a Zn^{2+}-ion-dependent catalysis mechanism. Acidic amino acids and histidines play a role in the formation of the putative catalytic pocket.

Materials and Methods

rTbMP42 preparation

Recombinant TbMP42 was prepared at denaturing conditions as described (Brecht et al., 2005). Protein preparations were dialyzed against 20mM HEPES pH7.5, 30mM KCl, 10mM $Mg(OAc)_2$, 5mM $CaCl_2$, 0.1mM $ZnSO_4$ and 2M urea, except for the modification reactions when urea was omitted. The Leishmania orthologue of TbMP42 (LC-7b) was purified as a C-terminal maltose binding peptide (MBP) fusion protein expressed in *E. coli* DH5α containing plasmid pMalc2x_Lt7b. Cells were lysed in 20mM Tris/HCl pH7.4, 0.2M NaCl, 1mM EDTA and 10mM (ß-mercaptoethanol using repetitive freeze/thaw cycles and sonication. The lysate was incubated with 0.5mL amylose resin for 2hrs at 4ºC. The resin was washed with 12mL buffer and the recombinant protein was eluted with the same buffer containing 10mM maltose. Eluted LC-7b/MBP was dialyzed to assay conditions.

Protein modification

To modify aspartic acid and glutamic acid residues, renatured rTbMP42 was incubated for 1hr at 27ºC in the above described buffer adjusted to pH6.2. 1-Ethyl-(3,3-dimethylaminopropyl)-carbodiimide (EDC) and glycine ethyl-ester were added up to a 275-fold molar excess. Histidine residues were derivatized in the same buffer at pH7.5 with a 50-fold molar excess of diethylpyrocarbonate (DEPC) for 1hr at 27ºC. Arginines were modified with phenylglyoxal in a 50-fold molar excess and cysteines with N-ethylmaleimide in a 80-fold molar excess. Following incubation, 50mM solutions of the different amino acids were added to stop the reactions. Finally, modified rTbMP42 was dialyzed to assay conditions.

Endo/exoribonuclease assay

RNA and DNA substrates were synthesized using solid phase phosphoramidite chemistry. The following sequences were synthesized: pre-mRNAs- U5-ds13: GGGAAAGUUGUGAUUUUUGCGAGUU AUAGCC, U5-ds10: GGGAAUGUGAUU UUUGCGAGUAGCC, U3-ds10: GGGAA UGUGAUUUGCGAGUAGCC, U1-ds10:

GGGAAUGUGAUGCGAGUAGCC. U5-ds7: GGGGUGAUUUUUGCGAGCC, T5-ds10: d(GGGAATGTGATTTTTGCGAGTAGCC), dU5-ds10: GGGAAUGUGAdUdUdUdUdUGCGAGUAGCC. gRNA sequences: gU5-ds13: GGCUAUAACUCGCUCACAACUUUCC, g(U5,dU5,U3) and gU1-ds10: GGCUACUCGCUCACAUUCCC, gU5-ds7: GGCUCGCUCACCCC, gT5-ds10: GGCTACTCGCTCACATTCCC. Oligonucleotide concentrations were determined by UV absorbancy measurements at 260 nm using extinction coefficients (ε_{260}, L mol^{-1} cm^{-1}) calculated from the sum of the nucleotide absorptivity as affected by adjacent bases (Puglisi and Tinoco, 1989). RNAs were radioactively labeled, purified and annealed as in Brecht et al., 2005. Annealed RNAs (50fmol, specific activity 0.3µCi/pmol) were incubated with 50-80pmoles of rTbMP42 or 10pmoles of rLC-7b/MBP in 30µL 20mM HEPES pH7.5, 30mM KCl, 10mM $Mg(OAc)_2$, 5mM $CaCl_2$, 0.1mM $ZnSO_4$, 0.2mM DTT, 0.5mM ATP, 0.04mM UTP and 1M urea for up to 3h at 27ºC. Competition binding assays were performed for 2hrs at 27ºC in a 30µL reaction volume. Samples contained 50pmoles refolded rTbMP42, 0.5pmoles radiolabelled U5-ds10 and increasing amounts (30fmoles to 7µmoles) of non-radioactive U5-ds10 or U1-ds10. Reaction products were analyzed as above.

Structure probing, RNA modeling and MD simulation

Radioactively labeled U5-ds10 was subjected to RNase A (0.03ng/µL) or RNase P (2mU/µL) treatment for up to 30 minutes. Reaction products were separated in denaturing polyacrylamide gels (18% w/v, 8M urea) and analyzed by phosphorimaging. Molecular dynamics calculations were performed using junction minimization of nucleic acids (JUMNA, Lavery et al., 1995). Images were rendered using PyMOL (Delano, 2002; http://www.pymol.org).

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CHAPTER THREE

Kinetoplastid RNA editing involves a nucleotidyl phosphatase activity

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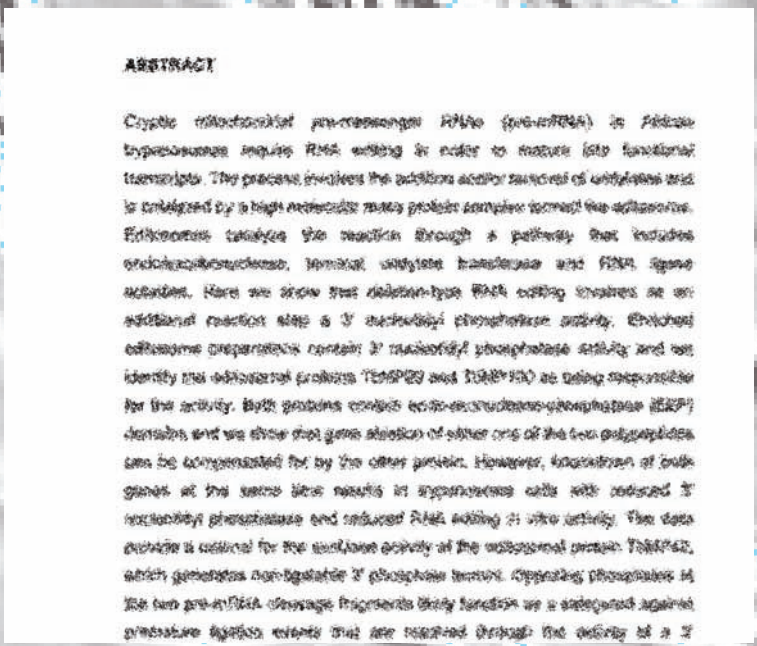

ABSTRACT

Cryptic mitochondrial pre-messenger RNAs (pre-mRNAs) in African trypanosomes require RNA editing in order to mature into functional transcripts. The process involves the addition and removal of uridylate and is catalyzed by a high molecular mass protein complex termed the editosome. Editosomes catalyze the reaction through a pathway that includes endoribonucleolysis, terminal uridylate transferase and RNA ligase activities. Here we show that deletion-type RNA editing involves as an additional reaction step a 3′ nucleotidyl phosphatase activity. Enriched editosome preparations contain 3′ nucleotidyl phosphatase activity and we identify two editosomal proteins, TbMP29 and TbMP30, as being responsible for the activity. Both proteins contain exonuclease–phosphatase (EEP) domains and we show that gene ablation of either one of the two polypeptides can be compensated for by the other protein. However, knockdown of both genes at the same time results in trypanosome cells with reduced 3′ nucleotidyl phosphatase and reduced RNA editing in vivo activity. The data provide a rationale for the nucleotidase activity of the editosomal protein TbRGG2, which generates non-ligatable 3′ phosphate termini. Opposing phosphatase to ligase pre-mRNA cleavage fragments may function as a safeguard against premature ligation events that are required during the editing of 3′

Kinetoplastid RNA editing involves a nucleotidyl phosphatase activity

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Abstract

Cryptic mitochondrial pre-messenger RNAs (pre-mRNA) in African trypanosomes require RNA editing in order to mature into functional transcripts. The process involves the addition and/or removal of uridylates and is catalyzed by a high molecular mass protein complex termed the editosome. Editosomes catalyze the reaction through a pathway that includes endo/exoribonuclease, terminal uridylate transferase and RNA ligase activities. Here we show that deletion-type RNA editing involves as an additional reaction step a 3' nucleotidyl phosphatase activity. Enriched editosome preparations contain 3' nucleotidyl phosphatase activity and we identify the editosomal proteins TbMP99 and TbMP100 as being responsible for the activity. Both proteins contain endo-exonuclease-phosphatase (EEP) domains and we show that gene ablation of either one of the two polypeptides can be compensated by the other protein. However, knockdown of both genes at the same time results in trypanosome cells with reduced 3' nucleotidyl phosphatase and reduced RNA editing in vitro activity. The data provide a rational for the exoUase activity of the editosomal protein TbMP42, which generates non-ligatable 3' phosphate termini. Opposing phosphates at the two pre-mRNA cleavage fragments likely function as a safeguard against premature ligation events that are resolved through the activity of a 3' nucleotidyl phosphatase.

Introduction

RNA editing in kinetoplast protozoa is a post-transcriptional processing event that modifies mitochondrial transcripts by the insertion and/or deletion of exclusively uridylyl residues. The reaction pathway is mediated by a unique ribonucleoprotein (RNP) complex, termed the editosome. The editing machinery consists of about 20 peptides (Panigrahi et al., 2001a), and provides a platform for the individual steps of the reaction cycle (reviewed in Madison-Antenucci and Hajduk, 2002; Simpson et al., 2004; Carnes and Stuart, 2008). Editosomes are guided by small, non-coding RNA molecules, termed guide RNAs (gRNA), which provide specificity to the editing cycle. The reaction pathway starts with the hybridization of a pre-edited messenger RNA (pre-mRNA) to its cognate gRNA, which is catalyzed by matchmaking-type RNA/RNA annealing factors (Müller et al., 2001; Blom et al., 2001; Müller and Göringer, 2002; Aphasizhev et al., 2003a; Schumacher et al., 2006). The resulting pre-mRNA/gRNA hybrid is characterized by three domains. A short duplex is located immediately 3' to

an editing site. The actual editing site contains mismatches between the two RNA molecules and thereby defines the uridylyl insertion and/or deletion events. 5' proximal to the pre-mRNA's editing site an imperfect duplex is formed involving the gRNAs 3' oligo(U)-tail (Blum et al., 1990; Seiwert and Stuart, 1994; Blum and Simpson, 1990, Alatortsev et al., 2008). The pre-mRNA is processed by an endoribonuclease, which is dictated by the gRNA/pre-mRNA mismatches at the editing site. Based on gene knockout studies it was shown, that in insertion editing this function is carried out by TbMP61 (Carnes et al., 2005) and in deletional editing by TbMP90 (Trotter et al., 2005). In the case of an insertion event, uridylyl residues are added by the terminal uridylyl transferase (TUTase) TbMP57 (Ernst et al., 2003, Aphasizhev et al., 2003b). In deletion editing, uridylyl residues are removed by a 3' to 5' specific exoribonuclease (exoUase). Several candidate proteins have been suggested to account for the exoUase activity (Brecht et al., 2005; Kang et al., Rogers et al., 2007). The last step in the reaction cycle is the re-ligation of the pre-mRNA fragments, which is catalyzed by the RNA editing ligases TbMP48 and TbMP52 (McManus et al., 2001; Schnaufer et al., 2001; Rusché et al., 2001). TbMP48 is suggested to act in insertion editing and TbMP52 appears to play a role in deletion editing (Huang et al., 2001; Cruz-Reyes et al., 2002; Gao and Simpson, 2003). The redundancy within the editosomal protein inventory is not understood, but has lead to the suggestion that insertion and deletion editing are catalyzed by distinct subcomplexes (Schnaufer et al., 2003).

Here we focus on the exoUase activity of the editing reaction cycle. Despite the identification of exoUase activities in mitochondrial extracts of *Leishmania* and *Trypanosoma* (Aphasizhev and Simpson, 2001; Igo et al., 2002), an assignment to one (or more) of the individual peptide(s) remained elusive. The high redundancy of putative exoUases is rendering RNAi-mediated gene ablation experiments inconclusive and difficult to interpret (Kang et al., 2005). Three proteins have been shown to execute exoUase activity *in vitro* and are implicated to contribute to the *in vivo* activity (Brecht et al., 2005; Kang et al., 2005; Rogers et al., 2007). TbMP100 and TbMP99 are highly identical proteins (29% identity/46% similarity on the amino acid level) and both proteins possess a 5'-3' exonuclease domain as well as an endo-exo-phosphatase (EEP) domain (Panigrahi et al., 2003; Worthey et al., 2003). Both proteins execute a single-strand (ss), U-specific 3' to 5' exoUase activity *in vitro*, which is abolished by defined point mutations within the EEP domain (Rogers et al., 2007). It has also been shown, that the 5' to 3' exonuclease domain is not needed for the uridylyl removal reaction. However, these investigations were carried out with ssRNA molecules terminating in a single uridylyl residue. Gene ablation of TbMP100 through RNA interference (RNAi) resulted in a severe growth defect phenotype (Kang et al., 2005) and the analysis of TbMP100-deficient mitochondrial extracts showed reduced *in vitro* pre-cleaved insertion and deletion activities 200 hours post-induction. However, at this time point, the cells have stopped dividing and the 20S editing complexes have started to disassemble (Kang et al., 2005). No *in vivo* data regarding knockout/knockdown studies of TbMP99 have been published.

