University of South Bohemia

Faculty of Science

Department of Molecular Biology

Master thesis

Functional analysis of subunit MRB3010 of the mitochondrial binding complex 1 in *Trypanosoma brucei*

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Annotation:

This thesis concerns a putative protein complex called the Mitochondrial Binding Complex 1 (MRB1), subunits of which have essential functions in the RNA metabolism of both procyclic and bloodstream forms of *T. brucei*. The focus of this study is on one of the subunits, called MRB3010. It was shown that this subunit of MRB1 complex is essential in both stages of *T. brucei* and the MRB3010 silencing affects the maxicircle encoded edited and pre-edited transcripts. However, the downregulation of this protein appears not to affect the assembly of other putative subunits into MRB1.

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I hereby declare that all the work summarized in this thesis was performed on my own or in collaboration with Michelle Ammerman and Laurie Read (State University of New York at Buffalo) and only using the cited literature.

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České Budějovice, April 21, 2010

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Novotná Lucie

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Special thanks go to Zdeňka Čičová my co-worker who worked with me for many years and helped me with my project. Thanks again.

And finally never enough thanks to my family.

Content

1. Introduction

1.1. *Trypanosoma brucei*

Trypanosoma brucei is a member of the protozoan order Kinetoplastida. This parasitic flagellate causes Human African trypanosomiasis (HAT), the clinical name for sleeping sickness. The disease is endemic in some regions of Sub-Saharan Africa, covering 36 of African´s 52 countries (Barrett et al., 2003). We can recognize three subspecies of *T. brucei. T. brucei gambiense* and *T. brucei rhodensiense* are responsible for chronic or acute infection in humans, respectively. A person can be infected for months or years without major symptoms, or the disease can develop very rapidly. The symptoms of the first stage infection are fever, headaches and itching. The second stage begins when the parasite crosses the blood-brain barrier and invades the central nervous system. Without any treatment, HAT is fatal. The last subspecies *T. brucei brucei* causes animal Trypanosomiasis called Nagana in many wild and domestic animal species.

T. brucei has very interesting life cycle that includes three main stages: bloodstream (in mammalian hosts), procyclic (in the mid-gut of the vector - Tsetse *Glossina* species) and metacyclic (in the Tsetse salivary gland) (Fig.1.1.). The procyclic (PS) and bloodstream (BS) stages can be very easily cultivated in liquid media. Because of this property, its status as a pathogen, and also other unique features like polycistronic transcription and almost excusive trans-splicing of nuclear transcripts, as well as the massive kinetoplast DNA network and RNA editing in the mitochondrion, *T. brucei* is used as a model organism in laboratories.

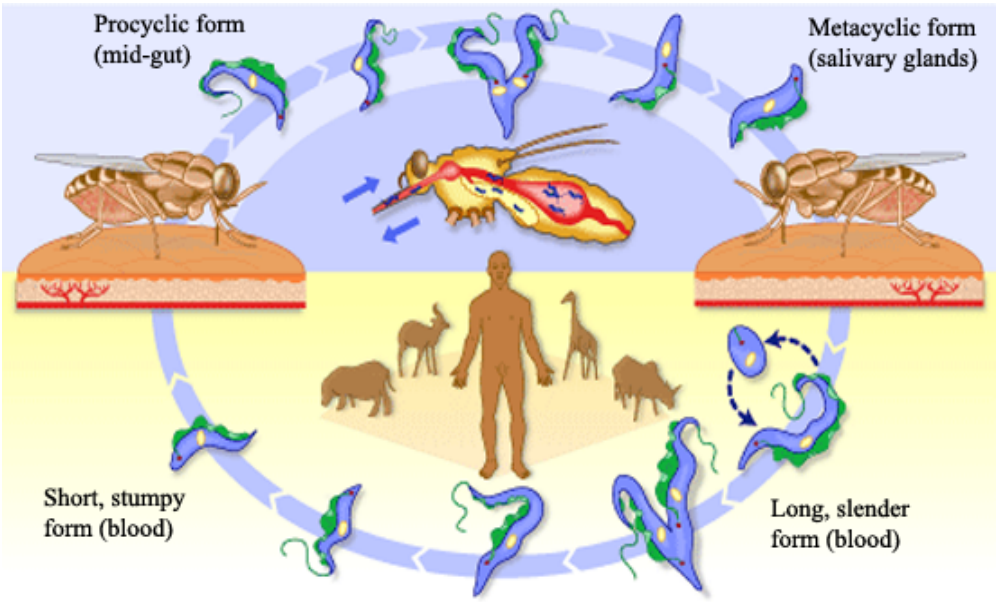


Figure 1.1. Life cycle of *Trypanosoma brucei* (adapted from http://homepages.ed.ac.uk/nsavill/withinhost.html).

Figure 1.2. The difference between *T. brucei* procyclic´s and bloodstream´s mitochondria.

As previously mentioned, *T. brucei* switches between two different settings in its life cycle, during which the parasite´s morphology, biochemistry and expression of some surface markers undergo rapid changes. In the PS, the single mitochondrion runs from the posterior to the anterior of the cell as a reticulated structure with many cristae. The situation is different in the BS form, where the organelle is a simple tubular structure with very few cristae (Fig1.2.). The mitochondria contains kinetoplastid DNA (kDNA), which is giant catenated network composed of two types circular DNAs – maxicircles and minicircles (Fig.1.3.) (Lukes et al., 2002).

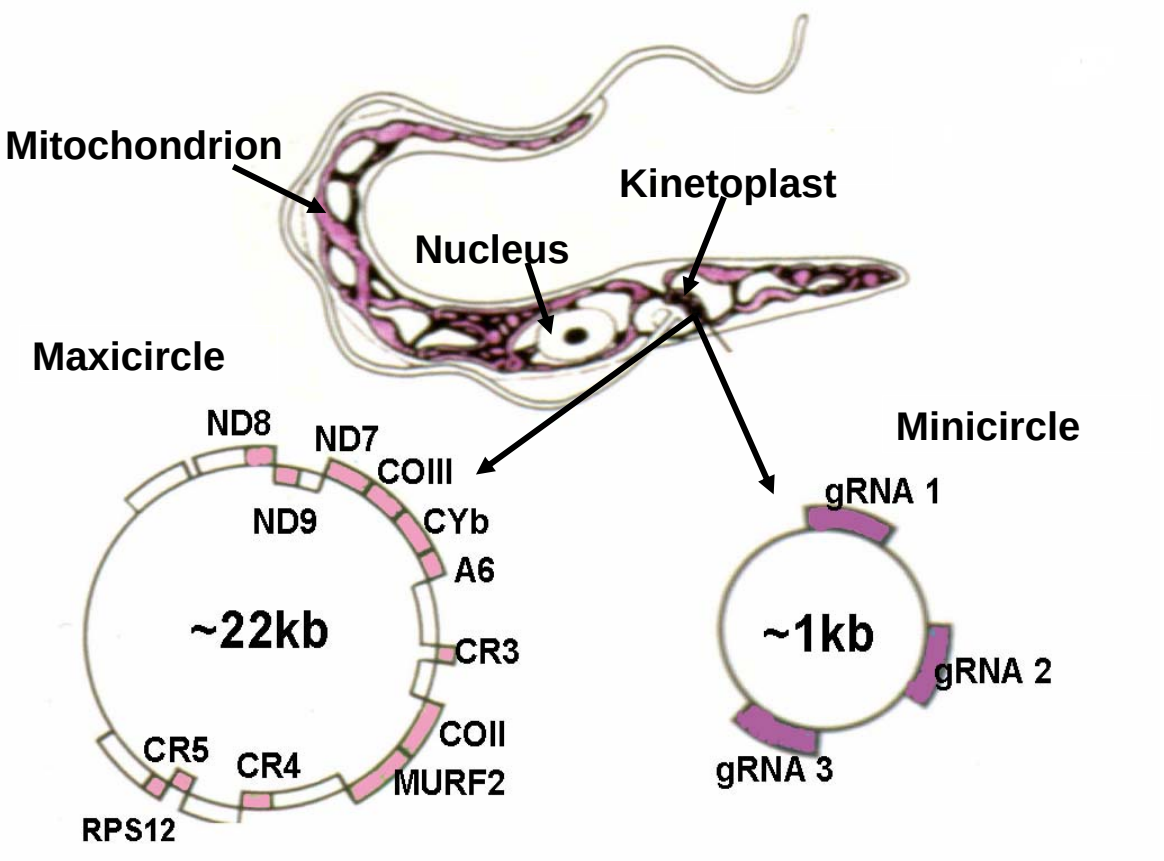


Figure1.3. structure of mitochondrial kDNA

Maxicircles are in dozens of identical copies, their size is approximately 22 kb, and they mostly encode mitochondrial genes for respiratory chain and ribosomal 9S and 12S (Simpson et al., 1987). In contrast, minicircles are in thousands copies that are heterogenous in their sequences and their size is about 1 kb. These molecules contain genes that encode guide RNAs (gRNAs), which are small (50 - 70 nucleotide) primary transcripts that provide information for uridine addition and deletion during mRNA editing.

1.2. RNA editing

RNA editing is a massive post-transcriptional mRNA maturation step that involved uridine (U) insertion or deletion. This process is essential for kinetoplastids and is critical for the production of conventional mitochondrial proteins including subunits of the mitochondrial respiratory chain and the ATP synthase. As previously mentioned, RNA editing is directed by guide (g) RNAs . These molecules contain the so-called anchor region on their 5´ end, which is complementary to the pre-edited mRNA (Sturm and Simpson 1990). On the 3´ end is the postranscriptionally add oligo (U) tail, which is proposed to stabilize the gRNA pre-mRNA complex. The third guiding region of the gRNA provides information for where and how many uridines will be inserted and/or deleted at the specific site in the mRNA (Fig.1.4.).

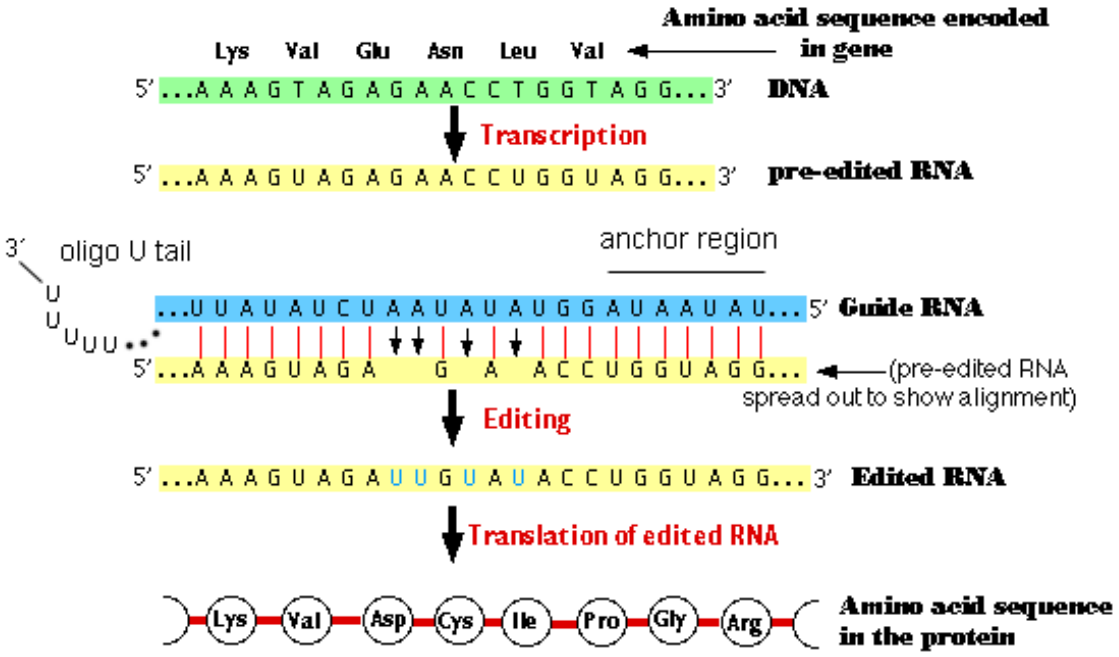


Figure 1.4. RNA editing mechanism
(adapted from http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/R/RNA_Editing.html).

Four enzymatic activities play an important role in this highly specific mechanism: at first an endonuclease cleaves the mRNA then a U-specific exonuclease (exoUase) or terminal uridylyl transferase (TUTase) deletes or inserts uridines from/into the appropriate site within mRNA sequence; finally, an RNA ligase rejoins fragments after the editing events dicated by the gRNA is completed (Stuart et al., 2005). These core enzymes are part of a multiprotein complex called the RNA editing core complex (RECC), which catalyzes U-insertion/deletion editing of mRNAs. The RECC is comprised of ~ 20 proteins and on the glycerol gradient sediments at 20 Svedburg units (S), and thus is also named the 20S editosome (Stuart et al., 2005).

It has been shown that there are three different RECCs, differentiated in part by the contained endonuclease (Carnes et al., 2008). REN1 (RNA editing endonuclease) specifically cleaves insertion editing sites and REN2 specifically cuts deletion sites. A third editosome endonuclease called REN3 specifically cleaves coxII insertion editing, which is special because its gRNA is present on the 3´-UTR of the coxII mRNA (Fig.1.5.) (Golden and Hajduk, 2005). All of these endonucleases are essential for the normal growth of PS and BS form cells (Carnes et al., 2008).

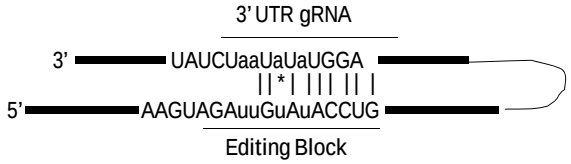


Figure 1.5. CoxII RNA editing (adapted from Zdeňka Čičová MSc. thesis).

1.3. Other proteins involved in RNA editing

A large number of proteins are involved in RNA editing and metabolism. One way how to identify a protein involved in this process is to look for their direct binding to gRNA molecules (Köller et al., 1994). RBP16 (RNA-binding protein of 16 kDa) was found in this way (Hayman and Read, 1999). This protein is a member of Y-box protein family, which contains a highly conserved domain that facilitates binding nucleic acids. RNA silencing of RBP16 has shown that it is essential in the PS form of *T. brucei* affecting the stability of some never-edited and edited mRNAs (Pelletier et al., 2003), while not affecting growth in BS (Fisk et al., 2009). Furthermore, RBP16 stimulates RNA editing activity *in vitro* (Miller et al., 2006).

