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DEAD-box RNA helicase is dispensable
for mitochondrial translation
in *Trypanosoma brucei*

RNDr. Thesis

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Annotation

Although the *Trypanosoma brucei* mitochondrion belongs among one of the best studied organelles, translation in this organelle is difficult to study for two reasons. First, in *T. brucei* (unlike in most organisms) part of the cytosolic translation is resistant to cycloheximide, making the examination of mitochondrial translation very challenging. Second, mitochondrial-encoded proteins of kinetoplastid protists do not enter the gels under standard conditions, due to their extreme hydrophobicity.

We present here the functional analysis of a potential protein component of *T. brucei* mitoribosomes, which is a DEAD-box RNA helicase (RH). RH was down-regulated in the procyclic and bloodstream stage by RNA interference.

For the analysis, we used a modified two-dimensional gradient blue-native/SDS PAGE gel system, in which the newly synthesized highly hydrophobic mitochondrial proteins can be separated.

Although ablation of the RH transcript leads to a weak growth phenotype in the procyclic cells, the protein does not seem to be essential for mitochondrial translation.

Declaration [in Czech]

Prohlašuji, že svoji rigorózní práci jsem vypracovala samostatně pouze s použitím pramenů a literatury uvedených v seznamu citované literatury. Prohlašuji, že v souladu s § 47b zákona č. 111/1998 Sb. v platném znění souhlasím se zveřejněním své rigorózní práce, a to v nezkrácené podobě elektronickou cestou ve veřejně přístupné části databáze STAG provozované Jihočeskou univerzitou v Českých Budějovicích na jejích internetových stránkách, a to se zachováním mého autorského práva k odevzdanému textu této kvalifikační práce. Souhlasím dále s tím, aby toutéž elektronickou cestou byly v souladu s uvedeným ustanovením zákona č. 111/1998 Sb. zveřejněny posudky školitele a oponentů práce i záznam o průběhu a výsledku obhajobě kvalifikační práce. Rovněž souhlasím s porovnáním textu mé kvalifikační práce s databází kvalifikačních prací Theses.cz provozovanou Národním registrem vysokoškolských kvalifikačních prací a systémem na odhalování plagiátů.

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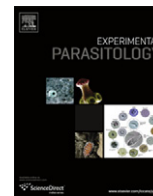
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Thank you all...



Research Brief

DEAD-box RNA helicase is dispensable for mitochondrial translation in *Trypanosoma brucei*Lenka RichtEROVÁ^{1,2}, Zuzana VÁVROVÁ², Julius LUKEŠ*

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ABSTRACT

DEAD-box RNA helicase, a putative subunit of the mitochondrial ribosome of *Trypanosoma brucei*, has been down-regulated in the procyclic and bloodstream stage by RNA interference. Although ablation of the transcript leads to a weak growth phenotype in the procyclic cells, the protein does not seem to be essential for mitochondrial translation under standard cultivation conditions, as shown by an assay that allows visualization of the *de novo* synthesized proteins. Furthermore, we show that synthesis of cytochrome *c* oxidase subunit I and cytochrome *b* does not occur in the mitochondrion of the bloodstream stage.

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In every eukaryotic cell the mitochondrion contains translation machinery needed to synthesize proteins from mitochondrial transcripts, with *Trypanosoma brucei* being no exception (Horváth et al., 2000a). However, until recently, several unique features of this process in kinetoplastid flagellates hindered its study. In most organisms, cytosolic translation can be specifically inhibited by cycloheximide, allowing the study of chloramphenicol-sensitive prokaryote-type translation in the organelle. However, in *T. brucei* and *Leishmania tarentolae* about 10% of cytosolic translation is resistant to the drug, making the examination of mitochondrial translation very challenging (Nabholz et al., 1999; Horváth et al., 2002). Moreover, due to their extreme hydrophobicity, mitochondrial-encoded proteins of kinetoplastid protists do not enter the gel under standard conditions. A breakthrough came when it was demonstrated that these highly hydrophobic proteins can be separated in a modified two-dimensional gradient blue-native/SDS PAGE gel system, where they migrate off the main protein diagonal (Horváth et al., 2000a,b, 2002). Furthermore, by direct sequencing these authors were able to verify the identity of never-edited cytochrome *c* oxidase subunit I (coxI) and cytochrome *b* (cyB), which is derived from an edited mRNA.

Although the *T. brucei* mitochondrion belongs to the best studied organelles, and we know a lot about replication and maintenance of its mitochondrial (=kinetoplast; k) DNA, as well as

about its transcription, RNA editing and other RNA processing (Lukeš et al., 2005), translation in this organelle remains poorly understood and has so far been examined only in the procyclic stage. While the composition of mitoribosomes of *T. brucei* and *L. tarentolae* has recently been established to great detail (Maslov et al., 2007; Zíková et al., 2008) as yet no functional analysis has been performed for any of their protein component.

