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Functional Analysis of Mitochondrial Ribosome of *Trypanosoma brucei*

(Master thesis)

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Annotation:

The aim of this work was to investigate the effect of RNA interference (RNAi) against three mitochondrial ribosomal subunits on mitochondrial translation. The procyclic stage cells of *Trypanosoma brucei* have been transfected with a vector producing double stranded RNA against putative mitochondrial ribosomal helicase LR, and mitoribosomal proteins MRPS5 and MRPL21. Moreover, bloodstream stage RNAi knock-down has also been prepared for the LR gene, and procyclic cells with targeted mitochondrial RNA polymerase I (mtRNAPI) have been used as a control. The growth phenotype and the effect of down regulation have been investigated by ^{35}S labelling of de novo synthesized mitochondrial proteins in all genetically manipulated cells. The down-regulation of the LR or MRPL21 proteins did not affect mitochondrial translation, while the ablation of the MRPS5 and mtRNAP1 proteins caused a decrease and termination of mitochondrial translation, respectively. Only the latter phenotype was lethal. These results lead us to the conclusion that mitochondrial translation in procyclics of *T. brucei* is essential but, unexpectedly, decrease of the tested mitoribosomal subunits is not.

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I hereby declare that I have worked out this master thesis by myself, using the cited literature.

I declare that in accordance with § 47b of the Act No. 111/1998 of the Collection of Laws as amended I agree to publication of my master thesis in an unabridged form by the Faculty of Science, electronically, in the part open to public of STAG database, run by the University of South Bohemia in České Budějovice on its Internet pages.

České Budějovice, 30 April, 2009 ………………………

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Contents

1. Introduction 1

1.1. *Trypanosoma brucei* 1

1.2. Mitochondrion of *T. brucei* 2

1.3. Mitochondrial ribosome of *T.brucei* 3

1.4. Tb 927.4.2720 putative mitoribosomal helicase (further LR) 5

1.5. MtRNA polymeraseI 6

1.6. S5 and L21 mitoribosomal proteins 6

1.7. Mitochondrial translation in *T. brucei* 7

2. Goals 9

3. Material and methods 10

3.1. Plasmid construction for RNAi 10

3.2. Cultivation, transformation, selection and growth phenotype analysis of *T. brucei* 11

3.3. Northern analysis 13

3.4. Two-dimensional polyacrylamide glycine-SDS gels 16

3.5. Overexpression of LR helicase 19

3.6. Used Kits 20

4. Results 21

4.1. Overexpression 21

4.2. RNAi of LR 22

4.3. ^{35}S labelling of de novo synthesised mitochondrial proteins assay in RNAi procyclic line for LR 24

4.4. ^{35}S labelling of de novo synthesised mitochondrial proteins assay in RNAi procyclic line for mtRNA polI 25

4.5. RNAi of MRPS5 and MRPL21 26

4.6. ^{35}S labelling of de novo synthesised mitochondrial proteins assay in RNAi procyclic line for MRPS5 and MRPL21 28

5. Discussion 30

6. Conclusions 33

7. References 34

1. Introduction

1.1. *Trypanosoma brucei*

Trypanosoma brucei is a flagellated protozoan belonging to the class Kinetoplastea, ranked currently into the eukaryotic superdomain Excavata (Keeling et al., 2005). This parasite is a causative agent of human sleeping sickness and ruminant nagana. The parasites are transmitted from the tsetse fly to their mammalian host, where they reside first in the bloodstream, lymphatic system and interstitial spaces, and then during the latter stages of the infection in the central nervous system. In the mammal the bloodstream trypanosomes multiply as a “long slender” form and eventually differentiate into a non-dividing “short stumpy” form. The “short stumpy” forms are thought to be pre-adapted for their subsequent transformation into the procyclic forms when they are ingested by a tsetse fly during its blood meal. This differentiation is accompanied by morphological changes (enlarging of the mitochondrion) as well as biochemical changes, most notably a shift from ATP generation via glycolysis in the bloodstream organisms to a dependence on the mitochondrial respiratory chain in procyclic organisms. After multiplication in the fly's midgut, the procyclic organisms migrate to the salivary glands, forming epimastigotes which continue to multiply and finally the non-dividing metacyclic organisms. Metacyclic trypanosomes are then transmitted during the fly bite to complete the life cycle (El-Sayed et al., 2000). **(Fig. 1.)** *T. brucei* has become a widely used model organism, because it is among the few protists amenable to all main approaches of reverse genetics (Roberts and Janovy, 2005). The completion of the genome sequences of the major pathogenic *Trypanosoma brucei* has presented a new way of investigating the functionality of their genomes, providing invaluable data about unknown aspects of their biology, distinct modes of pathogenic action, different strategies to evade the immune response of the host and novel therapeutic targets (Balana-Fouce and Reguera, 2007). The accidental discovery of RNA interference (RNAi) as a mechanism of gene silencing, and its successful application in *T. brucei*, has provided a robust tool to assess the functional importance of a particular gene in the development or survival of an organism(Ngo et al., 1998).

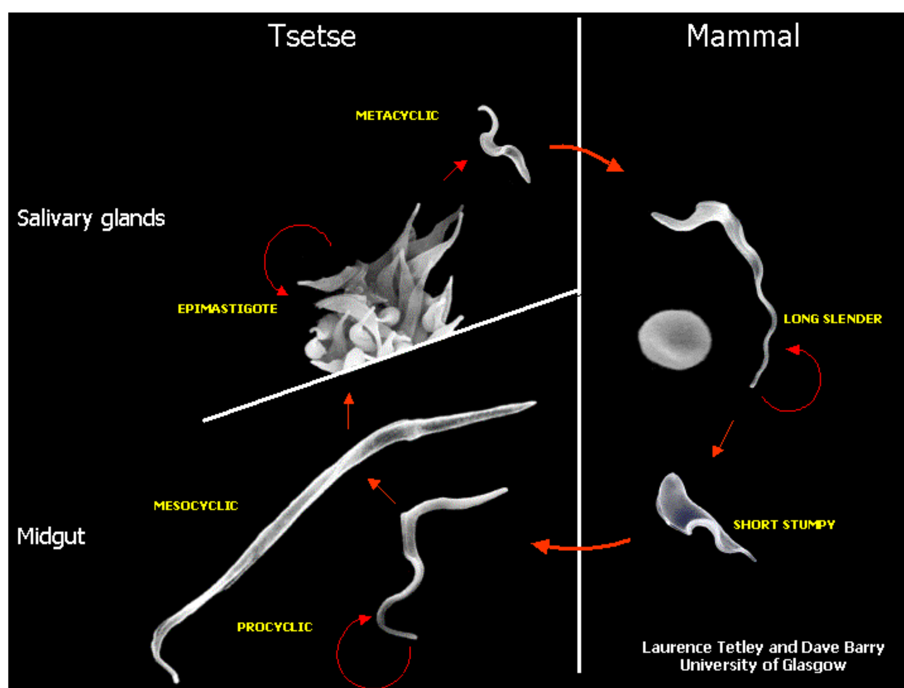

Fig. 1. Life cycle of *T. brucei* (Barry, Wellcome Centre for Molecular Parasitology)

1.2. Mitochondrion of *T. brucei*

T. brucei has a rather complex life-cycle, with developmentally regulated functions of its single mitochondrion as it alternates from a metabolically active form in the tsetse fly to a reduced and largely inactive form in human bloodstream (Lukeš et al., 2005). The reticulated mitochondrion contains a highly complex mitochondrial DNA (also termed kinetoplast DNA), composed of thousands of mutually interlocked circles, which has to be duplicated precisely in each round of the cell cycle in order for the parasite to replicate (Schneider, 2001). In kinetoplastids, mitochondrial gene expression is the combined result of a complex set of processes that include polycistronic transcription, cleavage to release monocistrons followed and/or accompanied by U-insertion/deletion type of RNA editing (Carnes et al., 2008). Final mRNA are Polyadenylated while small molecules called guide RNAs and mitoribosomal RNAs have poly-uridine extensions added to their 3′end (Hashimi et al., 2008,YU and Koslowsky, 2006, Adler and Hajduk, 1997). After exhaustive efforts, direct sequencing of proteins specified by the unedited and edited mRNAs provided compelling evidence of mitochondrial translation (Horváth et al., 2000 a). Twenty protein-coding genes and two ribosomal RNA (rRNA) genes are usually found in the maxicircle component of the kinetoplast DNA. The encoded proteins include subunits I, II, and III (COI, COII, and COIII) of cytochrome c oxidase (respiratory complex IV), apocytochrome b of ubiquinol-cytochrome c oxidoreductase (complex III), subunit 6 of oligomycin-sensitive ATPase

(complex V), several subunits of NADH dehydrogenase (complex I), ribosomal protein S12, and a number of putative proteins with unknown function (Horváth et al., 2000 a, Benne, 1994). These genes are expected to be translated on the mitochondrial ribosome.

1.3. Mitochondrial ribosome of *T.brucei*

All ribosomes are composed of rRNAs and ribosomal proteins. Mitochondrial ribosomes are of the prokaryotic-type and their rRNAs have been minimised during evolution. Whereas the 16S rRNA and 23S rRNA in *Escherichia coli* are 1542 and 2904 nucleotides in length, the homologous molecules in human mitochondria have been reduced to a length of 953 and 1555 nucleotides. In *T. brucei* this reduction is taken much further, the 9S and 12S rRNA being only 611 and 1150 nucleotides long, respectively, so that several domains and stem-loop structures are entirely missing (Cruz et al., 1985, Sloof et al., 1985). This makes them the shortest rRNAs known to date, with the possible exception of the rRNA fragments encoded by the apicomplexan mitochondrial genomes (Gillespie et al., 1999, Schneider, 2001). Unlike in most other organisms, all mitochondrial tRNAs are imported from the cytosol and therefore are of the eukaryotic type (Schneider and Marchal-Drouard, 2000). This results in a paradoxical situation. Mitochondrial translation in trypanosomatids, representing one of the most derived prokaryotic-type translation systems known, somehow has to function exclusively with the eukaryotic-type tRNAs imported from the cytosol (Schneider, 2001).
As visualized by electron microscopy, the kinetoplastid mitoribosome retains the shape reminiscent of bacterial and other eukaryotic mitoribosomes (Lukeš et al., 2005, Maslov, personal communication). The protein composition of *T. brucei* mitoribosome was determined by tandem affinity purification followed by mass spectrometry. The large ribosomal subunit sediment at 50S, while the small subunit sediments at 30S (Zíková et al., 2008). The only other kinetoplastid flagellate with characterised mitoribosome is *Leishmania tarentolae,* known to contain large and small mitoribosomal subunits sedimenting at 40S and 30S, respectively (Maslov et al., 2006). The very detailed analysis identified 133 proteins in the *T. brucei* mitochondrial ribosome, of which 77 were associated with the large subunit and 56 with the small subunit (Zíková et al., 2008). The sets of *L. tarentolae* and *T. brucei* mitoribosomal proteins were overlapping to a large degree, however, numerous subunits are either species-specific or have been missed in one of the studies. Comparisons of this set of proteins with the bacterial and mammalian mitoribosomal proteins identified homologs of L2-L4, L7/L12, L9, L11, L13-17, L20-24, L27-30, L33, L38, L43, L46, L49, L52 and S5, S6, S8, S9, S11, S15-18, S29, S34, although the degree of conservation varied widely. Sequence

characteristics of some of the component proteins indicated apparent functions in rRNA modification and processing, protein assembly and mitochondrial metabolism, implying possible additional roles for these proteins. Nevertheless, most of the identified proteins have no homologs outside Kinetoplastida, which underscores their putative function as subunits of a mitoribosome (Zíková et al., 2008) **(Fig. 2.-3.).** So far, no functional analysis of any subunit of mitochondrial ribosome in the kinetoplastid flagellates has been published.