TbMP42 has no canonical nuclease consensus motif (Panigrahi et al., 2001b). The protein is characterized by two C_2H_2-type Zn-fingers at its C-terminus and a putative oligonucleotide/oligosaccharide-binding domain (OB-fold) at its C-terminus (Panigrahi et al., 2001b). Recombinant

(r) TbMP42 has been shown to bind nucleic acid substrates with nanomolar affinity and it executes both endo- and exoribonuclease activities *in vitro* (Brecht et al., 2005). TbMP42-deficient mitochondrial extracts show reduced pre-cleaved insertion editing activity, as well as reduced endo- and exoribonuclease activity. All three activities can be restored through the addition of rTbMP42 to TbMP42-depleted extracts (Brecht et al., 2005). The catalytic activity resides within the OB-fold at the C-terminus of the protein (Brecht et al., 2005). It was further shown, that rTbMP42 acts as a topology-dependent ribonuclease that is sensitive to base-stacking (Niemann et al., 2008). The enzyme relies on Zn^{2+}-ions and likely follows a metal-ion catalysis reaction pathway (Niemann et al., 2008).

Here we present an analysis of the uridylyl removal reaction that is catalyzed by rTbMP42. We demonstrate that the enzyme releases 3' nucleoside monophosphates from a 3' oligo(U)-overhang of a pre-cleaved deletion editing substrate (Igo et al., 2002). The resulting 3' phosphate at the 5' cleavage fragment prevents premature ligation of the pre-mRNA. Thus, the completion of the editing reaction cycle depends on a 3' specific nucleotidyl phosphatase activity. The activity can be identified in mitochondrial extracts and is associated with the editing complexes. Two candidate proteins, TbMP100 and 99 possess an endo-exo-phosphatase (EEP) domain. Neither TbMP100- nor TbMP99-depleted mitochondrial extracts display reduced phosphatase activity, however, if both proteins are down-regulated, the phosphatase activity is lost. The data indicate that both TbMP99 and 100 can function as 3' specific nucleotidyl phosphatases *in vivo* and are able to complement each other. Together, we demonstrate that RNA editing in African trypanosomes requires an additional enzymatic activity to complete a deletion editing reaction cycle.

Results

TbMP42 creates 3' phosphate termini

Starting point of our analysis was the characterization of the exoribonucleolytic activity of the editosomal protein TbMP42 (Brecht et al., 2005). TbMP42 is a single strand (ss)-specific endo/exoribonuclease that is sensitive to base-stacking (Niemann et al., 2008). The enzyme likely follows a two-metal-ion reaction pathway and recombinant (r) TbMP42 has been shown to cleave and process synthetic gRNA/pre-mRNA hybrid RNAs in a distributive fashion (Brecht et al., 2005; Niemann et al., 2008). In order to analyze the biochemical characteristics of the TbMP42-directed trimming reaction we examined the reaction products of the exonucleolytic activity of rTbMP42. For that we used a 55nt long RNA oligonucleotide (RNA55) that was transcribed in the presence of α-(^{32}P)-CTP (Fig. 1A). Following transcription, RNA55 was digested with rTbMP42 and compared to control digests with RNase P1 and a cocktail of RNase A, RNase T1 and RNase T2. RNase P1 has been shown to generate 5' nucleoside monophosphates (5' NMP; pN) (Kirsebom, 2002), while RNase A, RNase T1 and RNase T2 all generate 3' NMP's (Np) (Takahashi and Moore, 1982; Raines, 1998; Deshpande, 2002). Digestion products were separated by two-dimensional (2D) thin layer chromatography (TLC) (Bochner and Ames, 1982) and Fig. 1B shows a representative result. Due to its cleavage specificity RNase P1 only generates radioactive 5' CMP. In the case of the 3' NMP-generating RNases the radioactive label is "transferred" to the 3' neighboring nucleoside thereby generating all 4

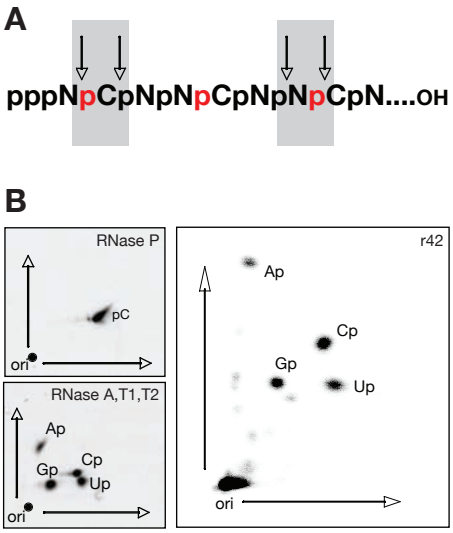

Figure 1: Nucleotide analysis of the r42–mediated trimming reaction.
(**A**) schematic representation of the RNA55 substrate. The RNA is internally labeled with α-^{32}P-CTP. A ribonuclease generating 3' nucleoside monophosphates produces only radioactive 3' cytosin monophosphate (pC), whereas a ribonuclease generating 5' nucleoside monophosphates produces all four radioactive 5' nucleoside monophosphates (Np). Red = ^{32}P. (**B**) The described RNA molecule was incubated with different ribonucleases and the products were resolved by 2D thin-layer chromatography (TLC) and visualized by phosphorimaging.

radioactive 3' NMPs. Digestion of RNA55 with rTbMP42 shows the same specificity, which demonstrates that TbMP42 proceed through a reaction mechanism that generates 3' nucleoside monophosphates (Np).

Editosomes contain 3' nucleotidyl phosphatase activity

The above described result has two consequences for the editing reaction cycle. First, it implies that the 5' pre-mRNA cleavage fragment after the exonucleolytic trimming reaction by rTbMP42 must carry a 3' phosphate. Second, since the 3' pre-mRNA cleavage fragment has been shown to be 5' phosphorylated (Seiwert et al., 1996) this creates a situation of two opposing phosphate groups, which are no substrate for the subsequent ligation step (Deng et al., 2004). As a consequence, we investigated, whether enriched editosome preparations contain a 3' specific nucleotidyl phosphatase activity to release the 3' terminal phosphate from the 5' pre-mRNA fragment.

For our analysis we used a chemically synthesized RNA substrate with a length of 31 or 8nt (RNA-p). The RNA is characterized by a hydroxylated 5' terminus and was 3' phosphorylated through the ligation of radioactively labeled (5'-^{32}P) pCp. RNA-p was incubated with 20S editosome preparations for up to 3 hours and the generated reaction products were analyzed by gel electrophoresis. Figure 2A shows a representative result. Over time, a radioactive product with a decreased electrophoretic mobility is generated, which co-migrates with a control sample where RNA-p was 3' dephosphorylated using alkaline phosphatase (AP). This confirms that 20S editosomes have 3' nucleotidyl phosphatase activity. Since 3' nucleotidyl phosphatases frequently are acidic enzymes and require bivalent cations for their activity (Jilani and Ramotar, 2002; Desphande and Wilson, 2004) we analyzed the editosome-associated nucleotidyl dephosphorylation activity with respect to these criteria. Interestingly, the activity is maximal at two pH values, pH 5.5 and pH 6.7 (Fig. 2B). Zn^{2+}-cations are required for the dephosphorylation reaction and Ca^{2+}-ions inhibit the reaction at concentrations > 5mM (data not shown). As above, Mg^{2+}- ion titration and EDTA chelation experiments identified two optima at 5mM and 20mM (Fig. 2C), which is suggestive of two separate activities.

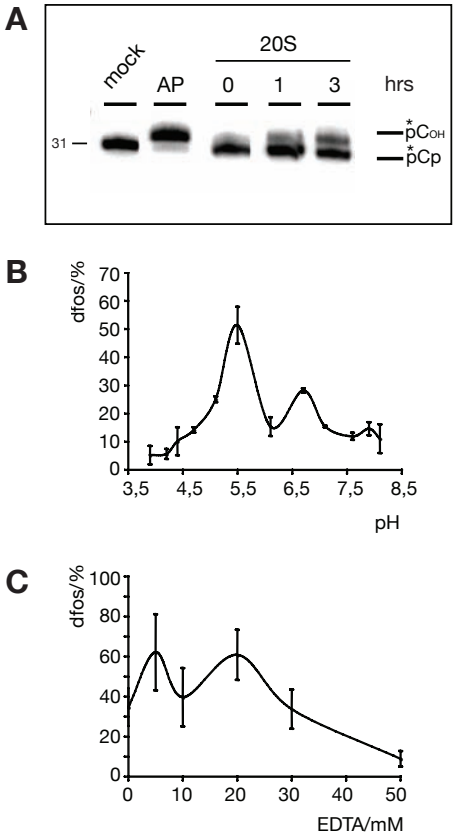

Figure 2: Characterization of the 3' nucleotidyl phosphatase activity of 20S editosomes.
(**A**) A ssRNA molecule was radioactively labeled at its 3' end using α-^{32}P-mononucleoside 3',5' bisphosphate (depicted by *) and incubated for up to 3h with 20S editosomes. Reaction products were resolved in urea containing polyacrylamide (PAA) gels (8M urea, 18% w/v PAA). The dephosphorylated RNA species shows a decreased electrophoretic mobility due to the loss of negative charge. Alkaline phosphatase (AP) served as a positive control. pH optima of the dephosphorylation activity (**B**) and bivalent cation requirements (**C**).

TbMP99 and TbMP100 as candidate nucleotidyl phosphatases

The inventory of editosomal proteins contains two candidate proteins for the above-described activity, which have been termed TbMP99 and TbMP100 (Panigrahi et al., 2003, Worthey et al., 2003). Both proteins carry an endo-exophosphatase (EEP) domain and a 5' to 3' exonuclease domain. The proteins have a high degree of amino acid identity/similarity (29%/46%) and have been shown to possess *in vitro* exoUase activity (Kang et al., 2005; Rogers et al., 2007). In order to investigate if the two proteins contribute to the described 3' nucleotidyl phosphatase activity we generated conditional knockdown (kd) trypanosome cell lines using RNAi (Wang et al., 2000). Three RNAi strains were constructed: individual single knockdown (skd) cells for TbMP99 (99 skd) and TbMP100 (100 skd) and a double knockdown cell line (99/100 dkd), which allows down regulation of both genes at the same time. All three strains were analyzed by RT-PCR to confirm gene ablation after induction with tetracycline (tet). Fig. 3A shows that 6 days post-induction the transcript level of all 3 mRNAs is at or below the level of detection. Although TbMP99 and TbMP100 share a high degree of similarity, RNAi-mediated down regulation is specific for the individual mRNAs. Analysis of the growth behaviour of the three cell lines demonstrated no growth rate phenotype for 99 skd cells, while 100 skd trypanosomes multiplied with a reduced cell doubling starting at around 150h post-induction (Fig. 3B). The 99/100 dkd cell line showed the strongest phenotype with a reduced growth rate already apparent after 100h of RNAi (Fig. 3B). As a follow up we determined the integrity of editing complexes in mitochondrial extracts of all three cell lines. This was done by monitoring the distribution of the two core components of the 20S editosome, the RNA ligases TbMP52 (KREL1) and TbMP48 (KREL2) (Stuart et al., 2005). Down regulation of TbMP99 caused a complete loss of TbMP52 (Fig. 3C), although TbMP48 was still present in

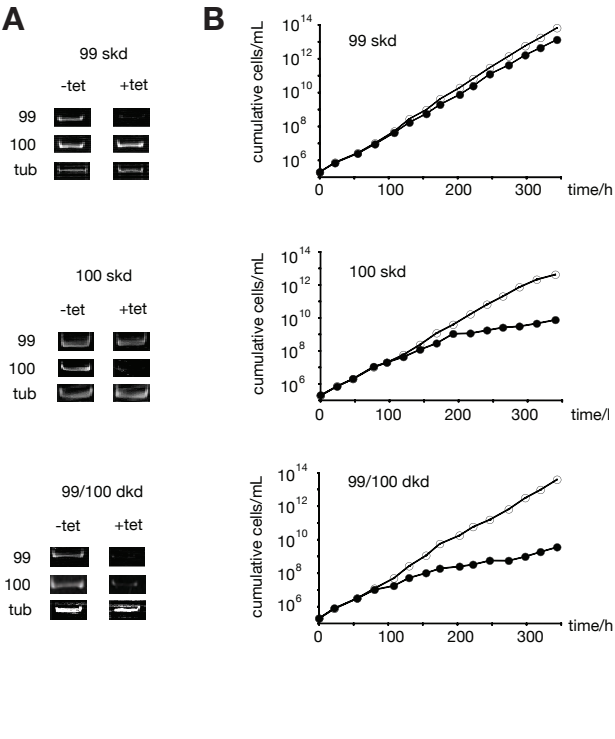

Figure 3: Consequences of the RNAi-mediated down-regulation of *TbMP99* and/or *TbMP100*.
(**A**) RT-PCR verified the efficiency of the mRNA down-regulation of the TbMP99 single knockdown (99 skd), the TbMP100 single knockdown (100 skd) and the double knockdown of both TbMP99 and TbMP100 (99/100 dkd). Upon induction for 6 days with tetracycline (tet) the remaining mRNA of either transcript was ≤5%. The α-tubuline (tub) transcript was used as internal control. (**B**) Growth behavior of 99 skd, 100 skd and 99/100 dkd cells. (**C**) Autoadenylation of mitochondrial extracts fractionized in linear 10-35% (v/v) glycerol gradients. Wild type (WT) extracts were compared to extracts derived from 99 skd, 100 skd and 99/100 dkd cells prepared 6 days post-induction

complexes ≥20S. Knockdown of TbMP100 did not affect the distribution of the two RNA ligases and extracts from 99/100 dkd cells showed a distribution pattern identical to the 99 skd extracts (Fig. 3C).