To start filling-in this gap in our knowledge, we decided to study the function of ribosomal helicase (Tb 927.4.2720) (here labeled RH), using mitochondrial RNA polymerase as a control. The RH subunit was identified in frame of an extensive mass spectrometry analysis of the mitoribosome of the procyclic trypanosomes, as it co-purified with 4 out of 6 TAP-tagged proteins of the large ribosomal subunit (A. Zíková, personal commun.). This protein, annotated as a homologue of the bacterial DEAD/DEAH helicase, belongs to the superfamily II of DNA and RNA helicases (Zíková et al., 2008). The RH protein was also found as part of the whole *T. brucei* mitochondrial proteome (Panigrahi et al., 2009), however, it was conspicuously missing from the *L. tarentolae* mitoribosome (Maslov et al., 2007). Cryo-electron microscopy reconstruction of this ribosome with minimal RNA showed that it is uniquely porous, contains less inter-subunit bridges but more tunnels, and recruits mRNAs and interacts with tRNAs in a manner strikingly different from ribosomes in other organisms (Sharma et al., 2009).

Using GeneDB RNAinterference (RNAi) primer software, we designed primers that amplified from total DNA of the *T. brucei* 29–13 strain a 428 bp-long fragment of the RH gene, which was cloned into the p2T7–177 RNAi vector. Upon electroporation into the 29–13 procyclic and 920 bloodstream strains, one

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representative clone selected for each stage as described elsewhere (Hashimi et al., 2009) was used for further studies. First, Northern blot analysis with a probe derived from the cloned RH gene fragment was used to monitor the extent of RNAi. As shown in Fig. 1A, the RH transcript is present in both the procyclics and bloodstreams. Within 48 h of RNAi induction, the target mRNA was virtually eliminated from the procyclic cells (Fig. 1A; left panel). Next, the growth of the parental 29–13 cells, as well as the non-induced and RNAi-induced procyclics was followed for 14 days, revealing a very slight growth phenotype that was apparent since day 4 of RNAi induction (Fig. 1B).

Since the RH protein was found in the mitoribosomes of procyclic *T. brucei* and is thus associated with mitochondrial translation, we decided to examine the effect of RH ablation on *de novo* synthesis of the organellar proteins in the procyclic cells. To visualize products of mitochondrial translation in the 29–13 parentals, the non-induced RH cells and those 4 days of RNAi induction, the S^{35} labeling method was performed as described elsewhere (Horváth et al., 2002; Týč et al., 2010). Unexpectedly, mitochondrial translation was equally efficient in the parental cells (data not shown), the non-induced cells (Fig. 2A) and those depleted for the RH mRNA (Fig. 2B). As a control for the translation assay, we have used *T. brucei* procyclics, in which mitochondrial RNA polymerase (mtRNAP) was ablated by RNAi. MtRNAP was shown to be the canonical polymerase responsible for the transcription of maxicircles (Grams et al., 2002) and minicircles (Hashimi et al., 2009), making it indispensable for the survival of both procyclic and bloodstream trypanosomes (Hashimi et al., 2009). We have therefore predicted that in cells, in which the mtRNAP transcript is targeted by RNAi, organellar translation will be disrupted due to the absence of mRNAs and cognate guide RNAs. Indeed, comparison of mitochondrial translation in the non-induced (Fig. 2C) and RNAi-induced mtRNAP cells (Fig. 2D) shows that in the latter cells mitochondrial translation virtually ceases, validating the used assay.

Finally, we wondered whether the above-described labeling protocol will allow visualization of any products of mitochondrial translation in the *T. brucei* bloodstream stage. While the two unambiguously identified mitochondrial-encoded proteins, *coxI* and *cyB*, were predicted to be absent from the mammalian stage, multiple lines of evidence, albeit indirect, suggested the synthesis of at least subunit 6 of F_1F_0 -ATP synthase (A6) occurs in this life cycle stage (Schnauffer et al., 2005; Lai et al., 2008; Hashimi et al., 2010). We have performed the S^{35} labeling with 5×10^7 bloodstream cells following the protocol used for procyclic *T. brucei* (Fig. 3). Although residual cycloheximide-resistant cytosolic trans-