TABLE I

List of T. brucei proteins that share the similarity to bacterial and/or mammalian ribosomal proteins

++, *E*-value is less than 1e−03; +, *E*-value is more than 1e−03; √, protein identified by MS analysis in purified ribosomal complex; *, proteins identified in 45 S SSU*-related ribosomal complex in *L. tarentolae* (26); superscript TAP_LSU1,2,6 and TAP_SSU1–3, proteins used as a bait in this study; tRNAsyn, tRNA synthetase. Dashes (—) indicate that the protein was not identified by MS analysis in purified ribosomal complex.

E. coli	Human	*T. brucei*	*E*-value	TAP tag	GeneDB	New name
L2	MRPL2	Tb927.5.3360	++	√	50 S L2	TbMRPL2
L3	MRPL3	Tb927.3.5610 TAP_LSU1	++	√	50 S L3	TbMRPL3
		Tb927.4.1800	+	—	HP	60 S L3
L4	MRPL4	Tb11.02.3810	++	√	HP	TbMRPL4
L7/L12	MRPL7/12	Tb927.7.4550	++	√	60 S-like	TbMRPL7/12
L9	MRPL9	Tb927.5.3410	+	√	HP	TbMRPL9
L10	MRPL10	Tb11.01.3520	+	—	HP	—
L11	MRPL11	Tb927.2.4890	++	√	L11	TbMRPL11
		Tb927.2.4740	++	—	L11	TbMRPL11
L13	MRPL13	Tb927.4.1070 TAP_LSU2	++	√	50 S L13	TbMRPL13
L14	MRPL14	Tb927.4.930	++	√	50 S L14	TbMRPL14
L15	MRPL15	Tb927.5.3980	++	√	HP	TbMRPL15
L16	MRPL16	Tb927.7.3960	++	√	50 S L16	TbMRPL16
L17	MRPL17	Tb927.8.5860	++	√	50 S L17	TbMRPL17
L20	MRPL20	Tb11.01.1930	++	√	HP	TbMRPL20
L21	MRPL21	Tb927.7.4140	++	√	HP	TbMRPL21
L22	MRPL22	Tb927.7.2760	++	√	HP	TbMRPL22
L23	MRPL23	Tb11.03.0260	++	√	HP	TbMRPL23
L24	MRPL24	Tb927.3.1710	++	√	HP	TbMRPL24
L27	MRPL27	Tb11.02.1110	+	√	HP	TbMRPL27
L28	MRPL28	Tb927.6.4040	+	√	HP	TbMRPL28
L29	—	Tb10.70.7650 TAP_LSU6	+	√	HP	TbMRPL29
L30	MRPL30	Tb09.211.0230	+	√	HP	TbMRPL30
L33	MRPL33	Tb927.4.1810	+	√	[illegible]	TbMRPL33
	MRPL38	Tb11.01.6620	+	√	HP	TbMRPL38
	MRPL39	Tb927.5.1090	++	—	tRNAsyn	—
	MRPL40	Tb927.5.1120	+	—	HP	—
	MRPL43	Tb927.4.4600	+	√	HP	TbMRPL43
	MRPL46	Tb927.7.4710	++	√	HP	TbMRPL46
	MRPL47	Tb09.160.5240	++	√	HP	TbMRPL47
	MRPL49	Tb927.5.3110	+	√	HP	TbMRPL49
	MRPL52	Tb11.02.2250	+	√	HP	TbMRPL52
S5	MRPS5	Tb10.70.0530* TAP_SSU3	++	√	HP	TbMRPS5
S6	MRPS6	Tb10.70.4850	+	√	HP	TbMRPS6
S8	—	Tb10.389.0130*	+	√	30 S S8	TbMRPS8
S9	MRPS9	Tb927.8.3110*	+	√	HP	TbMRPS9
S11	MRPS11	Tb10.406.0510* TAP_SSU1	++	√	HP	TbMRPS11
S15	MRPS15	Tb927.1.1200*	+	√	HP	TbMRPS15
S16	MRPS16	Tb11.02.5670	+	√	HP	TbMRPS16
S17	MRPS17	Tb09.211.2580* TAP_SSU2	++	√	30 S S17	TbMRPS17
S18	MRPS18	Tb10.6k15.3900*	+	√	HP	TbMRPS18
	MRPS29	Tb927.6.1250*	++	√	HP	TbMRPS29
	MRPS34	Tb927.8.5280	+	√	HP	TbMRPS34

Fig.2. Proteins with similarity to bacterial and/or mammalian ribosomal proteins. Both proteins analyzed within the framework of this study are highlighted in green frames (MRPS5 and MRPL21) (Table taken from Zíková et al., 2008).

TABLE II

List of T. brucei proteins found in tagged LSU complexes that show some homology and/or recognizable motif to other proteins outside Kinetoplastida or are unique for Kinetoplastida mitochondria (last row)

Superscript TAP_LSU3–5, proteins used as a bait in this study; tRNAsynt, tRNA synthetase; GTP bind, GPT binding; PPIase, peptidyl-prolyl cis-trans isomerase; PseudoU_synth, pseudouridine synthase; TPR, tetratricopeptide repeat; *C. elegans*, *Caenorhabditis elegans*.

Gene ID	GeneDB	Homology search (*E*-score)	Domain search (*E*-score)
Tb927.1.1160	HP	*Arabidopsis* PPR protein (4e−06)	PPR (0.66)
Tb927.4.2720	HP	Bacterial DEAD/DEAH helicase (9e−08)	Helicase (5e−07); Helicase C (0.0084)
Tb927.6.2480	Chaperone	Heat shock protein DnaJ (3e−10)	DnaJ (1e−13); DnaJ (9.2e−24)
Tb927.6.3600	HP	Bacterial methyltransferase (1e−07)	Methyltransferase (9e−13)
Tb927.7.1640	GTP bind	Bacterial GTP binding (3e−52)	EngA2 GTPase (2e−41); MMR-HSR1 (2.2e−28)
Tb927.7.2630	HP	Bacterial GTP binding (6e−14)	YihA_EngB GTPase (1e−36); MMR-HSR1 (2.5e−21)
Tb927.7.3430	PPIase	*Arabidopsis* peptidyl-prolyl cis-trans isomerase (4e−37)	Cyclophilin ABH_like (2e−49)
Tb927.8.2760	HP	Bacterial GTP binding (5e−10)	YihA_EngB GTPase (1e−30); GTPase (9e−11)
Tb927.8.3170	HP	*Arabidopsis* PPR protein (0.001)	PPR (0.00035)
Tb09.160.2000	HP	Bacterial LSU pseudouridine synthase C (7e−25)	PseudoU_synth (3e−28)
Tb09.211.0810	GTP bind	Yeast mitochondrial GTPase (7e−21)	YlqF GTPase (2e−39); MMR-HSR1 (9.7e−05)
Tb09.211.3800	HP	Bacterial tRNA/rRNA methylase (2e−11)	SpoU-methylase (2e−07)
Tb10.70.0780	tRNAsynt	Bacterial LSU pseudouridine synthase B (1e−20)	S4 domain (0.0079)
Tb11.01.1215	PPIase	*C. elegans* cyclophilin (2e−25)	Proisomerase (5.3e−44)
Tb11.01.8760	HP	*Rickettsia* GTP binding protein (8e−15)	GTPase (2e−18)
Tb11.02.3800	HP	*Homo* pseudouridylate synthase (4e−06)	PseudoU_synth (3e−13); DUF167 (0.26)
Tb09.211.4580	HP		Lipase_3 (0.009)
Tb927.6.3930	HP		TPR-2 (0.00047)
Tb927.6.4200	HP		DUF390 (0.08)
Tb927.7.3460	HP		TPR-2 (1.9)
Tb927.7.6800	HP		eRF1 2 (0.25)
Tb10.389.1710	HP		TPR (0.32)
Tb10.70.7960[TAP_LSU5]	HP		PPR (0.00013)
Tb11.01.7140	HP		HTH-12 (0.035)
Tb11.02.3670	HP		L36 (0.56)
Tb927.3.820, Tb927.4.4610, Tb927.5.2070, Tb927.5.3870, Tb927.5.4120[TAP_LSU4], Tb927.6.1440, Tb927.6.4080, Tb927.7.2990, Tb927.7.3510, Tb927.7.7010, Tb927.8.1880, Tb927.8.3300, Tb09.160.2250, Tb10.26.0600, Tb10.26.1000, Tb10.6k15.3290, Tb10.70.5970, Tb11.01.0500, Tb11.01.1600, Tb11.01.1810, Tb11.01.1840, Tb11.01.2340[TAP_LSU3], Tb11.01.3500, Tb11.02.3230, Tb11.55.0016			

Fig.3. Proteins found in the TAP-tagged large subunit complex that shows some homology and/or recognizable motif to other proteins outside Kinetoplastida, or are unique for Kinetoplastida mitochondria. The putative LR helicase analyzed within the framework of this study is highlighted in the red frame (Tb 927.4.2720) (Table taken from Zíková et al., 2008).

1.4. Tb 927.4.2720 is a putative mitoribosomal helicase (further LR)

The LR gene is 2220-bp long and is located on chromosome 4. The predicted protein has 83.6 kDa. Its orthologues were found in *Leishmania major* and *Trypanosoma cruzi* where they have 57% and 70% sequence identity, respectively. Analysis of sequence revealed that the probability of import into the mitochondrion is 63% (MitoProt). The LR protein was found during mass spectrometry analysis of the mitoribosome of *T. brucei*. It co-purified with 4 out of 6 TAP-tagged proteins from the large ribosomal subunit (Zíková, personal commun.) and was annotated as a homologue of the bacterial DEAD/DEAH helicase (Zíková et al., 2008). Moreover, this protein was also found within the framework of a study analyzing the whole *T. brucei* mitochondrial proteome, and was labelled as SrmB, belonging to the superfamily II DNA and RNA helicases (Panigrahi et al., 2009) **(Fig. 4.).** In *E. coli* the SrmB helicase is

involved in the assembly of 50S ribosomal subunits and deletion of this gene causes a slow-growth phenotype at low temperature (Charollais et al., 2003, Iost and Dreyfus, 2006).

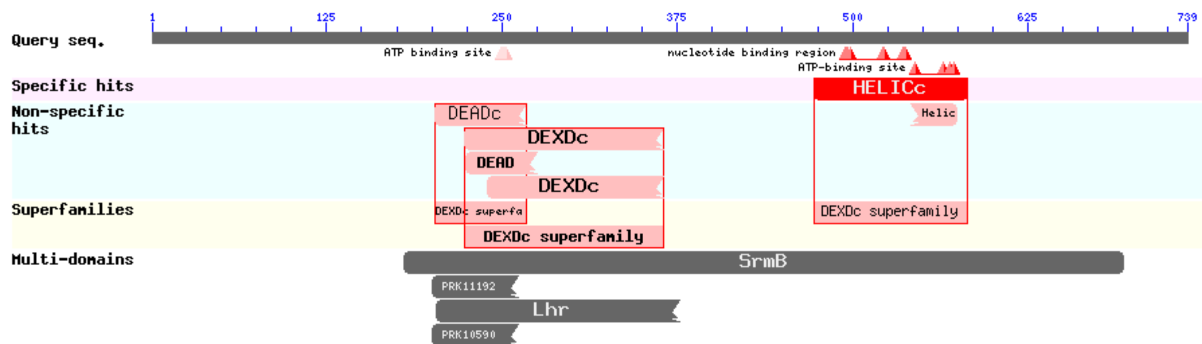

Fig.4. NCBI analysis of Tb 927.4.2720 gene.

1.5. MtRNA polymerase I

In contrast to the multisubunit RNAPs of the nucleus, in all organisms identified to date the catalytic domains of mtRNAPs are related to the single subunit RNA polymerase, similar to the T7 bacteriophage polymerase (Grams et al., 2002, Cermakian et al., 1996). Since in the mitochondrion of *T. brucei* mtRNAPI is indispensable for the transcription of all maxicircle-encoded mRNAs (Grams et al., 2002), as well as minicircle-encoded guide RNAs (Hashimi et al., 2009), it represents a suitable positive control for the disruption of mitochondrial gene expression. Mitochondrial RNAPI is required in active mitochondria, and functional knockout of the candidate gene is lethal for the procyclic stage trypanosomes. Indeed, cells depleted for this protein show a specific decrease of mitochondrial transcription (Grams et al., 2002).