Editosomes from 99/100 dkd cells have reduced editing and reduced nucleotidyl phosphatase activity

Enriched 20S editosome preparations from all three RNAi cell lines were analyzed for their RNA editing *in vitro* activity. U-deletion and U-insertion RNA editing were monitored independently (Seiwert and Stuart, 1994; Kable et al., 1996) and the two skd-cell lines (99 skd, 100 skd) showed activities identical to 20S editosome preparations derived from uninduced cells (data not shown). However, 20S editosome fractions from the mitochondria of the 99/100 dkd cell line were severely affected in their ability to faithfully process exogenously added substrate pre-mRNAs. Insertion editing went down to a value of ≤40% (Fig. 4A) and deletion editing dropped to ≤10% of the activity derived from uninduced cells (Fig. 4B). The same holds true for the 3' nucleotidyl phosphatase activity. 20S mitochondrial fractions from the two skd-cell lines were fully competent to dephosphorylate substrate RNAs at their 3' ends (data not shown). However, 20S fractions

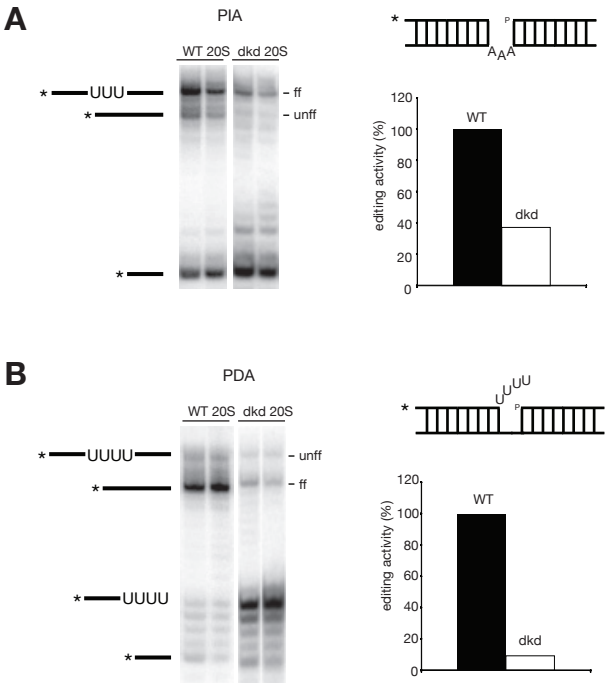

Figure 4: Pre-cleaved RNA editing activity of TbMP99/100-depleted extracts.
(**A**) 20S fractions of wild type (WT) and TbMP99/100 double knockdown (99/100 dkd) mitochondrial extracts were compared in precleaved insertion (PIA) and deletion (PDA) (**B**) assays. Representations of the two pre-mRNA/gRNA substrates are depicted at the right of (A) and (B). A * indicates the radiolabel. Reaction products were separated in denaturing polyacrylamide gels (18% w/v) containing 8M urea. In the PDA (A), the electrophoretic mobilities of the unfaithfully edited (unff) product, the faithfully edited (ff) product, the pre-mRNA 5' fragment and the exoUase processed 5' pre-mRNA fragment are given on the left (top to bottom). In the PIA assays (B), the electrophoretic mobilities of the faithfully edited (ff) product, the unfaithfully edited product (unff) and the 5' pre-mRNA fragment are given on the right (from top to bottom). The graph represents the quantification of the faithfully edited (ff) product in the autoradiograph.

from the 99/100 dkd cell line had 3' nucleotidyl phosphatase activities of only ≤5% (Fig. 5) when compared to WT extracts. This suggests that TbMP99 as well as TbMP100 can act as nucleotidyl phosphatases. Thus, whithin the context of the editing complex, either one of the two proteins is sufficient.

ExoUase activity in 99/100 dkd cells is not reduced

Recombinant TbMP99 and TbMP100 have been shown to execute exoUase activity *in vitro* (Kang et al., 2005; Rogers et al., 2007). In order to analyze, whether the two proteins contribute to the exoUase activity that is associated with the 20S editosome we again utilized the 99/100 dkd cell line. For the assay we used a non-ligatable deletion-type RNA editing substrate (Fig. 6A). The gRNA/pre-mRNA hybrid lacks a 5' phosphate at the 3' pre-mRNA cleavage fragment and thus cannot be ligated. Fig. 6B shows a representative result of a deletion-exoUase (deleX) assay. As expected, no ligation takes place and thus the deleX assay monitors exoUase activity independently of any ligase event. 20S editosome fractions from WT trypanosomes exhibit exoUase activity and faithfully process the 4 ss U nucleotides from the 5' pre-mRNA cleavage product. Mitochondrial extracts derived from the 99/00 dkd cell line also contain exoUase activity, however, the activity sediments below 20S.

Discussion

The final reaction step in both, deletion and insertion-type RNA editing is the

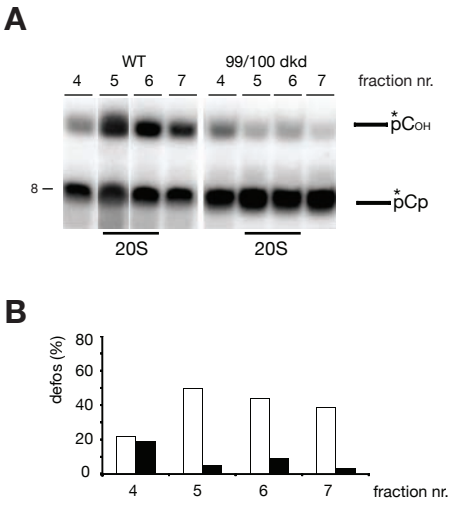

Figure 5: 3' nucleotidyl phosphatase assays of wild type (WT) and TbMP99/100-depleted (99/100 dkd) 20S editosomes.
(**A**) Autoradiograph of a phosphatase assay. A * represents the position of the radioactive label. The reduced electrophoretic mobility of the dephosphorylated RNA species is depicted on the right. Individual glycerol gradient fractions are indicated. (**B**) Quantitative analysis of the assay shown in (A).

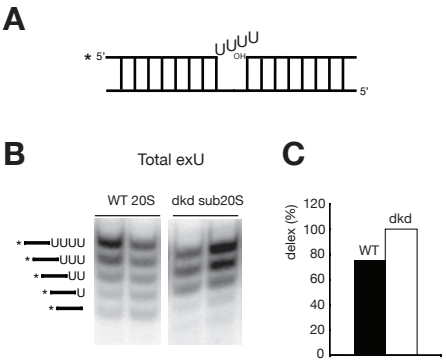

Figure 6: ExoUase activity of TbMP99/100-depleted editosomes.
(**A**) Graphical representation of the deletion-exoUase (deleX) pre-mRNA/gRNA substrate. The 3' cleavage fragment of the pre-mRNA contains a 5' hydroxyl (OH), thus preventing ligation of the pre-mRNA. (**B**) Autoradiograph of the deleX RNA substrate incubated with wild type (WT) 20S glycerol gradient fractions and TbMP99/100-depleted (99/100 dkd) sub-20S glycerol gradient fractions. Reaction products were separated in denaturing 18% (w/v) polyacrylamide gels containing 8M urea. Electrophoretic mobilities of 5' pre-mRNA fragments with no U, 1U, 2Us, 3Us and 4Us removed are given on the left (top to bottom). (**C**) Quantification of the total amount of uridylyl residues removed in WT 20S and 99/100 dkd sub-20S fractions.

re-ligation of the two processed pre-mRNA fragments. This requires the presence of a 3' hydroxyl and a 5' phosphate terminus at the editing site. The generation of 5' phosphate ends during the initial endonucleolytic cleavage of the pre-mRNA was demonstrated by Seiwert et al., 1996. This lead to the assumption that any exoribonucleolytic activity releases 5' nucleoside monophosphates (Rusché et al., 1997). In this study, we demonstrated that TbMP42, a protein component of the editing complex with endo- and exoribonucleolytic *in vitro* activity (Brecht et al., 2005), proceeds through a reaction cycle that endoribonucleolytically produces 3' phosphate and 5' OH termini. Furthermore, we verified that the exoribonucleolytic activity of TbMP42 generates 3' nucleoside monophosphates. Thus, we suggest that although rTbMP42 has both endo- and exoribonuclease activity *in vitro*, it appears that *in vivo* the endoribonucleolytic step is carried out by a different protein or a different set of proteins. Recent studies (Trotter et al., 2005; Carnes et al., 2005) suggest that the editosomal proteins TbMP61 and TbMP90 contribute to the endoribonucleolytic cleavage reaction. Mutations of single amino acids within the RNaseIII domains of the two proteins revealed discrete roles in both, deletion and insertion RNA editing. The absence of either TbMP61 or TbMP90 did not alter the sedimentation behavior of the editing complex and the endoribonucleolytic activity was reduced although TbMP42 was detected by Western blotting. Neither TbMP61 nor 90 were shown to have ribonuclease activity *in vitro*, but

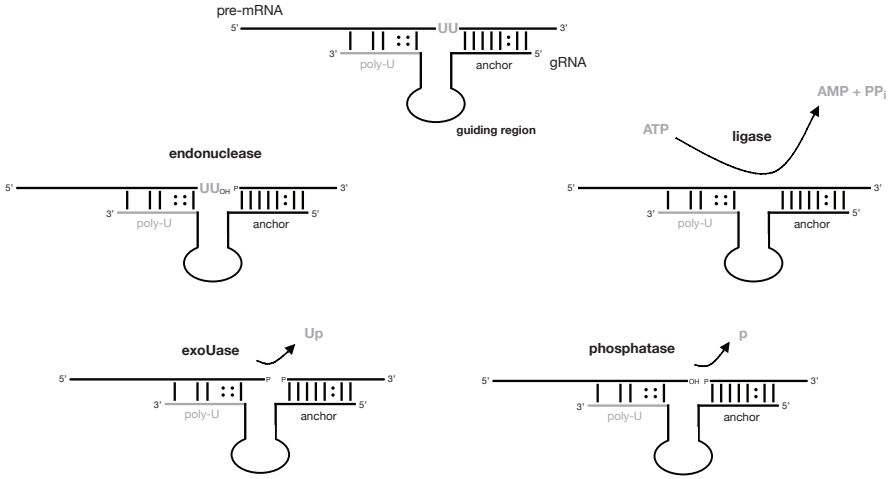

Figure 7: Revised model of the editing reaction cycle including a phosphatase (altered from Ochsenreiter and Hajduk, 2008).

the data indicate that TbMP61 is relevant for insertion editing and TbMP90 for deletion editing. TbMP67, another protein with an RNaseIII motif, was shown to play a role in the endoribonucleolytic processing of exclusively COII transcripts (Carnes et al., 2008).

However, the 3' nucleoside monophosphates removed from a deletional editing site by rTbMP42 are genuine. Moreover, the electrophoretic mobility of 3' uridylyl overhangs processed by rTbMP42 compared to editing active fractions of mitochondrial extracts is identical (data not shown). This suggests that either TbMP42 is the only active exoUase of the 20S editosome or that other enzymes follow the same reaction mechanism. Otherwise, one should be able to detect mixed populations of semi-processed 3' uridylyl-overhangs in PDA or deleX assays, which is not the case (e.g. Fig. 5). Taken together, the data are indicative of a scenario where TbMP42 acts as 3' to 5' exoUase and its intrinsic endoribonuclease function found *in vitro* is secondary.

RNA editing reloaded

In the emerging picture, a faithfully processed deletion-editing site is subject to a endoribonucleolytic cleavage reaction, which generates a 5' phosphate at the 3' cleavage fragment (Seiwert et al., 1996). The exoUase activity of the editosome then creates 3' phosphate termini thereby introducing a roadblock that prevents premature ligation. This roadblock is removed by a 3'-specific, acidic nucleotidyl phosphatase activity, which is present in mitochondrial extracts of African trypanosomes and is associated with a 20S high molecular mass complex. In that sense, the described reaction cascade serves as a protection against unfaithful editing events. Further evidence for the described scenario can be derived from an analysis of the reaction products in the pre-cleaved *in vitro* deletion editing assay (PDA). The dominant ligation product is the "faithfully" edited mRNA with all four Us removed and the two mRNA fragments properly ligated. The second most abundant product is the unedited mRNA prod-

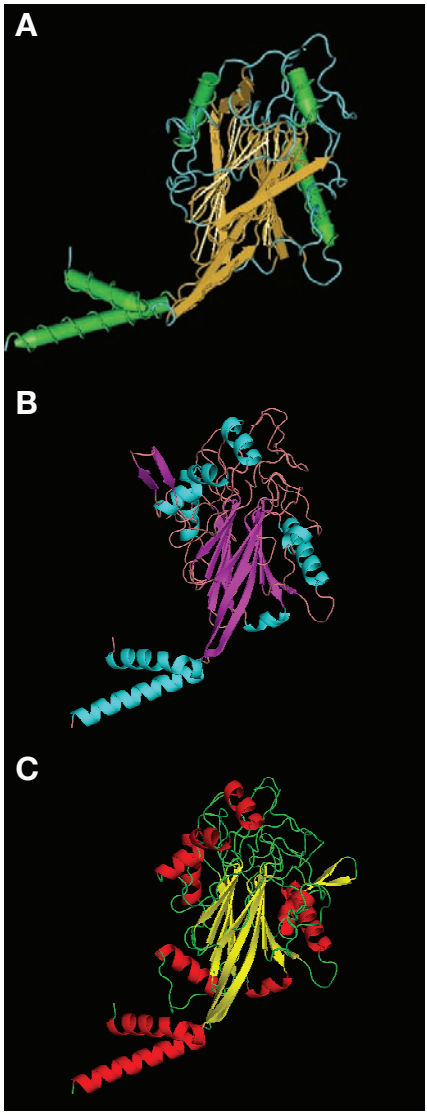

Figure 8: Crystal structure (**A**) of the phosphatase synaptojanin and homology models of the two putative phosphatases TbMP99 (**B**) and TbMP100 (**C**).

uct. No U-residue is removed from the editing site but the two mRNA fragments are ligated. All other ligation products (-3U, -2U, -1U) are below the level of detection. One possible explanation could be that the recruitment of the 3'-specific nucleotidyl phosphatase is in concert with the dissociation of the exoUase activity. Another explanation would be an exoUase with a processive mode of action. However, TbMP42 was shown to act in a distributive fashion (Brecht et al., 2005). These findings indicate a contribution of one previously not recognized enzymatic function to the editing reaction cycle, reloading the current mechanism of the RNA editing reaction cycle. A revised version of the RNA editing reaction cycle that includes the 3' nucleotidyl phosphatase is depicted in Fig. 7.

