



## RNA editing and mitochondrial activity in promastigotes and amastigotes of *Leishmania donovani* <sup>☆,☆☆</sup>

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### ABSTRACT

Kinetoplast maxicircle DNA sequence organisation was investigated in *Leishmania donovani*, strain 1S LdBob. Gene arrangement in the coding (conserved) region of the maxicircle is collinear with that of most trypanosomatids, with individual genes showing 80–90% nucleotide identity to *Leishmania tarentolae*, strain UC. The notable exception was an integration of a full-size minicircle sequence in the ND1 gene coding region found in *L. donovani*. Editing patterns of the mitochondrial mRNAs investigated also followed *L. tarentolae* UC patterns, including productive editing of the components of respiratory complexes III–V, and ribosomal protein S12 (RPS12), as well as the lack of productive editing in five out of six pan-edited cryptogenes (ND3, ND8, ND9, G3, G4) found in these species. Several guide RNAs for the editing events were localised in minicircles and maxicircles in the locations that are conserved between the species. Mitochondrial activity, including rates of oxygen consumption, the presence and the levels of respiratory complexes and their individual subunits and the steady-state levels of several mitochondrial-encoded mRNAs were essentially the same in axenically grown amastigotes and in promastigotes of *L. donovani*. However, some modulation of mitochondrial activity between these developmental stages was suggested by the finding of an amastigote-specific component in complex IV, a down-regulation of mitochondrial RNA-binding proteins (MRP) and MRP-associated protein (MRP-AP) in amastigotes, and by variations in the levels of RPS12, ND3, ND9, G3 and G4 pre-edited transcripts.

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### 1. Introduction

The life cycle of dixenous *Trypanosoma* and *Leishmania* spp. includes two types of hosts (vertebrate and invertebrate) which represent drastically different types of environments. Adaptations that evolved in *Trypanosoma brucei* to meet the challenges for survival and propagation in its hosts include well documented reversible changes in mitochondrial metabolism, and are accompanied by far less understood changes in mitochondrial gene activity, including editing of mRNA (recently reviewed by Hannaert et al., 2003; Lukeš et al., 2005; Stuart et al., 2005; Bringaud et al., 2006; Fenn and Matthews, 2007). Thus, while insect stage trypanosomes have a fully functional oxidative phosphorylation system, in blood-stream trypanosomes the cytochrome *c* oxidase and *bc*<sub>1</sub> complexes are no longer present and the function of ATP synthase functions to

hydrolyse ATP in order to maintain a transmembrane potential (Clarkson et al., 1989; Bienen et al., 1991; Schnauffer et al., 2005; Vertommen et al., 2008). On the contrary, relatively little is known about changes in mitochondrial activity that might accompany differentiation of *Leishmania* during the insect (promastigote) and mammalian (intracellular amastigotes) stages of the life cycle. An early report indicated that promastigotes and lesion-derived amastigotes in *Leishmania mexicana* had similar oxygen uptake rates and sensitivity to inhibitors of the respiratory enzymes, indicating that oxidative phosphorylation was active in both developmental stages (Hart et al., 1981). The current view is generally that the metabolic changes between the stages of *Leishmania* are much less pronounced compared with *T. brucei* (Oppenheimer and Coombs, 2007). Although a number of genes with stage-specific expression patterns were identified earlier (Bahr et al., 1993; Joshi et al., 1993; Charest and Matlashewski, 1994; Kar et al., 2000; Nugent et al., 2004; Walker et al., 2006), the recent genome-wide transcriptome and proteome analyses revealed that only ~3.5% of genes demonstrate a stage-specific expression pattern (Holzer et al., 2006; Leifso et al., 2007; Morales et al., 2008).

There is still some controversy with respect to the presence and function of NADH dehydrogenase (complex I) in the respiratory chain at any stage in these organisms (Santhamma and Bhaduri,

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1995; Bermúdez et al., 1997; Oppendoes and Michels, 2008). The investigated nuclear and mitochondrial genomes of several *Leishmania* species encode subunits of this complex (Simpson et al., 1998; Hertz-Fowler et al., 2004; Peacock et al., 2007), although expression of these genes has not yet been verified. In spite of the presence of these genes, the complex could not be visualised by Blue Native gel analysis of mitochondrial lysate from promastigotes of *Leishmania amazonensis* and *Leishmania tarentolae* (Maslov et al., 2002). Moreover, complex I is not detected in *L. tarentolae* promastigotes by in gel activity staining or in vitro NADH-ubiquinone oxidoreductase activity measurements (A. Horváth, personal communication). Finally, a disruption of productive editing of several genes due to the loss of minicircle-encoded guide RNAs did not affect the viability of *L. tarentolae* cells in culture (Thiemann et al., 1994). These results suggest that, if this complex is indeed present, it is a small amount and/or is dispensable for proliferation of promastigotes, at least in culture. This also leaves open the possibility that the complex might be required in amastigotes or in some other life cycle stages, such as metacyclic promastigotes.