Other proteins involved in RNA editing mechanism are mitochondrial binding proteins (MRPs) 1 and 2. Both proteins are essential for PS, but have no effect on the growth of BS (Vondrušková et al., 2005,

Missel et al., 1997). They also mediate the stability of a specific subset of edited and never-edited mRNAs (Vondrušková et al., 2005) and RNAi silencing of MRP1 and 2 leads to the impairment of the construction of the respiratory complexes (Zíková et al., 2006). These proteins play a role in mRNA-gRNA annealing (Zíková et al., 2008).

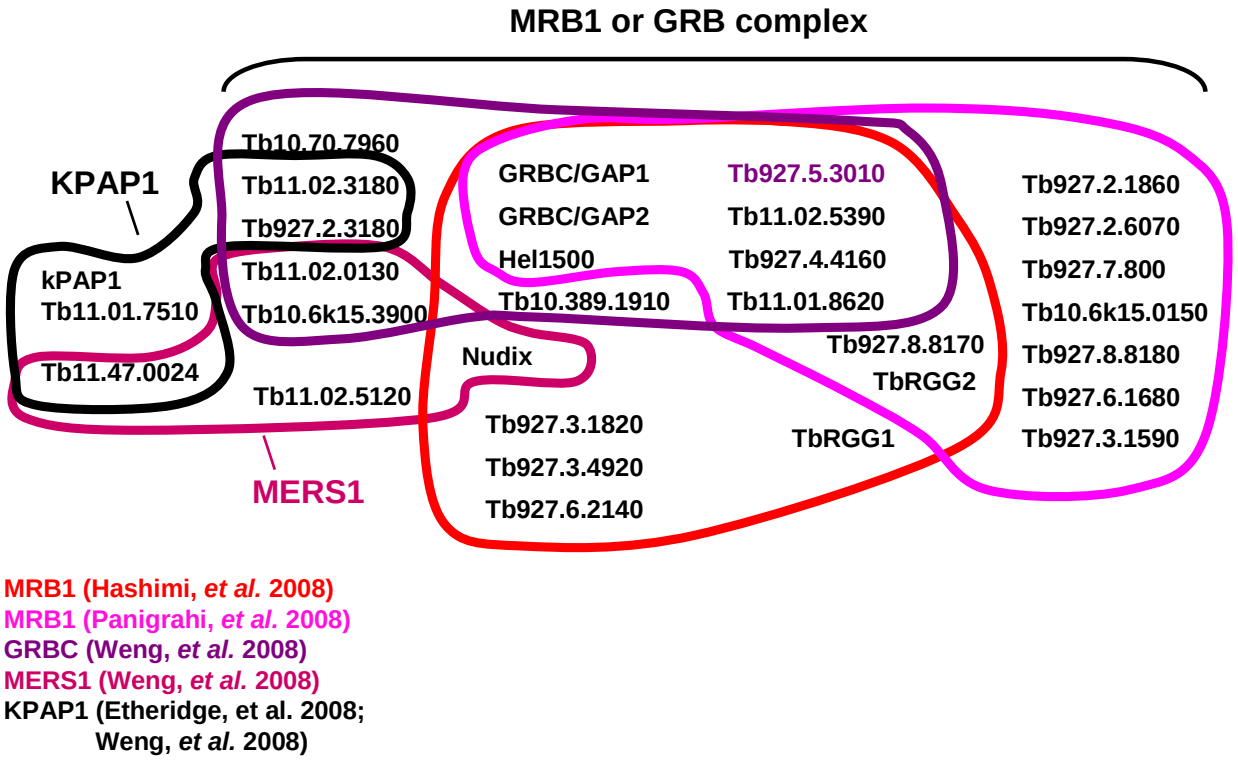
Some proteins can directly bind to oligo(U) sequences *in vitro,* including a protein termed TbRGG1. It contains a RGG (arginine-glycine-glycine) repeat in its N-terminus (Vanhamme et al., 1998), which facilitates RNA binding, and is essential in the PS stage of *T. brucei* (Hashimi at al., 2008). Glycerol gradient co-sedimentation with *in vitro* RNA editing activities suggested that this protein may play a role in RNA editing (Vanhamme et al., 1998). However, direct interaction with the RECC has not been demonstrated, and its down-regulation does not appear to affect the level of gRNAs (Hashimi at al., 2008). By using the tandem affinity purification (TAP) approach, which allows the isolation of tagged protein with its putative binding partners *in vivo,* this protein was identified to associate with a putative complex named the mitochondrial RNA binding complex 1 (MRB1) in a RNA mediated manner (Hashimi at al., 2008). The functional analysis of one of the subunits of MRB1, Tb927.5.3010 (called MRB3010) is the main subject of my thesis, although I also address the organellar localization of two other subunits MRB5390 and MRB8620.

1.4. The MRB complex 1

The isolation of the MRB1 complex in our laboratory revealed composition of 14 subunits (Hashimi et al., 2008), although this composition varies from that of other MRB1 purifications (Weng et al., 2008; Panigrahi et al., 2008). Other complexes of overlapping composition with MRB1 are those associated with the mitochondrial edited mRNA stability factor (MERS), which is also known as Nudix hydrolase and discussed below (Weng et al., 2008), and a poly(A) polymerase (KPAP1) (Enteridge et al., 2008) (Fig 1.6.). It is not known yet if all subunits that are illustrated in Fig.1.5. make up one complex, or if they represent a few small complexes and/or monomers that are somehow associated dynamically and/or via RNA interactions. The latter situation seems to be the most likely case because individual proteins appear to play different roles in RNA metabolism. Some of the proteins have domains or motif involved in RNA metabolism and protein-protein interactions, such as the DExD-box helicase and Nudix hydrolase, as well as several proteins bearing pentatricopeptide (PPR) motifs and the previously mentioned RGG domain, whereas others have no known motifs.

Our group is concentrated on the subunits that are involved almost in all mentioned complexes, which we provisionally call the core subunits. Recently we described three of them, in addition to Nudix hydrolase. The guide RNA associated proteins (GAPs) 1 and 2 are paralogs sharing sequence similarity.

They associate together to form a complex, which has been labeled the guide RNA binding complex (GRBC) (Weng et al., 2008; Hashimi et al., 2009). They are essential for PS and BS stages, and their downregulation also leads to decrease of steady state level of gRNAs in both stages of *T. brucei* (Hashimi et al., 2009). The predicted RNA helicase is also essential for PS stage, and its RNAi silencing also decreases the level of gRNAs (Hashimi et al., 2009, Hemandez et al., 2009). The downregulation of Nudix hydrolase affects growth in PS and was found to destabilize almost all maxicircle transcripts (Hashimi et al., 2009) or only edited mRNAs (Weng et al., 2008), leading the authors of the latter study to name this protein MERS.



MRB1 (Hashimi, *et al.* 2008)
MRB1 (Panigrahi, *et al.* 2008)
GRBC (Weng, *et al.* 2008)
MERS1 (Weng, *et al.* 2008)
KPAP1 (Etheridge, et al. 2008; Weng, *et al.* 2008)

Figure 1.6. Overlapping composition of the MRB1 complex. Individual complexes isolated from three different groups are labeled by different colored lines. The accession numbers were used for unknown subunits, whereas others are named because of their predicted motifs: guide RNA binding complex (GRBC), guide RNA associated protein (GAP), RNA helicase, Nudix hydrolase TbRGG1 and 2 (containing the RGG domain).

The TbRGG2 protein (also known as TbRGGm) contains the previously mentioned RGG domain,(Panigrahi et al., 2003). It has been reported that it is essential for BS and PS stages, and its silencing appears to affect the protein complex stability (Fisk et al., 2008; Acestor et al., 2009). Two more subunits, thus far known only by their gene DB (www.genedb.org) accesion numbers Tb11.02.5390 and Tb927.6.1680, were described recently (Acestor et al., 2009). Tb11.02.5390 appears to be one of the core MRB1 subunits, and it has no known motif. Tb927.6.1680 has a C2H2 zinc finger motif, which can mediate interactions with nucleic acids or proteins. These proteins are essential for PS form of *T. brucei*, but their exact role in the RNA metabolism of the mitochondrion has still not been finalized.

The topic of my study was to contribute to the general knowledge of one of the core MRB1 proteins, thus far referred to by its gene DB accesion number, Tb927.5.3010 (MRB3010), since it has no known motif or orthologs in other organisms. In addition, some data will be presented addressing the composition of MRB1 in general.

1.5. Mitochondrial RNA processing

The mitochondrial genome present on the maxicircle DNA encode two ribosomal RNAs (rRNAs), 12S and 9S, as well as several subunits of the respiratory chain and one of the mitoribosome: NADH dehydrogenase (ND), cytochrome oxydase (Co), cytochrome b (CyB), ribosomal protein subunit 12 (RPS12) (Fig.1.7.). In addition, they contain genes of unknown function, such as MURF2 (maxicircle unidentified reading frame), or those that undergo such extensive editing that their identity remains mysterious (the so called G-rich domains). Maxicircle genes are transcribed polycistronically (Read et al., 1992) and require some processing steps to generate mature RNA: RNA editing of some RNAs, polyadenylation of mRNAs (Bhat et al., 1992) and polyuridilation of rRNAs (Adler et al., 1991) (Fig 1.8.).

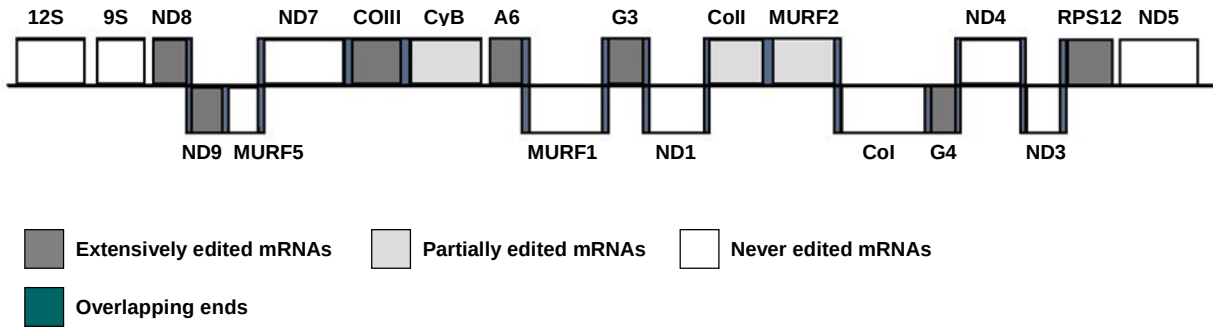


Figure 1.7. Maxicircle encoded genes. MURF1 = ND2 (Kannan and Burger, 2008).

RNA editing is a complicated process that can create start codons, stop codons and open reading frames to make translatable RNAs. Polyadenylation is also a part of the mRNA maturation process, in which a poly(A) tail is added to the 3´end of the mRNA. Almost all eukaryotic mRNAs are polyadenylated. The poly(A) tail on nuclear mRNAs is important for nuclear transport and mRNA stabilization (Manley and Proudfoot, 1994). The poly(A) tail on mitochondrial mRNAs also plays a role in its stabilization. For example human mitochondrial mRNAs with the poly(A) tail are stabile, whereas deadenylated mRNAs are not (Nagaike et al., 2005). In plant mitochondria (Gagliardi et al., 2004) and bacteria (Kushner, 2004), the poly(A) tail is a signal for degradation. Yeast mtRNAs are not polyadenylated. In the *T. brucei* mitochondrial mRNAs contain two different poly(A) sizes.

The short tail (20-25 nucleotides) is present on pre-edited molecules, whereas the never-edited and edited mRNAs have short or long (120-250 nucleotides) poly(A) tails (Bhat et al., 1992). The short poly(A) tail stabilize the pre-edited mRNAs, but the same tail destabilizes the edited molecules (Aphasizheva and Aphasizhev, 2010). Edited and never edited mRNAs with the long A/U tail are presumed to be marked for translation (Etheridge et al., 2008). Nuclear polyadenylation is accomplished by macromolecular complex that contains a poly(A) polymerase termed PAP (Proudfoot, 2004), but this catalytic complex was not observed in *T. brucei* mitochondria. Etheridge and colleagues described a mitochondrial complex with its own poly(A) polymerase, named kinetoplast poly(A) polymerase (KPAP1), which plays the same role as the nuclear enzyme (Etheridge et al., 2008). This complex adds short poly(A) and long A/U tails to the mitochondrial mRNAs, and it is essential for parasite variability and mitochondrial function.

Polyuridylation is postranscriptional process in which uridine residues are added to the 3´end of maxicircle encoded rRNAs and minicircle encoded gRNAs (Adler et al., 1991; Blum and Simpson, 1990).
The poly(U) tail length of the 9S and 12S rRNAs is different, the former having a tail of precisely 11 Us, while the latter has a tail of a heterogenous number (2-17 nucleotides). The gRNAs poly(U) tails are also heterogenous in length (5-27 nucleotides) (Blum and Simpson, 1990). Uridines (Us) are added to the 3´end of the molecule by the terminal uridylyl transferase (TUTase) named RNA-editing TUTase 1 (RET1) (Aphasizhev at al., 2003; Aphasizheva et al., 2009, Aphasizheva and Aphasizhev, 2010).