1.6. S5 and L21 mitoribosomal proteins

The MRPS5 and MRPL21 proteins are conserved mitoribosomal proteins present across the prokaryotic and eukaryotic ribosomal domains. They were found in *E. coli, H. sapiens, D. melanogaster, C. elegans, S. cerevisiae* as well as in the kinetoplastids *L. major, L. tarentolae* and *T. brucei*. Their omnipresence qualifies them as suitable comparative controls.
The MRPS5 protein is the first ribosomal protein whose function and sequence have been shown to be conserved between procaryotes and eucaryotes (All-Robin et al., 1990). It forms part of the exit (E) site on the 40S ribosomal subunit in yeast and is essential for cell viability **(Fig. 5.).** Replacement of the yeast S5 protein with its human homolog yielded a viable yeast strain with a 20%–25% decrease in growth rate (Galkin et al., 2007, Ignatovich et al., 1995). Mutations in the gene encoding the *E. coli* ribosomal protein S5 have been shown to increase

translational error frequencies leading to general ambiguity of translation and suppression nonsense as well as missense and frameshift mutations. Alterations in S5 affect the 16S rRNA conformation, which in turn affects translational accuracy (All-Robin et al., 1990).

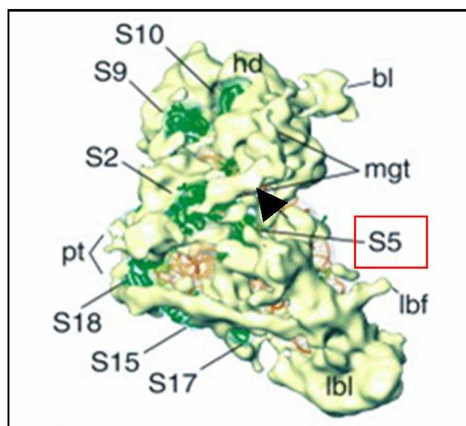

Fig.5. Location of MRPS5 within the head of the *E. coli* 30S ribosomal subunit. The arrow points to the mRNA-entry site (Sharma et al., 2003).

The MRPL21 protein is known to bind to the 23S rRNA in the presence of L20 in the large ribosomal subunit in *E. coli* (Heiland and Wittmann-Liebold, 1979, Sugimoto et al., 2009). It belongs to a group of six proteins present in the ribonucleoprotein core of the 50S subunit (Kühlbrandt and Garrett, 1978). In reconstitution experiments it was found that the 50S particle without protein L21 retains full activity in the poly-(U)-dependent polyphenyalanine synthesis (Spillmann et al., 1977). It was also found that protein L21 is not essential for the peptidyltransferase activity (Heiland and Wittmann-Liebold, 1979).

1.7. Mitochondrial translation in *T. brucei*

As compared to cytosolic translation, mitochondrial translation is always by one or two (or even less) orders of magnitude lower (Nabholz et al., 1999). Therefore, it is usually a challenge to detect mitochondrially translated proteins, which is a necessary prerequisite to follow and manipulate organellar translation. ^{35}S labelling of *de novo* synthesized mitochondrial proteins is a method of choice used to identify mitochondrial encoded proteins. In fungi, mammals, and plants differential sensitivity of the mitochondrial and cytosolic translation systems to cycloheximide and chloramphenicol provide a reliable criterion to distinguish one system from another (Horváth et al., 2002, Kroon and De Jong, 1979, Leaver et al., 1983).

Unfortunately, the situation is less straightforward in kinetoplastid flagellates. In *T. brucei* cytosol-specific translation inhibitor is cycloheximide, while the mitochondria-specific translation inhibitors are chloramphenicol and erythromycin, and the general translation inhibitor is puromycin. Because cycloheximide inhibits just 90% of cytosolic translation in *T. brucei*, in this organism it is impossible to sufficiently examine mitochondrial translation by selectively blocking cytosolic translation (Horváth et al., 2002, Nabholz et al., 1999, Neboháčová et al., 2004). This shortcoming and the resistance of highly hydrophobic proteins to enter the gel rendered organellar translation in kinetoplastids virtually inaccessible until the breakthrough discovery of Horváth et al. in 2000. These authors showed that it is possible to separate the extremely hydrophobic *de novo* synthesized proteins in a modified 2D gel system, off the main protein diagonal. Isolation of respiratory complexes followed by 2D gel electrophoresis allowed direct sequencing of two peptides from mitochondria-encoded proteins, one derived from cytochrome b and the other from cytochrome oxidase subunit I. Both peptides had sequences predicted from their corresponding mitochondrial genes. In summary, the described experiments have shown that a translation system capable of functioning with edited and unedited mRNA exist in the trypanosomatid mitochondria (Horváth et al., 2000 a b, Horváth et al., 2002).

Despite the fact that the mitochondrion of *T. brucei* belongs to the best studied organelles, and we know a lot about its mitochondrial DNA, transcription, RNA editing and other processing, we know still very little about translation in this organelle (Schneider, 2001). While two mitoribosomes were recently characterized (Zíková et al., 2008, Maslov et al., 2006), as briefly described above, there is as yet no functional analysis known about any of their component proteins. To fill-in this gap in our knowledge, I decided to disrupt, by RNA interference, selected mitoribosomal proteins, and to describe the resulting phenotypes. As shown in the presented work, the mitochondrial translation of *T. brucei* still retains numerous secrets, yet we have already embarked at the path to uncover them.

2. Goals

1. Overexpression of N-terminally His-tagged LR helicase in *E. coli* using pET100 vector. Purification of the overexpressed protein and its use for preparation of specific antibodies.

2. Preparation of RNAi knock-downs for the LR helicase in procyclic and bloodstreams stages of *T. brucei* using the p2T7-177 vector. Measurement of ensuing growth phenotypes.

3. Testing, by Northern blot analysis, whether double stranded RNA for LR is produced in RNAi-induced cells.

4. ^{35}S labelling of *de novo* synthesized mitochondrial proteins in procyclic RNAi lines for LR.

5. ^{35}S labelling of de novo synthesized mitochondrial proteins in procyclic RNAi lines for mtRNAP1 as a control.

6. Preparation of RNAi knock-downs for two conserved mitochondrial ribosomal subunits S5 (MRPS5) and L21 (MRPL21) in procyclic *T. brucei* using p2T7-177 vector.

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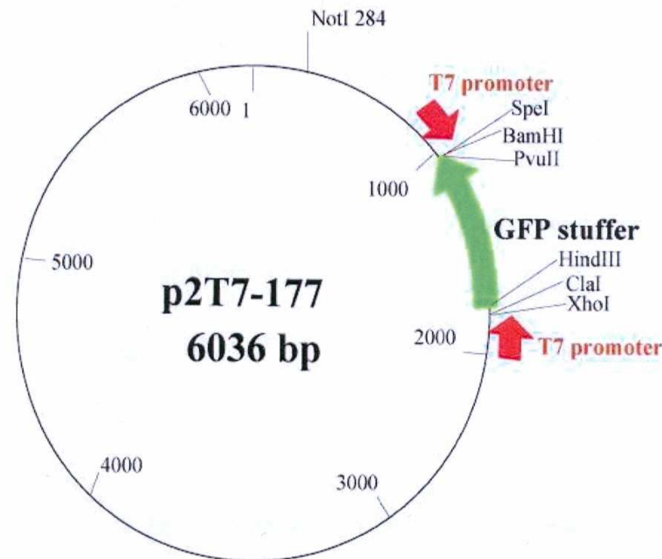
7. Testing, by Northern blot analysis, whether double stranded RNA for MRPS5 and MRPL21 is produced in RNAi-induced cells and establish the growth of RNAi-induced clonal cell lines.

8. ^{35}S labelling of *de novo* synthesized mitochondrial proteins in procyclic RNAi lines for MRPS5 and MRPL21 clonal cell lines.

3. Material and methods

3.1. Plasmid construction for RNAi

For RNAi in the procyclic and bloodstream forms of *T. brucei*, I used the p2T7-177 vector (Wickstead et al., 2002).



The PCR amplified fragment of the target gene was inserted between the *BamHI* and *XhoI* restriction sites, and verified by sequencing. For electroporation, about 10 μg of the construct was prepared and linearized by *NotI*.
The primers for preparation of this RNAi construct were designed using the GeneDB RNAi primer design software.

<u>For Tb927.4.2720</u>

LR RNAiF: GGATCC/CGCTGTGCGCATTCCCTC
LR RNAiR: CTCGAG/CACGCACGGCCTTTGCTTC
fragment: 428bp

<u>For MRPS5</u>

MRPS5 RNAiF: GGATCC/ACAACCAGTGGCGGTCTAAC
MRPS5 RNAiR: CTCGAG/TCTAAGTGCCTTGACCGCTT
fragment: 500bp

<u>For MRPL21</u>

MRPL21 RNAiF: GGATCC/TCACCAGTACAAAGTTGCGG
MRPL21 RNAiR: CTCGAG/CATTCTCCATGGCCTCCTTA
fragment: 428bp

3.2. Cultivation, transformation, selection and growth phenotype analysis of *T. brucei*

Cultivation of procyclic *T. brucei*

The procyclic cells of *T. brucei* strain 29-13 (Wirtz et al., 1999) were cultivated in SDM-79 medium with the addition of 10% of inactivated fetal bovine serum (FBS), penicillin (2.5 U/ml), streptomycin (2.5 μg/ml), neomycin (15μg/ml) and hygromycin (50μg/ml) at 27°C. Phleomycin (2.5 μg/ml) was used for selection and tetracycline (1μg/ml) for induction of RNAi.

SDM-79 (1 l)

2 g Grace′s media with L- glutamin/without $NaCO_3$, 1 g glucose, 8 g HEPES, 5 g MOPS, 2 g $NaHCO_3$, 100 mg pyruvic acid, 20 mg L-alanin, 100 mg L-arginin, 300 mg L-glutamin, 70 mg L-methionin, 80 mg L-fenylalanin, 600 mg L-prolin, 60 mg L-serin, 160 mg L-taurin, 350 mg L-treonin, 100 mg L-tyrozin, 10 mg adenozin, 10 mg guanozin, 50 mg D-glukosamin-HCl, 4 mg folic acid, 2 mg p-aminobenzoic acid, 0,2 g biotin, 6,24 g S-DEM, 8 ml DEM AA (50x), 6 ml DEM-non-esencial AA (100x), 750 μl hemin(7,5 μg/ml), 10 ml pen-strep (100x), MiliQ H_2O to 900 ml, 100 ml inactivated FBS.

Cultivation of bloodstream *T. brucei*

The bloodstream cells of *T. brucei* SM (Wirtz et al., 1999) were cultivated in HMI-9 medium with the addition of 10% of inactivated FBS, penicillin (0.125U/ml), streptomycin (0.125 μg/ml) and neomycin (0.5 μg/ml) under 5% CO_2 at 37°C. Phleomycin (0.2 μg/ml) was used for selection and tetracycline (1μg/ml) for induction of RNAi.

HMI-9(1l)

3,024 g Sodium bicarbonate, 17,66 g IMDM (GIBCO), 0,136 g Hypoxanthin, 0,11 g Sodium Piruvate, 39 mg Thymidine, 28,2 g Bathocuprone sulfate, 0,5 ml 20 mM β-mercapto-ethanol, 0,5 ml 150 mM l-Cys.HCl.H_2O, MiliQ H_2O to 890 ml, 100 ml heat inactivated FBS, 10 ml pen-strep (100x)

Transformation of procyclics *T. brucei.*

1. 10 ml of mid-long pf culture was harvested (10-20 x 10xE6 cells per ml) by centrifugation at 1300 g at 4°C. The cells were in a good shape in terms of their morphology and growth.
2. The cell pellet was washed once with 10 ml ice-cold CytoMix buffer

3. The cells were resuspended in 0.5 ml ice-cold CytoMix

4. The cuvettes were filled (0.2 cm gap) with 10 µg of linearized, sterile DNA and cooled on ice.

5. 0.5 ml cell suspension was added to the cuvettes and mixed by pipetting.