The mitochondrial genetic system, including RNA editing, has been well characterised only in one species of *Leishmania*, *L. tarentolae*, a parasite of geckos. Unfortunately, this organism has not been propagated as amastigotes, and that renders it unsuitable for investigation of mitochondrial adaptations during the life cycle. However, axenic cultivation of amastigotes of several human pathogenic species is possible by exposing the organisms to acidic pH and increased temperature (Bates, 1993). One such strain is a clonal derivative (LdBob) of the *Leishmania donovani* strain 1S (MHOM/SD/62/1S-Cl<sub>2</sub>) (Joshi et al., 1993; Goyard et al., 2003). This species represents an important agent of human visceral leishmaniasis in the Old World. In addition, a large collection of minicircle sequences from this and closely related species (*Leishmania infantum*, *Leishmania chagasi*) is already available in public databases and can be used to search for guide RNAs once the sequences of homologous pre-edited and edited maxicircle mRNA sequences are determined. The work presented below describes the initial characterisation of the mitochondrial genome and RNA editing of *L. donovani* 1S LdBob, as well as an investigation of the possibility of mitochondrial gene regulation during the life cycle of these parasites.

## 2. Materials and methods

### 2.1. *Leishmania* cultures and isolation of mitochondria

Promastigotes of *L. donovani* 1S clonal line LdBob were grown at 26 °C in M199 medium, while amastigotes of the same strain were cultivated at 37 °C with 5% CO<sub>2</sub> in the 'amastigote' medium as described previously (Goyard et al., 2003). Promastigotes of *L. tarentolae* UC strain were cultivated in brain heart infusion medium supplemented with 10 µg/ml hemin (Simpson and Braly, 1970). Mitochondria from all types of cells were isolated by hypotonic lysis followed by Renografin density gradient centrifugation (Braly et al., 1974). Typically 1–2 L cultures with cell densities of 20–40 × 10<sup>6</sup> cell ml<sup>-1</sup> were used to obtain ~0.5 g of isolated mitochondria (wet weight).

### 2.2. Measurement of respiration rate

Oxygen uptake by *L. donovani* cells was measured with a biological oxygen monitor, YSI 5300, equipped with the YSI 5331 oxygen probe. The rate (expressed as µmol of O<sub>2</sub> consumed per cell per min) was calculated by assuming the oxygen content of air-saturated Ringers solution of 0.227 mM at 28 °C or 0.2 mM at 37 °C (according to the YSI 5300 manual). KCN was used at 1 mM, and

salicylhydroxamic acid (SHAM) at 0.1 mM. The cell concentration was 20–30 × 10<sup>6</sup> ml<sup>-1</sup> for promastigotes and 2–6 × 10<sup>6</sup> ml<sup>-1</sup> for amastigotes.

### 2.3. Protein electrophoretic and immunochemical procedures

Samples were analysed by single-dimension Tris–glycine SDS–polyacrylamide gels (Laemmli, 1970) and two-dimensional Blue Native/Tris–tricine SDS–polyacrylamide gels (Schägger et al., 1994). Gel loading was normalised by using 70 × 10<sup>6</sup> cells or 50 µg of mitochondrial proteins, as appropriate. The resolved polypeptides were transferred onto nitrocellulose membranes by semi-dry blotting, as described previously (Horváth et al., 2000). After electrophoresis, the gels were stained either with Coomassie Brilliant Blue R250 (Sigma) or SYPRO Ruby (Molecular Probes). For autoradiography, dried gels were exposed to low energy screens and analysed by using the PhosphorImager (Molecular Dynamics). Antibodies used were described previously: mouse antibody against *L. tarentolae* Rieske iron–sulphur protein of cytochrome bc<sub>1</sub> (Neboháčová et al., 2004), the rabbit antibodies against *L. tarentolae* subunit IV of cytochrome c oxidase (Maslov et al., 2002), *L. tarentolae* p18 protein (subunit b of mitochondrial ATP synthase) (Bringaud et al., 1995), *L. tarentolae* MRP1/2 complex (formerly named Ltp26/28 complex) (Aphasizhev et al., 2003). Rabbit polyclonal antibodies against *Leishmania major* adenylate kinase 2 (AK2) were provided by D. Nierlich (University of California – Los Angeles, Los Angeles, USA). Mouse antibody against the amastigote-specific protein A2 family (Zhang et al., 1996) was provided by S. Beverley (Washington University, Missouri, USA). Western blots were processed by using the SuperSignal West Pico chemiluminescent system (Pierce).

### 2.4. DNA isolation, cloning and sequence analysis procedures

Kinetoplast DNA was isolated by sedimentation through a CsCl cushion as described previously (Simpson and Berliner, 1974). The maxicircle component was purified after release from kinetoplast DNA networks by digestion with EcoRI followed by equilibrium centrifugation in CsCl–Hoechst 33258 gradients (Simpson, 1979). In the initial stage of the project, the maxicircle DNA was randomly sheared to fragments of average size of 3 kb using a GeneMachine HydroShear, a computer-controlled repetitive syringe-driven device with an occlusion of about 10 µm (Thorstenson et al., 1998). The sheared fragments were cloned into the pCR2.1 vector (Invitrogen) using standard procedures. Subsequently, oligonucleotide primers were designed based on the sequence conservation between *L. donovani* and *L. infantum* and used to amplify several missing regions of the maxicircle. Gaps between the contigs were joined using direct sequencing of the respective PCR products. The final sequence was assembled using the ContigExpress program of Vector NTI (Invitrogen). Both strands of the maxicircle DNA were sequenced. The sequences were analysed using Vector NTI programs. Guide RNA genes were searched for using the UWGCG program BESTFIT as previously described (Simpson et al., 1994; Maslov and Simpson, 2007).