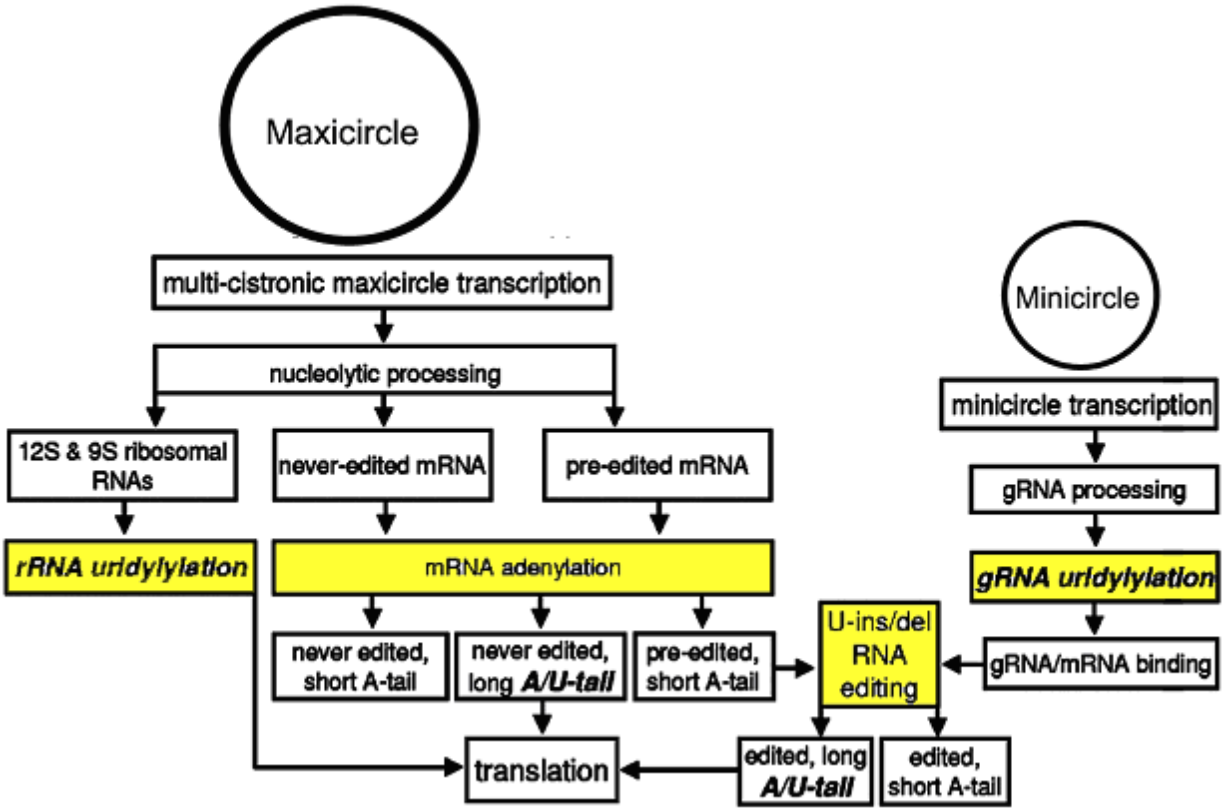


Figure 1.8. Mitochondrial RNA processing (Adapted from Aphasizheva and Aphasizhev, 2010).

2. Materials and Methods

2.1. RNAi silencing

2.1.1. Preparation of primers and Polymerase Chain Reaction PCR

A BamH I restriction site was added to the 5´-end of the forward (Fw) primer and the Xho I site was added to the reverse (Rv) primer (Tab.2.1; sites are indicated in bold).

	Tb927.5.3010 (MRB3010)
Fw	GGATCCCTAAGCAAGAGGTTCGCCAC
Rv	CTCGAGGTCTCCCCTGCATCCAGTAA

Table 2.1. Primers for the PCR amplification of the MRB3010 fragment. In bold are the sequences recognized by the restriction endonucleases BamH I and Xho I.

PCR reaction program

1. denaturation	96°C 5 minutes	
2. denaturation	94°C 1 minute	25x
3. primers extension	55°C 1 minute	
4. polymerase	72°C 1,5 minutes	
5. polymerase	72°C 10 minutes	
6. hold at 4° C		

2.1.2. PCR – TOPO cloning

The TOPO TA Cloning® kit (Invitrogen) was used according to the manufacturer's instructions (Plasmid depicted in Fig. 2.1.)

- Transformation (add cells *E.coli* DH5α and leave on ice 15-20 minutes, heat shock 40 sec 42°C, leave 2 min on ice, add 150 μl SOC and shake 45 minutes at 37°C)
- Add IPTG (final concentration 0,1 mM) and X-gal (final concentration 40 mg/ml) to the agarose plates because blue-white selection
- Choose only white colonies for plasmid isolation

- Verify the cloning by restriction reaction with enzymes BamH I and Xho I and separate the fragments on an agarose gel

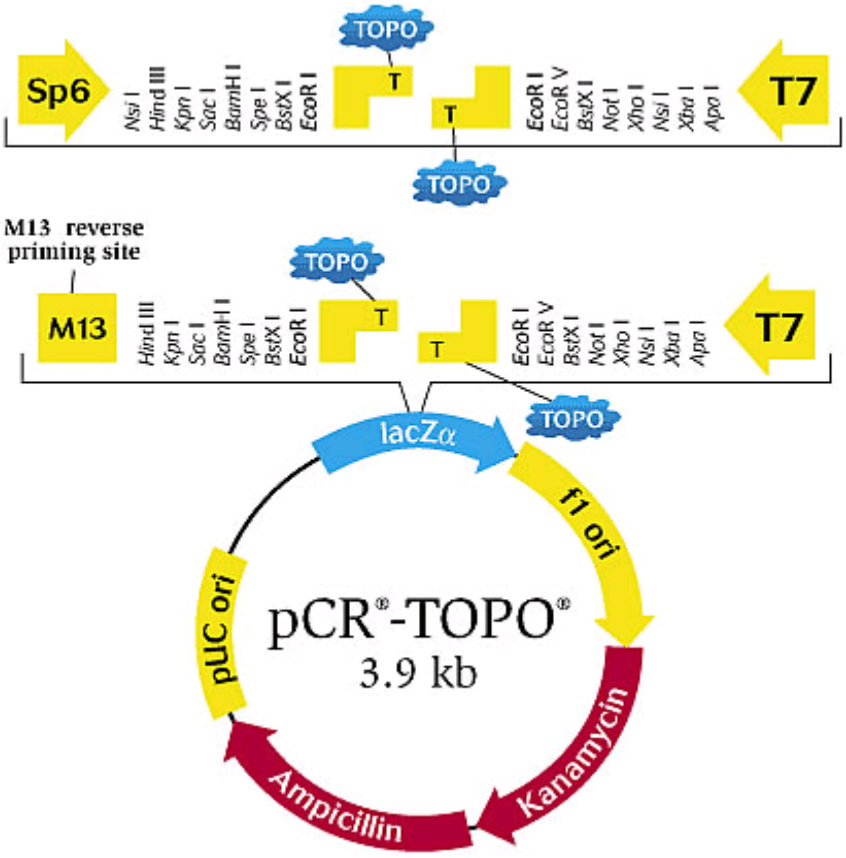


Figure 2.1. Map of TOPO TA Cloning® kit (Invitrogen).

2.1.3. Gel extraction

Performed according to the QIAquick Gel Extraction Kit (QIAGEN) protocol

2.1.4. Ligation to p2T7-177 vector (Fig. 2.2.)

- According to instructions (Invitrogen)
- Transformation and selection on ampicilin plates

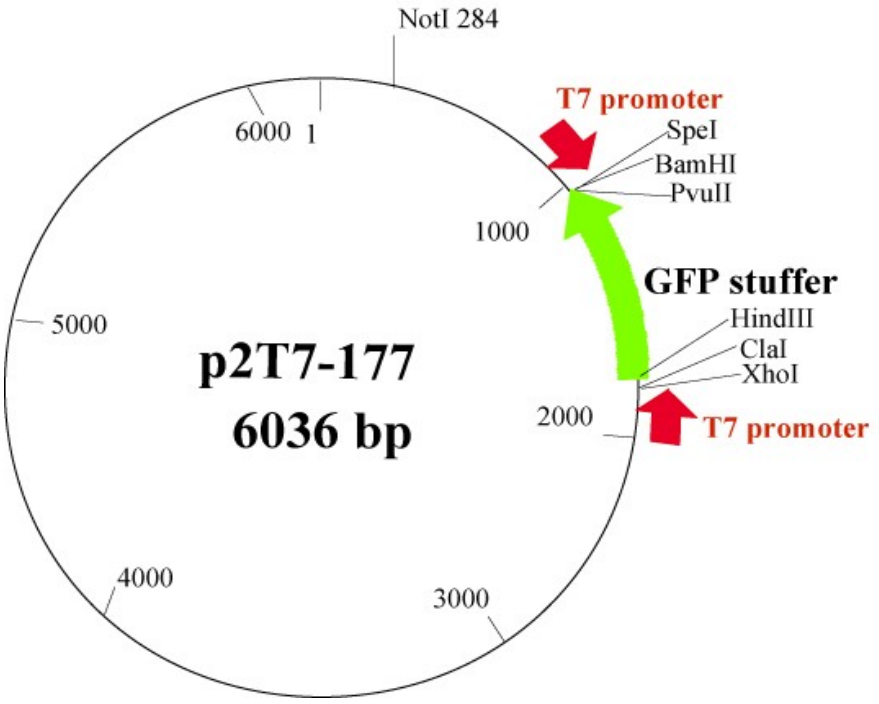


Figure 2.2. Map of p2T7-177 vector (Wickstead et al.,2002).

2.1.5. Isolation of plasmid DNA for transfenction

This procedure was performed according to the Midi-prep Kit (QIAGEN) protocol

2.1.6. Linearize plasmid for tranfection

- 10 µg DNA
- 10 µl 10x buffer
- 10 U (units) Enzyme (Not I)
- Final volume – 100 µl
- incubate at 37°C for 2-3 hrs
- To isolate the linearized plasmid add ½ volume of phenol and ½ volume of chloroform supplemented with isoamyl alcohol in a ratio (1:20)
- Vortex 1 minute
- Centrifuge at 4°C and 16100 g 10 minutes and take the upper aqueous phase to a new tube
- Precipitate with 1/10 volume Na Acetate and 2.5 X volume Ethanol (100%)
- Incubate at - 80°C for at least 30 min
- Spin down 30 min at 16100 g and 4°C

- Wash with 180µl ice cold 70% ethanol
- Air dry for 10 min in TC hood and resuspend in 400 µl of sterile cytomix for subsequent electroporation

Cytomix	120 mM KCl
	0,15 mM $CaCl_2$
	10 mM K_2HPO_4 / KH_2PO_4
	2 mM EDTA
	6 mM glukose
	5 mM $MgCl_2$
	25 mM HEPES

2.1.7 Transfection of PS *T. brucei*

- Harvest 10 ml of mid-log culture (1 - 2 x 10^6 cells per ml) by centrifugation at 1300 g, 4°C
- Wash the cell pellet twice with 10 ml ice cold cytomix buffer
- Resuspend cells in 400 µl of cytomix with linearized plasmid
- Load cuvettes (0.2 cm gap) with 10 µg of linearized sterile DNA and 1 – 2x10^7 cells
- Electroporate with ECM650 (BTX) machine with two pulses using the settings Settings: 1500 V, 25 Ω, 50 µF, 10s, 1700 V, 25 Ω, 50 µF
- Resuspend electroporated cells in 10 ml of medium supplemented with hygromycin (H) final concentration 50 mg/ml and G418 (G) to final 15 mg/ml, and incubate O/N
- Add 2,5 µg/ml phleomycin into flask and then distribute into plate for semi-cloning of transformants by limiting dilution in SDM-79 in 24-well plates
    - Load the first row of wells with 1.5 ml of your culture
    - Load the second and third rows with 1 ml fresh SDM-79 (10% FBS, with drug)
    - Load the third row with 0.5 ml fresh SDM-79 (10% FBS, with drug)
    - Starting in the fourth column, transfer 0.5 ml of the first well into the second, pipette up and down, transfer 0.5 ml to the third well, and so on (Fig.2.3.)
- Keep the plate at 27°C until mock transformants are eliminated by phleomycin selection

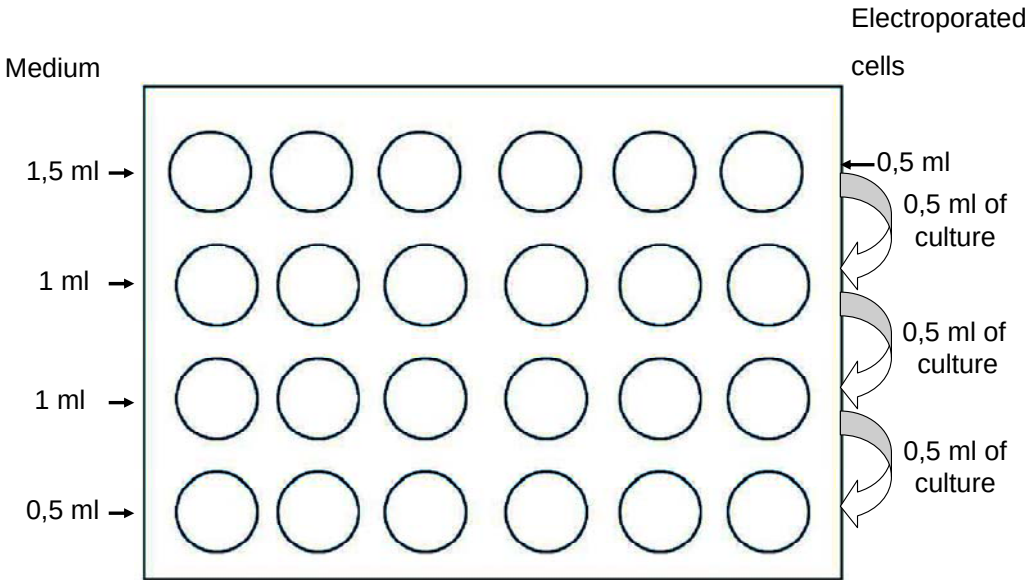


Figure 2.3. Dilution of electroporated cells.