lation was visualized at about the same rate as in the procyclic stage, no labeled protein migrating outside off the main diagonal was observed on the two-dimensional gradient blue-native/SDS PAGE gel (Fig. 3). To avoid dilution of the S^{35} labeled-cysteine due to the presence of 1.5 mM cysteine in the HMI9 cultivation medium, the 2-h long incubation was performed in the same medium lacking cysteine. In an alternative approach, bloodstream cells were labeled in the SoTe buffer (Neboháčová et al., 2004) complemented with 10 or 33 mM glucose (Duszenko et al., 1988). However, all these alternative protocols produced labeling pattern indistinguishable from the one in Fig. 3. To the best of our knowledge, these results provide first direct evidence that *coxI* and *cyB*, abundantly present in the insect stage, are missing from the stage parasitizing mammals. However, the essentiality in the *T. brucei* bloodstreams of RNA editing (Schnauffer et al., 2001) and RNA processing (Fisk et al., 2008; Hashimi et al., 2009) implies that mitochondrial translation is an ongoing process also in this life cycle stage. Based on the indispensability of the F_1F_0 -ATPase complex, the absence of other respiratory complexes (Schnauffer et al., 2005; Hashimi et al., 2010) and by the easy loss of the kDNA (Lai et al., 2008; Jensen et al., 2008), it was hypothesized that A6 might actually be the only protein translated by the mitochondrial machinery of the bloodstream stage (Lai et al., 2008). Since the A6 protein was so far not identified even in the procyclics, no final conclusion can be reached regarding its presence or absence in the bloodstreams, as the protein may either not enter this type of gel, or may co-migrate with the cytosolic proteins.

Although RNAi was less efficient in the 920 bloodstream cells, and double stranded RNA was to some extent synthesized due to leaky transcription, elimination of the targeted RH mRNA occurred (Fig. 1A; right panel). The consequent decrease of the RH protein, however, did not result in a growth phenotype under standard conditions even after 14 days (data not shown). Due to the failure of the labeling assay to visualize mitochondrial synthesis in the bloodstream stage, no assay is currently available that would allow following organellar protein synthesis in this life cycle stage.

We conclude that under standard cultivation conditions the RH protein, predicted by mass spectrometry to be a putative subunit of the *T. brucei* mitochondrial ribosome, is non-essential for translation in the procyclic and bloodstream stages. Since in the *Escherichia coli* ribosome orthologue of this protein is involved in the assembly of 50S ribosomal subunit, and its deletion causes a slow growth phenotype at low temperature (Charollais et al., 2003; Iost and Dreyfus, 2006), the possibility that RH is needed in the metacyclic stage or only under stress conditions remains open and shall

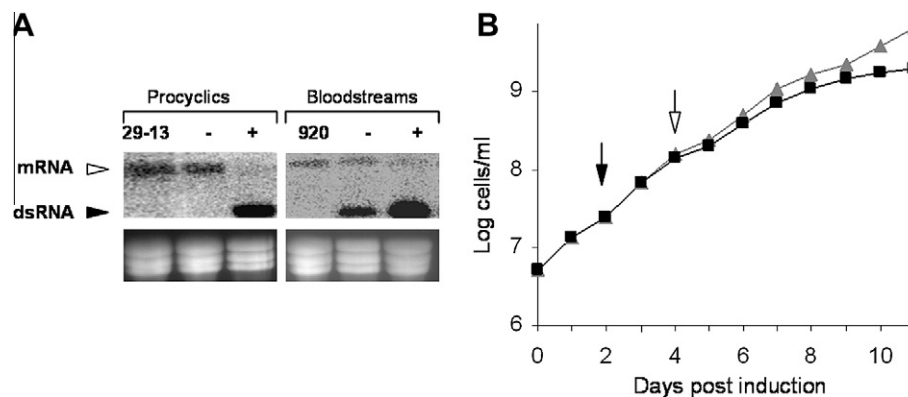


Fig. 1. Effect of RH RNAi on mRNA levels and cell growth. (A) RH mRNA levels were analyzed by blotting total RNA extracted from the parental *T. brucei* procyclics (29–13), non-induced RH RNAi cells (–), and cells 48 h of RNAi induction (+). The position of the targeted mRNA and the dsRNA synthesized following induction are indicated with arrows. As a control, the gel was stained with ethidium bromide to visualize rRNA bands. (B) Numbers of non-induced (gray triangles) and RNAi-induced RH procyclics (black squares) are shown. Cell densities were measured daily using a Beckman Z2 Coulter. Black and white arrow indicate time points at which RNA was sampled and translation assay was performed, respectively.