The role of TbMP99 and 100

TbMP99 (REX2) and TbMP100 (REX1) are proteins with high sequence similarity and identity (Panigrahi et al., 2003; Worthey et al., 2003). Both contain a 3'-5' exonuclease motif, an endo-exo-phosphatase (EEP) motif and recombinant TbMP99 and TbMP100 have been shown to display a U-specific exoUase activity (Kang et al., 2005; Rogers et al., 2007). Together with a recombinant RNA editing ligase, either protein is able to direct deletional editing *in vitro*. However, neither TbMP99- nor TbMP100-minus mitochondrial extracts show a reduced deletion RNA editing activity or reduced exoUase activity. Furthermore, Mian et al., 2006 showed by homology modeling that both proteins can be folded according to DNaseI, which is an archetypical EEP-family member with nuclease activity. However, the modeling data are inconclusive since the two proteins can equally well be folded along the structure of synaptojanin, which is a phosphoinositide phosphatase involved in signaling (Tsujishita et al., 2001; Maehama et al., 2001) (Fig. 8). Since the proteins possess both a 5'-3' exonuclease motif and an EEP-motif, it is possible, that the two proteins can carry out both an ex-

oUase and a phosphatase activity. If both proteins possess exoUase and phosphatase activities *in vitro* and can fully complement each other *in vivo*, this conundrum cannot be resolved with the tools at hand.

Conclusion

From our analysis we can conclude a mode of action for TbMP42. The exoUase activity of the protein produces 3' nucleoside monophosphates, which at a faithfully deletion editing site results in two opposing phosphate residues. In order to resolve this situation for the subsequent ligation step, a 3' specific nucleotidyl phosphatase is required. Such an activity is present in mitochondrial extracts of African trypanosomes and it co-sediments with the RNA editing activity. Moreover, its pH optimum and its cofactor requirement suggest that the enzyme(s) belong to the group of 3' acidic phosphatases. The characterization of the activity identified two potential candidates, TbMP99 and TbMP100. Both proteins carry an endo-exo-phosphatase motif (Panigrahi et al., 2003; Worthey et al., 2003) and have been shown to execute exoUase activity *in vitro* (Kang et al., 2006; Rogers et al., 2007). However, whether this is of relevance for the *in vivo* activity remains to be seen. We propose, that at least one peptide executes a nucleotidyl phosphatase activity and contributes this activity to the editing reaction cycle. In the light of an emerging picture of separate insertion and deletional editing (sub)complexes, it is tempting to speculate that the two enzymes contribute their function to different particles.

Taken together, the data provide a new facet to the biochemical consequences of TbMP42's function as an exonuclease in the RNA editing reaction cycle. By carrying out its activity, premature ligation is prevented through the generation of a 3' phosphate at the 5' mRNA cleavage fragment. Thus, the protein provides a form of quality control after the ribonucleolytic trimming reaction.

Acknowledgements

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Material and Methods

Trypanosome cell growth and preparation of mitochondrial vesicles

Insect stage *Trypanosoma brucei brucei* (*T. b. brucei*) strains 427 (Cross, 1975) and 29-13 (Wirtz et al., 1999) were grown in SDM-79 medium (Brun and Schönenberger, 1979). Mitochondrial vesicles were isolated, lysed and fractionated in linear 10-35% (v/v) glycerol gradients as described previously (Göringer et al., 1994).

Endo/exoribonuclease assay

Recombinant (r)TbMP42 was prepared at denaturing conditions as in Brecht et al., 2005. gRNA/pre-mRNA model substrates were chemically synthesized, radioactively labeled, annealed and purified as described (Niemann et al., 2008). pre-mRNA sequence - U5-10: GGGAAUG UGAUUUUUGCGAG UAGCC. gRNA sequence - gU5-10: GGGCUACUCGCUCA CAUUCCC. Annealed gRNA/pre-mRNA hybrid molecules (1pmole, specific activity 2-5µCi/pmol) were incubated with rTbMP42 as described (Niemann et al., 2008). Reaction products were separated

by gelelectrophoresis and analyzed by phosphorimaging.

RNA editing assays

RNA substrates for the pre-cleaved insertion assay (PIA) and the pre-cleaved deletion assay (PDA) were prepared, radioactively labeled and purified as described (Seiwert and Stuart, 1994; Kable et al., 1996; Brecht et al. 2005). After gel-extraction, the RNA was dissolved in TE pH7.5. Prior to assaying, 50fmoles radioactively labeled 5'CL mRNA-fragment (specific activity ~0.3µCi/pmole) was incubated with 1pmol of cognate gRNA and 1pmol 5' phosphorylated 3'CL mRNA-fragment for 5 minutes at 65°C. The three RNAs were annealed by subsequent incubation at RT for at least 10 minutes. Insertion assays (PIA) were carried out for 3h at 27°C using 1-2.5µg protein of fractionized mitochondrial lysate in editing buffer (EB) (20mM HEPES/KOH pH 7.5, 30mM KCl, 10mM $Mg(OAc)_2$) containing 0.2mM DTT, 0.5mM ATP and 0.04µM UTP, whereas UTP was omitted from deletion assays (PDA). Reaction products were resolved in denaturing polyacrylamide gels (18% w/v, 8M urea) and visualized by phosphorimaging. Product formation was quantified densitometrically using ImageGauge V4.23 (Fuji Film Science Lab 2003). RNA sequences for PIA: 5' CL 18 - GGAAG UAUGAGACGUAGG, 3' CL 13 – AUUGG AGUUAUAG-NH_2 and gPCA6 – CUAUAA CUCCGAUAAACCUACGUCUCAUACU-UCC. PDA: 5'CL22_del – GGAAAGGGA AAGUUGUGAUUUU, 3'CL15_del – GC GAGUUAUAGAAUA-NH_2, gA6(14)PC_del – GGUUCUAUAACUCGCUCACAACUU UCCCUUUCC.

Phosphatase assay

0.5pmoles of (5'-^{32}P)-pCp-labeled (spec. activity ~0.04µCi/pmol) phosphatase RNA substrate (31nt: 5' GGAAAGU UGUGAUUUUUGCGAGUUAUAGCG 3'; 8nt: 5' GCGCUCCC 3') were incubated with equal volumes of fractionized mitochondrial lysate in EB at 27ºC for up to 3 hours. In the pH optimization experiment, MES substituted for HEPES in a range of pH3.8 to 8.0. Bivalent cation requirements were studied using EDTA, EGTA and 1-10 phenanthroline ranging from 0.1 to 50mM. Reaction products were separated in 18% (w/v) polyacrylamide gels containing 8M urea, analyzed by phosphorimaging and quantified using ImageGauge V4.23 (Fuji Film Science Lab 2003). Phosphatase pre-cleaved insertion assays (PPIA) used the setup as described for PIA with the radioactive label introduced 3' at the 5'CL18 mRNA fragment using (5'-^{32}P)-pCp (3000mMCi/mmol) and T4-RNA ligase resulting in 5' hydroxyl- and 3' phosphate containing 5'CL18. To obtain 5'/3' hydroxyl-bearing 5'CL18, the pCp-labeled RNA was treated with 10U alkaline phosphatase for 1h at 37°C.

Nucleotide analysis

A 55nt long RNA was produced by in vitro transcription as described in Homann and Göringer (1999) with the exception of using (α-32P)-CTP instead of (α-32P)-UTP to introduce the radioactive label. The RNA transcript was purified by size exclusion chromatography, EtOH precipitation and subsequent gel purification. 0.5pmoles RNA were incubated two times with 10µg rTbMP42 for 4h at 37ºC. Reaction products were resolved by 2D thin layer chromatography on PEI-cellulose plates (20x20cm) (Bochner and Ames, 1982). 1st dimension: 50:28.9:1.1 iso-butyric acid: H_2O:NH_4OH (25% v/v) for 5h. 2nd dimension: 100:60:2 (v/w/v) $Na_xH_yPO_4$ (pH 6.8):$(NH_4)_2SO_4$:n-propanol for 4h. Plates were dried before running the second dimension and prior to phosphorimaging.

Gene silencing through RNAi and characterization of 99 skd, 100 skd and 99/100 dkd cell lines

Gene ablation of TbMP99 and TbMP100 was carried out based on the inducible RNAi system of Wang et al. (2000) using the hairpin-vector. For the TbMP99 single knockdown (99 skd), a 374bp DNA fragment (ORF position 1150-1524) was used. For the TbMP100 single knockdown (100 skd), a 361bp DNA fragment (ORF position 1119-1480) was used and the bler-cassette of that vector was replaced with the blasticidinr (blast) cassette. Trypanosome strain 29-13 (Wirtz et al., 1999) was transfected independently with both the 99 skd and the 100 skd RNAi constructs as described (Brecht et al., 2005). Clonal cell lines were established by limited dilution. To obtain the 99/100 double knockdown (dkd) cell line, the clonal 99 skd cell line was transfected with the 100 skd RNAi construct and cloned out. To maintain transgenic parasites, the cells were incubated with 1µg/mL bleocin (bleo) and/or 10µg/mL blast. RNAi was induced by the addition of 1µg/mL tetracycline (tet). For each cell line, a total of 109 cells were harvested after 6 days of tet induction. Control cells were grown in the absence of tet. Total RNA extraction was carried out according to Chomczynski and Sacchi (1987). Transcript abundance was determined by RT-PCR using the primer pair GGCATCAA GAATTTTCGGTGGAC and TTGTCACAA CAACCTGTAGCACAC for TbMP99 gene silencing. TbMP100 gene silencing was monitored with primers TAAGAAGGCGAA GGAAGGGG and CGCATAATGCAGCAA GATGAC. The transcript of the α-tub gene served as an internal control. PCR products were resolved in nondenaturing 5% (w/v) polyacrylamide gels and visualized by EtBr-staining.

Autoadenylation assay

To analyze the presence of the RNA editing ligases TbMP52 (KREL1) and TbMP48 (KREL2) in glycerol gradient fractions (Sabatini and Hajduk, 1995), 2-5µg protein was incubated for 1h at 27°C in EB in the presence of 3µCi (α-32P)-ATP (3000Ci/mmol). Samples were acetone precipitated and separated in SDS-containing 12% (w/v) polyacrylamide gels prior to phosphorimaging.

ExoUase activity assay

To determine the exoUase activity of fractioned mitochondrial lysates a non-ligatable version of the PDA was performed. The same RNA substrates were used, except for the 3'CL 15_del mRNA fragment lacking the 5' phosphate. Reaction conditions and sample treatment was identical to the editing assays described above. Reaction products were resolved in denaturing polyacrylamide gels (18% w/v, 8M urea) and visualized by phosphorimaging. Data were quantified densitometrically using ImageGauge V4.23 (Fuji Film Science Lab 2003).

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CHAPTER FOUR

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Research Paper

Identification of novel guide RNAs from the mitochondria of *Trypanosoma brucei*

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Abbreviations: ncRNA, non coding RNA; oU-RNA, oligo-uridylated RNA; gRNA, guide RNA; kDNA, kinetoplast DNA; mtRNA, mitochondrial RNA; CO3, cytochrom oxidase 3; RPS12, ribosomal protein subunit 12; ND3, 7, 8, NADH dehydrogenase subunit 3,7,8; CR3,4, C-rich region 3 and 4; ATP6, ATPase subunit 6

Key words: non-coding RNAs, guide RNA, trypanosomes, RNA editing, alternative editing

Identification of novel guide RNAs from the mitochondria of *Trypanosoma brucei*

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Abstract

The majority of mitochondrial mRNAs in African trypanosomes are subject to an RNA editing reaction, which is characterized by the insertion and/or deletion of U nucleotides only. The reaction creates functional mRNAs and is catalyzed by a high molecular mass enzyme complex, the editosome. Editosomes interact with a unique class of small non-coding, 3'-oligo-uridylated (oU) RNAs, so-called guide RNAs (gRNAs). Guide RNAs function as trans-acting templates in the U deletion/insertion reaction and thus, represent key components in the reaction cycle. Furthermore, by utilizing different gRNAs, alternative editing events can take place, thereby expanding the protein diversity in the mitochondria of the parasites. In this study, we have analyzed small, non-coding mitochondrial transcripts from *Trypanosoma brucei*. By generating cDNA libraries from size-selected RNA populations we identified 51 novel oU-RNAs. For 29 of these RNAs we were able to predict cognate mRNA targets. By Northern blot analysis, we verified the expression of 22 of these oU-RNAs and demonstrate that they share all known gRNA characteristics. Five of these 51 putative gRNAs are characterized by single mismatches to their cognate, fully edited mRNA sequences suggesting that they could act as gRNAs for alternative editing events.

Introduction

Trypanosomes are protozoan parasites that belong to the order of Kinetoplastida. All kinetoplastid species are characterized by a single mitochondrion that contains a unique assembly of mitochondrial DNA, known as kinetoplast (k)DNA. kDNA consists of thousands of catenated circular DNA molecules, which have been grouped into 2 classes: minicircle and maxicircle DNA elements (reviewed in Simpson, 1987). The mitochondrial genome of *Trypanosoma brucei* contains about 50 maxicircles (~20kb) and 5.000-10.000 minicircles (~1kb). Maxicircles code for a number of respiratory chain proteins in addition to small and large subunit ribosomal RNA (rRNA) molecules. In contrast to other eukaryal mitochondria, the majority of maxicircle genes are encoded as so-called cryptogenes. Cryptogenes require RNA editing in order to generate translatable open reading frames (ORFs). The "kinetoplastid" form of editing is characterized by the insertion and/or deletion of uridine (U) nucleotides exclusively (reviewed in Stuart et al., 2005)

and of the 18 maxicircle-encoded protein-coding genes in *T. brucei*, 12 pre-mRNAs are subject to editing. The extent of editing can differ significantly. It ranges from 4 inserted uridines into the cytochrome c oxidase II pre-mRNA to hundreds of U's added and dozens of uridines deleted into the cytochrome c oxidase III pre-mRNA (reviewed in Seiwert, 1995).