### 2.5. RNA isolation and RT-PCR

Total cell RNA was isolated by Trizol extraction (Invitrogen) following the manufacturer's protocol. The same protocol was used for isolation of RNA from purified mitochondria. The isolated RNA was additionally treated with RNase-free DNase I (La Roche). cDNA was synthesised and amplified using SuperScript™ III One-Step reverse transcriptase (RT)-PCR System (Invitrogen). The amplification conditions included cDNA synthesis at 45 °C for 45 min, initial denaturation at 94 °C for 2 min, followed by 40 cy-

cles composed of incubations at 94 °C for 15 s, 45 °C for 30 s and 65 °C for 1 min 30 s, concluding with incubation at 65 °C for 5 min. The oligonucleotides used for the amplification of *L. donovani* mitochondrial mRNA are listed in Supplementary Table S1. The amplified DNA was resolved in agarose gels, appropriate size products were extracted from electrophoretic gels using a QIAquick gel extraction kit (Qiagen), and cloned in a pT7Blue plasmid vector (EMD Bioscience). The recombinant plasmids were purified using a PureLink™ Quick Plasmid Miniprep kit (Invitrogen). DNA was sequenced by the UC Riverside Institute for Integrative Genome Biology Core Instrumentation facility (Riverside, California, USA) with an Applied Biosystems 3730xl DNA Sequencer.

## 2.6. Quantitative RT-PCR

The oligonucleotide primers were designed using Integrated DNA Technologies' Primer Quest online design tool (<http://bio-tools.idtdna.com/Scitools/Applications/Primerquest/>). Sequences of primers can be found in Supplementary Table S1. Four micrograms of total RNA were reverse transcribed by using random hexamers and SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen) according to the manufacturer's protocol. Quantitative RT-PCR was performed by using an ABI Prism 7900HT sequence detection system (Applied Biosystems) and SYBR Green detection (QuantiTect SYBR Green PCR Kit, Qiagen) in triplicate according to the manufacturer's protocols. The PCR was performed as a 15-min step at 94 °C and 50 cycles of 94 °C for 15 s and 57 °C for 1 min, ending with a slow heating step from 55 to 95 °C to generate the melting curve data. cDNA levels in each sample were calculated using standard curves generated from serial fivefold dilutions of pooled RNA. Each sample of cDNA was analysed separately and normalised to endogenous controls, such as the *L. donovani* 24S $\alpha$  ribosomal RNA gene (designated Ld24S) and the *L. infantum* 40S subunit ribosomal protein S12 gene (designated Li40Srp). The data are presented as a scattergram with the mean and the standard errors indicated on the graph. The ANOVA *t*-test was performed using Statview (version 5.0) (Abacus Concepts, Berkeley, CA) to compare differences among groups in gene expression. The differences were considered statistically significant at  $P < 0.05$ . The final data are expressed as an average relative expression of amastigotes versus promastigotes.

## 3. Results

### 3.1. Mitochondrial gene organisation

The determined sequence of the maxicircle kinetoplast DNA of the strain *L. donovani* 1S LdBob covers almost the entire coding region of the molecule. The gene order with the 12S rRNA gene on one side and the ND5 gene on the other is collinear with that found in other trypanosomatids (Simpson et al., 1998). Gene sequences in *L. donovani* were, as expected, highly similar to *L. tarentolae*, with the nucleotide identity levels averaging close to 90%. Sequence conservation also included small pre-edited regions of 5'-edited and internally edited mitochondrial cryptogenes, such as ND7, COIII, Cyb, COII and MURF2, found in the same positions in both species. The high genomic sequence conservation extended to extensively edited pan-edited cryptogenes identifiable as intergenic G-rich regions: G1 (=ND8), G2 (=ND9), G3, G4, G5 (=ND3) and G6 (=RPS12), as well as the extensively edited 5'-region of A6, which were ~80–90% identical, with RPS12 and A6 being the most conserved.

The only notable exception from the overall highly conserved architecture of the determined sequence was a full-length minicircle insertion (positions 7913–8697 of GenBank™ entry FJ416603)

found in the 3' region of the ND1 gene (see Supplementary information). The insertion is 99.1% identical to one of the known minicircle sequences from *L. infantum* (GenBank™ L19877) (Tripp et al., 1993). The putative integration sites on both molecules do not have any significant sequence similarity, leaving no obvious clue as to what might have triggered this recombination event. Such inserts have not been observed in other trypanosomatids studied to date. Moreover, PCR amplification and size analysis of the respective maxicircles region from *L. donovani* strains KA-Jeddah, THAK35 (LG10), GILANI (LG12), and *L. infantum* strains LPN114 (LG2), PM1 (LG3), IMT260 (LG6) (Lukeš et al., 2007), as well as a partial DNA sequencing of the amplicons from the first two of these, showed that the minicircle insert is absent in these strains (see Supplementary information). Therefore, this insert represents a peculiar feature of the strain 1S LdBob.