6. The cells were electroporated in the BTX electroporator using settings 1500 V, 25Ω, and 50 µF for the first pulse and 1700 V, 25 Ω, and 50 µF for the subsequent second pulse.

7. The cells where resuspended in 5 ml of conditioned medium and incubated for 18 hours at standard conditions.

8. 5 ml of SDM-79 containing the appropriate antibiotic (at 2× higher concentration) was added

9. Clones were obtained by limiting dilution in SDM-79 in 24-well plates.

A/ the first row of wells was loaded with 1.5 ml of culture

B/ the second and third rows were loaded with 1 ml fresh SDM-79

C/ the fourth row was loaded with 0.5 ml fresh SDM-79

D/ starting with the first column, I transferred 0,5 ml of the first well into the second one, pipetting up and down, transferring 0,5 ml to the third well, pipetting up and down and transferring 0,5 ml to the fourth well.

10. The plates were kept under 5% CO_2 at 27°C. The clones were diluted when the concentration reached 2×10^7 cells per ml.

<u>CytoMix for procyclics transfection:</u>

25 mM HEPES pH 7.6, 120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/ KH_2PO_4 pH 7,6, 2 mM EDTA, 6 mM glucose, 5 mM $MgCl_2$

<u>Transformation of bloodstreams *T. brucei*</u>

1. 90 ml HMI-9 medium with 10% FBS was prepared, and penicilline/streptomycin/neomycin was added. The medium was distributed among three Falcon tubes as follows: 30 ml in tube A, and 27 ml in tubes B and C.

2. 3×10^6 cells were harvested by centrifugation at 1300 g for 10 min at RT

3. The cells where resuspended in 100 µl of Amaxa Human T-cell solution at 4°C.

4. 10 µg of DNA (in 5 µl) was added to the cuvette and 100 µl cells were added immediately. The cap of the cuvette was replaced and the cells were electroporated.

5. The entire cell-DNA mixture was transferred to tube A, containing 30 ml of the prepared medium (cell density = 1 $\times 10^7$ cells/ml).The tube was inverted several times to mix the cells properly.
6. 3 ml of cells from tube A were transferred to tube B and again inverted several times (cell density = 1×10^6 cells/ml). The procedure was repeated for tube C (cell density = 1×10^5 cells/ml).
7. 1 ml aliquots of each dilution were distributed to three 24-well plates and incubated under 5% CO_2 at 37°C for 16hrs.
8. 75 ml of HMI-9 medium was prepared with the selective drug at double the normal concentration. 1 ml aliquot was added to each well of the three 24-well plates. Transformed cells were visible on the 5th or 6th day after transfection.

Growth curves

The growth curves were measured in three different ways. For the procyclic cells with RNAi for LR, the cells where diluted 10 times after reaching the concentration of 2×10^7. For the bloodstream form of RNAi for LR, the cells were diluted every day to the same concentration of 6×10^6. For the procyclic cells with RNAi for MRPS5/MRPL21, the cells where diluted every other day to the same concentration of 5×10^6.

3.3. Northern analysis

RNA isolation

1. The cells in 1 ml of Tri-Reagent were taken out of -80°C and thawed on ice, then kept at RT for 5 min.
2. 200 µl of chloroform was added and vortexed for 15 s. The solution was kept for 2 min at RT to settle down.
3. The reaction was centrifuged for 15 min at 4°C, 16100 g. The upper layer was transferred into a new tube.
4. 500 µl of isopropanol was added and vortexed for 15 s and kept at RT for 10 min to sediment.
5. The reaction was centrifuged for 10 min at 4°C and 16100 g. The supernatant was discarded.
6. 1ml ice-cold 75% ethanol was added. The tube was centrifuged for 10 min at 4°C at 16100g.
7. Ethanol was removed and the pellet was air dried.

8. The pellet was resuspended in 30 µl of water and kept on ice.

9. RNA was incubated for 10 min at 60°C water bath.

10. Concentration of RNA was measured using spectrophotometer.

RNA sample preparation

1. Appropriate volume of 1.5× sample buffer was added to 10 µg of RNA.

2. The sample was incubated for 10 min at 65°C in water bath.

3. Subsequently, it was transferred for 3 min on ice and 2 µl of loading solution was added.

4. The electrophoresis was run at 60V for 3 hours with the buffer mixed every 30 min.

5. The gel was inspected under UV and photographed after the run.

RNA blotting

1. 4 pieces of filter paper (11 x 9 cm) and one shaped like glass + 2 cm on each side were cut out, membrane (11 x 9 cm) was cut out, and paper towels were prepared.

2. 5× SSC was put on a filter paper and into the bath, with the following layers: 1 small filter paper, gel upside down, membrane, 3x filter paper, 5x SSC was poured after each layer and air bubbles were eliminated by rolling a glass stick.

3. Folded paper towels were put on top, covered with a glass plate, on top of which a weight was placed, and finally it was wrapped with a foil on the edges.

4. The transfer was allowed to run for 24 hours.

5. Membrane was checked under UV and marked by cutting incisions according to the ladder, and the membrane was labelled to recognize the RNA side.

6. The RNA was immobilised on membrane under UV cross linking (UV Stratalinker, Stratagene) and kept wrapped at 4°C.

Dot blot-probe testing

1. The DNA midiprep was serially diluted as follows:

1 µl into 99 µl of H_2O-concentration A (1: 100)

10 µl of A into 90 µl of H_2O-concentration B (1: 1000)

10 µl of B into 90 µl of H_2O-concentration C (1: 10 000)

10 µl of C into 90 µl of H_2O-concentration D (1. 100 000)

2. 3 µl of each concentration was put on the membrane and allowed to partially dry.

3. The membrane was then put for 3 min in the denaturing solution (1.5 M NaCl, 0.5 M NaOH).

4. Next, it was transferred for 3 min in the neutralisation solution (1.5 M NaCl, 0.5 M TRIS-HCl pH 7).
5. Next, it was transferred for 3 min to 3× SSC, this step being repeated twice.
6. The DNA was immobilized on membrane under UV cross linking as described above.
7. The membrane was pre-hybridized at 55°C for 2 hrs in the NAPI solution.

Preparation of the probe
1. PCR of RNAi fragment was performed using the same primers as for preparation of RNAi vector. Genomic DNA was used as a template.
2. Purification of PCR product was performed using the GeneElute PCR CleanUp Kit (Sigma).
3. The elution was checked on gel.
4. Radioactive labelling by random priming using the HexaLabel DNA labelling Kit (MBI Fermentas) was performed as follows:
-100 ng of purified DNA
-10 µl Random hexamer buffer
- 29 µl H_2O (44 µl total volume)
- 10 min 100°C
- 2 min on ice
- 3 µl dUTP, dGTP, dCTP mix + 1 µl Klenow enzyme + 2 µl α-^{32}P dATP (3000 Ci/mmol, Amershram) was added
- the reaction was incubated at 37°C for 1 hour
- the reaction was purified in centrifugation column containing the G-25 Sephadex beads
- spin column was placed in an eppendorf tube with the lid cut off
- the volume of the radioactively labelled reaction was brought up to 100 µl and applied onto the centrifugation column
- the reaction was centrifuged for 2 min and the filter was scanned for radioactivity
- the liquid from the eppendorf tube was removed into a new tube and scanned for radioactivity: When the radioactivity of filter was higher than of the liquid in the eppendorf tube, the filter was washed again with 100 µl dd H_2O and centrifuged for another 2 min
- then it was scanned again
- the probe was heated in a 100°C water bath for 3 min, immediately transferred on ice, added to 5 ml of the NAPI solution and poured into the cylinder containing the membrane with pre-

hybridized NAPI (membrane pre-hybridization: wetted in NAPI, shakes for 2 hrs at 60° C), then the cylinder with membrane was kept moving at 60°C for 16 hrs.

Membrane washing

1. The cylinder with membrane was taken out of 60°C; the probe was poured into a 50 ml Falcon tube and kept in -21°C.

2. 5 ml of 2x SSC + 0.1% SDS was added into the cylinder and kept moving at RT for 20 min.

3. The liquid was poured into a radioactive waste container and 5 ml of 2x SSC + 0.1% SDS was added, with the cylinder kept shaking at 55°C for 20 min.

4. The liquid radioactive waste was poured into the container, the membrane was placed on a filter paper and the radioactivity was measured; in case of a strong signal, step 3 was repeated.

5. Washing liquid was discarded, membrane was taken out of the cylinder, wrapped into a Saran wrap and placed into the phosphoimager casette, with RNA on top, and the membrane was kept in the cassette for 16 hrs.

6. The result was viewed by a Typhoon Phosphoimager.

RNA gel (1%) 100 ml

10 ml 10x MOPS, 1g agarose, 72 ml double distilled (dd) H2O, 18 ml formaldehyde

1.5× Sample buffer

600 µl Formamide, 210µl Formaldehyde 37%, 156 µl 10x MOPS, 5 µl Ethidium bromide (wrap into aluminium foil and kept at 4°C)

Running Buffer (500 ml)

50 ml 10x MOPS, 450 ml ddH_2O

3.4. Two dimensional polyacrylamide glycine-SDS gels

Cell labelling

1. 5×10^7 cells were harvested, washed 2× with SoTE and resuspended in 90 µl SoTE

2. 1 µl 100mM DTT and 1 µl of 100 mg/ml cycloheximide was added.

3. The cells were transferred to 27°C for 10 min and shaken in a 50 ml Falcon tube.

4. 9 µl of ^{35}S was added and the cell suspension was put for 2 hrs in 27°C, shaking in 50 ml Falcon tubes.

5. The cells have been centrifuged in an eppendorf tube.

6. The pellet was washed with100 µl SoTE, the supernatant was discarded and the pellet was frozen in –80°C.

Insoluble protein extraction with TX 100

1. The pellet was resuspended in 50 µl of 0.05% TX100 by pipetting + 15 sec vortex

2. The sample was put for 30 min on ice.

3. Than centrifuged for 20 min (16100 g, 6°C)

4. The supernatant was removed and the pellet was kept

5. 50 µl of 0.05% TX100 was added (pellet was resuspended by pipetting) and 50 µl of 2× SB was added to the pellet and vortexed for 15 sec.

6. The sample was incubated for 30 min at 37°C

First dimension

1. The sample was loaded on a 9.5% glycine gel (9.5% resolving gel - 6.75 ml AA, 15.45 ml H_2O, 7.5 ml lower buffer, 300 µl 10%APS, 18 µl TEMED) (stacking gel - 1 ml AA, 6.4 ml H_2O, 2.5 ml upper buffer, 100 µl 10%APS, 10 µl TEMED)

2. The electrophoresis was run for 20-28 A in the reservoir buffer.

Charge

1. The gel was taken out; the line without the stacking part was cut out

2. The gel was charged for 45 min in 25 ml 4× Upper buffer, 10 ml 10% SDS, 1 ml mercaptoethanol, and 64 ml H_2O

3. Finally, it was washed for 20 min in 1× Upper buffer; the buffer was changed 3×

Second dimension

1. The slide was put between glasses, and stacking gel – 1 ml AA, 6.4 ml H_2O, 2.5 ml upper buffer, 100 µl 10%APS, 10 µl TEMED was added

2. 14% resolving – 10.5 ml AA, 11.7 ml H_2O, 7.5 ml lower buffer, 300 µl 10% APS, 18 µl TEMED was added

3. 100 µl of 2× Sample buffer was loaded on top of the gel

4. The electrophoresis was run at 20-28 A in the reservoir buffer

Fix, stain, intensify, dry, expose

1. The gel was fixed in 10% acetic acid, 50% methanol

2. Staining was for 40 min in the staining solution

3. Destaining was for 5–9 hours in the destaining solution.

4. The gel was washed for 20 min in ddH_2O, the ddH_2O was changed 3×

5. The gel was intensified for 40 min in 1M sodium salicilate

6. Next, it was dried in vacuum gel dryer at 50°C for 5 hrs, the dried gel was photographed and used as a loading control

7. Finally the dry gel was exposed in a cassette with film in -80°C for 14 days

EXPRE^{35}S^{35}S Protein Labeling Mix Easy Tag^{TM} (Perkin Elmer)

A mixture of approximately 73% [^{35}S]-methionine and 22%L-[^{35}S]-cysteine prepared directly from the hydrolysate of *E. coli* grown in the presence of carrier-free $^{35}SO_4$ with added stabilizer.