The sequence information required for the editing reaction is provided in *trans* by small oligo(U)-tailed mitochondrial transcripts, known as guide RNAs (gRNAs). Most gRNAs are encoded on the minicircle DNA elements, with each minicircle encoding 3-5 gRNA genes. A much smaller number of gRNAs are maxicircle-derived (Pollard et al., 1990; Simpson, 1997). It has been estimated that at least 200 gRNAs are required to direct all editing sites in *T. brucei* (Corell, 1993). Guide RNAs are base complementary to fully edited mRNAs. They are characterized by a high A/U content and an average length of 50-70nt (Schmid et al., 1995). The molecules are 3' oligo-uridylated and adopt a common secondary (2D) structure, which consists of two hairpin elements (Schmid et al., 1995; Hermann et al., 1997; Schumacher et al., 2006). This structure becomes progressively unfolded as the editing reaction proceeds, ultimately generating a fully basepaired gRNA/mRNA hybrid molecule. In these gRNA/mRNA hybrids, canonical as well as non Watson/Crick-type base pairs can be found (Corell et al., 1993; Blum et al., 1990; Read et al., 1992; Souza et al., 1992; Koslowsky et al., 1992; Riley et al., 1994). From a functional point of view, the primary sequence of a gRNA can be divided into three domains. The 5' end contains a so-called anchor sequence, which hybridizes to the pre-edited mRNA 3' of an editing site. The anchor sequence has a length of 4-18nt and its formation is essential for the initiation of the editing reaction (Blum et al., 1990; Bhat et al., 1990; Koslowsky et al., 1990; Sturm et al., 1990). The second domain is located immediately 3' to the anchor sequence and is called information or guiding domain. This sequence dictates the insertion and/or deletion of the U nucleotides via antiparallel base pairing. The third domain is the 3'-oligo(U)-tail ranging in size from 5-25nt with an average length of 15nt (Blum and Simpson, 1990; Pollard and Hajduk, 1991)

The minicircle network exhibits a much higher complexity than previously anticipated. Currently, about 200 different minicircle classes coding for more than 1.200 gRNA sequences have been identified (Ochsenreiter et al., 2007; Ochsenreiter et al., 2008). Although not all of these sequences have been verified on the level of RNA, redundant gRNAs have been described that differ in their primary sequence but target the same editing site. While some gRNAs exhibit single or more mismatches to their cognate mRNA targets (Koslowsky et al., 1992; Corell et al., 1993; Riley et al., 1994; Ochsenreiter et al., 2007; Ochsenreiter et al., 2008) other gRNAs are characterized by extensive mismatches, thus bringing into question whether they are able to direct proper editing (Gao et al., 2001). However, gRNAs that have retained a perfect anchor sequence but exhibit mismatches within the guiding domain might actually direct alternative editing reactions. In *T. brucei* and *Leishmania tarentolae*, mRNAs exhibiting alternative editing events have been reported for several genes (e.g. CO3, RPS12 or ND3) (Maslov and Simpson, 1992; Maslov et al., 1992; Sturm et al., 1992) but have been interpreted as misediting products (Sturm et al., 1992). However, the recent discovery of gRNA L07 in *T. brucei*, which mediates an alternative editing reaction within the CO3 gene, has been

shown to generate a novel open reading frame that is translated into a unique protein (Ochsenreiter and Hajduk, 2006). This suggests a function for alternative editing reactions (Ochsenreiter and Hajduk, 2006; Ochsenreiter et al., 2008) and implies that some of the redundant gRNAs that target the same editing site but exhibit mismatches within their guiding sequence contribute to expand the protein diversity in kinetoplast mitochondria (Ochsenreiter et al., 2008).

In this study we have analyzed the small mitochondrial transcripts from *Trypanosoma brucei* by generating cDNA libraries encoding small non-coding RNA (ncRNA) species from 15-600nt in length. We identified 51 novel, metabolically stable, oU-tailed minicircle encoded ncRNAs representing potential gRNAs.

Results

Library construction and sequence analysis of cDNA clones

The aim of this study was to characterize small, non-coding (nc) RNA transcripts of *Trypanosoma brucei* mitochondria. For that we isolated mtRNA from purified mitochondrial vesicles and generated cDNA libraries encoding size-selected ncRNAs in the range from ~15nt to 600nt. A total of 1.200 sequences were analyzed. The majority of the cDNA clones was assigned to nuclear-encoded RNAs (Fig. 1). About 25% represented nuclear-encoded tRNAs known to be imported from the cytoplasm into the mitochondria (Schneider and Maréchal, 2000). 59% represented cytoplasmic ncRNAs, which are not known to be imported, such as rRNAs or small nucleolar RNAs (Fig. 1). The high content of cytoplasmic RNA sequences suggests a contamination of the mitochondrial RNA preparation with cyto-

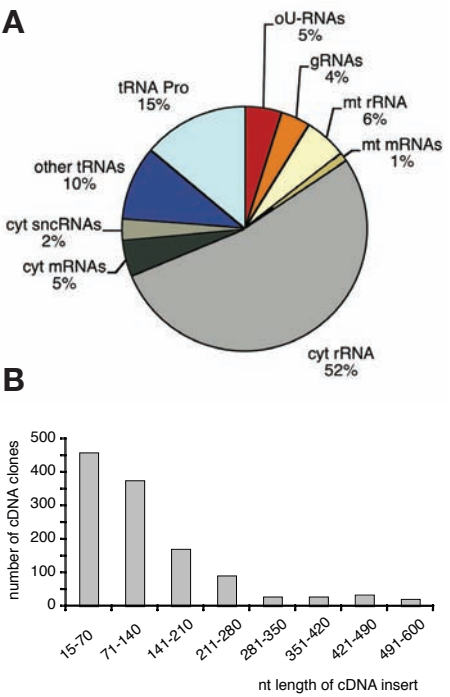


Figure 1: *Sequence analysis of ~1.200 cDNA clones representing different RNA species derived from the kinetoplast genome of Trypanosoma brucei.* **(A)** *cDNA clones were grouped into different classes, depending on their genomic location and are shown as percent of the total number of clones. Oligo(U) RNAs: oligo-uridylated RNAs.* **(B)** *Graphical representation of the cDNA length distribution.*

plasmic RNAs. This can be explained by cytoplasmic ribosomes being attached to the outer membrane of the mitochondria, since polysomes associated with mRNAs encoding mitochondrial proteins have been shown to co-isolate with mitochondrial fractions (Margeot et al., 2002; Kaltimbacher et al., 2006). Since the cells were disrupted at isotonic conditions, this provides a plausible explanation. About 11% of the RNAs derived from the kinetoplast genome mapped to rRNA, mRNA and gRNA genes (Fig. 1). Five percent of the clones corresponded to oligo-uridylat-

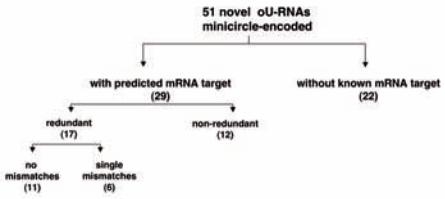


Figure 2: *Schematic overview and classification of 51 novel oU-RNAs candidates; (N): number of candidates.*

ed RNAs (oU-RNAs) and could not be mapped to any known genomic location. Therefore, it is likely that these RNAs are encoded on the minicircle DNA elements of the mitochondrial genome, which have not been fully sequenced to date. We selected these oU-RNAs for further investigations as potential candidates for novel gRNAs.

Characterization of novel oU-RNAs derived from the kinetoplast genome of Trypanosoma brucei.

The minicircles of *Trypanosoma brucei* encode from 3 to 5 gRNA genes per minicircle, which are flanked by imperfect 18bp repeats separated by about 110bp (Pollard et al., 1990; Simpson, 1997). Transcription of gRNAs initiates at the first purine within the conserved sequence motive 5´-RYAYA-3´ (Pollard et al., 1990). The 18bp repeats are thought to be essential for transcription initiation of gRNAs (Pollard et al., 1990). Through this screen, we identified 51 novel oU-RNAs (Fig. 2; Table 1, 2). 25 of these oU-RNAs initiate with a 5´-RYAYA-3´ sequence as observed for canonical gRNA genes, while the remaining sequences do not initiate with that sequence. However, 20 oU-RNA species, represented by single cDNA clones, are shorter than canonical gRNAs. It is likely that they do not represent full-length clones and therefore the 5´-RYAYA-3´ element might be missing from these sequences.

Identification of oU-RNA target sequences

In order to assign a potential function to the cloned oU-RNA species, we first analyzed whether they possess cognate mRNA targets and thus might act as canonical gRNAs. We used the Kinetoplastid Insertion and Deletion Sequence Search Tool (KISS), which predicts interactions of gRNAs with pre-edited and edited mRNAs (Ochsenreiter et al., 2007). For 29 oU-RNAs, cognate mRNA targets could be identified (Table 1, 2; Fig. 3). We identified novel gRNA target sequences to different loci within the pan-edited ND7, ND8 or CO3 genes, the C-rich region 3 (CR3), the C-rich region 4 (CR4), the ATP6 and the RPS12 gene (Table 1). 17 gRNAs represent redundant gRNAs, which differ in sequence compared to previously identified gRNAs, but cover the same editing sites. The remaining 12 oU-RNAs target to editing sites for which no gRNA has been identified to date (Fig. 2; Table 1).

A characteristic feature of gRNA/mRNA duplexes is that the anchor sequence usually consists of Watson/Crick base pairs, while the duplex formed between the guiding template of the gRNA and the edited part of the mRNA contains G:U base pairs. The duplex is probably unwound by an RNA helicase activity (Missel and Göringer, 1994; Missel et al., 1997) and the released gRNA is then replaced by a second, upstream gRNA (Stuart et al., 2005; Maslov and Simpson, 1992). The majority of the oU-RNAs identified through this screen are able to form Watson/Crick-type duplexes (Fig. 3). For the remaining 22 oU-RNAs, it was not possible to predict cognate mRNA targets (Fig. 2; Table 2, suppl. Table). For 7 of these 22 oU-RNAs, the cloned sequences were too short to assign a gRNA/mRNA interaction (suppl. Table).

Table 1. Minicircle-encoded oU-RNAs with predicted mRNA target location

Name	Sequence	cDNA clones	Northern blot	Target prediction –KISS/Remarks
gND7-72	**ATATA**CAAATGTAAAGAAGCTATCAGAGGTA ATATAAGTGATATAATTTTTTTTTTTTTT	2	+	ND7: 37-72; redundant
gND7-371	CTACGAGTAGATTCTATGATTGATGAACGTG TAAATTTTTTTTTTTTTT	1	+	ND7: 337-371; non-redundant
gND7-428	GGAGATGAGCAATTTAGATTCAGAGTTATAT GTGATTTTTTTTTTTTT	1	+	ND7: 394-428; non-redundant
gND7-496	**ATATA**CAAATGCTACGACGACTATGATATAA GTTAAAGAATGATGTCAATTTTTTTTTTTTT	3	+	ND7: 460-496; redundant (1)
gND7-576	**ATATA**TATCAACAACAGTGAAAAGTCAACGA GATTAGAGATAGAATTATTTTTTTTTT	1	n.d.	ND7: 534-576; redundant
gND7-663	**ATATA**GATGACAAACCACGTAGACGTAGAT AGAGTGAATGATTGTATTTTTTTTTTTTT	2	+	ND7: 623-663; non-redundant
gND7-1075	**ATATA**AAATAAACGAGAATATAAACTGATGT AGAGATATAGTGATAAGTATTTTTTTTT	2	+	ND7: 1033-1075; redundant
gND8-342	**ATATA**TAACGACGATGAATCAGTGTAAATTG GTACGTGAAATGATGGTTTTTTTTTTT	3	+	ND8: 298-342; redundant (2)
gCO3-123	**ATATA**TAATAACAATAGCAGGTAAAGGTAAG AAAGTGAAGATATCATTTTTTTT	1	+	CO3: 78-123; non-redundant
gCO3-141	AAATAACAACAAACGAGATGACCAATACACA GTGGTGATGGTATATATTTTTTT	1	n.d.	CO3: 101-141; non-redundant
gCO3-226a	**ATAAA**TAAATACAAAAATCGACAGAGAGAAA AGTAGGATTGTGATTAATTTTTTTTTTTTT	6	+	CO3: 179-226; redundant
gCO3-226b	**ATATA**TATACAAACAGATACAGAAGCCAACG AGAAAGAAAGTGAAATTTTTTTTTT	4	n.d.	CO3:189-226; redundant
gCO3-386	AAAGGTAAGCATAGACTAAGTGATATAATGA TGAATTAATAGTTATATTTTTTTTTTTTT	1	+	CO3: 351-386; redundant (1)
gCO3-680	**ATAAT**AGAACACCACAGCTTAATGTAGTAGA TGGCAGTGTAAATTTTTTTTT	3	+	CO3: 643-680; non-redundant
gCO3-802	**ATATT**AAACAAAACGTGTATTCATATGTAGG AAGTTAAGTGAATGATTTTTTTTTTT	1	+	CO3: 763-802; non-redundant
gCO3-888	AAATATAAAACATCAAGATAAATGGATTGTG ATAGAGAAAGTTAAATTTTTTTTTTT	3	n.d.	CO3: 844-888; redundant
gCR3-191	**ATATT**ATAAACATAATAATAGATTCATAGTGA GTGAGAAATATTTTTTTTTTTTT	2	+	CR3: 155-191; redundant (1)
gCR4-178	GGAGTGATGAGATAGATGAAATATGCTGTAT TAAGCAGTATAAATTTTTTTTTTTT	1	-	CR4: 155-178; redundant
gCR4-301	AAACACAAAGCGAGATAAAAGAGGGAAGTA AATAGAGTATGCTGGAATAATTTTTTTTT	2	n.d.	CR4: 264-301; redundant
gCR4-469	**TATA**CAATAACAACAATCGCGAGTAAAGATA GATGTAAGTGAGAAATTTTTTTTTTTT	2	+	CR4: 429-469; redundant
gATP6-207	ATAAACAAACACAAATCAGTAGACGAGTACA AGTTAGATGGACGTATCTTTTTTTT	1	n.d.	ATP6: 166-207; redundant
gATP6-230	AACAGCATAAACTATAGCAGTGAAGATAGAT GTGAATTAATTTTTTTTTTTT	1	+	ATP6: 191-230; redundant
gATP6-337	**ATAT**GACAAAACAAAAATAAGAATGTGATAT ACGGTAGAAGGATGATATTTTTTTTTT	1	n.d.	ATP6: 294-337; non-redundant
gATP6-453	**ATATA**TAACGACACAATAGAGAAAGATGCTC TGAGAGATGAATTATTTTTTTTTTTTT	1	+	ATP6: 418-453; non-redundant
gATP6-483	**ATATT**ACAAAACAGACGTAAAGATGTCGATG AATGGTGGTATAATTTTTTTT	1	n.d.	ATP6: 442-483; non-redundant
gATP6-693	**ATATA**CAACTGCAAGATCATATTATAGAAGG TGAATGATTGTAATTTTTTTT	1	n.d.	ATP6: 564-693; non-redundant
gRPS12-127	ACAAATAACTGGCAATCGTGGATTAGTGTAG TGATGTAAAGTGAATATTTTTTT	1	+	RPS12: 91-127; redundant (1)
gRPS12-132	**ATATA**TAACTGGACAATCGTAGGCTTGTATG ATGAGATGAGATGAGTAAATTTTTTTTTTTT	4	n.d.	RPS12: 89 -132; redundant (1)
gRPS12-154	**ATA**CTTACAATACACGTTGGTTATCGGAGTT AGATGATTGTGACTTATTTTTT	1	n.d.	RPS12: 117 -154; non-redundant