2.1.8. Transfection of BS *T. brucei*

- Harvest 10 ml of mid-log culture (1 - 2 x 10^6 cells per ml)
- Continue according to protocol for PS transfection

2.2. *T. brucei* Cultivation

- Procylic stage *T. brucei* are grown in SDM-79 medium (Brun and Schonenberger, 1997) at 27°C
  - Hygromycin (50 μg/ml), neomycin (G418) (15 μg/ml) - (H,G) for wild type (WT-29-13)
  - Hygromycin, neomycin, phleomycin (2,5 μg/ml) – (H,G,P) for stable cell line (non-induced cells)
  - Hygromycin, neomycin, phleomycin, tetracyclin (1 μg/ml) – (H,G,P,tet) for stable cell line (induced cells)
- BS are grown in HMI medium at 37°C and under CO_2
  - Neomycin (G418) (2,5 μg/ml) for wild type (WT 427)
  - Neomycin and phleomycin (1,25 μg/ml) for stable cell line (non-induced cells)
  - Neomycin, phleomycin and tetracyclin (1 μg/ml) for stable cell line (induced cells)
- Density of the culture is measured by the Beckman Coulter Z2 Particle Counter

2.3. Isolation of RNA

- Spin down the cells at 700 g 10 min, 4°C, discard supernatant
- Resuspend the pelleted cells in 1 ml of RNA Blue (TopBio) or Tri-Reagent (Invitrogen) and continue according to protocol
- Resuspend the RNA pellet in appropriate volume of water according to pellet size (usually 30 ul)
- Measure the concentration of RNA prior to its running on a gel using spectrophotometer (Eppendorf) or NanoDrop (Thermo SCIENTIFIC)

2.4. Northern analysis

2.4.1. Gel preparing and Solutions

Prepare 1% agarose gel in MOPS buffer with formaldehyde in the final concentration 6,6%

Running buffer is 1X MOPS

Load 10 ng of RNA

1.5 x Sample Buffer	
Formamide	600 ul
Formaldehyde 37%	210 ul
10x MOPS	156 ul
Ethidium Bromide	5 ul

10x MOPS 100 ml	
0,5 M MOPS	40 ml
3 M Sodium Acetate	1,67 ml
0,5 M EDTA pH 8	2 ml
milliQ water	56,33 ml

2.4.2. Blotting (Fig. 2.4.)

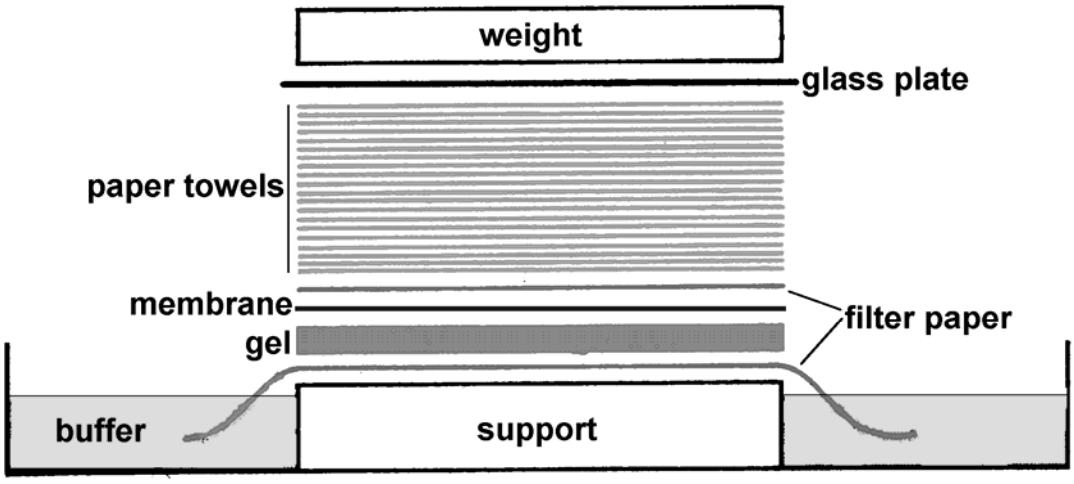


Figure 2.4. Blotting apparatus.

- Use 5X saline-sodium citrate buffer (SSC)
- Let blot for 24 hours
- Immobilize the RNA on membrane by UV crosslinking (UV Stratalinker, Stratagene)

2.4.3. Pre-hybridization

- Pre-incubate the membrane in NaPi (sodium phosphate buffer) in cylinder
- Rotate at 60°C at least 2 hours

2.4.4. Radioactive labeling by random priming using the HexaLabel DNA labeling Kit or DecaLabel (MBI Fermentas) according to protocol using 100 ng of purified PCR product as a template

- Purify the probe in spin column (MicroSpin G-50 Sephadex-GE Healthcare)
- Heat the probe at 100°C 3 min, put on ice and then add to approximately 5 ml of NaPi and pour into the cylinder with the pre-hybridized membrane
- Rotate at 55-60°C O/N

2.4.5. Membrane washing

Remove hybridization solution

- Add 5 ml 2x SSC + 0,1% SDS into the cylinder and rotate 20 min
- Pour the liquid out to the radioactive container and add 5 ml of 0,2x SSC + 0,1% SDS. Rotate at 55°C for 20 min
- Discard the washing solution
- Put the membrane on a filter paper, wrap into foil and place into the phosphoimager cassette, RNA on top and keep in the cassette O/N
- View in a Typhoon Phosphoimager (Amersham)

2.4.6. Dot Blot (Probe testing)

- Serially dilute MIDIprep of RNAi fragment
- 1: 100 1:1000 1: 10 000 1: 100 000
  1ul midi + 99 ul 10 ul + 90 ul 10 ul + 90 ul 10 ul + 90 ul
- 3 ul of each onto membrane, dry a bit soak the membrane for 3 min in denaturing solution (1,5 M NaCl, 0,5 M NaOH)
- Wash for 3 min in neutralizing solution (1,5 NaCl, 0,5 M Tris-HCl pH = 7)

- Wash for 3 min in 3x SSC, repeat twice
- UV crosslink
- Prehybridization in NaPi and then hybridize with probe

2.5. Growth Curves

The aim is to follow a growth of cell cultures upon induction of RNAi to see whether RNAi-silencing of the target gene results in growth inhibition
Use Beckman Coulter Z2 Particle Counter for measuring the cell culture

2.5.1. Growth Curves for PS stage of *T. brucei*

Cells are grown in 1ml media and their density is measured every 24 hours using the Beckman Coulter Z2 Particle Counter for 14 days. Starting cell density is $2x10^6$ cells/ml. Cells are diluted every other day to the starting concentration $2x10^6$ cells/ml.

2.5.2. Growth Curves for BS stage of *T. brucei*

Cells are grown in 1ml media and their density is measured every 24 hours using the Beckman Coulter Z2 Particle Counter for approximately 10 days. Starting cell density is 10^5 cells/ml. Cells are diluted every day to the starting concentration 10^5 cells/ml.

2.6. Quantitative real time PCR

2.6.1. General rules

- Use filter tips for every step to avoid contamination with gDNA, RNases, PCR Products etc. and do all subsequent steps in separate room than where plasmid DNAs are manipulated.
- Use dedicated water
- Use dedicated reagents only for qPCR
- Use a dedicated set of pipettes that are used from the “DNase digestion” step
- Pipette the qPCR reactions in an environment where NO genomic DNA is isolated, no qPCR tubes are opened, etc., to avoid contamination
- Touch reagents only with gloves
- Grow cells, isolate your RNA and make your cDNA at the same time for all your samples

2.6.2. Harvest of cells

- Harvest 10^8 cells at a concentration of 1-2x10^7 cells/ml

2.6.3. DNase Digestion using TURBO DNA-free™ Kit (Ambion)

- From here on use dedicated set of pipettes
- Prepare DNase Master Mix (MM) (Tab 2.2.)
- Take 20-25ug RNA to 50ul with miliQ H_2O

DNase							
		MM					
	1 round (µl)	3,5x round					
RNA (ug)	*50*	--		Mix 25 µg of RNA with 50 ul of MM			
10 X buffer	10	35					
Turbo Dnase	1	3,5					
ddh20	39	136,5					

Table 2.2. Calculation for DNase assay.

- Be sure to enter your values into the yellow boxes
- Mix 50ul RNA sample with 50ul Master Mix, incubate 30 min at 37°C
- Add another 1ul of DNase to the reaction, incubate for 30 min at 37°C
- Add 10 ul of DNase inhibitor (resuspend thoroughly first!), flick constantly for 2 min
- Spin down at full speed for 2 min
- Take 90ul of the supernatant to the new tubes
- precipitation :
    - 250ul EtOH (96%)
    - 10 ul 3M NaOAc pH 5.2
    - 1 ul glycogen
    - Mix well, keep at -20°C for 1 h or overnight
    - Spin for 30 min at full speed at 4°C
    - Wash with 70% EtOH
    - Spin again for 15 min at full speed at 4°C
    - Decant supernatant, short spin, dry on air then resuspend in 20 ul miliQ water
- Measure RNA concentration by nanodrop (Thermo SCIENTIFIC)

- Verify RNA integrity on 1% agarose gel

2.6.4. cDNA /Reverse Transcription (Superscript III™) (Invitrogen)

- Transcribe 4-5 ug of DNase treated total RNA to cDNA using random hexamer primers
- Prepare two reactions each with Reverse Transcriptase (RT), one without; call the RT+ reactions A and B and RT- (without reverse transcriptase)
- Switch on the thermocycler, choose program 59 (HHRT)
- Prepare cDNA Master Mixes 1, 2+ and 2- (MM1, MM2+ and MM2-) (Tab. 2.3.)

4	ug RNA				
			RNA(ng/µl)	µl RNA	µl H2O
sample	+	A	*896,7*	4,46	4,54
	+	B			
	-				

		MM1
	1 round (µl)	11x round
250 ng/ul RandomHex	1	11
10 mM dNTPs	1	11
RNA	9	-

		MM2	MM3
		RT+	*RT-*
	1 round (µl)	7x round	3,5x round
5X Buffer	4	28	14
0.1 M DTT	1	7	3,5
RnaseOUT	1	7	3,5
Superscript III	1	7	--
ddH20	3	14	10,5
	10	63	31,5

Table 2.3. Calculation for cDNA assay.

- Prepare RNA in 200 ul PCR tubes according to table 2.3. (need 3 samples of 4-5ug RNA) Mix 2 ul each of Master Mix 1 with each of your 9 ul RNA sample
- Put the samples into the thermocycler, unpause. (Step 1: Heat to 65°C for 5 min)

- During Step 2 (1 min 4°C), add 9ul of the corresponding MM2 to the samples (Be careful not to add the wrong MM!), carefully pipette up and down
- Put the samples back to the thermocycler and let the program run to the end:
    - Step 3 : 25°C for 5'
    - Step 4: 50°C for 60'
    - Step 5: 70°C for 15'
    - Step 6: Keep at 4°C
- Take the volume of your cDNA to 200ul with 180ul H_2O MQ
- Prepare 1:50 dilutions of your RT+ samples (= A and B)

2.6.5. Primers for qPCR

Gene	Forward	Reverse	Amplicon (bp)
MBP3010	CCGCTTTTCCTACTGTTGTG	ATATTGCGAGCAGAGAGGTG	130

Table 2.4. Primers for Real Time PCR analysis for MBP3010 designed using. http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome

Gene	Forward	Reverse	Amplicon (bp)
A6 P	TTGCCTTTGCCAAACTTTTAGAAG	ATTCTATAACTCCAAAATCACAACTTTCC	90
A6 E	TGTTAGTTGGTGATAGTTTTATGGATGT	AAAAACGTCGACAAACAACGA	100
RPS12 P	GATTTTAAGATTGGCTTTGATTGA	AATATAAAATCTAGATCAAACCATCACA	68
RPS12 E	CGTATGTGATTTTTGTATGGTTGTTG	AATATAAAATCTAGATCAAACCATCACA	98
MURF2 P	GATTTTAAGATTGGCTTTGATTGA	AATATAAAATCTAGATCAAACCATCACA	97
MURF2 E	GATTTTAATGTTTGGTTGTTTTAATTTAG	AATATAAAATCTAGATCAAACCATCACA	126
ND4	CAATCTGACCATTCCATGTGTGA	TTTCAGCACAATACTTGCTAATAAAACA	89

Table 2.5. Oligos for Real-Time PCR analysis of edited (E) and pre-edited (P) maxicircle RNAs (Carnes et al., 2005).

2.6.6. qPCR

- To the three qPCR tubes (Quigen) pipette 2 μl of cDNA RT+ (A) and to the to the three different qPCR tubes pipette 2 μl of cDNA RT+ (B) and to the one tube cDNA RT- as a control
- Add 18 μl of mastermix (Tab. 2.6.) with 18 S primers to the each tube
- Choose cDNA RT+ A or B for further analysis analysis based on which tet+ and tet- sample from each pair exhibits the most similar C_T values
- To the three different qPCR tubes pipette 2 μl of chosen cDNA and add 18 μl of mastermix. Do the same for all primers see Tab. 2.4. and 2.5.
- Do the negative control with cDNA RT-

Mastermix	Rounds	
	1	17
2X Sybr Green MM (Applied Biosystem)	10	170
cDNA	0	0
1,5uM primers	8	136

Table 2.6. calculation of mastermix for qPCR reaction.

- Use Rotor-Gene RG300 (Corbett research)
- PCR reaction program

Denaturation	94°C 2 min	
Denaturation	94°C 20 sec	40X
Annealing	60°C 30 sec	
Elongation	72°C 30 sec	

Heat on 72°C – 95°C (as a control to determine the presence of the correct amplicon according to melting temperature)

Data taken from Rotor Gene Application Software version 6.1 71 were analyzed by Pfaffl method (Pfaffl, 2001) (principle of which is a relative quantification of transcripts based on the relative expression of a target gene versus an unaffected reference gene).