Stabilized aqueous solution: Activity Concentration 11 [mCi/ml], 1000Ci (37.0TBq)/mmol.

SoTE

0.6 M sorbitol, 20 mM Tris-HCl, pH 7.5, 2 mM EDTA

TX 100

50nM NaPi pH 7,4

Lower gel buffer 4x

181,5 g Tris base, HCl to pH 8,8, TV 1l

Upper gel buffer 4x

60 g Tris base, HCl to pH 6,8, TV 1l

AA 40%

389,48 g Acryl, 1,052 g bis-acryl, TV 1l

Reservoir-running buffer 5x

Tris base 30g, Glycine 144g, (0,1% SDS in final solution), TV 1l

Sample buffer- denaturing buffer

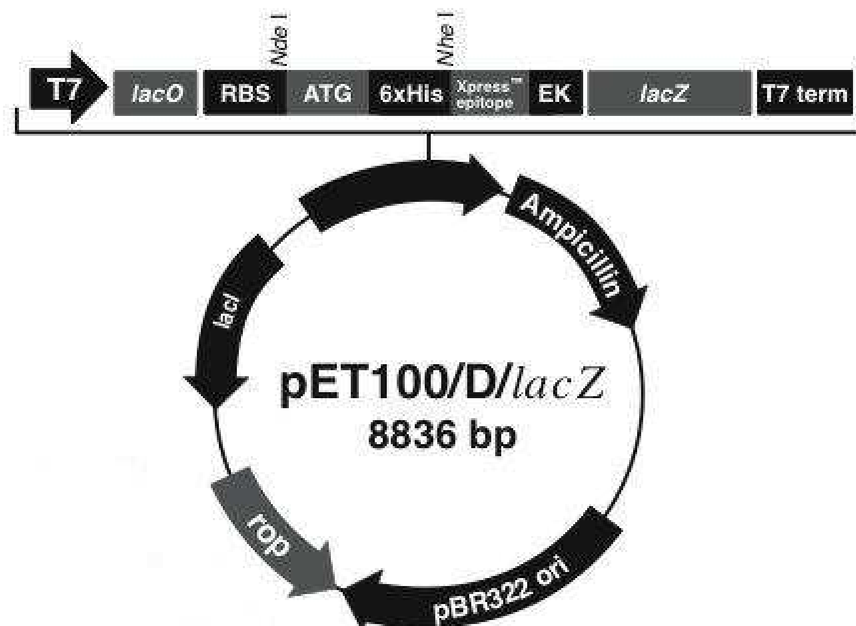
2 ml 2% SDS, 0,1 ml BPBlue, 2,7 Glycerol, 5 ml UGBuffer, 0,2 ml mercapto-ethanol

Staining solution

2,5 g Coomassie Blue R250, 454 ml MeOH, 92 ml acetic acid, 454 ml ddH_2O, TV 1l

3.5. Overexpression of LR helicase

For overexpression Champion pET Directional TOPO Expression Kit (K100-01) was used.



<u>Primers for overexpression</u>
Tb927.4.2720 F: CACC/CAACGAAATGTCATACAATC
Tb927.4.2720 R: TTATTTATTACTCTTCATACCATAAAGTGCTGC

<u>Primers for sequencing</u>
601vnitrni F: GATAAAGGTAGCATGGC
1192 F: CATGCGAACTGTTGCTTG
1678vnitrni R: CCTCTGATTCATGTAGC
T7: AATACGACTCACTATAG
T7 Reverse: TAGTTATTGCTCAGCGGTGG

<u>Strategy</u>
LR was amplified using Tb927.4.2720 F/ Tb927.4.2720 R by the Platinum polymerase.
The fragment was cloned into the pET TOPO vector and transformed into chemically competent *E. coli.*
The selected clones where analysed by sequencing using the following primers: T7 /601vnitrni F /1192 F /1678vnitrni R / T7 Reverse.
One clone without mismatches with annotated protein sequence for Tb927.4.2720 was picked for further use.
The purified plasmid from this clone was transformed into BL21 Star expression *E. coli* cells.
The pilot overexpression was performed using the following conditions:

21°C and 1mM IPTG, 21°C and 1.5mM IPTG, 21°C and 2mM IPTG
29°C and 1mM IPTG, 29°C and 1.5mM IPTG, 29°C and 2mM IPTG,
37°C and 1mM IPTG, 37°C and 1.5mM IPTG, 37°C and 2mM IPTG.
I picked 7 time points (0h, 1, 2, 3, 4, 5, 6 hrs) after the addition of IPTG, when I took the samples for protein SDS-PAGE gel electrophoresis and subsequent western blot analysis.

Western blot analysis
The SDS-PAGE gel was 12% AA.
21°C and 2mM IPTG, 29°C and 2mM IPTG, and 37°C and 2mM IPTG conditions were picked for western blot analysis.
The gels were stained with the Coomassie blue and after de-staining blotted to membrane Hybond^{TM} –P (Amersham).
The membrane was incubated with mouse anti-His antibody and then with the anti-mouse secondary antibody.
The membrane was visualized using PIERCE ECL Blotting Substrate (Thermo scientific)

LB-liquid
for 100 ml of LB: 1g NaCl, 1g Tris-base, 0,5 g yeast extract, 25 mg/ml ampicillin

LB-for plates
for 100 ml of solution: 1g NaCl, 1g Tris-base, 0,5 g yeast extract, 1,5g Bacto-agar, 25 mg/ml ampicillin

3.6. Used Kits

QIAprep Spin Miniprep Kit(Qiagen), QIAprep Spin Midiprep Kit(Qiagen), QIAquick Gel extraction Kit (Qiagen), GeneElute PCR Clean Up Kit(Sigma-Aldrich), DNAeasy Tissue Kit(Qiagen), Champion pET Directional TOPO Expression Kit (Invitrogen), HexaLabel DNA labelling (Fermentas), EXPRE^{35}S^{35}S Protein Labelling Mix Easy Tag^{TM}(PerkinElmer Life Sciences), PIERCE ECL Blotting Substrate (Thermo scientific)

4. Results

4.1. Overexpression

The gene Tb927.4.2720 (LR) was cloned into pET100/D/*lacZ* expressional vector according to the commercial protocol (Invitrogen). Five clones were completely sequenced using following primers: T7, 601vnitrni F, 1192 F, 1678vnitrni R, T7 Reverse. The results where analysed and one clone without mismatches with annotated protein sequence for Tb927.4.2720 was picked for further use. The purified plasmid from this clone was transformed into BL21 Star expression *E. coli* cells. Nine conditions for over expression were picked. **(Fig. 1.)**

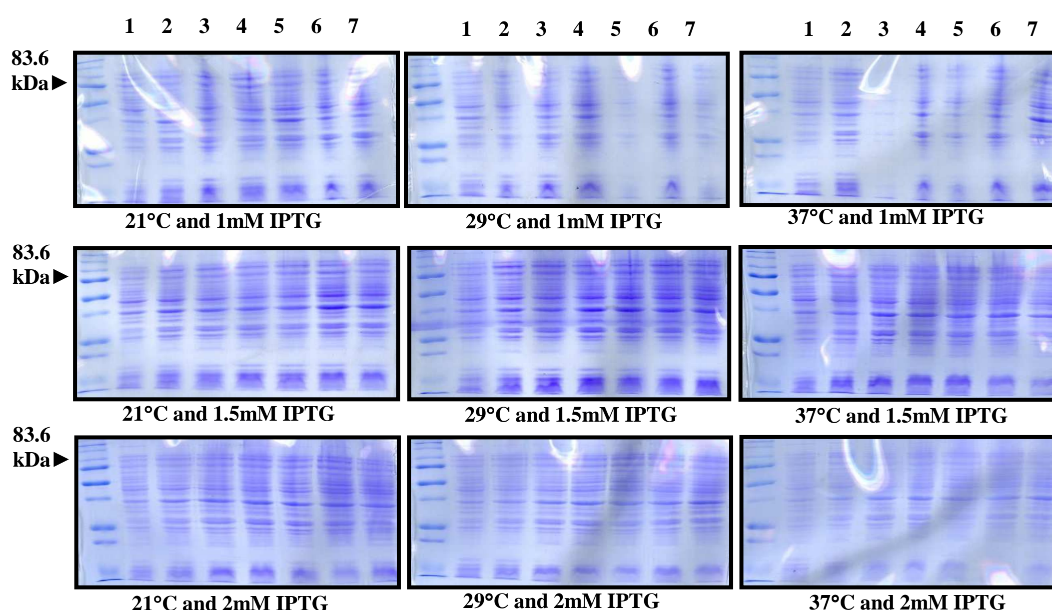


Fig. 1. SDS-PAGE gels whole cells lysates: **1**-time 0 before adding of IPTG, **2**-1h after induction, **3**-2h after induction, **4**-3h after induction, **5**-4h after induction, **6**-5h after induction, and **7-**6 h after induction. Arrows show expected size of LR tagged protein.

In the protein gels under none of the conditions the increase of protein of the required size were observed. For the Western analysis, we decided to use the conditions with the highest amount of IPTG (2mM).

Three of the conditions: 21˚C 2mM IPTG, 29˚C 2mM IPTG and 37˚C 2mM IPTG were picked for Western blot analysis using mouse anti-HIS Ab as a primary Ab and anti-mouse Ab as a secondary Ab. The membrane was visualized using PIERCE ECL Blotting Substrate (Thermo scientific). **(Fig. 2.)**

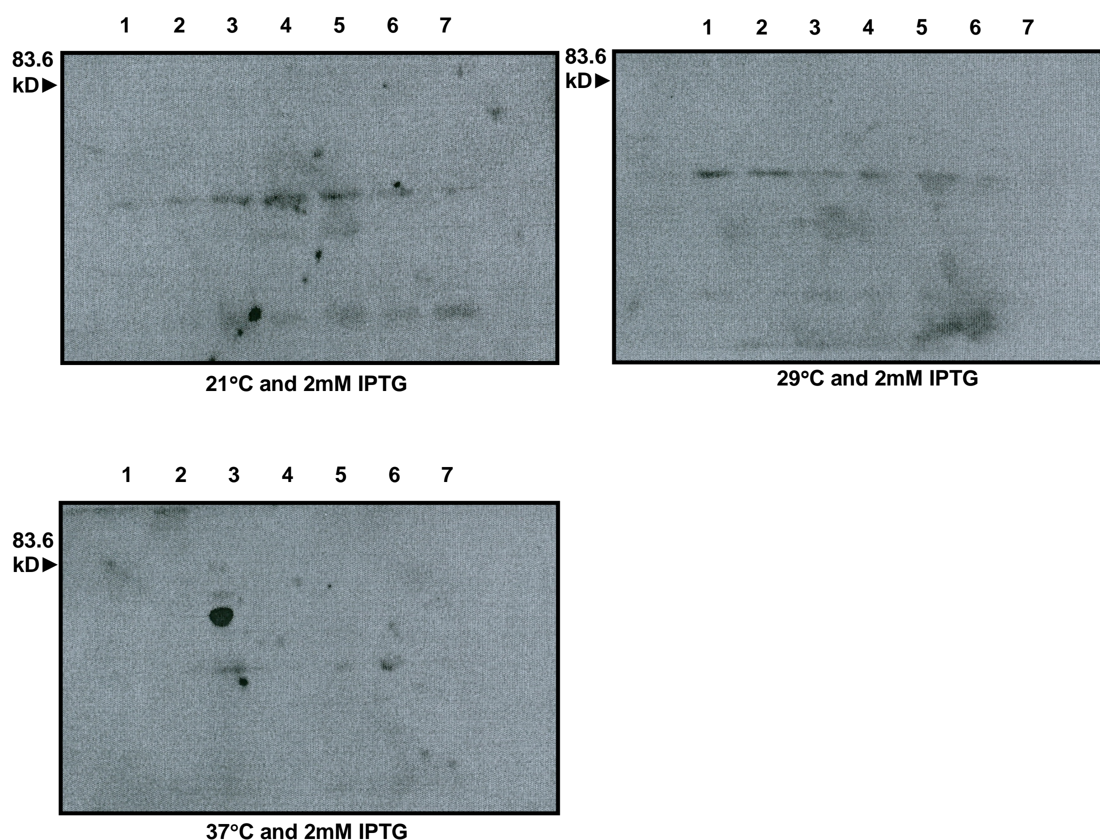


Fig. 2. Western blot analysis: **1**-time 0 before adding of IPTG, **2**-1h after induction, **3**-2h after induction, **4**-3h after induction, **5**-4h after induction, **6**-5h after induction, **7**-6h after induction. Arrows show expected size of LR tagged protein.