Name: oU-RNA species corresponding to the site of interaction with respective mRNA; cDNA clones: number of independent cDNA clones identified from each RNA species; Northern blot: the presence or absence of hybridizations signals in northern blot analysis is indicated as (+) or (-); Target prediction-KISS, exact site of interaction of oU-RNA and mRNA as predicted by KISS (Ochsenreiter et al., 2006); (N) number of mismatches between oU-RNA and mRNA; n.d., not determined; conserved sequence boxes in bold indicate potential transcription initiation sites.

Table 2. Minicircle-derived oU-RNAs without known mRNA targets

Name	Sequence	cDNA clones	Northern blot
Tbmin1	**ATAT**CCAATAAACAAGGAGTAGATTCGAATAGATGATTAATT TTTTTTTTTTTT	2	+
Tbmin2	TCACAGTATAAATAGGGTTTTCAGGGTCTCCAGGATAGAAA AATAGAATAATTTTTTTTTTTTT	1	-
Tbmin3	**ATATA**TAAAACATGCTAGAGTGTAGTAAGTTCAGTGAAAGT GATATAGTTTTTTTTTTTT	1	+
Tbmin4	**ATATA**TAAAATAAATTAATTTGATGTAGATGATAGTGTGTATA ATTTTTTTTTTTT	2	+
Tbmin5	GCAGACTTTCATGATTGTGATTTWATAGAGATAATTTTTTTTT TTTTTTT	1	n.d.
Tbmin6	**ATAT**TATTTTAACTCTAGGGTAGTCAAGGAGGAAGGAATTAA ATAGTAAATTTTTTTTTTTT	2	n.d.
Tbmin7	ATTTTAGGTAAGAGTAGTTAGATATCTTGTTGAGTAGTGAGG GAAATGGAATAATTTTTTTTTTT	1	n.d.
Tbmin8	**ATAT**TTAGAAGATGCTTAAAATAGTGCTATAAAGTGCTTAGA GTTGGGAGAATTTTTTTTTTTT	1	n.d.
Tbmin9	AACGAGATATTATTTTAACTCTAGGGTAGTCAAGAAGTTAAT TTTTTTTTT	1	n.d.
Tbmin10	**ATAT**GAAAGTGAGAAGTTGGAGTGTAAAGGGATTATGAATT TTAGGGAATTTTTTTTT	1	n.d.
Tbmin11	ATAGTAACTTCGAGTCAGAGTATGAGATTAAATTTTT	1	-
Tbmin12	TCGTTATGAGAAATAGAATATGAGAAATTTTTTTATTTTTTT	1	+
Tbmin13	GATGACAGGTATAAGTTTGGATGGGTACTTTTTTTTTTTTTT	1	-
Tbmin14	AAGGGTTTCTAGGATAAGAAAAAATTTTTTTTTTTTT	1	n.d
Tbmin15	GATGTAAGGTATTGATATCAGTGATTTAATTTTTTTTTTTT	1	-

Name: oU-RNA species; cDNA clones: number of independent cDNA clones identified from each RNA species; Northern blot: the presence or absence of hybridizations signals in Northern blot analysis is indicated as (+) or (-); n.d., not determined; W stands for an A or T; conserved sequence boxes in bold indicate potential transcription initiation sites.

Supplementary Table Minicircle-derived truncated oU-RNAs too short to assign mRNA targets

Name	Sequence	cDNA clones	Northern blot
Tbmin16	GATTGTGAGTTAATTAGTTATATTTTTTTTTT	1	n.d
Tbmin17	AAAGAGAGAGATTTAAATGTTTTTTTTT	1	n.d
Tbmin18	TAAGATTGTGAGAGTTAAGTTTTTTTTTTTTT	1	n.d
Tbmin19	ATAAGAGAAATTTGTTACATTTTTTT	1	n.d
Tbmin20	GTGAATTCGTATATGATAATTTTTTTT	1	n.d
Tbmin21	GGATCTGATGATTTAAATTTTTT	1	n.d
Tbmin22	CGAGGATTCGTTATAATTTTTTTTTTT	1	n.d

Name: oU-RNA species; cDNA clones: number of independent cDNA clones identified from each RNA species; Northern blot: the presence or absence of hybridizations signals in Northern blot analysis is indicated as (+) or (-); n.d., not determined.

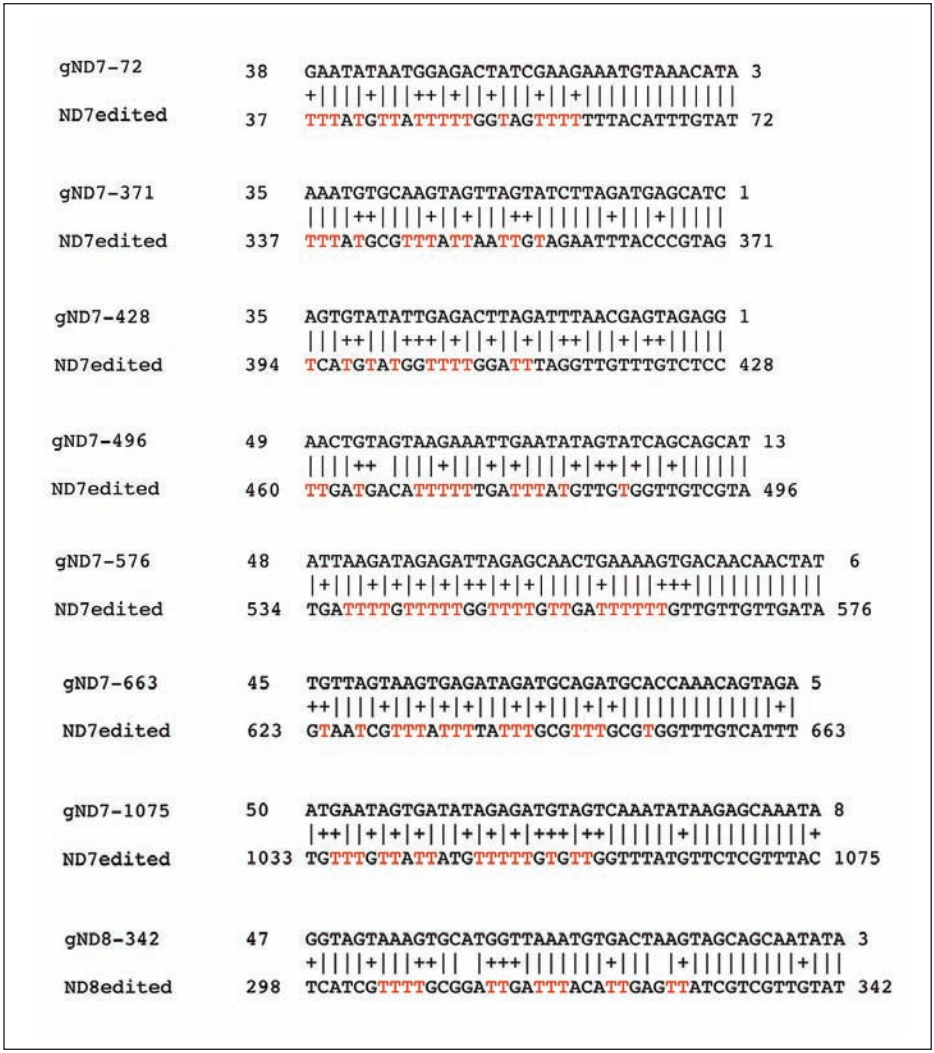


Figure 3: *Potential base-pairing interaction of gRNA candidates and mRNAs as determined by the computational gRNA target search tool KISS (Ochsenreiter et al., 2007); mRNA sequences are in 5´-3´orientation, gRNA sequences are in 3´-5´orientation.*

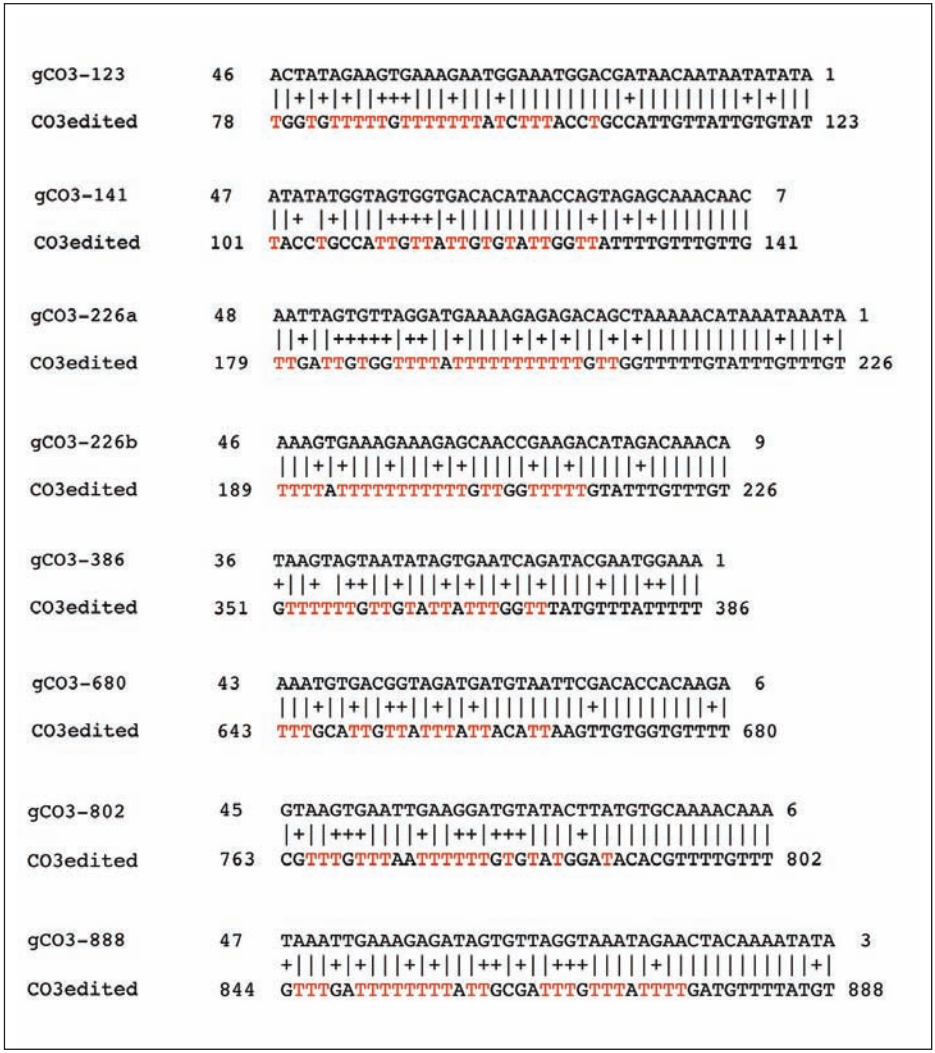
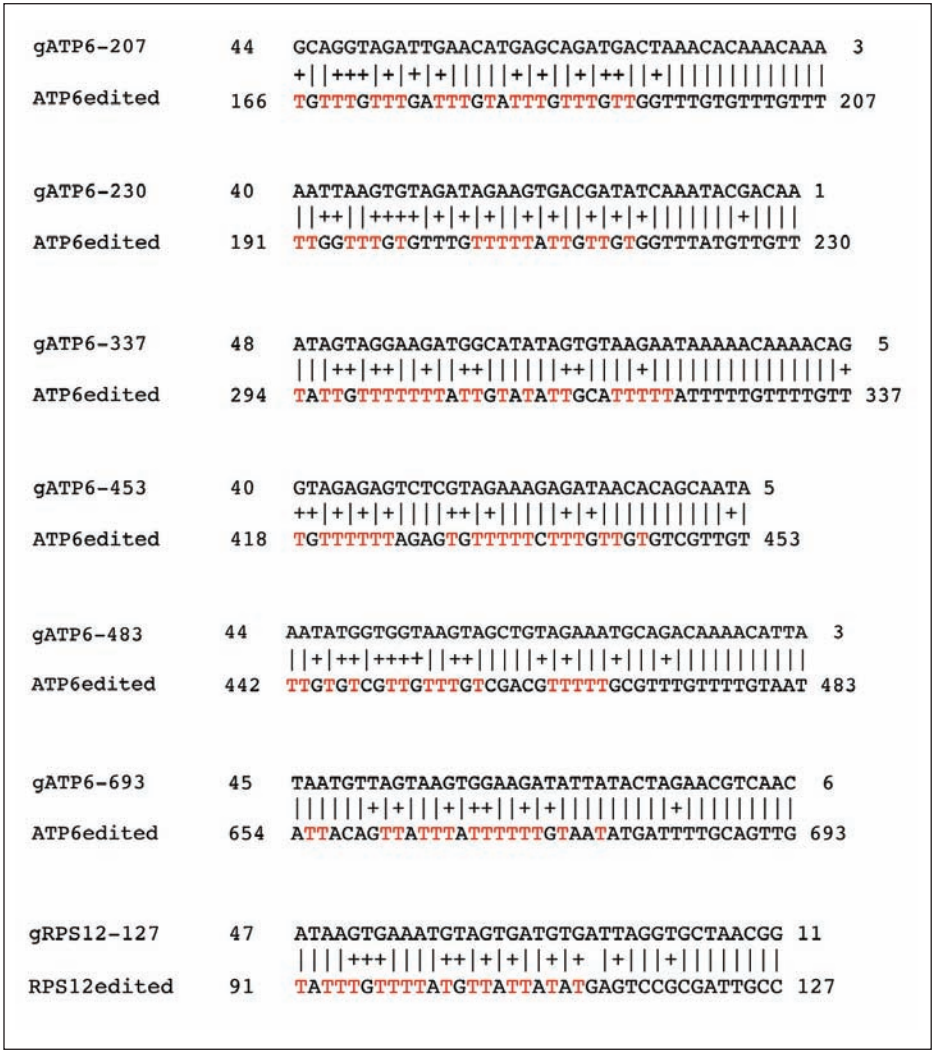