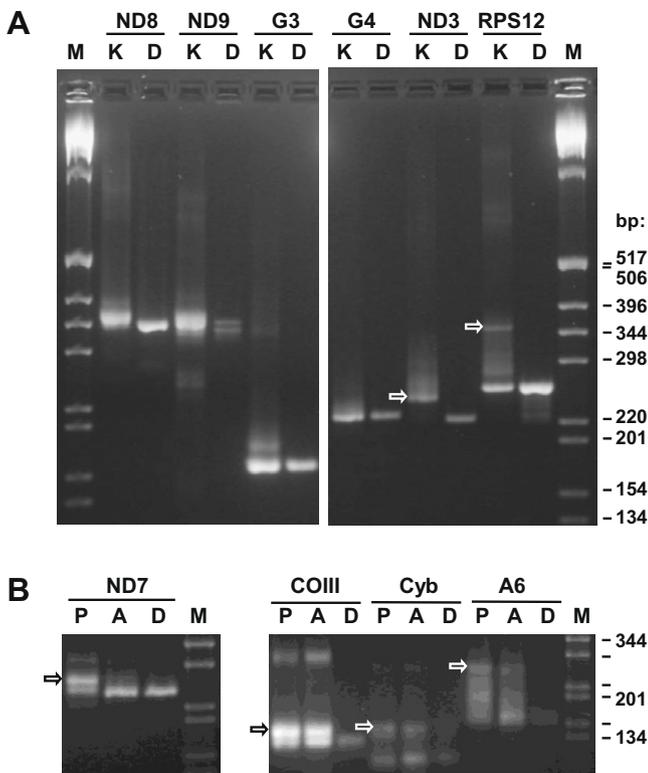
The integrated minicircle has all typical features including conserved sequence blocks (CSB) 1–3, a discernable bend sequence region and a guide RNA gene that is properly positioned with respect to the CSB-3 region (Ray, 1989; Simpson, 1997). By showing a match with the edited sequences of *L. tarentolae*, this guide RNA might represent gND9-XIV. However, this could not be verified, because the cognate edited sequence is not available (see below).

The insertion interrupts the open reading frame (ORF) of the ND1 gene at the position 51 amino acids upstream of the conserved termination codon. Instead, the reading frame continues from the upstream coding sequence into the minicircle sequence for 92 amino acid residues and then terminates at a TAA codon. It is not known whether the resulting polypeptide product is functional. An ND1 gene product has not yet been detected in any trypanosomatid species. It also remains to be investigated how this insertion affects the processing of the ND1 mRNA.

### 3.2. Editing of mRNA

RNA editing patterns of several maxicircle transcripts have been investigated in promastigotes by RT-PCR amplification, cloning and sequencing using the experimental approach summarised previously (Simpson et al., 1996; Maslov and Simpson, 2007). In brief, the putative pre-edited regions were delineated by sequence comparison with maxicircle DNA from *L. tarentolae*. Oligonucleotide primers flanking pre-edited regions were used to obtain cDNA clones representing a mixture of pre-edited and various edited molecules. Edited sequences were aligned according to the known 3'-to-5' progression of editing, and an edited sequence consensus was derived whenever possible. When translation of an edited consensus yielded a conserved amino acid sequence, that consensus was regarded as representing a mature (fully) edited mRNA pattern.

The fact that the editing leads to a noticeable net size increase of the mRNA allows identification of a sub-population of the PCR products enriched in edited cDNA molecules (Fig. 1). Edited RPS12, COIII, Cyb, ND7 and A6 cDNA molecules (Fig. 1A and B, arrows) were present in a relatively large amount among the amplified products, and the respective consensus edited patterns could be easily derived from the cloned sequences. Because the edited patterns and the respective derived amino acid products in each case were highly conserved with those in *L. tarentolae*, these patterns most likely represented functional mature edited mRNAs (Fig. 2A–E). A non-canonical initiation codon AUU (Ile) is present in the edited RPS12 mRNA of *L. donovani* instead of a canonical AUG (Met) observed in *L. tarentolae* (Fig. 2A). Putative non-canonical initiation codons were observed previously (Shaw et al., 1988; Landweber and Gilbert 1993; Maslov et al., 1999), although experimental proof of their utilisation is still required. Nonetheless, it is interesting to note that a conserved in-frame AUU is also observed



**Fig. 1.** Reverse transcriptase-PCR analysis of RNA editing of several mitochondrial transcripts in promastigotes and axenic amastigotes of *Leishmania donovani*. (A) Amplification of mRNA derived from pan-edited cryptogenes ND8 (G1), ND9 (G2), G3, G4, ND3 (G5) and RPS12 (G6) using RNA isolated from purified kinetoplast-mitochondria of promastigotes, K. Control amplifications of the respective gene regions, D, were used to identify pre-edited cDNA products. Arrows indicate bands with the sizes expected for fully edited products that were used for cloning and sequencing. A 1 kb DNA ladder (Invitrogen) was used as a size marker, M. The amplified products were resolved in 3% NuSieve agarose gels. (B) Amplification of mRNA derived from 5'-edited and internally edited mRNA encoded by the genes ND7, COIII, Cyb and A6 using total cell RNA isolated from promastigotes (P) and axenically grown amastigotes (A).

in the *L. tarentolae* RPS12 mRNA sequence upstream of the AUG codon (Fig. 2A).

In the cases where a fully edited cDNA amplicon could be obtained by standard RT-PCR, this amplicon was observed with both promastigote and amastigote total cell RNA, although some difference in the relative amount was noticed for ND7 and, to a lesser extent, A6 (Fig. 1B). In the case of fully edited RPS12, efficient amplification of the full-length transcript (Fig. 1A) was only possible using mitochondrial mRNA.

Several putative guide RNA genes were identified using these edited mRNA patterns. Genes for both guide RNAs required for Cyb mRNA editing (gCyb-I and gCyb-II) and one gene for ND7 mRNA editing (gND7-I) were located in identical maxicircle loca-

tions as in *L. tarentolae* (Blum et al., 1990) and *C. fasciculata* (Van der Spek et al., 1991). The second guide RNA gene for ND7 editing (gND7-II) is expected to localise in a conserved position upstream of the 12S gene outside of the sequenced region. This was confirmed by investigating the respective locus of the maxicircle sequence from a closely related species, *L. infantum* (S. Beverley, personal communication). For each mRNA, the editing pattern observed is highly similar to that of *L. tarentolae* in (Fig. 2B and D).