2.7. Isolation of Mitochondria

- PS *T. brucei* are grown in suspension at 27°C in a SDM-79 medium. Cells are harvested at a density $2*10^7$ cells/ml to the final amount $7.5 \times 10^9 – 10^{10}$
- Spin cells at 4^0C for 10 min at 6000 g
- After centrifugation immediately remove the medium
- Resuspend pellets in 35 ml of 1x SBG, combine pellets in one bottle, and spin for 10min at 7000 g
- Resuspend cell pellet in 35 ml of DTE (hypotonic lysis buffer)
- Homogenize in a glass Dounce Tissue Homogenizer (push 10X up and down), quickly add 5,8 ml of 60% sucrose. Mix well to re-establish isotonic conditions
- Spin lysate at 4^0C for 10 min at 15800 g
- Poure out supernatants and resuspend pellets by vortexing in 6,8 ml of 1x STM buffer
- Add 21 μl 1M $MgCl_2$ a 0,1 μl 0,1M $CaCl_2$ and solid DNase I (2mg/ml) (Ambion) to 0.1 mg/ml final
- Incubate for 45 min on ice
- Add 6,9 ml of STE buffer
- Spin at 4^0C for 10 min at 15800 g. The resulting pellet will be very soft
- Pellets can be keep in -80°C

2.8. Glycerol Gradient

2.8.1. Buffers and Material

SBG buffer:	20 mM glucose
	150 mM NaCl
	1,4 mM NaH_2PO_4
	8,6 mM Na_2HPO_4

DTE buffer:	1 mM Tris pH = 8,0
	1 mM EDTA

STM buffer:	20 mM Tris pH = 8,0
	2 mM $MgCl_2$
	250 mM saccharose

STE pufr:	20 mM Tris pH = 8,0
	2 mM EDTA
	250 mM saccharose

Buffer A (10% glycerol)	10 mM Tris, pH = 7.2
	10 mM $MgCl_2$
	10 mM KCl
	10% glycerol

Buffer B (30% glycerol)	10 mM Tris, pH = 7.2
	10 mM $MgCl_2$
	10 mM KCl
	30% glycerol

Lysis buffer:	10 mM Tris, pH = 7.2
	10 mM $MgCl_2$
	200 mM KCl

T. brucei cells or mitochondria
Glycerol Gradient Buffers A & B
RNAse-free glycerol
14 x 95 mm Ultraclear tubes
Lysis Buffer
Ultracentrifuge and SW rotor at 4 °C
Hoefer SG50 gradient pourer and accessories
10% Triton-X 100
Pefabloc SC; AEBSF, 4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochlorid

2.8.2. Pouring the gradients

Note: Instructions are for an 11 ml gradient

- Have all buffers on ice or at 4°C
- To the ultracentrifugation tube pour at first 5.5 ml of buffer B and carefully covered with 5.5 ml of buffer A
- Leave at - 80°C for at least 12 hours
- Thaw at 4°C before use (few hours)

2.8.3. Lysing the cells or mitochondria and running the gradient

- Resusped the *T. brucei* mitochondria in 500 – 700 µl of lysis buffer with TritonX - final concentration 1%
- Spin down 15 min in 4°C
- Resuspend the cell pellet in lysis buffer and spin down

- Resuspend the cure mitochondria in 1 ml of lysis buffer

  Note: Always keeps mitochondria and mitochondrial lysate on ice or at 4 °C!
- Cool SW 40 rotor at 4° C
- Very carefully load 1 ml cleared mitochondrial lysate onto the top of the glycerol gradient
- Balance weight of opposite tubes with appropriate volume of lysis buffer
- Put the tubes in the rotor buckets and centrifuge at 38 000 rpm for 12 hours at 4°C
- Collect 500 μl fractions from the top of the gradient to the bottom very carefully

2.8.4. Prepare Glycerol Gradient Sample for SDS PAGE

- 80μl of glycerol gradient fraction + 20 μl of 5X sample buffer
- Load on 12% acryl amide gel

5X Sample buffer	
	10% sodium dodecyl sulfate
	10mM Dithiotraitol
	0,2M Tris-HCl pH = 6,8
	0,05% Bromophenol Blue

2.8.5. Blot the gel to the PVDF membrane (Polyvinylidene Fluoride) Amersham Hybond™ - P (use according to the manual)

2.8.6. Western analysis (Molecular Cloning a Laboratory Manual – Sambrook and Russell)

2.9. Imunofluorescence Protocol

2.9.1. 4% paroformaldehyde

- Prepare about 2 hours before experiment
- Heat PBS to ~ 65°C, add paraformaldehyde and mix
- Cool to room temperature before use

2.9.2. Mitotracker staining of PS cells

Protocol for either Mitotracker Green FM (M-7502) or Red CMX Ros (M-7512)

- according to manual, green does not require membrane potential while red does

- Resuspend commercial tubes with DMSO (dimethyl sulfoxide)
    - 94 ul per Red tube
    - 75 ul per Green tube
- Dilute to working concentration
    - 50 uM Red (or 20 uM)
    - 100 uM Green
- Add to 1-2 mls cells proper dilution of working solution
    - Final conc 50 nM (20 nM)-Red (1:1000)
    - 500 nM Green (1:200)
- Incubate 15-20 minutes at 26°C
- Spin down 4500 g 90 sec and aspirate supernatant
- Wash with 1 ml PBS (repeat step 6)
- leave ~50-100ul and spot onto slide
- let dry in humid chamber ~ 5 minutes
- Place in 4% Paraformaldehyde for 5 minutes
- Wash 1 X in PBS 5 minutes rocking
- Wash 2 X in PBS + 0.1 M glycine (0.75 g per 100 ml) as in step 11
- Mount slide in Vectashield with or without DAPI or continue IFA protocol

Prepare humid chambers at least 2 hours before experiment

2.9.3. Imunofluorescence protocol for coverslip sample preparation

- Spin the cells down (4500 g, 90 sec, RT) and wash them with PBS, resuspend cells with PBS
- Note: cells can be washed and resuspended with cytomix
- Spot cells on "+" side of Superfrost slide (5-10 min in humid chamber)
- Fix cells in 4% paraformaldehyde for 5 min at room temperature
- Wash with PBS 5 min, then PBS+0.1 M Glycine for 2X each for 5 min.
- Permeablize by -20°C methanol for more than 1 hour
- Wash by PBS for 2X for 5 min.
- Apply block for 60 min (10% goat serum in PBS)
- Apply 1˚ antibody for 60-90 min. (Antibody is diluted by PBS+10% Goat Serum)
- Wash the slide by PBS, 3X for 5 min
- Apply 2˚ antibody, 30-60 min. (Antibody is diluted by PBS+10% Goat Serum)

- Wash the slide by PBS, 3X for 5 min
- Mount slides in Vectashield with or without DAPI

  -or-
- Add 1 μg/ml DAPI, stain for 30-60 sec (DAPI is in a stock solution of 5 mg/ml stored at –20˚C)
- Wash the slide by PBS 2 × 5 min
- Mount the slide in Vectashield or other appropriate reagent

2.10. Native Gel

2.10.1. Solutions for polyacrylamide pel

	2% (8 ml)	4% (8 ml)	15% (7 ml)
AB-mix	0,35 ml	0,7 ml	2 ml
Gel solution (3x)	2,66 ml	2,66 ml	2,33 ml
H_2O	4,99 ml	4,64 ml	2,67 ml
10% APS	45 µl	45 µl	39 µl
TEMED	4,5 µl	4,5 µl	3,9 µl

AB-mix	48% acrylamid
	1,5% bis acrylamid

Gel solution (3x)	75 mmol/l imidazol-HCl pH 7,0
	1,5 mol/l aminokaprónová acid

Cathode buffer (10X)	0,5 mol/l tricine
	0,02% CBB (Comassie G-250)
	75 mmol/l imidazol-HCl pH 7,0

Anode solution (10X)	0,25 mol/l imidazol-HCl pH 7,0

Sample buffer	0,5 mol/l aminocaprone acid
	5% CBB G-250

2.10.2. Pouring of Native Gel (Fig. 2.5.)

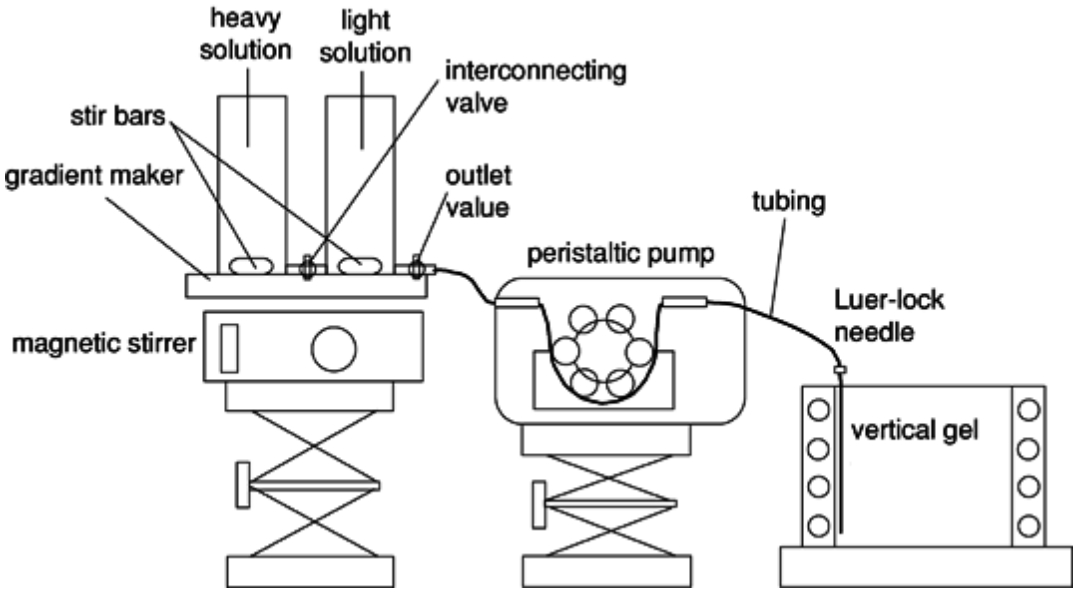


Figure 2.5. Pouring of native gel.
(adapted form http://media.wiley.com/CurrentProtocols/PS/ps1918/ps1918-fig-0002-1-full.gif)

2.10.3. Sample preparation

- Load 50-100 µg of proteins resuspended in 1 M aminocaprone acid to the final volume 20 µl, add appropriate volume of sample buffer
- Run the gel for 1,5 W overnight at 4°C

2.10.4. Blot the gel to the PVDF membrane (Amersham Hybond™ - P)

2.10.5. Western analysis

3. Results

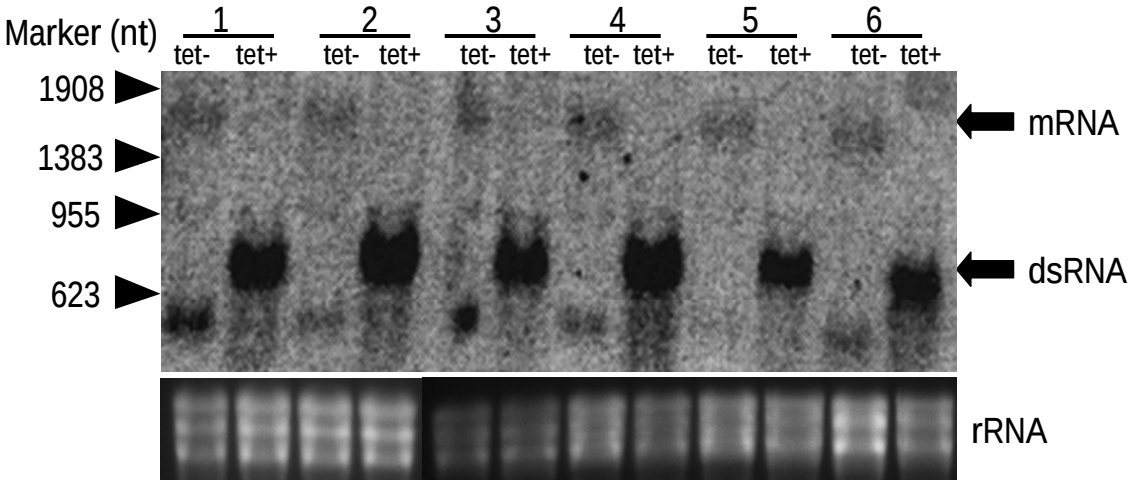
3.1. Generation of MRB3010 Procyclic and Bloodstream Knockdown Cell Lines

The vector prepared as described in Materials and Methods was transfected into either BS (strain 427) and PS (strain 29-13) cells. After obtaining stable transformants by selection for phleomycin resistance, the cells were induced by tet to a final concentration of 1μg/ml and collected after a 48 h period for Northern analysis to verify the induction of dsRNA production and downregulation of MRB3010 mRNA. As a control, non-induced cells (tet-) were grown and processed in parallel (Fig. 3.1).

Clone 1 of PS transfectants was chosen for further analysis, because this clone shows the most tightly regulated MRB3010 mRNA downregulation and the least leaky dsRNA synthesis. All clones displayed mRNA downregulation. Observed mRNA and dsRNA are at their expected sizes, which are 1551 bp for mRNA transcript and 650 bp for dsRNA.

The 3010 mRNA is very difficult to detect in the BS by Northern analysis. This situation may be because in the BS of *T. brucei* rRNAs may represent a higher proportion of total RNA; when the total RNA is loaded on the gel, the mRNAs may be represent a lower portion of the total RNA and thus be present below the level of detection by this method. Nevertheless, clone 2 was chosen because the expected size of dsRNA (650 bp) was detected in the induced sample. Downregulation of the MRB3010 mRNA in this clone was verified directly by quantitative real time PCR (qPCR) analysis (3.3. section) and indirectly by measurement of growth (3.2. section).