Figure 3 (cont.)


gATP6-207 44 GCAGGTAGATTGAACATGAGCAGATGACTAAACACAAACAAA 3
ATP6edited 166 TGTTTGTTTGATTTGTATTTGTTTGTTGGTTTGTGTTTGTTT 207
gATP6-230 40 AATTAAGTGTAGATAGAAGTGACGATATCAAATACGACAA 1
ATP6edited 191 TTGGTTTGTGTTTGTTTTTATTGTTGTGGTTTATGTTGTT 230
gATP6-337 48 ATAGTAGGAAGATGGCATATAGTGTAAGAATAAAAACAAAACAG 5
ATP6edited 294 TATTGTTTTTTTATTGTATATTGCATTTTTATTTTTGTTTTGTT 337
gATP6-453 40 GTAGAGAGTCTCGTAGAAAGAGATAACACAGCAATA 5
ATP6edited 418 TGTTTTTTAGAGTGTTTTTCTTTGTTGTGTCGTTGT 453
gATP6-483 44 AATATGGTGGTAAGTAGCTGTAGAAATGCAGACAAAACATTA 3
ATP6edited 442 TTGTGTCGTTGTTTGTCGACGTTTTTGCGTTTGTTTTGTAAT 483
gATP6-693 45 TAATGTTAGTAAGTGGAAGATATTATACTAGAACGTCAAC 6
ATP6edited 654 ATTACAGTTATTTATTTTTTGTAATATGATTTTGCAGTTG 693
gRPS12-127 47 ATAAGTGAAATGTAGTGATGTGATTAGGTGCTAACGG 11
RPS12edited 91 TATTTGTTTTATGTTATTATATGAGTCCGCGATTGCC 127

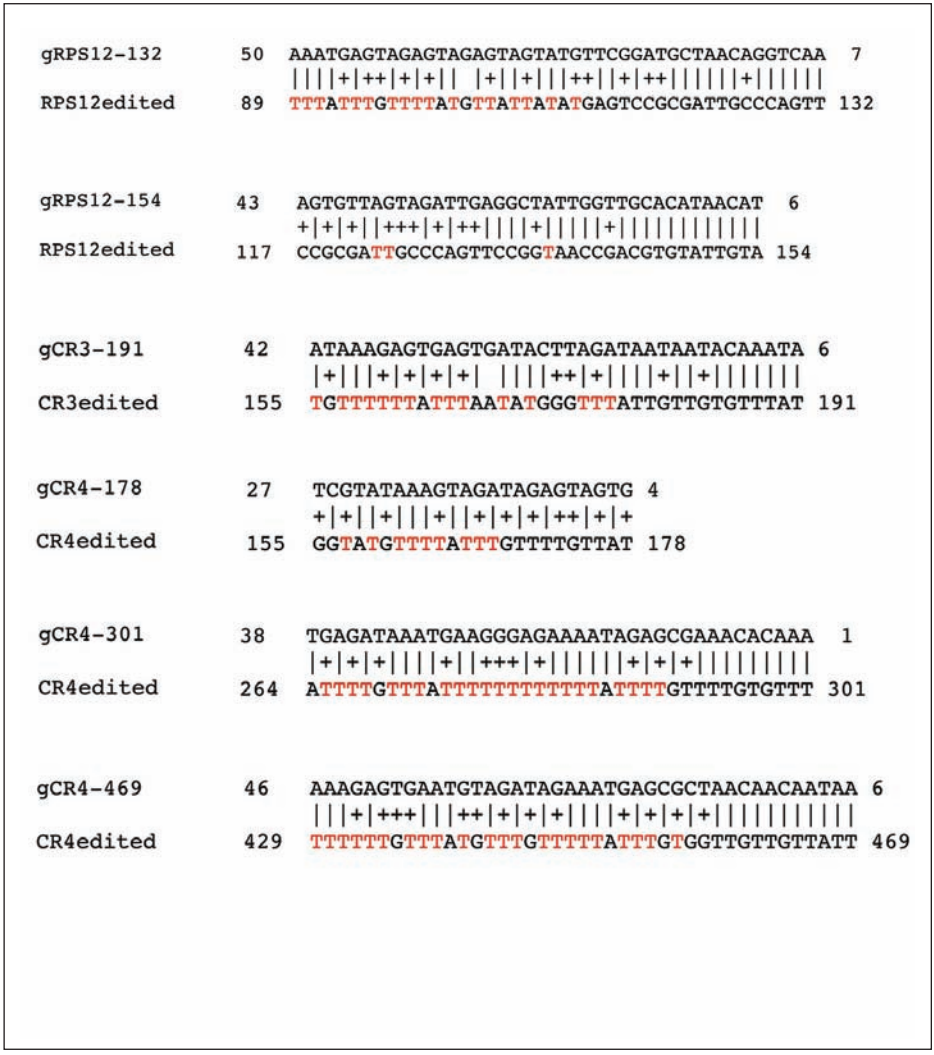


Figure 3 (cont.)

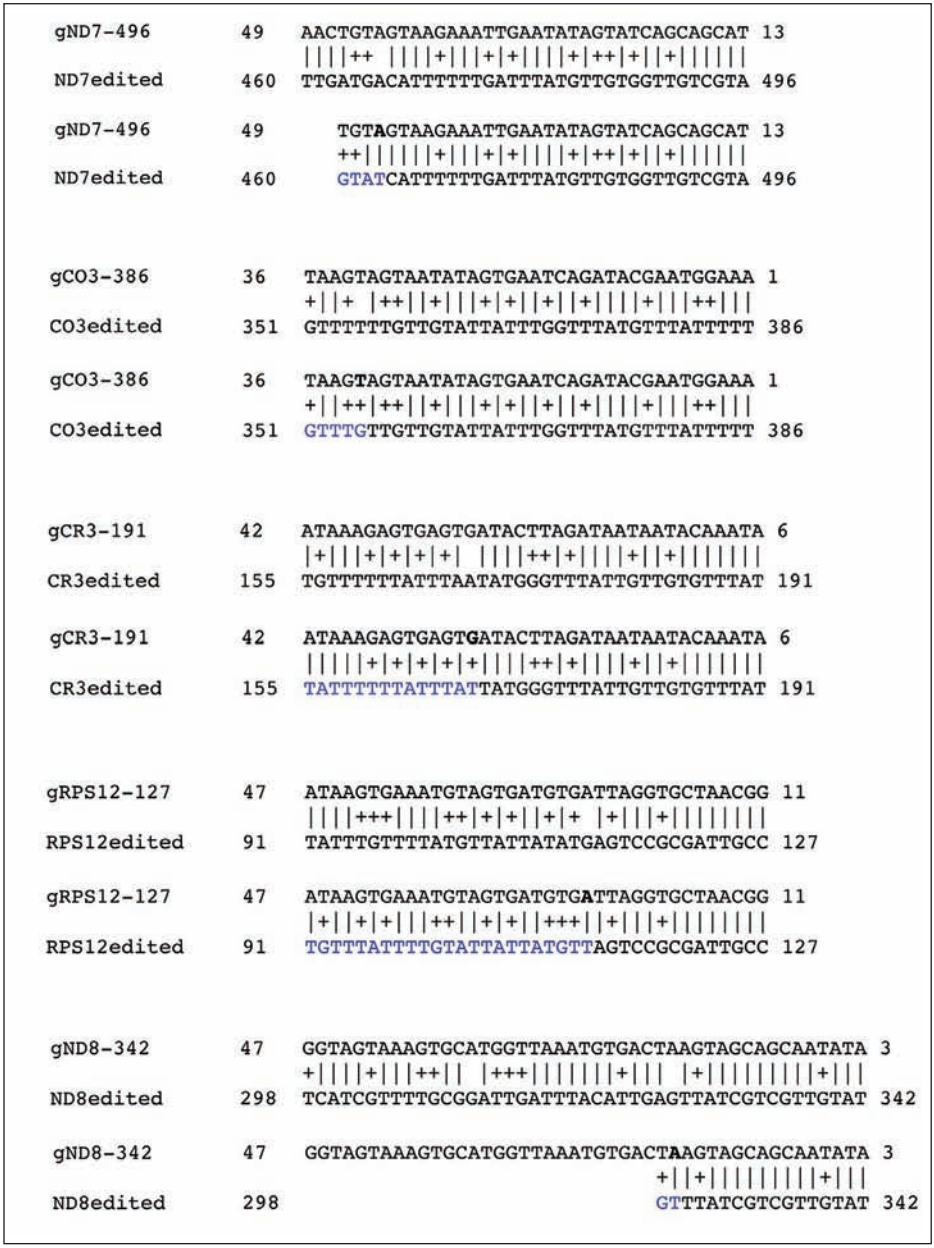


Figure 4: *Potential base-pairing interaction of gRNA candidates and mRNAs as determined by the computational gRNA targets search tool KISS (Ochsenreiter et al., 2007) and proposed alternative mode of editing. mRNA sequences are shown in 5´-3´orientation. gRNA sequences are in 3´-5´orientation; alternatively edited sequences are in blue; a bold letter in the gRNA sequence indicates the start of alternative editing.*

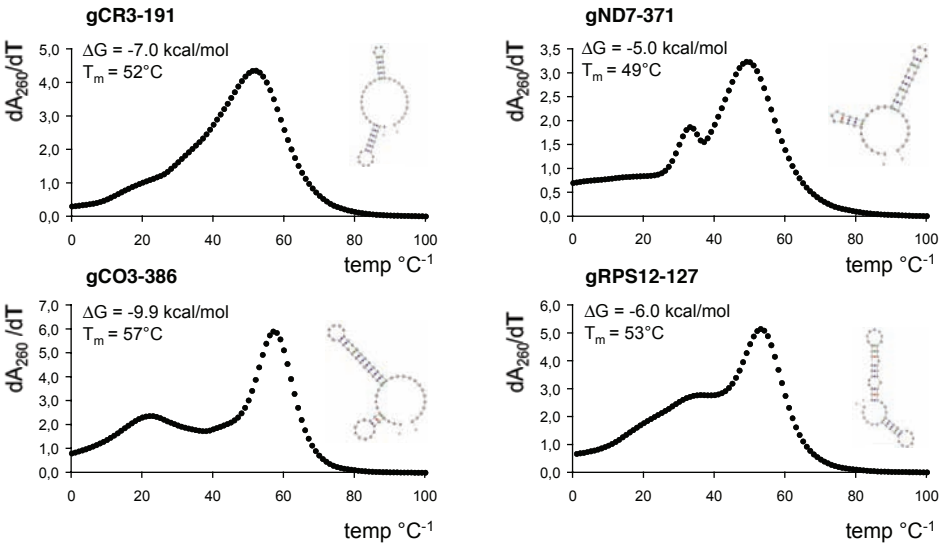


Figure 5: *Predicted melting curves (1st derivatives) for gCR3-191, gND7-371, gCO3-386 and gRPS12-127. T_m and ΔG values as well as 2D structure predictions are indicated in the graph.*

Interestingly, six of the oU-RNAs exhibit mismatches to the edited sequence of their cognate mRNAs (Fig. 4). From these, four gRNA candidates are represented by multiple cDNA clones of identical sequence in the cDNA library arguing against the possibility that the mismatched nucleotides are a result of sequencing errors (Table 1). Six candidates (gND7-496, gCO3-386, gCR3-191, gRPS12-127, gRPS12-132, gND8-342) exhibit single mismatches to the canonically edited mRNA sequences. Thereby, editing directed by these gRNAs differs from the canonical editing patterns of cognate mRNAs as summarized in Fig. 4. These alternatively edited mRNAs might be further edited by upstream gRNAs, which possess the ability to recognize a novel anchor sequence generated by alternative editing. The presence of alternatively edited mRNAs directed by these gRNAs needs to be further elucidated.