In the Western blott analysis we saw the production of HIS-tagged protein in about half of the size of the tagged LR protein. Due to the fact that the overexpression of 83.6 kD LR protein failed in nine different conditions we decided to continue with other approaches to investigate the role of LR putative subunit of *T. brucei* mitochondrial ribosome on mitochondrial translation.

4.2. RNAi of LR

The mRNA for LR was downregulated by RNAi using p2T7-177 vector. The RNAi primers were designed using GeneDB RNAi primer design software. The RNAi fragment was 428bp long. The procyclics and bloodstream cells were transfected with 10µg of *Not I* linearized vector. Five clones of procyclics and five clones of bloodstreams were selected on phleomycin. The clones were induced into the media by adding tetracycline. Two days after induction the cells were harvested and RNA was isolated. The isolated RNA was analysed by Northern blot analysis. **(Fig. 3.)**

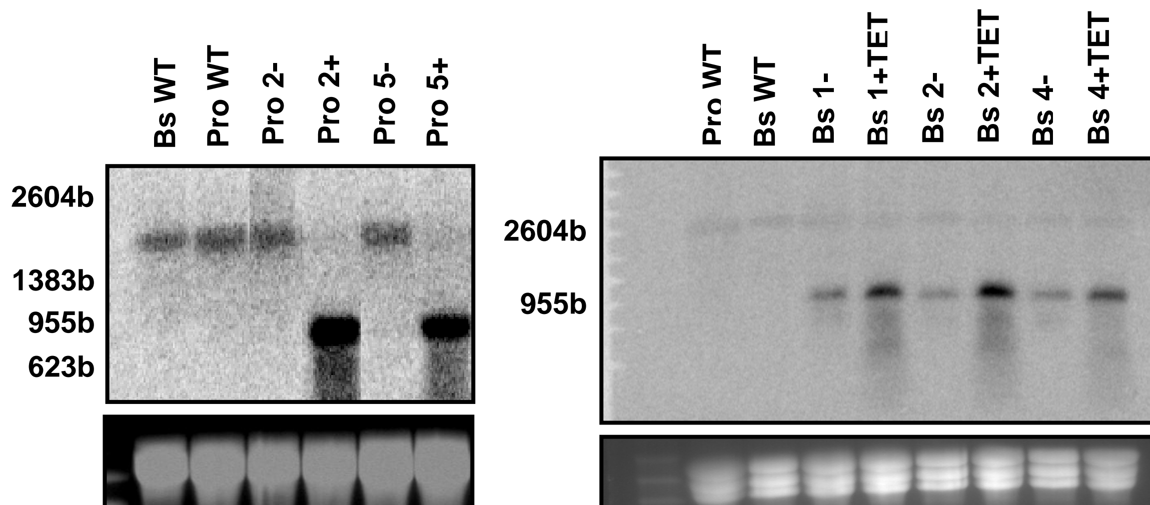


Fig. 3. Northern blot analysis:10µg of RNA/well. **A:** BsWT, ProWT, clon2-TET 2. day after induction, clon2+TET, clon5-TET, clon5+TET 2. day after induction.
B: Pro WT, Bs WT, Bs clon1 –TET, Bs clone 1 +TET 2.day after induction, Bs clone 2 –TET, Bs clone 2 +TET 2.day after induction, Bs clone 4 –TET, Bs clone 4 +TET 2.day after induction.

The Northern blot analysis revealed the production of dsRNA the second day after induction and disappearance of mRNA for LR by the same time in procyclics and bloodstreams. The dsRNA is unfortunately also visible in bloodstreams in non-induced cells, which is a sign of vector leakage. The growth analysis was done and two clones are presented. **(Fig.4.-5.)**

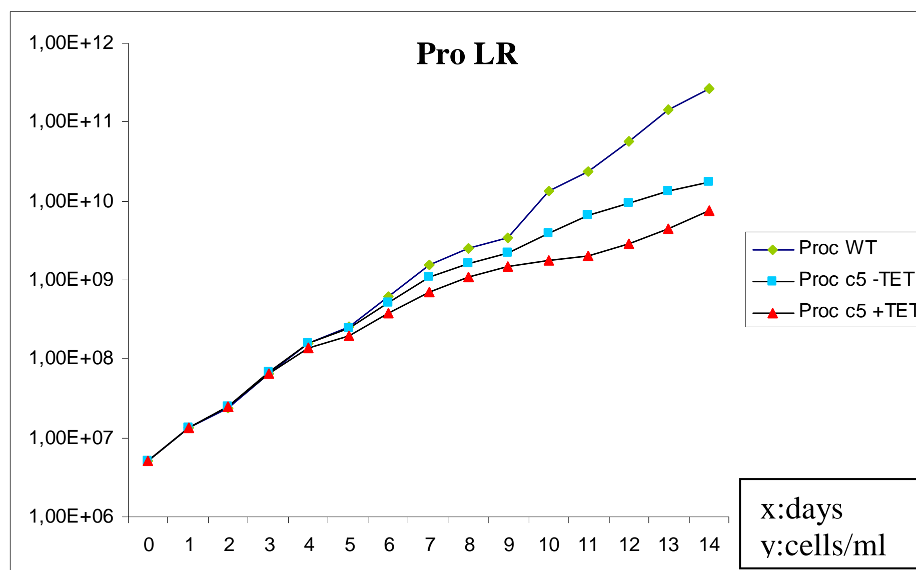


Fig. 4. Growth curve of RNAi procyclic cell clone 5 for LR: Proc WT in green, Proc c5 –TET in blue, and Proc c5+TET in red.

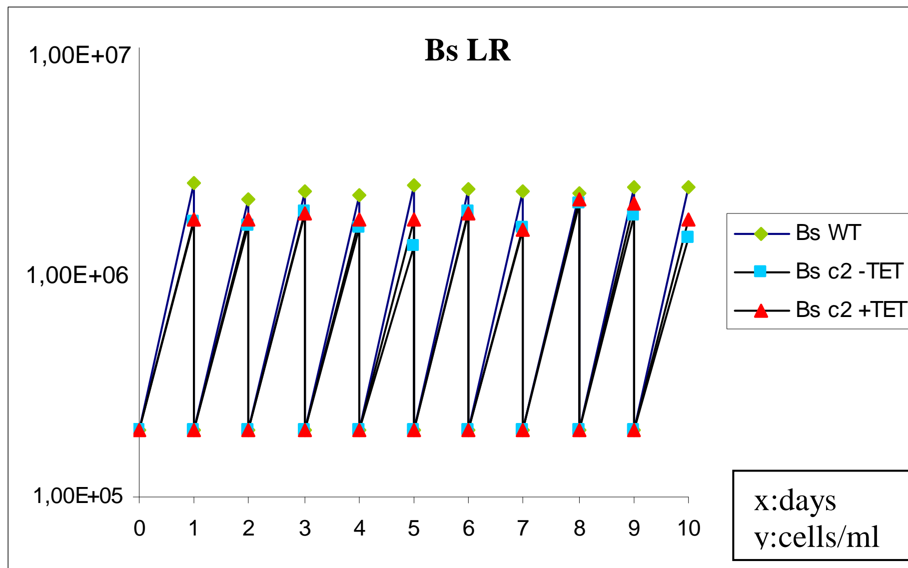
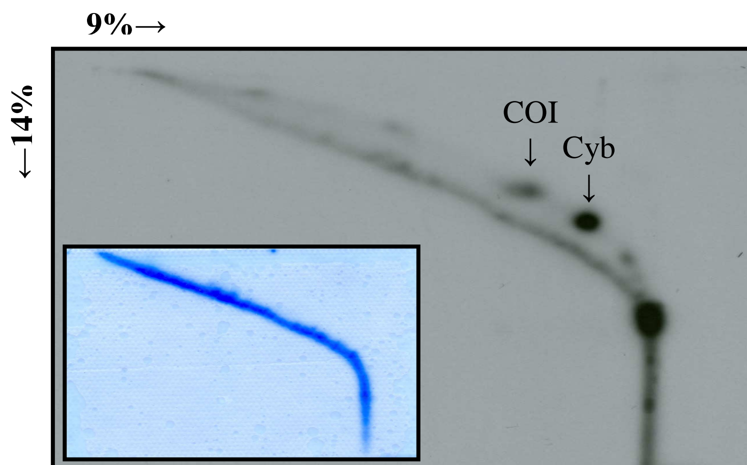


Fig. 5. Growth curve of RNAi blood streams cell clone 2 for LR: Bs WT in green, Bs c2-TET in blue, Bs c2 +TET in red.

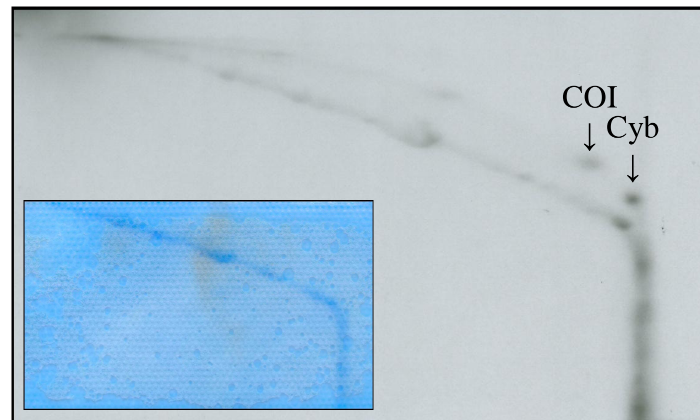
The growth curves revealed just a small slow growth phenotype in procyclic cells while in bloodstreams no decrease in growth rate in induced cells was observed.

4.3. ^{35}S labelling of de novo synthesised mitochondrial proteins assay in RNAi procyclic line for LR

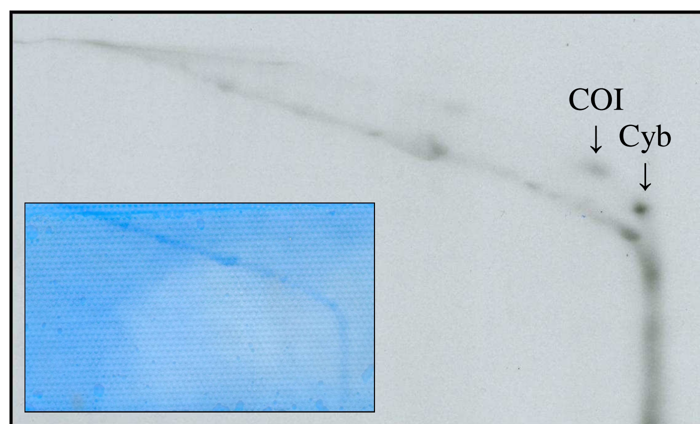
The RNAi procyclic cells for LR were ^{35}S labelled without presence of tetracycline and fourth days after addition of tetracycline into the media. Than the Two dimensional polyacrylamide glycine-SDS gels assay was performed and the dry gels where exposed in a cassette with film in -80°C for 14 days. **(Fig. 6.)**



A:



B:



C:

Fig. 6. Two dimensional polyacrylamide glycine-SDS gel assay: **A:** proc WT **B:** LR clone 5 without addition of tetracycline into media. **C:**. LR clone 5, 4. day after addition of tetracycline into media. Arrows are showing mitochondrially encoded cytochrome oxidase I and Cytochrome b.