A maxicircle-encoded putative COII guide RNA gene is identifiable at the 3'-end of the gene (not shown), as in other species, assuming that the edited COII pattern is as in *L. tarentolae*. Similarly, at least one of the two maxicircle-encoded MURF2 guide RNA genes (gMURF2-II), based on comparison with the edited *L. tarentolae* MURF2 mRNA sequence (not shown), is found in a conserved position.

We searched for minicircle-encoded guide RNAs using the edited mRNA sequences from *L. donovani* determined in this paper and minicircle DNA sequences from *L. donovani* and the closely related spp., *L. infantum* and *L. chagasi*, which are available from GenBank™. The identified genes included block I (GenBank™ entry AJ010082) and block II (X98347) guide RNAs for A6 mRNA editing (Fig. 2E), as well as guide RNA block III (AJ010079) and block V (multiple entries including AF167712–AF167717) of RPS12 mRNA (Fig. 2A). In each case a single guide RNA gene is located in a respective minicircle molecule downstream of the CSB-3 sequence (5'-GGGGTTGGTGA) at a distance of ~0.45 kb on the opposite strand, in an arrangement that is similar to that in minicircles of *L. tarentolae* (Simpson, 1997). It is interesting to note that the extent of guide RNA overlaps and positions of block boundaries closely parallel those observed in *L. tarentolae* (Maslov and Simpson, 1992).

To date, only pre-edited and minimally edited transcripts were found for the pan-edited cryptogenes, ND8, ND9, G3 and G4, in cultured promastigotes and amastigotes of *L. donovani*. Upon completion of editing, these transcripts are nearly double the size of pre-edited transcripts in the *L. tarentolae* LEM125 strain (Thiemann et al., 1994; Gao et al., 2001) and the same size increase is expected for *L. donovani*. However, no prominent cDNA band with such a significant size increase was found among the RT-PCR products even when purified mitochondrial RNA was used as template (Fig. 1A). The partially edited sequences obtained contained a variety of patterns that could not be reduced to a consensus (not shown). An interesting exception from this was ND3. It can be seen in the gel representing the respective cDNA products that most of those (Fig. 1A, arrow) are noticeably larger than the size of the respective pre-edited molecule. Cloning and sequencing showed that these transcripts contained an identical short editing pattern at the very 3'-end, apparently representing a single block of editing (Fig. 2F). The respective maxicircle-encoded guide RNA gene (gND3-I) was found in the conserved position upstream of the 12S rRNA gene. Various editing patterns were seen further upstream of the correctly edited block I, and one of these patterns was attributable to mis-editing by the putative gND9-XIV guide RNA encoded by

**Fig. 2.** Sequences of edited mitochondrial mRNA in *Leishmania donovani* (Ld) and their comparison with respective transcripts of *Leishmania tarentolae* (Lt). Genomically encoded nucleotides are shown in upper case, inserted uridylates with lower case u, and deleted residues are indicated with asterisks. Complete editing sequence patterns are underlined. Gaps (shown with dashes) were introduced for the purpose of aligning the sequences. The predicted amino conserved acid sequences from both species are shown in boldface. The identified guide RNA molecules (mc, minicircle encoded; Mc, maxicircle encoded) are shown above the edited sequences of *L. donovani* with canonic bp shown with vertical lines (|) and G–U bp with colons and A–C mismatches with dots. The 5' and 3' ends of the *L. donovani* transcripts are not mapped. (A) The entire edited region of the pan-edited mRNA of mitochondrial ribosomal protein S12 (RPS12). Domain connection sequences (DCS) I and II are boxed. Notice that the translation sequence of *L. tarentolae* includes three additional conserved residues (italicised) which precede the proposed N-terminal methionine. (B) 5'-edited region and internal edited region of the NADH dehydrogenase subunit 7 (ND7) mRNA. (C) 5'-edited region of the cytochrome c oxidase subunit III (COIII) mRNA. (D) 5'-edited region of the apocytochrome b (Cyb) mRNA. (E) A mature editing pattern of the first two out of six expected editing blocks of the 5'-edited mRNA of ATP synthase subunit 6 (A6). (F) Mis-editing of a partially edited transcript of the pan-edited ND3 mRNA. The transcript is correctly edited with cognate maxicircle-encoded gND3 block I guide RNA (gND3-I) and mis-edited further upstream by gND8 block XIV guide RNA (gND8-XIV) that is encoded by the minicircle insert discovered in the maxicircle of *L. donovani* 1S LdBob.



**Table 1**

Respiration of *Leishmania donovani* amastigote and promastigote cells. The oxygen consumption is expressed as nanomole per min by  $10^6$  cells.

Cells	No inhibitor	1 mM KCN	0.1 mM SHAM
Amastigotes	0.241 ± 0.063	Not detectable	0.144
Promastigotes	0.263 ± 0.018	0.008 ± 0.007	0.239

SHAM, salicylhydroxamic acid.

mis-edited (data not shown), indicating the lack of productive editing upstream of block I.

It is still unknown whether the apparent lack of productive editing of the ND8, ND9, G3 and G4 was due to technical problems, such as an inappropriate choice of the primer sequences, or to a low amount of fully edited products. However, it is likely that this, together with the ND3 case, represents another example of the situation first observed with the old laboratory strain *L. tarentolae* UC (Thiemann et al., 1994), whence the lack of productive editing was due to the loss of respective minicircle-encoded guide RNAs.