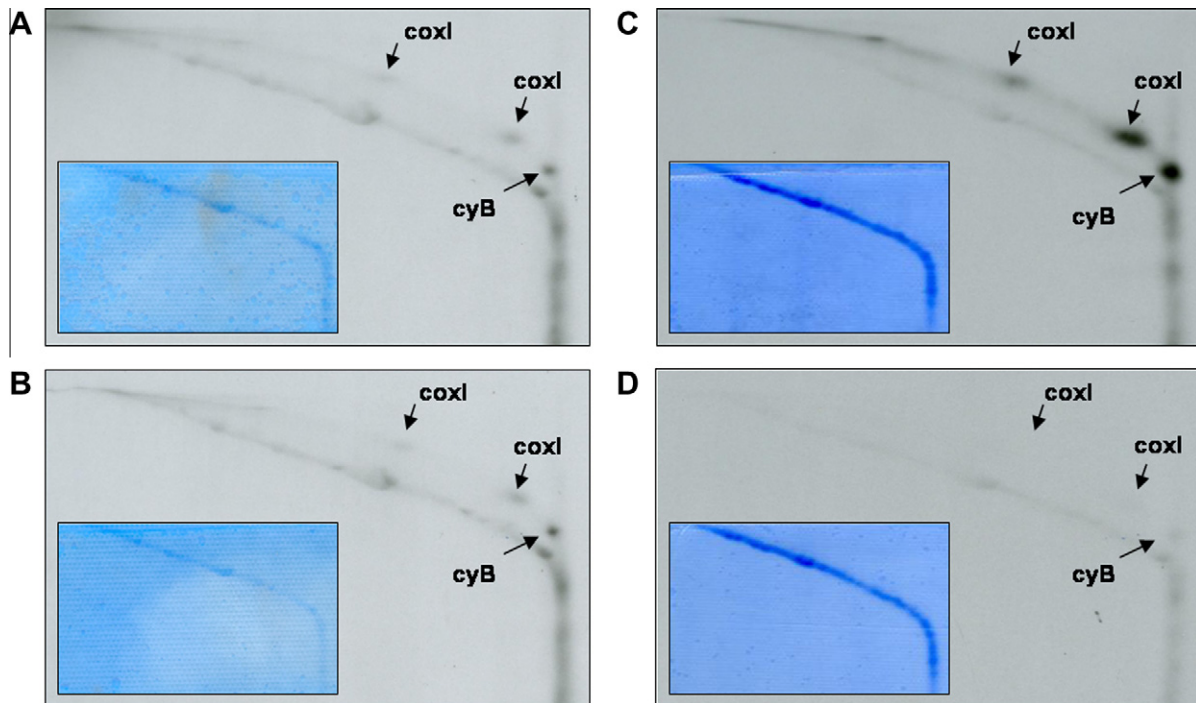


Fig. 2. Analysis of mitochondrial translation products in non-induced procyclics and those interfered against RH and mtRNAP. 5×10^7 cells harvested at day 4 after RNAi induction were labeled for 1 h with EXPRESS³⁵S labeling mix (PerkinElmer Life Sci.) in the presence of 100 μ g/ml cycloheximide, and the products were fractionated in 9.5%/14% two-dimensional SDS gels following a protocol described elsewhere (Horváth et al., 2000a). Insets show the corresponding Coomassie Brilliant Blue R250-stained gels. Arrows indicate the ³⁵S-labeled *de novo* synthesized cytochrome oxidase subunit I (coxI) and cytochrome *b* (cyB) visualized by autoradiography, which migrate off the main protein diagonal. (A) Non-induced RH cells; (B) RH cells at day 4 of RNAi induction; (C) Non-induced mtRNAP cells; (D) MtRNAP cells at day 4 of RNAi induction.

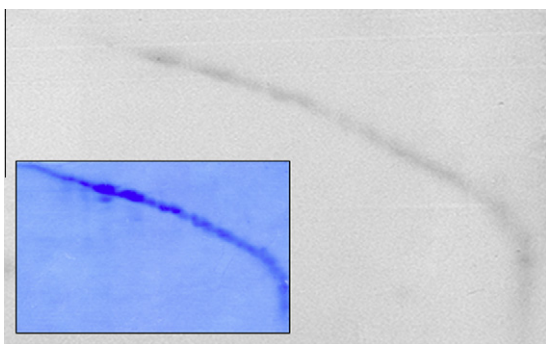


Fig. 3. Analysis of mitochondrial translation products in *T. brucei* 920 bloodstream cells. 5×10^7 cells cultivated in the HMI medium at 37 °C were labeled as described above for the procyclics with the following modification. Since the bloodstreams lose viability in the SoTE buffer, the labeling was performed in the cultivation medium and the autoradiograph was developed after 3 weeks in -80 °C.

be tested in subsequent studies. Alternatively, the protein may be required for some step(s) in ribosome assembly, but not for the actual translation.

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