The assay for de novo synthesised mitochondrial encoded protein revealed the following. In procyclic WT cells the strong production of subunits of respiratory complexes III (derived from its edited Cy*b* subunit) and IV (derived from never-edited COI subunit) were seen. In RNAi cells lines for LR the weaker but equal production of Cyb and COI could be observed. This observation leads us to the conclusion that mitochondrial translation is not affected by downregulation of LR.

4.4. ^{35}S labelling of de novo synthesised mitochondrial proteins assay in RNAi procyclic line for mtRNA polI

Cell line with pZJM RNAi constructs for mtRNApolI where use for ^{35}S labelling of de novo synthesised mitochondrial proteins assay(Wang et al., 2000, Grams et al., 2002). **(Fig. 7.)** These cells have strong lethal growth phenotype starting 3. day after induction (Grams st al.,

2002). On day 4 the cells are still alive, but not dividing any more. mRNA for mtRNApolI is disappearing on day 1 after induction and dsRNA is produced on the same day.

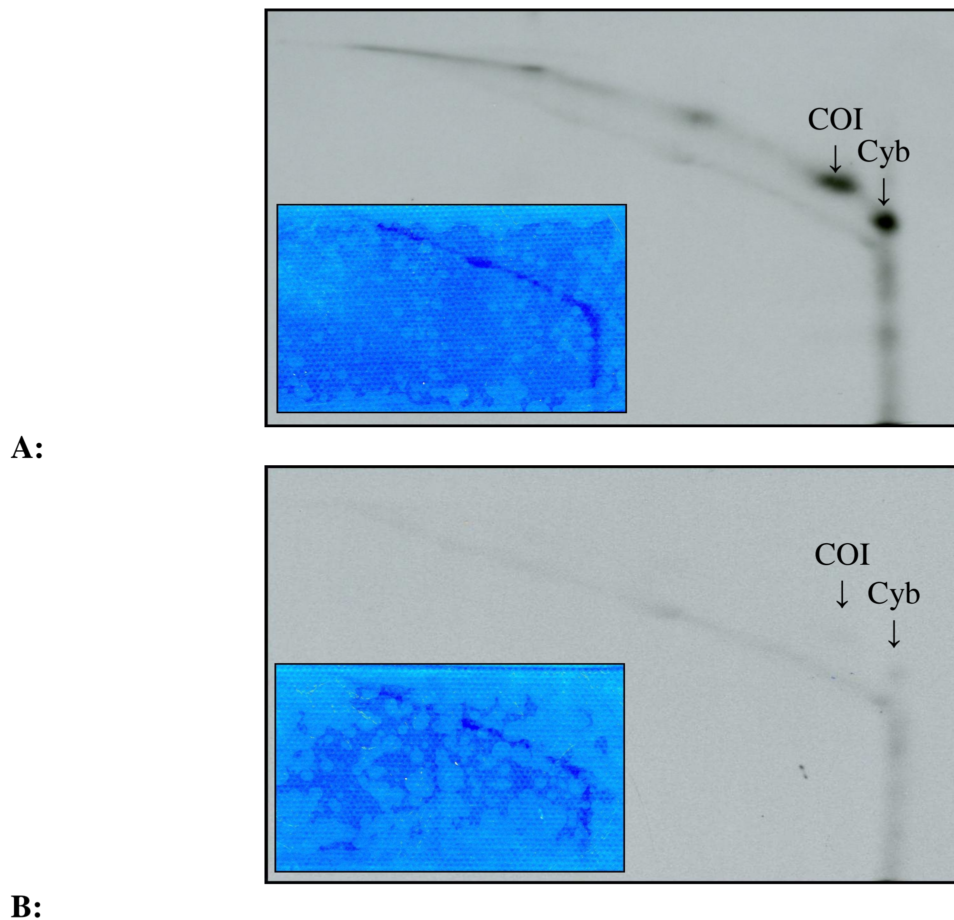


Fig. 7. Two dimensional polyacrylamide glycine-SDS gel assay: **A:**mtRNApolI without addition of tetracycline into media. **B:** mtRNApolI 4 days after addition of tetracycline into media.

In the case of RNAi of mtRNApolI we can see almost total disappearance of Cyb and COI on the fourth day after the induction with tetracycline. This corresponds with lethal growth phenotype later in this cell clones. The conclusion from this experiment is that mtRNApolI is essential for mitochondrial translation and its elimination leads to the end of mitochondrially encoded proteins production. Due to this fact the mtRNApolI could further serve as a positive control for the disruption of mitochondrial translation.

4.5. RNAi of MRPS5 and MRPL21

The mRNA for MRPS5 and MRPL21 was downregulated by RNAi using p2T7-177 vector. The RNAi primers where designed using GeneDP RNAi primer design software. The wild type procyclic cells have been transfected with *Not I* linearized vectors. The growth curves

have been measured in six selected clones for both ribosomal proteins. One clone from each is presented. **(Fig. 8.-9.)**

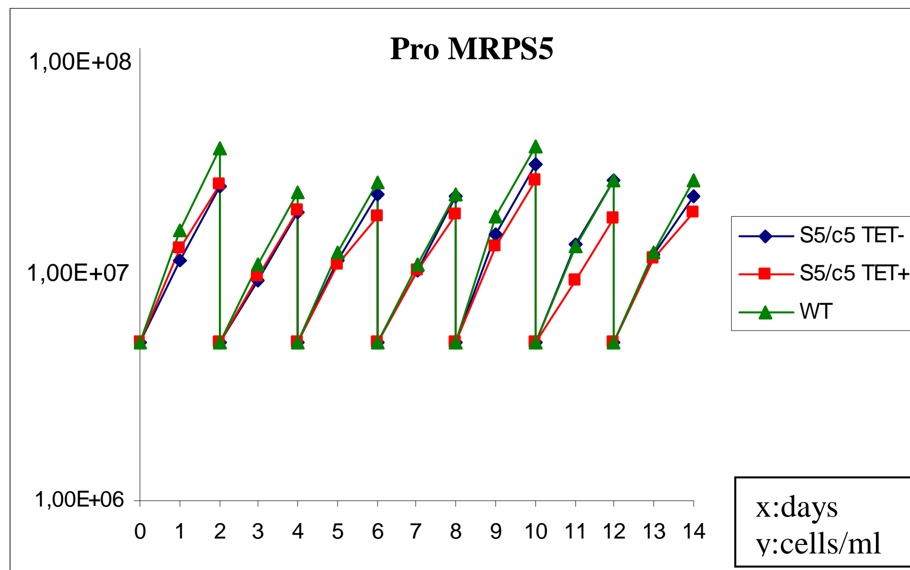


Fig. 8. Growth curve of RNAi cells line for MRPS5: Proc WT in green, Proc c5 –TET in blue, and Proc c5+TET in red.

The growth curves revealed just small slow growth phenotype in cells with RNAi of mRNA for MRPS5.

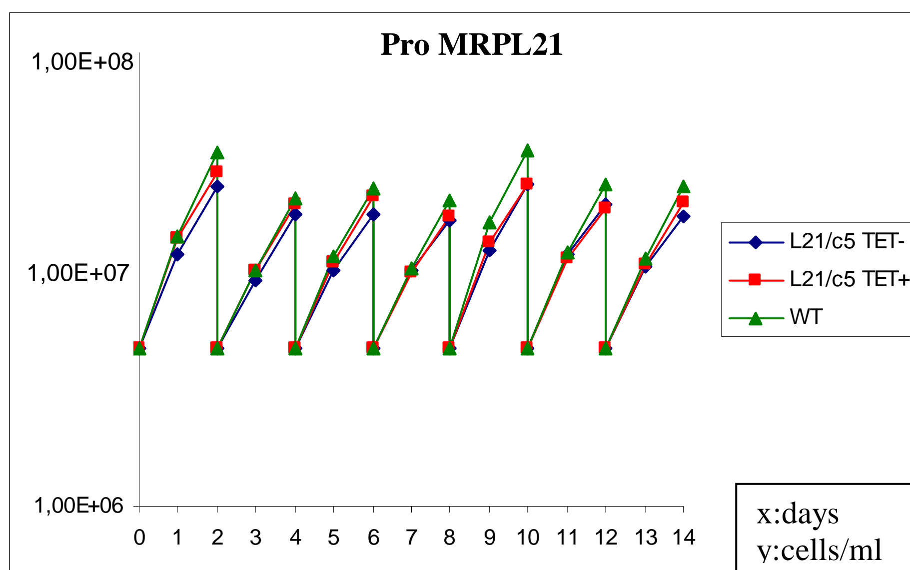


Fig. 9. Growth curve of RNAi cells line for MRPL21: Proc WT in green, Proc c5 –TET in blue, and Proc c5+TET in red.

The growth curves revealed no growth phenotype in cells with RNAi of mRNA for L21.

4.6. ^{35}S labelling of de novo synthesised mitochondrial proteins assay in RNAi procyclic line for MRPS5 and MRPL21

The RNAi procyclic cells for MRPS5 and MRPL21 were ^{35}S labelled without presence of tetracycline and fourteen days after addition of tetracycline into the media. Than the two-dimensional polyacrylamide glycine-SDS gels assay was performed and the dry gels were exposed in a cassette with film in -80°C for 14 days. **(Fig. 10.-11.)**

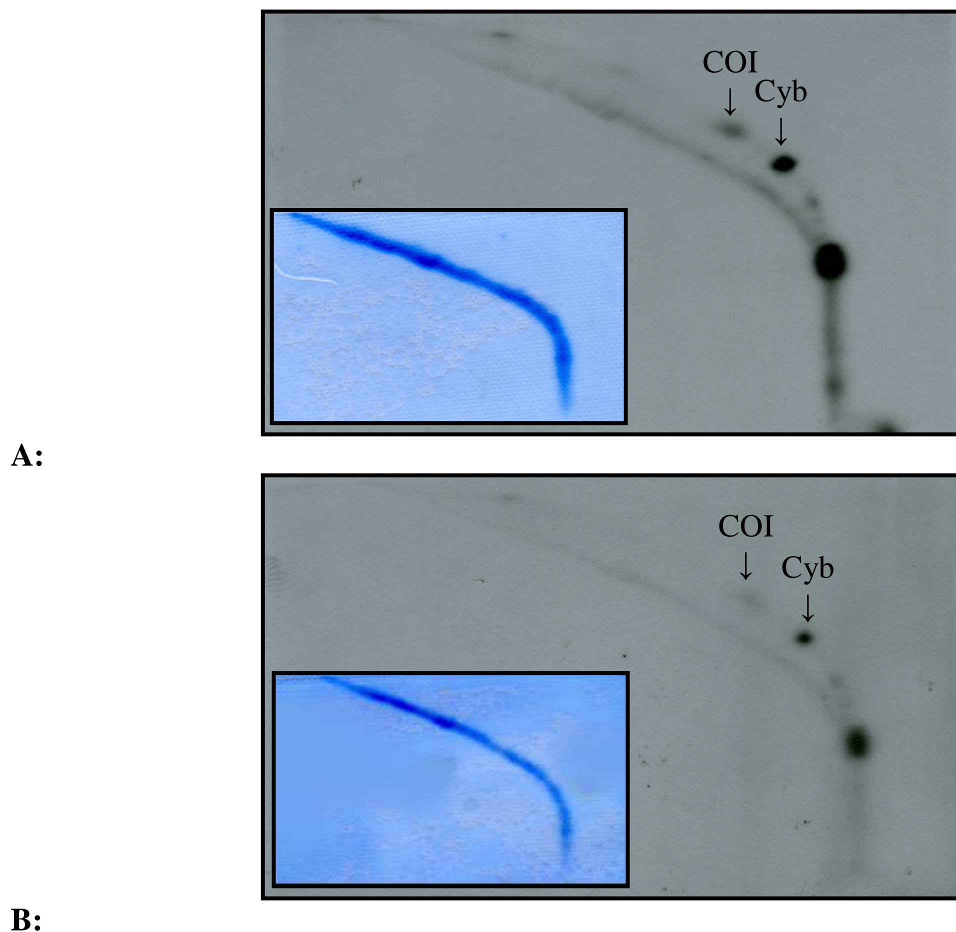


Fig. 10. Two-dimensional polyacrylamide glycine-SDS gels assay: **A:**MRPS5 without addition of tetracycline into media. **B:**MRPS5 14 days after addition of tetracycline into media.