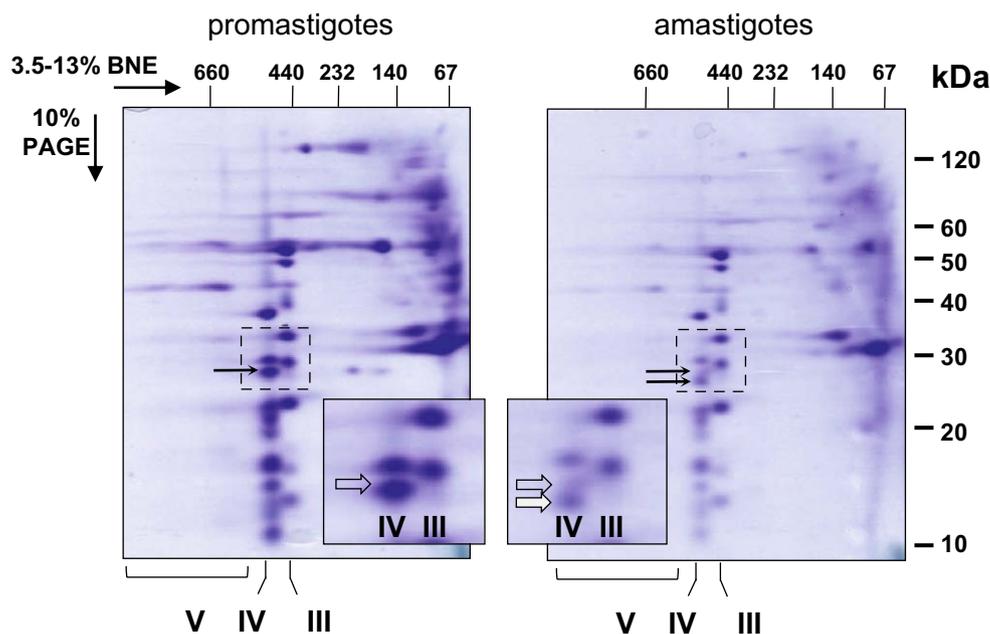
### 3.3. Mitochondrial respiratory complexes in promastigotes and amastigotes

Oxygen uptake by in vitro grown promastigotes and amastigotes was investigated with the aim to verify the presence of an active electron transport chain in mitochondria of these cell types. No substantial difference in uptake was detected, with both forms consuming approximately 0.250–0.300 nmol O<sub>2</sub> min<sup>-1</sup> per 10<sup>6</sup> cells (Table 1). Only a relatively small decrease in uptake (~16%) was observed in both forms in the presence of 0.1 mM SHAM, in line with the absence of a functional alternate oxidase in *Leishmania* (Opperdoes and Coombs, 2007). On the contrary, the respiration was completely (in amastigotes) or 95% (in promastigotes) inhibited by 1 mM KCN which, in accordance with earlier observations (Hart et al., 1981; Santhamma and Bhaduri, 1995; Bermúdez

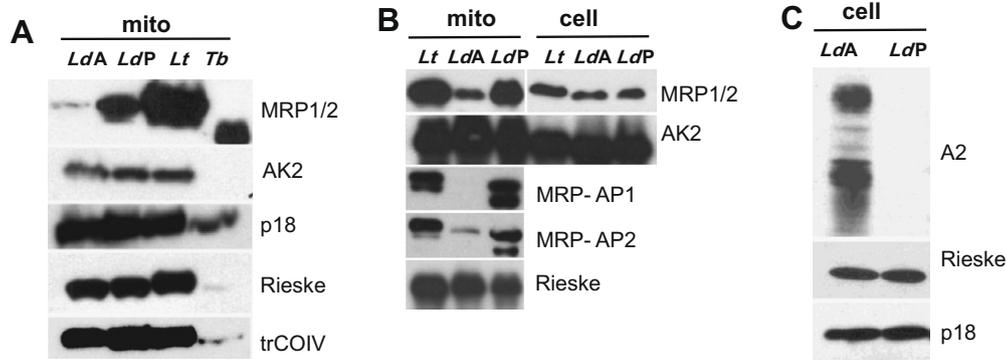
et al., 1997) indicated an exclusive role of cytochrome c oxidase in oxygen consumption.

The presence of respiratory complexes in isolated mitochondria from both types of cells was directly verified by two-dimensional Blue Native gel system (Schägger et al., 1994) (Fig. 3). The complexes observed were identified by their relative migration order in the native dimension and by characteristic subunit band patterns in the denaturing dimension, with both of these features being highly reminiscent of those observed in *L. tarentolae* and *L. mexicana amazonensis* (Maslov et al., 1999; Horváth et al., 2000; Maslov et al., 2002). The same set of complexes including cytochrome *bc*<sub>1</sub> (complex III), cytochrome c oxidase (complex IV) and ATP synthase (complex V, apparently oligomerizing) was detected in promastigotes and amastigotes. A noticeable difference between these cells was observed for the nuclear encoded subunit trCOVI of cytochrome c oxidase. This subunit represents the third largest visible band in the gel because the three mitochondrial-encoded subunits COI, COII and COIII, the largest in the complex, are virtually undetectable by staining (Horváth et al., 2000). Thus, the largest visible band is actually subunit IV (trCOIV) (Maslov et al., 2002). In amastigotes, the subunit VI band is reduced in amount relative to the other subunits of the complex. Also, a different, slightly faster migrating band appears close to the original band. It still remains to be investigated whether the new band represents a completely different polypeptide or is due to a proteolytic cleavage of the original polypeptide. Similarly, the appearance of an amastigote-specific band in cytochrome c oxidase has been observed in *L. mexicana amazonensis* (Dmitri A. Maslov, unpublished observations).