A



B

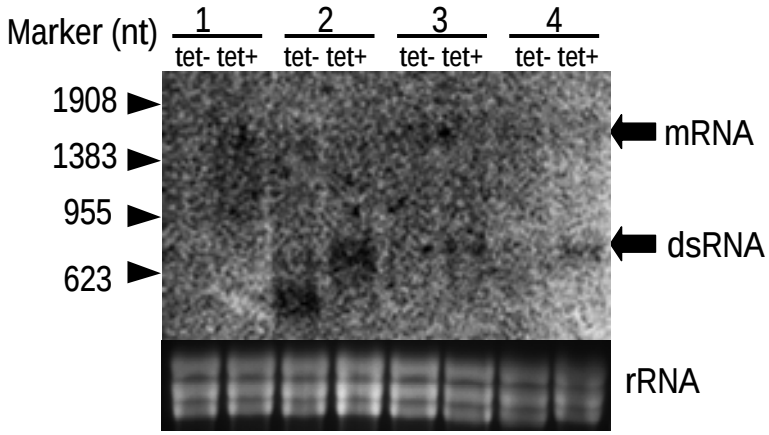
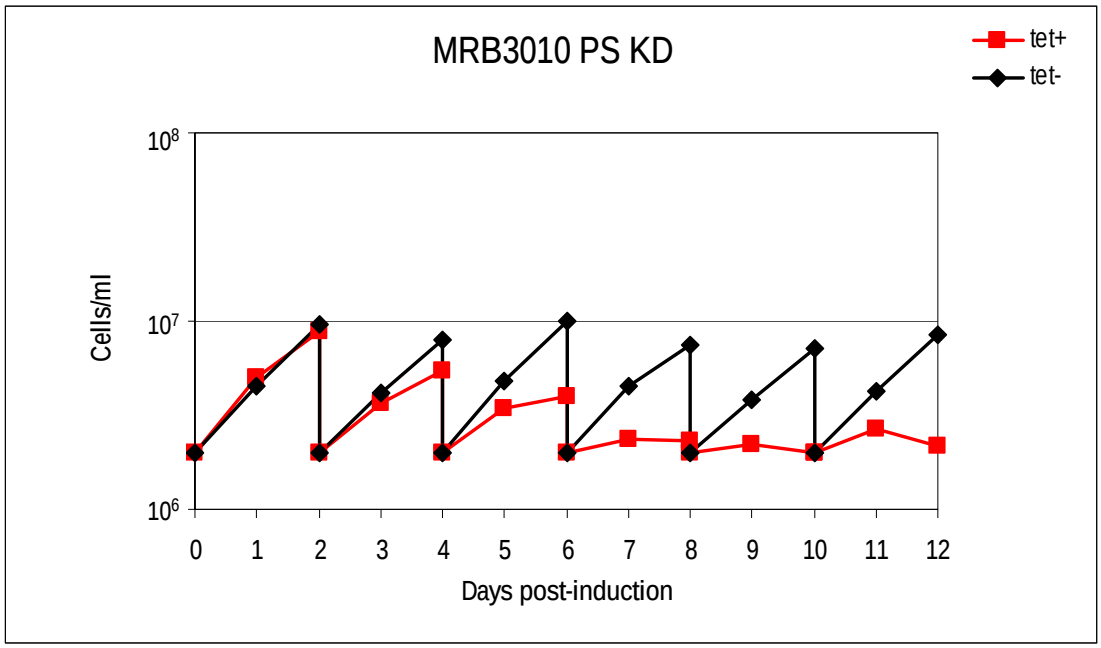


Figure 3.1. RNAi silencing of MRB3010 A) Cloned PS cell lines 1 – 6. MRB3010 mRNA (predicted size 1551 bp) is present in non-induced cells (tet-). The bands around 650 bp in induced cells (tet+) correspond to anti-MRB3010 dsRNA. According to this result, clone 1 was chosen for further analysis. B) Northern analysis for BS cell lines 1 – 4. MRB3010 mRNA was difficult to detect using this method, as was the presence of dsRNA in the induced samples. Clone 2 was chosen for further analysis because the induced sample has visible dsRNA (size 650 bp). Ethidium bromide stained rRNA is shown as a loading control in both panels.

3.2. Silencing of MRB3010 PS and BS inhibits growth

The stable cell lines were grown for 12 days for PS and 7 days for BS trypanosomes in the presence or absence of tet. The density (cells/ml) of the cell culture cultures was measured every 24 hours after tetracyclin induction (Fig.3.2.). Inhibition of growth in tet+ cells in both stages of *T. brucei* is apparent around the fourth day of tet induction as compared to non-induced cells. This time point was chosen for further analysis.

A



B

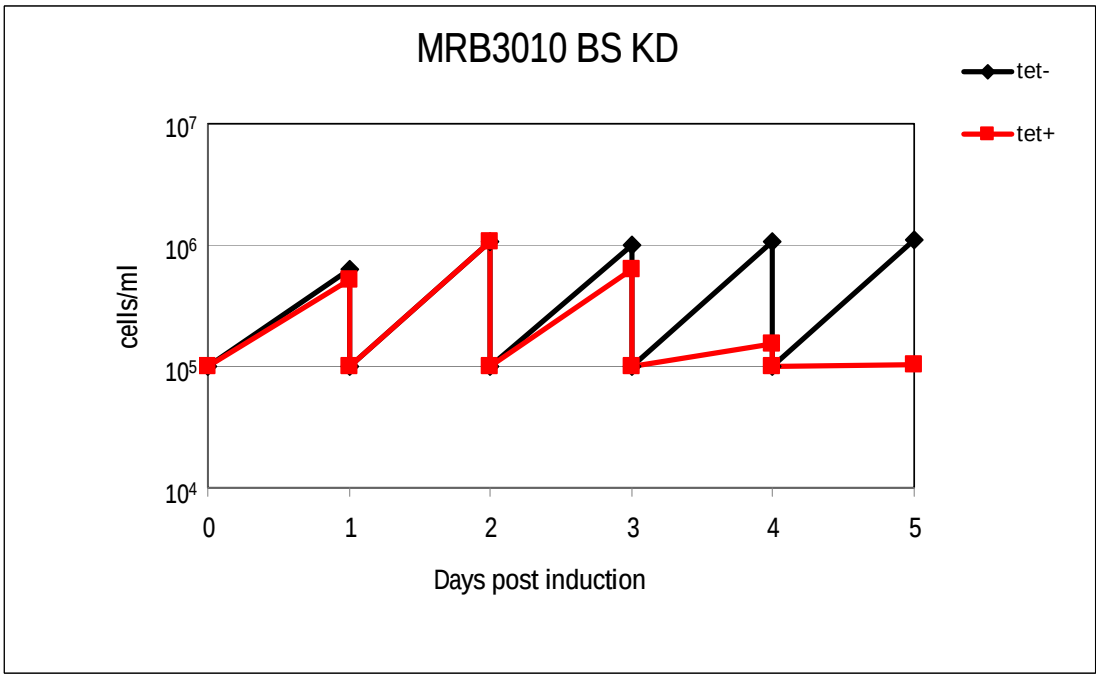
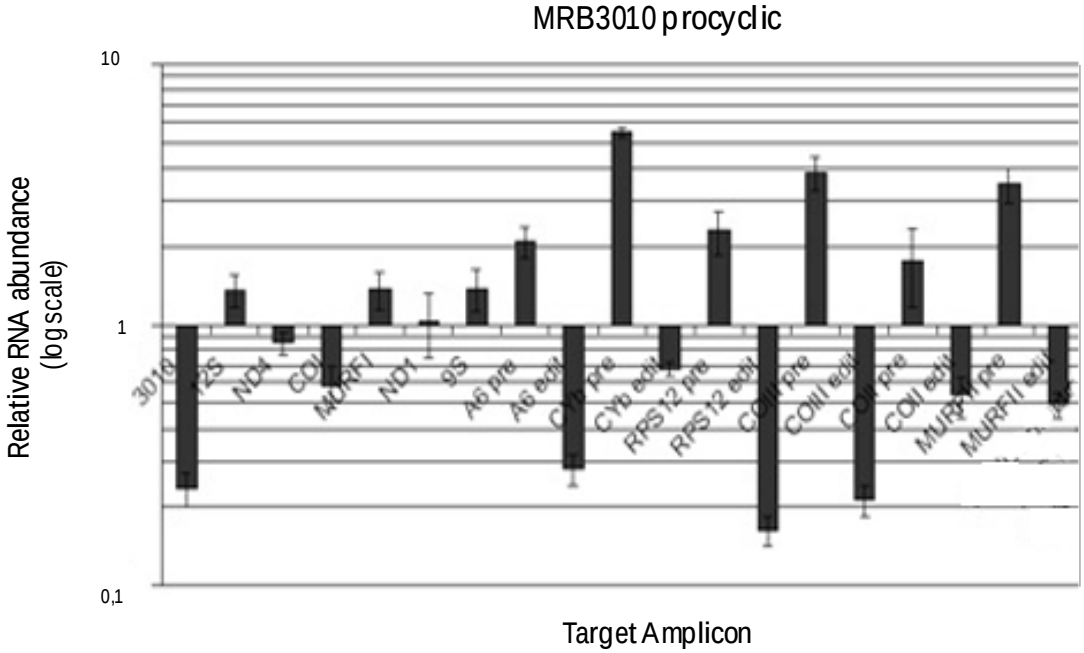


Figure 3.2. Downregulation of MRB3010 inhibits growth. Logarithmic graph plotting the density of non-induced (tet-) and induced (tet+) cells (cell/ml) against days after tetracyclin induction (final concentration of 1μg/m). A) Procyclic stage growth curves – the cells were diluted every second day to a concentration of $2x10^6$ cells/ml. B) Bloodstream stage growth curves– cell were diluted every day to a final concentration 10^5 cells/ml. In both cases, the inhibition of growth appears on the fourth day after induction.

3.3. Quantitative real-time PCR

Since some subunits of the MRB1 complex (GAP1, GAP2, TbRGG1, TbRGG2) are essential for the processing of maxicircle mRNAs, including RNA editing (Fisk et al., 2008, Hashimi et al., 2008, Hashimi et al., 2009), qPCR was used to determine a potential effect of silenced MRB3010 PS and BS trypanosomes on the stability and editing of mRNAs. The maxicircle encoded transcripts were chosen according to Fisk and colleagues (2008). The template for qPCR was cDNA generated from non-induced and induced cells of both stages of *T. brucei*, and several pre-edited, edited and never-edited RNAs were analyzed. This assay verified the RNAi silencing of MRB3010 in both PS and BS (3010 amplicon, Fig 3.3). The data were normalized to the measured level of cytoplasmic 18S rRNA, because it is not affected by RNAi, and calculated using the Pfaffl method (Pfaffl et al., 2001). The measured never-edited transcripts (12S, ND4, MURF1, 9S and ND1) were not affected by MRB3010 silencing in either stage. In PS and BS, the edited mRNAs decrease and almost all PS pre-edited transcripts increase, whereas the BS pre-edited A6, RPS12 and MURF2 are not influenced.

A



B

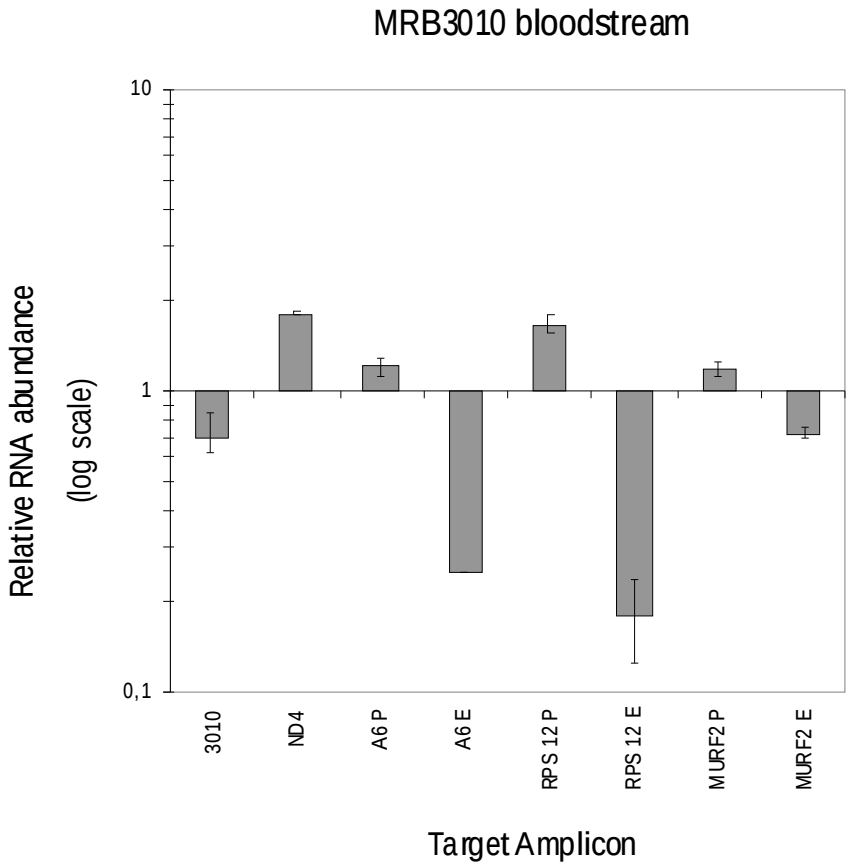


Figure 3.3. Quantitative real-time PCR analysis of pre-edited (P), edited (E) and neveredited mRNAs performed on knockdown of MRB3010. The RNA levels averaged from three replicates were normalized to 18S rRNA. The relative RNA abundance is in logarithmic scale and it shows the increase or decrease of the measured RNAs (maxicircle encoded genes or MRB3010), with bars above or below the median line respectively, after RNAi silencing of MRB3010. Never-edited transripts are not influenced after MRB3010 downregulation in both stages. Data from qPCR analysis of samples from (A) PS (taken from Laurie Read and Michelle Ammerman, unpublished data) and (B) BS.

3.4. Guanylyltransferase

Because of the real time PCR result (downregulation of edited maxicircle transcripts), the guanylyltransferase assay was performed by our collaborators from the Laurie Read laboratory. This enzyme specifically caps the 5´end of gRNAs with [α-^{32}P]GTP allowing the direct visualization of these molecules between tet+ and tet- MRB3010 knockdown RNA samples (fig. 3.4.). The result shows, that the level of gRNA was not affected after MRB3010 silencing.

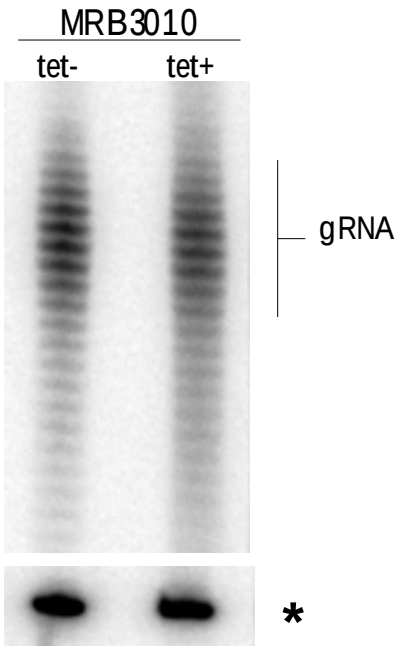


Figure 3.4. The gRNA in the MRB3010 knockdown was labeled by 5´capping guanylyltransferase and α32-P GTP. The level of gRNA was not affected by MRB3010 RNAi silencing. The (*) indicates a cytoplasmic RNA, that is also labeled by the reaction and is used as a loading control. 5mg of RNA was used per reaction and separated on denaturing 12% acrylamide gel (taken from Laurie Read, unpublished data).

3.5. Glycerol Gradient

The lysates from mitochondria isolated from induced and non-induced PS cells were separated on a 10 - 30% glycerol gradient (described in Material and methods). The odd fractions were loaded on a SDS-PAGE gel and analyzed by Western analysis with antibodies against GAP1 and TbRGG1, the previously mentioned proteins that are proposed to be part of the MRB complex1. As a control, these blots were also immunodecorated with antibody against KREL1, a subunit of the RECC complex that sediments at 20S (Fig. 3.5B), and which is not affected by downregulation of other MRB1 subunits (Fisk et al., 2008, Hashimi et al., 2008, Hashimi et al., 2009).