In the case of down regulation of MRPS5 we were able to see the decrease in quantity of production of mitochondrially de novo synthesised proteins with built-in radioactively labelled amino acids. The decrease corresponds with slow growth phenotype in these cell lines.

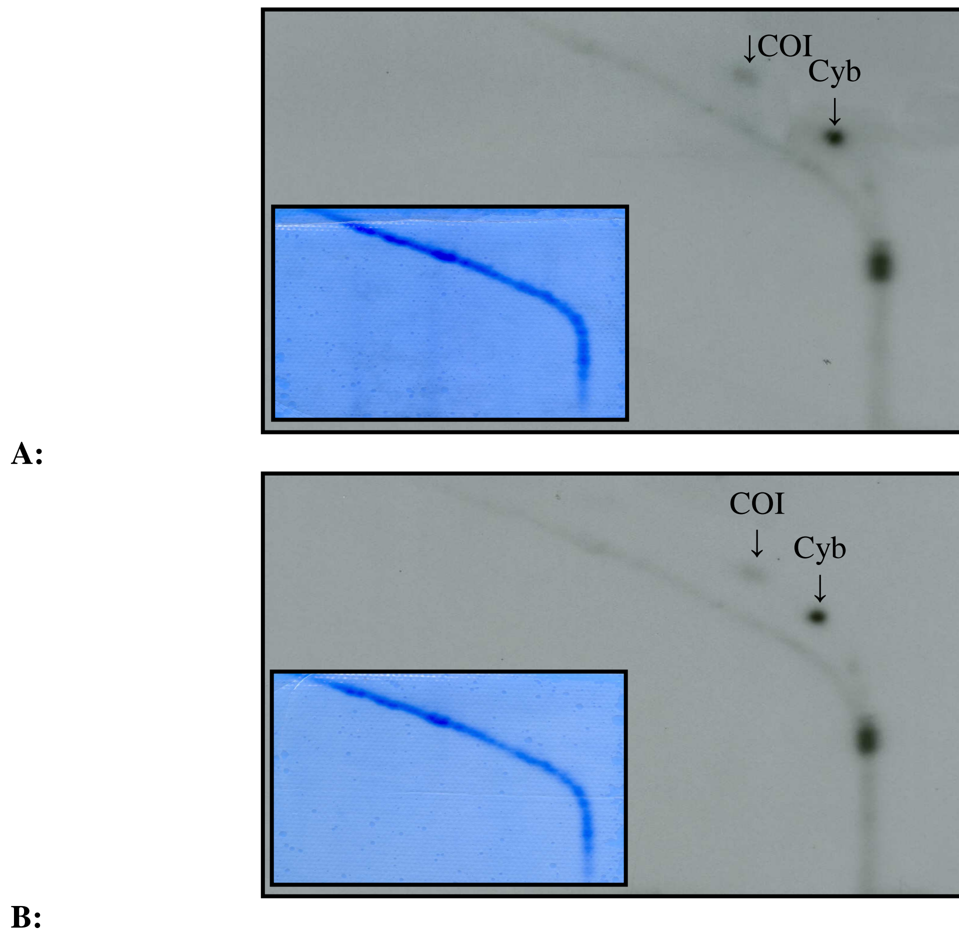


Fig. 11. Two-dimensional polyacrylamide glycine-SDS gels assay: **A:**MRPL21 without addition of tetracycline into media. **B:**MRPL21 14 days after addition of tetracycline into media.

MRPL21 was the fourth protein to test for the impact of its RNAi on mitochondrial translation. Neither did its downregulation have any affect on cells viability, nor did the 2D gel assay reveal the effect on mitochondrial translation. In induced and non-induced cells the level of Cyb and COI did not change.

5. Discussion

The goal of this work was to analyze the effect of down-regulation of several mitoribosomal subunits on mitochondrial translation in *T. brucei.* For that purpose I prepared RNAi knock-downs for several mitoribosomal subunits, and attempted characterization of ensuing phenotypes by numerous methods.

Attempts to generate antibodies against the putative mitoribosomal LR helicase failed due to lack of overexpression of the respective gene in *E. coli* under all tested conditions. Therefore it was not possible to visualize the ribosome by Western analysis or immunolocalization, as no antibodies are available against its other subunits. We focused our interest on the LR helicase which was, along with the mitoribosomal proteins S5 and L21, identified by mass spectrometry analysis as a putative component of the mitochondrial ribosome of *T. brucei* (Zíková et al., 2008). Cells in which mtRNAPI was ablated by RNAi were used as a control, since it was reasonable to assume that these cells are unable to perform mitochondrial translation due to the shutdown of transcription (Grams et al., 2002, Hashimi et al., 2009). While MRPS5 and MRPL21 represent conserved and omnipresent mitoribosomal subunits, LR helicase is an unexpected component of the ribosome, and we can only speculate about its function. In fact, so far no functional analysis has been performed on any component of any protein involved in mitochondrial translation in a trypanosomatid flagellate.

RNAi against LR and MRPS5 showed that the down-regulation of these subunits affects cells growth. The induced cells are still dividing, although the division rate slows down, as compared to their non-induced counterparts. The growth is not affected in cells with targeted MRPL21. The lack of growth phenotype in cell lines in which down-regulation of selected mitoribosomal subunits has been triggered can be explained by several ways. Prokaryotic ribosome, such as that of *E. coli*, which is the predecessor of mitochondrial ribosome, was shown to be able to self assemble *in vitro* without assembly-enhancing factors (Semrad and Green, 2002, Culver and Miller, 1999, Nierhaus and Dohme, 1974). For the assembly it is sufficient to simply increase the temperature in a medium with special ion composition. Moreover, even at lower temperatures, the elimination of proteins involved in the assembly of a prokaryotic ribosome results only in a slowed growth phenotype (Chaudhuri et al., 2008, Damell and Miller, 1993).

These findings are in good correlation with my observations that the ablation of putative LR helicase in *T. brucei* results in no more than a slower growth phenotype. Indeed,

the LR protein has a homology with the SmRB helicase superfamily, which is known to have a role in mitochondrial assembly in prokaryotes (Chaudhuri et al., 2008), and the ablation of which would likely trigger a similar phenotype. However, the slow growth phenotype observed in cells depleted for conserved subunit MRPS5, is more difficult to explain. We suppose that either the cells survive with basal levels of respective mRNA or are able to recycle the ribosomal subunits or could replace the targeted structural proteins with another mitoribosomal subunit (Shi et al., 2000; Ngo et al., 1998) The lacking of growth phenotype observed in cells with downregulated MRPL21 is not surprising while the non-essentiality of this subunit was previously described (Spillmann et al., 1977, Heiland and Wittmann-Liebold, 1979). Yet the robustness of the used experimental approach applied in this study is supported by the fact that in the same experimental setup, the mtRNAPI protein is clearly essential and no organellar translation occurs in the procyclic cells where this protein was down-regulated.

Since we are interested in the function of mitochondrial ribosomes and translation in both life cycle stages of *T. brucei*, we decided to eliminate the LR helicase mRNA also in the bloodstreams stage, which is the pathogenic form of the parasite. Any efficient and sensitive targeting of its proteins, sufficiently different from their homologues in the vertebrate host, has a potential to be used in pharmacotherapy. For that purpose, we used the same vector as the one used in both procyclic and bloodstream stages. The cells revealed no growth phenotype and neither we, and to the best of our knowledge nor any other laboratory, have so far been able to monitor organellar translation in the bloodstreams. The reason(s) for that remain(s) unclear, but may rather have methodological reasons (also see below). Since the leakage of dsRNA occurred in the non-induced cells, the experiment has to be repeated with another, hopefully more tightly regulated vector. Our conclusion at this point is that the down-regulation of LR in both stages of *T. brucei* has virtually the same – very mild - effect on cell viability.

We have made extensive use of a method designed to visualize mitochondrial translation in kinetoplastids (Horvath et al., 2000a b, 2002). Its description was a breakthrough almost a decade ago, as the classical approach of selective blockade of cytosolic and mitochondrial translations by cycloheximide and chloramphenicol, respectively, could not be used in kinetoplastid protists, most likely because of the absence of key binding sites in the mitochondrial ribosome.

In fact there is also residual cytosolic translation that is believed to be represented by the signal in the main diagonal on the two-dimensional (9 *versus* 14%) polyacrylamide glycine-SDS gel (Horváth et al., 2002). Under these conditions, the subunits of respiratory

complexes III (derived from its edited Cy*b* subunit) and IV (derived from never-edited COX1 subunit) are visualized as radioactively labelled proteins detected by autoradiography. They appear as a series of spots located off the main diagonal, a property that has been explained by abnormal electrophoretic migration and aggregation (Horvath et al., 2000). Aberrant electrophoretic migration of detected polypeptides in SDS gels further indicates that their charge to mass ratio is different from other proteins. This property can be explained by a high hydrophobicity, likely connected with high cysteine content, leading to an increased binding of SDS (Horváth et al., 2000).

Results of the *de novo* labelling assay of mitochondrial-encoded proteins in the cellular background with down-regulated putative LR helicase were unanticipated. Since the LR helicase was described as a subunit of mitoribosome of *T. brucei* (Zíková et al., 2008) (although it is significantly missing in the reconstituted mitoribosome of related *L. tarentolae* [Maslov D A 2006]), mitochondrial translation on day 4 of RNAi induction was unaffected. This could be explained by non-essentiality of the LR helicase for translation or by its wrong assignment to the ribosome.

The same assay performed with cells with down-regulated MRPS5 revealed a decrease of mitochondrial translation of Cy*b* and COI, but did not disrupt mitochondrial translation totally. The time point for labelling of RNAi-induced cells was shifted to 14 days of induction in order to eliminate the possibility of delayed effects on mitochondrial translation, when no strong growth phenotype was observed. As proposed earlier, this could be a consequence of cells ability to cope with the lack of this subunit or to survive with basal level of target mRNA, which is the intrinsic weakness of the RNAi approach. The mitochondrial translation assay performed with cells depleted for MRPL21, revealed no visible effect on mitochondrial translation. This result corresponds with non essential character of this subunit in *E. coli*. Unfortunately, the Northern analysis has been inconclusive in this case so far, so the possibility of failed RNAi cannot be ruled out at this point. Additional experimentation will be needed in order to ascertain that RNAi works optimally in the studied cell lines.

6. Conclusions

The RNAi-based down-regulation of three subunits of the mitochondrial ribosome in *T. brucei* revealed unexpectedly small effect on organellar translation, while the elimination of mitoribosomal RNA polymerase I by the same strategy resulted in total disruption of mitochondrial translation, followed by lethality. Based on these findings I conclude that the mitochondrial translation in *T. brucei* is essential.
The present findings will be further corroborated by the ongoing functional analysis of the MRPS5 and MRPL21 proteins in the bloodstream stage of *T. brucei*.

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