2D structure assessment of oU-RNA

All oU-RNA sequences were analyzed with respect to their nucleotide composition, and showed the expected A/U nucleotide bias (data not shown). The U-tails of the oU-RNAs averaged around 12nt in agreement with the expected oligoU-tail length of previously characterized gRNAs (Schmid et al., 1995; Hermann et al., 1997). Theoretical melting curves of the oU-RNAs show the expected helix/coil transition of two hair-pin elements, with T_m-values for the main transition ranging from 43° to 65°C. Figure 5 shows representative melting profiles for gCR3-191, gND7-371, gCO3-386 and gRPS12-127. To identify structural characteristics, we calculated theoretical 2D structures (Walter et al., 1992; Zuker, 2003). All oU-RNA sequences can be folded into structures consisting of two stem loops as observed for multiple gRNAs (Schmid et al., 1995;

Hermann et al., 1997; Schumacher et al., 2006). The calculated ΔG values range between -4.2 to -9.9kcal/mol, consistent with the observed Gibbs free energy of canonical gRNAs (Schmid et al., 1995).

Expression analysis of novel oU-RNAs

In order to analyze the expression of the different gRNA candidates, a Northern blot analysis for 26 of the RNAs was performed. For 22 oU-RNAs expression could be confirmed (Fig. 6). All hybridization signals were in the range of 60nt as expected for canonical gRNAs. Since oU-RNAs for which we have not been able to identify cognate mRNA targets could also be detected, this indicates that some of the oU-RNAs without known targets are also expressed as metabolically stable RNA species (Table 2). Shorter, truncated oU-RNAs lacking the RYAYA sequence element were not analyzed by Northern blotting (suppl. Table: Tbmin16-22).

Discussion

The analysis of the small, non-coding transcripts from insect stage *Trypanosoma brucei* mitochondria resulted in the identification of 51 novel oU-RNAs. A bioinformatic analysis did not reveal their genomic location, therefore, they are most likely derived from the minicircle DNA elements, which have not been fully sequenced as of today. The expression of 22 oU-RNAs was confirmed by Northern blotting including 4 oU-RNAs without a predicted mRNA target. From their A/U nt-bias, their predicted secondary structures and their theoretical melting profiles, these sequences resemble *bona fide* gRNAs. For 29 of the oU-RNAs, cognate mRNA targets could be assigned suggesting that they might act as canonical gRNAs. Six of the RNAs are characterized by single or double mismatches to their target mRNA sequences as observed for the redundant gRNA L07, which has been shown to direct an alternative editing event of the CO3 gene (Ochsenreiter and Hajduk, 2006). This suggests that these novel oU-RNAs might be involved in alternative editing. Further verification of this hypoth-

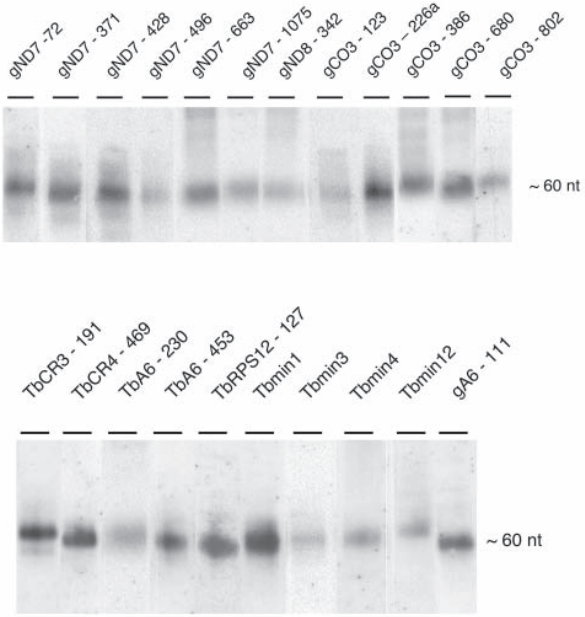


Figure 6: *Northern blot analysis of novel minicircle derived oligo(U) RNAs in comparison to gA6-111.*

esis will require a comprehensive compilation of alternatively edited mRNAs and their cognate gRNAs, as carried out recently in blood stream stage trypanosomes (Ochsenreiter et al., 2008). In contrast to this work, the present study reflects the steady-state gRNA population of procyclic stage trypanosomes. The fact that at these conditions less gRNAs were found as anticipated suggests a delicate regulation of gRNA steady-state expression levels. On the other hand, these gRNAs might compete with gRNAs directing "faithful" editing events within the same locus and thus might influence the efficiency of the reaction. In general, alternative editing seems to be a more common phenomenon than initially anticipated in line with the recent data of Ochsenreiter et al., 2008.

Acknowledgements

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Materials and Methods

Parasite cell growth, mitochondria isolation and RNA extraction

Insect stage *Trypanosoma brucei brucei*, strain 427 (Cross, 1975) were cultured in SDM79 (Brun and Schönenberger, 1979) and harvested at a cell density of 10^7 cells/mL. The parasites were disrupted at isotonic conditions by nitrogen cavitation (Hauser et al., 1996). Mitochondrial vesicles were isolated from the washed and DNaseI-treated cell lysate by isopycnic centrifugation in preformed linear 20-35% (v/v) percoll gradients in 20mM Tris-HCl/pH8, 2mM Na_2EDTA, 250mM sucrose for 40min at 24krpm and 4°C. Mitochondrial vesicles were lysed using an acidic phenol/$CHCl_3$ extraction in the presence of 4M guanidinium isothiocyanate and 2% (w/v) sodium-N-lauroyl sarcosinate (Chomczynski and Sacchi, 1987). Following extraction, the RNA material (mtRNA) was precipitated, washed and resuspended in 10mM Tris-HCl/pH7.5, 1mM Na_2EDTA.

Generation of a cDNA library encoding small RNA species

About 70µg of mtRNA was treated with DNase RQ1 (Promega) and was size-fractionated by denaturing 8% (w/v) polyacrylamide gelelectrophoresis (PAGE; 7M urea, TBE/pH8.3). RNAs in the size range between ~15-600nt were excised from the gel in three fractions: i) from ~600-90nt, ii) from ~90-70nt and iii) from ~70-15nt, passively eluted and ethanol-precipitated. Eluted RNA fractions were poly(C)-tailed. The C-tailing reaction was carried out in 50µL C-tailing buffer (50mM Tris-HCl/pH8, 200mM NaCl, 10mM $MgCl_2$, 2mM $MnCl_2$, 0.4mM EDTA, 1mM DTT), containing 2mM CTP and 2U of poly(A) polymerase. Poly(C)-tailed RNAs were treated with tobacco acid pyrophosphatase and ligated to a 5´-oligonucleotide linker as described (Madej et al., 2007). Poly(C)-tailed and 5´-adaptor ligated RNAs were reverse transcribed using SuperScript II reverse transcriptase and amplified by PCR, followed by cloning into a pGEM®-T vector (Promega). Gene-specific primers were used for the construction of the cDNA library encoding edited RNAs.

Sequence analysis of the cDNA library

cDNA clones were sequenced using the M13 reverse primer and the BigDye terminator cycle sequencing reaction kit (PE Applied Biosystems). Sequencing reactions were run on an ABI Prism 3100 capillary sequencer (Perkin Elmer). Sequences were analyzed using the LASERGENE sequence analysis program package (DNASTAR, Madison, USA), followed by a BlastN search against the GenBank database (http://www.ncbi.nlm.nih.gov/).

gRNA secondary structure predictions, UV-melting profiles and target predictions

RNA secondary structures were calculated using the mfold software (version 2.3) based on a free energy minimization algorithm (Walter et al., 1994; Zuker, 2003). Calculations were performed for 27°C, which is the optimal growth temperature for insect stage trypanosomes. Theoretical UV-melting curves were calculated using the RNAheat algorithm of the Vienna RNA package (Adms, 1979; McCaskill, 1990; Hofacker et al., 1994). Nucleotide bias calculation was performed with the help of the "composition" subroutine of the GCG software (GCG Wisconsin Package, Accelrys Software Inc.; http://www.accelrys.com). gRNA binding targets were predicted using the Kinetoplastid Insertion and Deletion Sequence Search Tool (KISS) at http://gmod.mbl.edu/kiss/ (Ochsenreiter et al., 2007)

Northern blot analysis

Five µg of total mitochondrial RNA was denatured for 1 min at 95°C, separated by 8% (w/v) denaturing PAGE (7M urea, TBE/pH8.3) and transferred onto a Hybond-N^+ membrane (Amersham Biosciences) and UV-cross-linked as described Madej et al., 2007). Oligonucleotides were 5´ end-labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase. Hybridization was carried out at 45°C in 1M $Na_xH_YPO_4$/pH6.2, 7% (w/v) SDS for 12hrs. Blots were washed (2x) at room temperature in 2xSSC buffer (20mM $Na_xH_YPO_4$/pH7.4, 0.3M NaCl, 2mM EDTA), 0.1% (w/v) SDS for 15 min followed by a wash at 58°C in 0.1xSSC, 0.5% (w/v) SDS for 1min.

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Zusammenfassung

Mitochondriale Transkripte in afrikanischen Trypanosomen durchlaufen eine als RNA-Editing bezeichnete posttranskriptionelle Modifizierungsreaktion. Der Prozess ist durch die Insertion und/oder Deletion von ausschließlich Uridylatresten in kryptische prä-mRNAs charakterisiert und transformiert diese zu funktionalen, translatierbaren mRNAs. Die Reaktion wird durch hochmolekulare, subzelluläre Maschinen katalysiert, sogenannte Editosomen. Editosomen sind Proteinkomplexe, die aus ca. 20 Komponenten bestehen und mit kleinen, nicht kodierenden RNA-Molekülen wechselwirken. Diese RNAs spezifizieren die Insertions- und Deletionsereignisse als *trans*-agierende Faktoren und werden *guide* RNAs genannt. TbMP42 (*Trypansoma brucei mitochondrial protein*, MW: 42kDa) ist ein integraler Bestandteil der editosomalen Maschinerie und in der vorliegenden Arbeit wird die Endo/Exoribonukleaseaktivität des Polypeptides beschrieben sowie sein Reaktionsmechanismus untersucht. Versuche mit rekombinantem TbMP42 zeigten, dass es sich um eine struktursensitive Ribonuklease handelt, die in der Lage ist, *base stacking* zu erkennen und eine Präferenz für einzelsträngige Uridylatreste zeigt. Die Ribonuklease-Aktivität von TbMP42 ist Zn^{2+}-Ionen abhängig und lokalisert im C-terminalen Bereich des Proteins, der eine Oligonukleotid/Oligosaccharid-Bindedomäne (OB-Fold) enthält. RNAi-vermitteltes *gene silencing* von *TbMP42* induziert Letalität in *T. brucei* sowie eine Reduktion der Endo/Exoribonuklease und RNA-Editing Aktiviät *in vitro*. Alle drei Aktivitäten können jedoch durch rekombinantes TbMP42 komplementiert werden. Die 3'-5' uridylatspezifische Exoribonukleaseaktivität (exoUase) von TbMP42 generiert 3' Nukleosidmonophosphate. Hieraus ergibt sich als biochemische Konsequenz die Beteiligung einer 3' spezifischen Nukleotidyl-Phosphataseaktivität am RNA-Editingzyklus. Eine solche Aktivität ist in mitochondrialen Extrakten nachweisbar und assoziiert mit editosomalen Komponenten. Die Charakterisierung der 3' spezifischen Phosphatase-Aktivität deutet auf die Beteiligung von zwei Proteinen hin: TbMP99 und TbMP100. Beide Polypeptide besitzen eine Endo-Exo-Phosphatase (EEP) Konsensus-Sequenz, RNAi-vermitteltes *gene silencing* von *TbMP99* oder *TbMP100* hat allerdings keinen Einfluss auf die *in vitro* Phosphataseaktivität. Der simultane *knockdown* von beiden Proteinen hingegen zeigt eine Reduktion in der Phosphataseaktivität *in vitro*. Das deuted darauf hin, dass zumindest eines der beiden Proteine Phosphataseaktivität besitzt.

Obwohl die RNA Editing Maschinerie sehr komplex aufgebaut ist und eine Notwendigkeit für korrekt edititerte Transkripte besteht, ist die Reaktion nicht auf Präzision hin optimiert. *In vivo* Studien zeigen, dass im *steady state* ein hohes Maß an mis-editierten Transkripten existiert. Tatsächlich finden sich eine Reihe von *guide* RNAs, die „alternative" Editing-Ereignisse dirigieren. Eine mögliche Erklärung ist, dass dieses „alternative" Editing eine Quelle für Proteindiversität darstellt. In diesem Teilaspekt der Arbeit wird der Versuch beschrieben, zusätzliche gRNAs zu identifizieren und alternative Editing-Prozesse nachzuweisen.

Eidesstattliche Erklärung

Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation selbstständig und nur mit den angegebenen Hilfsmitteln angefertigt habe. Ich habe bisher noch keinen Promotionsversuch unternommen.

Moritz Niemann

Darmstadt, den 30. Mai 2008

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Homebase – TDN, HAMMPS + Café Leo, RPA d-town HQ witchhill, Düçbag-Kicker, Röhn-Ringer i.w.S., DocBock and the Muppets, … was soll ich sagen? Steinbruch Kantina … wir hatten gute Zeiten, wir bleiben troY!

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So long, and thanks for all the fish!

Lebenslauf

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lab-meeting
monday morning, 0915...