The screening of the glycerol gradients shows that GAP1 and TbRGG1 in induced and non-induced cells sediment in the same fractions. Thus, MRB3010 silencing has no apparent effect on the assembly of these two proteins into the MRB1 complex (Fig 3.5A). As expected, KREL1 appears to maintain the same sedimentation properties in MRB3010 depleted cells (Fig 3.5B) (fractions 13 – 17).

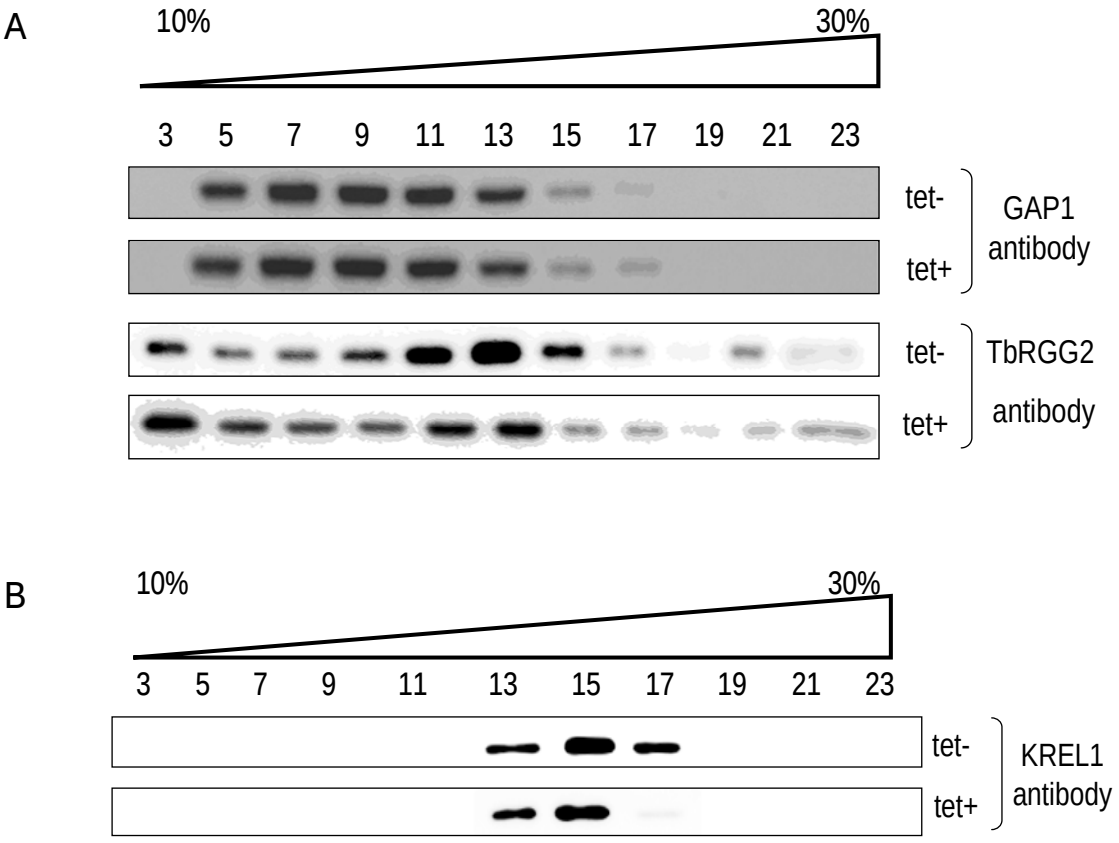


Figure 3.5. Western analysis of induced (tet+) and non-induced (tet-) MRB3010 knockdowns in which their mitochondrial lysates are separated on glycerol gradients. The gradient is divided into fractions and the concentration of glycerol increases from the left to right. Proteins from the gradient are separated on a 12% SDS-PAGE gel. A) Fractions probed with anti-GAP1 (antigen ~50 kDa) and anti-TbRGG1 (~32 kDa) antibodies. B) Control with KREL1 antibody (~52 kDa) against RECC sediments at 20S.

To further examine any effect on the assembly of GAP1 into the MRB1 complex upon silencing of MRB3010, odd fractions from the same gradient were also separated by gel electrophoresis under native conditions. The 3 – 14% blue native gel and was subsequently transferred to a membrane that was probed using the anti-GAP1 antibody (Fig.3.6.). Western analysis shows that the size of the complex containing GAP1 under the native conditions is about 150 kDa. This size appears to remain the same in all immunopositive fractions.

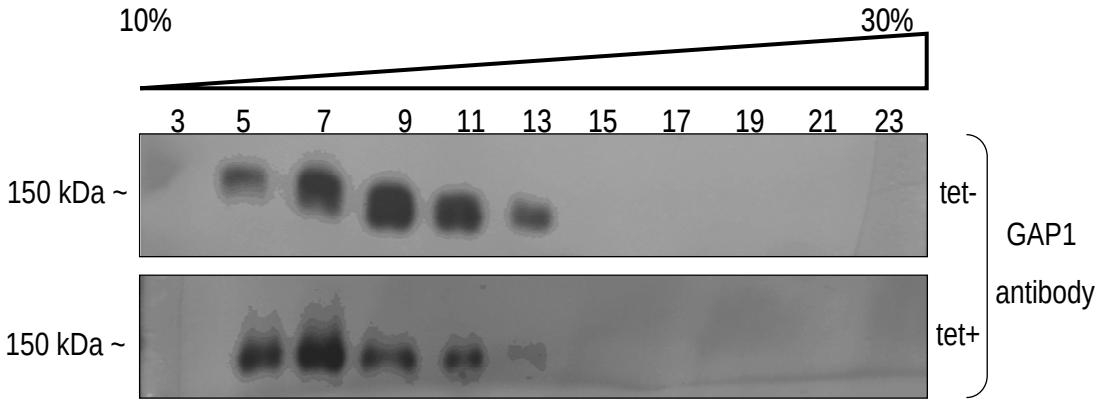


Figure 3.6. Western analysis of induced (tet+) and noinduced (tet-) cells of MRB3010 glycerol gradient fractions. The concentration of glycerol increases from fraction 3 to 23. Proteins from the gradient were separated on a blue native 3 – 14% acrylamide gel, which was then transferred onto a PVDF membrane and probed with the anti-GAP1 antibody.

3.6. Imunofluorescence

Previously published data (Hashimi et al., 2009) shows a punctuate localization of the MRB1 complex subunits GAP1 and GAP2 throughout the mitochondrion. To address whether other MRB1 subunits have the same localization, indirect imunofluorescence was performed using polyclonal rabbit antibodies against synthetic peptides of two subunit of MRB1 complex, Tb11.02.5390 (MRB5930) and Tb11.01.8620 (MRB8620). PS trypanosomes were fixed in 4% paraformaldehyde and stained with DAPI, green mitotracker (EGFP fluorescence) and the previously mentioned antibodies. As a secondary antibody was used a anti-rabbit antibody conjugated to the cy3 fluorophore, emitting red fluorescence. As a negative control, antibodies blocked by the appropriate antigenic peptide were also used on parallel slides (Fig. 3.7.). The localization of both proteins is uniform throughout the whole mitochondrion since the signal co-localizes with mitotracker, which specifically labels these organelles. No signal in negative control was due to blocking the anti-MRB5390 and anti-MRB8620 antibodies with appropriate peptides.

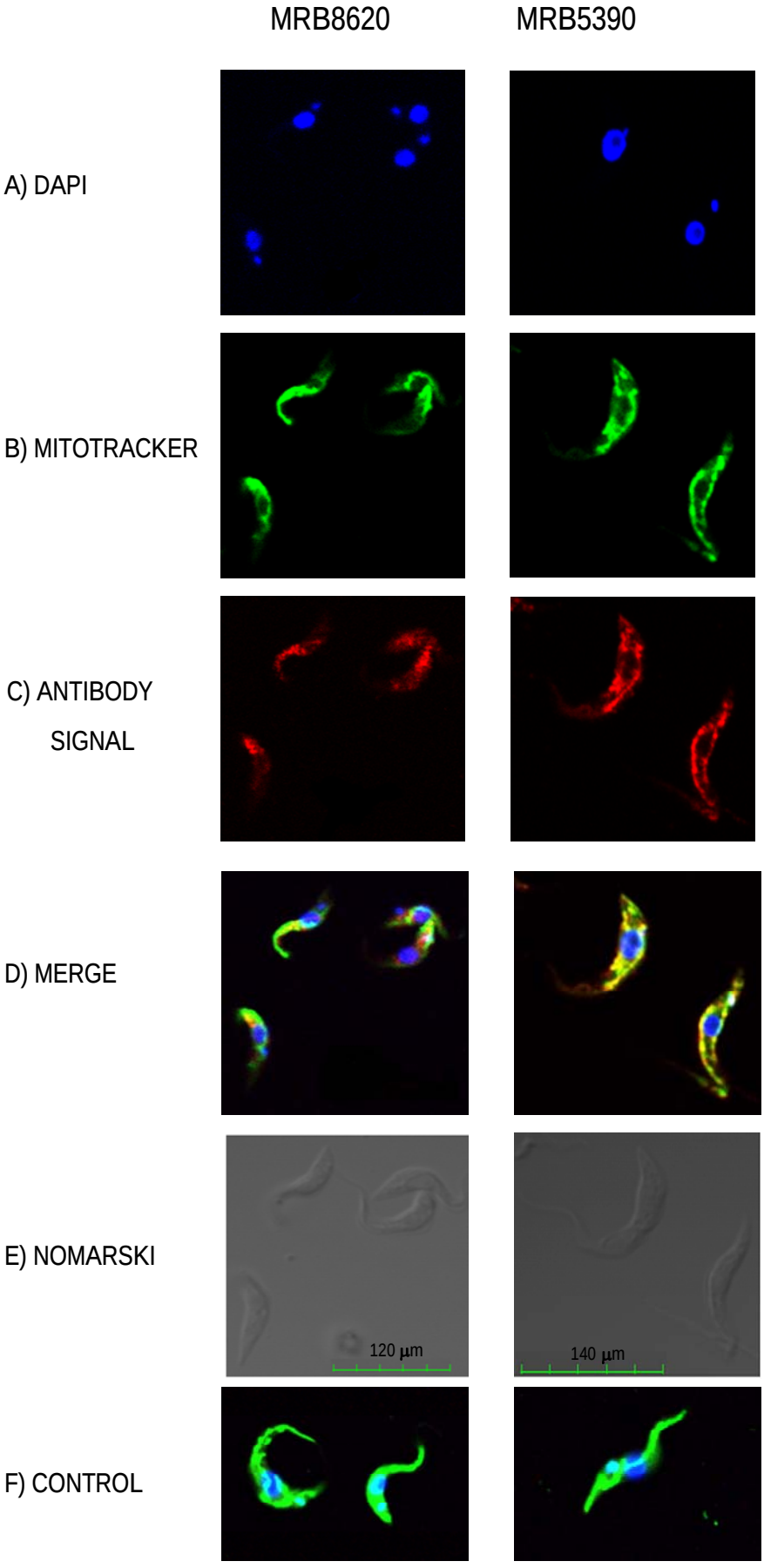

MRB8620
MRB5390
A) DAPI
B) MITOTRACKER
C) ANTIBODY SIGNAL
D) MERGE
E) NOMARSKI
F) CONTROL
120 µm
140 µm

Figure 3.7. Indirect imunofluorescence microscopy of PS T. brucei shows that both MRB5930 and MRB8620 are uniformly distributed in the mitochondrion. A) Staining with DAPI indicate the position of the nucleus and mitochondrial kinetoplast DNA. B) Labeling of the mitochondrion using green mitotracker. C) Localization of the protein using the appropriate antibody. D) Merge of mitotracker and cy3 signal (yellow fluorescence). E) Nomarski phase contrast image showing the normal morphology of the trypanosomes. F) Merged image of DAPI staining, green mitotracker and blocked antibody as a control, which exhibits no cy3 signal.

4. Discusion and conclusions

Mitochondrial RNA metabolism, including RNA editing, is very complicated and involves the participation of several different complexes. Recently published data (Fisk et al., 2009; Hashimi et al., 2009; Acestor et al., 2009) shows the functional analysis of some subunits of putative MRB1 complex by RNAi-silencing of several component proteins, indicating they play various roles in this process. This thesis is mainly concerned with the description of one of the MRB1 subunit called MRB3010. This subunit has no known motifs or orthologs outside of the sequenced kinetoplastids.

MRB3010 was RNAi silenced in procyclic and bloodstream stages of *T. brucei*. The RNA from induced and non-induced cells was analysed by Northern analysis. In PS, an evident downregulation of the mRNA transcript and synthesis of dsRNA was observed in the tetracycline induced cells. In BS, it was very difficult to detect this mRNA in the non-induced cells tet- and dsRNA in the induced samples tet+ by this method. One possible explanation is that a higher portion of the RNA that was loaded onto the formaldehyde gel is rRNA as compared to that loaded from the PS samples, resulting in a lower portion of mRNA that may be hard to detect. However, the silencing of MRB3010 in BS was confirmed by real time qPCR.

Because the MRB3010 appears to be one of the core subunit of MRB1 complex, I expected that this gene would be essential for both stages of *T. brucei*, as other core subunit GAP1 and 2, helicase, TbRGG2 (Hashimi et al., 2009; Fisk et al., 2008). This assumption was verified by the measurement of cell growth in both stages, where its inhibition of growth occurs on fourth day after tet induction. This time point was established for the collection of cells for further analysis.

According to previous data indicating that the MRB1 proteins play various roles affecting maxicircle transcripts (Hashimi et al., 2009; Fisk et al., 2009; Acestor et al., 2009), qPCR-based analysis was performed to assay these transcripts upon MRB3010 silencing. The PS result shows downregulation of extensively edited (A6, RPS12, CoIII) and partially edited (CyB, CoII, MURF2) maxicircle-encoded RNAs, whereas their pre-edited counterparts were upregulated. The never-edited genes (ND4, ND8, 12S, CoI, MURF1, 9S) were not affected after MRB3010 silencing. The same result for never edited ND4 was observed in the BS. While there was a decrease in the extensively edited RPS12 and A6 RNAs, the pre-edited RNAs were not significantly affected by MRB3010 downregulation as they were in the PS knockdowns. A further difference is that there was nearly no affect on the partially edited MURF2 RNA. The downregulation of the edited RNAs suggests that the MRB3010 may play a role in the RNA editing.