In order to estimate relative amounts of the respiratory complexes in amastigotes and promastigotes, normalised loads of proteins in whole cell and mitochondrial lysates were resolved by gel electrophoresis and probed with polyclonal antibodies against *L. tarentolae* nuclear encoded subunits trCOIV (cytochrome c oxidase), Rieske (cytochrome *bc*<sub>1</sub>), subunit b (p18) (ATP synthase) and the intermembrane space marker AK2 (Fig. 4). Expression of



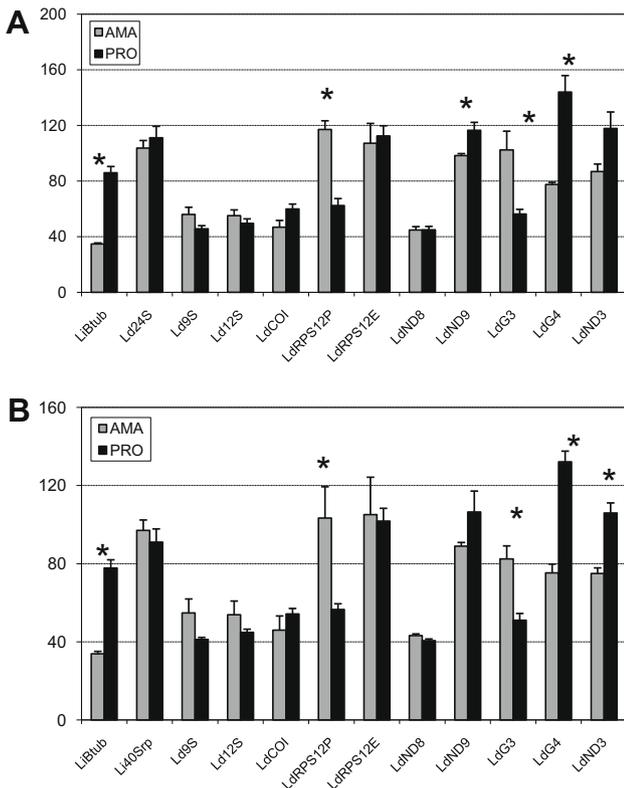
**Fig. 3.** Separation of *Leishmania donovani* respiratory complexes. Mitochondria were isolated from promastigote and amastigote cells, lysed with 1% dodecyl maltoside and fractionated in 3.5–13% gradient Blue Native/10% Tris–tricine–SDS two-dimensional polyacrylamide gel. The gel was stained with Coomassie Brilliant Blue R250. Positions of the respiratory complex V (ATP synthase), complex IV (cytochrome c oxidase) and complex III (cytochrome *bc*<sub>1</sub>) are shown below the gel. The inset panels represent the gel areas (bordered with a dotted line) containing an amastigote-specific band of complex IV (filled arrow). The promastigote-specific subunit trCOVI, which is highly reduced in amastigotes, is shown with a transparent arrow. The native gel dimension was calibrated with an HMW Native Marker Kit (GE Healthcare) and the denaturing dimension with BenchMark™ Pre-stained Protein Ladder (Invitrogen). The size markers are shown above and to the right of the gel panels.



**Fig. 4.** Protein levels of mitochondrial proteins in different trypanosomatids. (A) Mitochondrial extracts from *Leishmania donovani* amastigote (*LdA*), *L. donovani* promastigote (*LdP*), *Leishmania tarentolae* (*Lt*) and *Trypanosoma brucei* (*Tb*) cells were analysed by Western blot. The antisera used are shown to the right of each panel. (B) Mitochondrial extracts and whole cell lysates were analysed by Western blotting with antibodies against Mitochondrial RNA-binding proteins 1 and 2 (MRP1/2) and adenylate kinase 2 (AK2). In addition, mitochondrial extract were probed for MRP-AP1, MRP-AP2 and Rieske proteins. (C) Differentiation of *L. donovani* was verified by probing cell lysates for an amastigote-specific marker A2. Notice that this protein is represented by multiple bands (Charest and Matlashewski, 1994). Rieske and p18 proteins were probed as indicated. Total cell lysate loading was normalised by using the same number of cells ( $70 \times 10^6$ ) per lane and loading of mitochondrial lysates was normalised by using 50  $\mu\text{g}$  protein per lane.

the amastigote-specific A2 protein family (Zhang et al., 1996) was used as a differentiation control (Fig. 4C). No significant difference

in Western blot signal intensity was observed for the mitochondrial proteins tested, suggesting an approximately equal content of these components and, therefore, of the respective complexes in both types of cells.



**Fig. 5.** Quantitative reverse transcriptase-PCR analysis of nuclear and mitochondrial transcripts in amastigotes and promastigotes of *Leishmania donovani*. The columns represent averages of three replicates normalised to mRNA of *Leishmania infantum* cytoplasmic 40S subunit ribosomal protein S12 (A), designated Li40Srp, and *L. donovani* 24S $\alpha$  ribosomal RNA (B), designated Ld24S. Other designations for *L. donovani* transcripts are: Ld9S and Ld12S – mitochondrial ribosomal 9S and 12S RNA, respectively; LdRPS12P and LdRPS12E – pre-edited and edited mRNA, respectively, of mitochondrial ribosomal protein S12; LdCOI – cytochrome c oxidase subunit I mRNA; LdND3, LdND8 and LdND9 – subunits 3, 8 and 9 of NADH dehydrogenase mRNA; LdG3 and LdG4 – pan-edited cryptogenes G3 and G4 mRNA. LiBtub – *L. infantum*  $\beta$ -tubulin mRNA. The vertical axis shows the relative abundance of the respective mRNAs in amastigotes (grey) and promastigotes (black) with a standard error as shown. The asterisks positioned above some columns ( $\beta$ -tubulin, pre-edited mitochondrial RPS12, ND9, G3, G4 and ND3 transcripts) indicate that the observed differences in relative amount statistically significant (with  $P < 0.05$ , derived from Student's test).