Because gRNAs are an essential part of RNA editing, the next step was to look at the steady state levels of minicircle-encoded gRNA level in PS using the guanylyltransferase assay, which visualizes these molecules. The same time point as was described before was used for collecting of tet+ and tet- cells. Unlike in the knockdowns of the GAP proteins and RNA helicase (Hashimi et al., 2009, Weng et al., 2008), there was not any difference in the level of gRNAs between MRB3010–silenced cells and non-induced controls. Thus the downregulation of edited maxicircle RNAs was due another reason, such as a negative impact on the stability of these molecules.

Part of my study was to address the structural composition of the putative MRB1 complex after MRB3010 silencing. Glycerol gradient separation of macromolecular complexes was performed in RNAi-induced and non-induced cells. As a control, the sedimentation of one subunit (KREL1) of the RECC complex, which sediments in 20S, was examined. The signal in fractions 13 – 17 of the prepared gradient corresponds with previous results in our laboratory (Hashimi et al., 2009). The sedimentation of GAP1, a possible subunit of MRB1, and TbRGG1, a protein that associates with this complex in an RNA mediated manner, was examined in these knockdowns as well. The GAP1 signal was located in the fractions 5 – 15 in tet+ and tet- cells. The TbRGG1 signal is found throughout the whole gradient, with the main peak in the fractions 11 to 13; this pattern was observed previously. As was mentioned before, MRB3010 appears to be in the core of the MRB1 complex since it is one of the subunits that appears in all purifications of this complex (Fig [INTRO FIG-YOU FILL IN YPOURSELF!!!]) (Hashimi et al., 2008; Panigrahi et al., 2008; Etheridge et al., 2008; Weng et al., 2009). Thus, the expected result was a shift in the localization of GAP1 and/or TbRGG1 to the higher fractions, an indication that the complex is disturbed. This result was observed when the RNA helicase was silenced, which is another candidate core protein (Hashimi et al., 2009). The surprising result was that MRB3010 subunit has no affect on the MRB1 stabilization, at least in terms of the assembly of GAP1 or TbRGG1 into the complex. This result can also support the idea that all proteins described in introduction do not form one big complex, but instead represent a few small complexes that are somehow associated. Alternatively, some proteins may associate with several of these small complexes in a dynamic fashion.

For visualization of the GAP1/2 (GRBC) complex under native conditions, the same glycerol gradient fractions were separated by blue native gradient gel electrophoresis, and the Western blots of these gels was probed with the GAP1 antibody. Compared with the result of Weng and co-workers (2008), in which both of these proteins create tetramer that is about 200 kDa in size, my results shows a signal in the fractions 5 – 13 of about size 150 kDa in tet- and tet+ cells. This size is based on the migration of the protein ladder also run in the native gel, and should be treated as a

very approximate estimate. Clearly, the downregulation of MRB3010 does not affect the GAP protein containing complex.

The MRB1 complex is located in the mitochondrion. To address whether any of the other putative subunits of MRB1 have a similar pattern of localization within the organelle, the MRB5390 and MRB8620 proteins were visualized *in situ* by imunolocalization using antibodies against them. In contrast with GAPs 1 and 2, which has a punctuate pattern of localization throughout the mitochondrion, the signal of both antibodies was indicate a uniform distribution in the organelle. This result further supports the idea that MRB1 is a collection of smaller complexes that may interact in a dynamic fashion.

5. References

Acestor N, Panigrahi AK, Carnes J, Zikova A and Stuart KD (2009). The MRB1 complex functions in kinetoplastid RNA processing. RNA. 5:277-286.

Adler BK, Harris ME, Bertrand KI and Hajduk SL (1991). Modification of *Trypanosoma brucei* mitochondrial rRNA by posttranscriptional 3' polyuridine tail formation. Mol Cell Biol. 11:5878-84.

Aphasizhev R, Aphasizheva I and Simpson L (2003). A tale of two TUTases. Proc Natl Acad Sci U S A. 100:10617-10622.

Aphasizheva I, Ringpis GE, Weng J, Gershon PD, Lathrop RH and Aphasizhev R (2009). Novel TUTase associates with an editosome-like complex in mitochondria of Trypanosoma brucei. RNA. 15:1322-37.

Aphasizheva I and Aphasizhev R (2010). RET1-catalyzed uridylylation shapes the mitochondrial transcriptome in *Trypanosoma brucei*. Mol Cell Biol. 30:1555-1567.

Barrett MP, Burchmore RJ, Stich A, Lazzari JO, Frasch AC, Cazzulo JJ and Krishna S (2003). The trypanosomiases. Lancet. 362:1469-80.

Bhat GJ, Souza AE, Feagin JE and Stuart K (1992). Transcript-specific developmental regulation of polyadenylation in *Trypanosoma brucei* mitochondria. Mol Biochem Parasitol. 52:231-240.

Blum B and Simpson L (1990). Guide RNAs in kinetoplastid mitochondria have a nonencoded 3' oligo(U) tail involved in recognition of the preedited region. Cell. 62:391-397.

Brun R and Schonenberger M (1979). Cultivation and in vitro cloning or procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. Short communication. Acta Trop. 36:289-292.

Carnes J, Trotter JR, Ernst NL, Steinberg A and Stuart K (2005). An essential RNase III insertion editing endonuclease in *Trypanosoma brucei*. Proc Natl Acad Sci U S A. 102:16614-16619.

Carnes J, Trotter JR, Peltan A, Fleck M and Stuart K (2008). RNA editing in *Trypanosoma brucei* requires three different editosomes. Mol Cell Biol. 28:122-130.

Etheridge RD, Aphasizheva I, Gershon PD and Aphasizhev R (2008). 3' adenylation determines mRNA abundance and monitors completion of RNA editing in T. brucei mitochondria. EMBO J. 27:1596-1608.

Fisk JC, Presnyak V, Ammerman ML and Read LK (2009). Distinct and overlapping functions of MRP1/2 and RBP16 in mitochondrial RNA metabolism. Mol Cell Biol. 29:5214-5225.

Gagliardi D, Stepien PP, Temperley JR, Lightowlers RN and Chrzanowska-Lightowlers ZM (2004). Messenger RNA stability in mitochondria: different means to an end. Trends Genet. 20:260-7.

Golden DE and Hajduk SL (2005). The 3`-untranslated region of cytochrome oxidase II mRNA functions in RNA editing of African trypanosomes exclusively as a cis guide RNA. RNA. 11:29-37.

Hashimi H, Zikova A, Panigrahi AK, Stuart KD and Lukes J (2008). TbRGG1, an essential protein involved in kinetoplastid RNA metabolism that is associated with a novel multiprotein complex. RNA. 14:970-980.

Hashimi H, Cicova Z, Novotna L, Wen YZ and Lukes J (2009). Kinetoplastid guide RNA biogenesis is dependent on subunits of the mitochondrial RNA binding complex 1 and mitochondrial RNA polymerase. RNA. 15:588-599.

Hayman ML and Read LK (1999). *Trypanosoma brucei* RBP16 is a mitochondrial Y-box family protein with guide RNA binding activity. J Biol Chem. 274:12067-74.

Hernandez A, Madina BR, Ro K, Wohlschlegel JA, Willard B, Kinter MT and Cruz-Reyes J (2010). REH2 RNA helicase in kinetoplastid mitochondria: ribonucleoprotein complexes and essential motifs for unwinding and guide RNA (gRNA) binding. J Biol Chem. 285:1220-1228.

Kannan S and Burger G (2008). Unassigned MURF1 of kinetoplastids codes for NADH dehydrogenase subunit 2. BMC Genomics. 9:455.

Koller J, Norskau G, Paul AS, Stuart K and Goringer HU (1994). Different *Trypanosoma brucei* guide RNA molecules associate with an identical complement of mitochondrial proteins in vitro. Nucleic Acids Res. 22:1988-95.

Kushner SR (2004). mRNA decay in prokaryotes and eukaryotes: different approaches to a similar problem. IUBMB Life. 56:585-94.

Lukes J, Guilbride DL, Votypka J, Zikova A, Benne R and Englund PT (2002). Kinetoplast DNA network: evolution of an improbable structure. Eukaryot Cell. 1:495-502.

Manley JL and Proudfoot NJ (1994). RNA 3' ends: formation and function-meeting review. Genes Dev. 8:259-64.

Miller MM, Halbig K, Cruz-Reyes J and Read LK (2006). RBP16 stimulates trypanosome RNA editing *in vitro* at early step in the editing reaction. RNA. 12:1292-303.

Missel A, Souza AE, Norskau G and Goringer HU (1997). Disruption of a gene encoding a novel mitochondrial DEAD-box protein in *Trypanosoma brucei* affects edited mRNAs. Mol Cell Biol. 17:4895-903.

Nagaike T, Suzuki T, Katoh T and Ueda T (2005). Human mitochondrial mRNAs are stabilized with polyadenylation regulated by mitochondria-specific poly(A) polymerase and polynucleotide phosphorylase. J Biol Chem. 280:19721-7.

Panigrahi AK, Allen TE, Stuart K, Haynes PA and Gygi SP (2003). Mass spectrometric analysis of the editosome and other multiprotein complexes in *Trypanosoma brucei*. J Am Soc Mass Spectrom. 14:728-35.

Panigrahi AK, Zikova A, Dalley RA, Acestor N, Ogata Y, Anupama A, Myler PJ and Stuart KD (2008). Mitochondrial complexes in *Trypanosoma brucei*: a novel complex and a unique oxidoreductase complex. Mol Cell Proteomics. 7:534-545.

Pelletier M and Read LK (2003). RBP16 is a multifunctional gene regulatory protein involved in editing and stabilization of specific mitochondrial mRNAs in *Trypanosoma brucei*. RNA. 9:457-68.

Pfaffl MW (2001). A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29:e45.

Proudfoot N (2004). New perspectives on connecting messenger RNA 3' end formation to transcription. Curr Opin Cell Biol. 16:272-8.

Read LK, Myler PJ and Stuart K (1992). Extensive editing of both processed and preprocessed maxicircle CR6 transcripts in *Trypanosoma brucei*. J Biol Chem. 267:1123-8.

Simpson L, Neckelmann N, de la Cruz VF, Simpson AM, Feagin JE, Jasmer DP and Stuart JE (1987). Comparison of the maxicircle (mitochondrial) genomes of Leishmania tarentolae and *Trypanosoma brucei* at the level of nucleotide sequence. J Biol Chem. 262:6182-96.

Stuart KD, Schnaufer A, Ernst NL and Panigrahi AK (2005). Complex management: RNA editing in trypanosomes. Trends Biochem. Sci. 30:97-105.

Sturm NR and Simpson L (1990). Kinetoplast DNA minicircles encode guide RNAs for editing of cytochrome oxidase subunit III mRNA. Cell. 61:879-884.

Vanhamme L, Perez-Morga D, Marchal C, Speijer D, Lambert L, Geuskens M, Alexandre S, Ismaili N, Goringer U, Benne R and Pays E (1998). *Trypanosoma brucei* TBRGG1, a mitochondrial oligo(U)-binding protein that co-localizes with an in vitro RNA editing activity. J Biol Chem. 273:21825-21833.

Vondruskova E, Burg J, Zikova A, Ernst NL, Stuart KD, Benne R and Lukes J (2005). RNA interference analyses suggest a transcript-specific regulatory role for MRP1 and MRP2 in RNA editing and other RNA processing in *T. brucei*. J Biol Chem. 280: 2429-2438.

Weng J, Aphasizheva I, Etheridge RD, Huang L, Wang X, Falick AM and Aphasizhev R (2008). Guide RNA-binding complex from mitochondria of trypanosomatids. Mol Cell. 32:198-209.

Wickstead B, Ersfeld K and Gull K (2002). Targeting of a tetracycline-inducible expression system to the transcriptionally silent minichromosomes of *Trypanosoma brucei*. Mol Biochem Parasitol. 125:211-6.

Zíkova A, Horakova E, Jirku M, Dunajcikova P, Lukes J. The effect of down-regulation of mitochondrial RNA-binding proteins MRP1 and MRP2 on respiratory complexes in procyclic *Trypanosoma brucei*. Mol Biochem Parasitol. 149:65-73.

Zikova A, Kopecna J, Schumacher MA, Stuart K, Trantirek L and Lukes J (2008). Structure and function of the native and recombinant mitochondrial MRP1/MRP2 complex from *Trypanosoma brucei*. Int J Parasitol. 38:901-912.

6. List of Abbreviations

BS	bloodstream stage of *T. brucei*
Co	cytochrome oxidase
C_T	cycle treshold
CyB	cytochrome reductase subunit B
dsRNA	double-stranded RNA
exoUase	U-specific 3'→5` exonuclease
G	neomycin
GAP1	guide RNA associated protein 1
GAP2	guide RNA associated protein 2
GRBC	guide RNA binding complex
gRNA	guide RNA
H	hygromycin
HAT	Human African trypanosomiasis
kDNA	kinetoplast DNA
KPAP1	kinetoplast poly (A) polymerase 1 complex
KREN	kinetoplast RNA editing endonuclease
MERS1	mitochondrial edited mRNA stability factor 1
MM	mastermix
MRB1	mitochondrial RNA binding complex 1
MRP	mitochondrial RNA binding protein
mt	mitochondrial

nt	nucleotide
Nudix	nucleoside diphosphate linked to some other moiety x
P	phleomycin
PPR	pentatricopeptide repeat
PS	procyclic stage of *T. brucei*
qPCR	quantitatice Real-Time PCR
RECC	RNA editing core complex
REN	RNA editing endonuclease
RET	RNA editing TUTase
RGG	arginine-glycine-glycine
RNAi	RNA interference
RPS12	ribosomal protein S12
RT	reverse transcriptase
S	Svedburg units
TAP	tandem affinity purification
tet	tetracyclin
tet-	non-induced cells
tet+	inducend cells
TUTase	terminal uridylyl transferase
U	uridine
Us	uridines
WT	wild type