3.4. RNA editing and mRNA levels in promastigotes and amastigotes

The results described above have demonstrated that mitochondria remain active in amastigotes as they are in promastigotes. To investigate whether some modulation of mitochondrial gene expression still might take place during the development of these organisms, we compared the levels of several mitochondrial transcripts by quantitative RT-PCR (qRT-PCR). Since nuclear genome expression in trypanosomatids is largely constitutive (Holzer et al., 2006; Leifso et al., 2007; Morales et al., 2008), we used two nuclear genes encoding components of the cytoplasmic ribosomes, 24S $\alpha$  ribosomal RNA of *L. donovani* (GenBank™ entry L19408) and ribosomal protein S12 of *L. infantum* (GeneDB entry LinJ13\_V3.0460). The results, presented in Fig. 5A and B, were essentially the same with both normalisation procedures. Analysis of the  $\beta$ -tubulin gene (LinJ33.0830) used as a control showed a reduced level in amastigotes, as expected (Rainey et al., 1991; Mojtabedi et al., 2008). The levels of immature (pre-edited) mRNAs encoded by six pan-edited cryptogenes were found to display various patterns. One of these transcripts (ND8) remained unchanged, three transcripts (ND9, G4 and ND3) were up-regulated in promastigotes, and two transcripts (G3 and RPS12) were up-regulated in amastigotes. Remarkably, unlike the pre-edited mRNAs, the levels of the edited RPS12 transcripts were approximately equal in both stages. Edited sequences for the other pan-edited mRNAs are not available so their levels could not be investigated. The levels of the 12S and 9S mitochondrial ribosomal RNAs also remained unchanged, as were levels of the unedited ('never-edited') COI mRNA. Thus, in the four functional transcripts tested (RPS12 edited, COI, 12S and 9S), the levels were approximately constant. The levels of edited ND7 and A6 transcripts which might differ in promastigotes and amastigotes (Fig. 1B) were not investigated.

This constant level of the edited RPS12 mRNA together with the relative decrease in the ratio of pre-edited to edited RPS12 mRNA in promastigotes, along with the differences in levels of other pre-edited mRNAs, suggest a differential stability of pre-edited transcripts in these cells. The MRP1/2 complex has been implicated in the stability of several mRNA species in *T. brucei* (Zíková et al., 2006, 2008). To this end, we compared the levels of MRP1/2 and MRP-associated proteins 1 and 2 (AP1 and AP2) proteins in prom-

astigotes and amastigotes by Western blot analysis. There was no noticeable difference in the levels of the MRP1/2 proteins in total cell lysate (Fig. 4B), however, the levels in isolated mitochondria differed significantly, with a highly reduced amount of this complex in amastigotes (Fig. 4A and B). The mitochondrial levels of AP1 and AP2 proteins were also highly reduced in amastigotes compared with promastigotes (Fig. 4B). The levels in total cell lysates could not be investigated.

#### 4. Discussion

In this work, we investigated RNA editing and mitochondrial gene expression in *L. donovani*. To our knowledge, this represents the only medically important *Leishmania* species for which such analyses have been performed to date. We also employed axenically grown amastigotes of the strain 1S LdBob to investigate the possibility of mitochondrial gene regulation during the life cycle of these cells. Our main finding was that, although there are some differences between these two life cycle stages, the investigated aspects of mitochondrial activity are largely constitutive. Thus, both forms possess cyanide-sensitive respiration that is due to the constitutive presence of cytochrome *c* oxidase (complex IV), as well as the respiratory complexes III and V. The levels of several nuclear encoded subunits tested (trCOIV of cytochrome *c* oxidase, Rieske subunit of cytochrome *bc*<sub>1</sub>, subunit b (p18) of ATP synthase) also do not show noticeable changes in amastigotes compared with promastigotes. Although we have not directly investigated levels of the mitochondrial-encoded subunits of these complexes, we have obtained indirect evidence that their expression is also likely constitutive. The evidence represents the qRT-PCR data demonstrating that the components of mitochondrial ribosomes (12S and 9S rRNA, and edited mRNA for ribosomal protein S12), which are necessary for the synthesis of mitochondria-encoded polypeptides, are constitutively expressed, as is the mRNA for mitochondrial subunit COI of cytochrome *c* oxidase.

The functionality of the mitochondrial respiratory chain in promastigotes and amastigotes of *L. donovani* is further corroborated by the presence of fully edited transcripts for the subunits of these complexes: edited COIII, Cyb and A6 are present in both stages, although we have not compared their relative levels.

The finding of an amastigote-specific band among the nuclear subunits of cytochrome *c* oxidase indicates that, in addition to its constitutive functionality, some modulation of its activity might be taking place in amastigotes.

By the contrast with the subunits of complexes III–V, the expression of some mitochondrial-encoded products, especially the putative subunits of NADH dehydrogenase (complex I), has not been convincingly demonstrated in both promastigotes and amastigotes. In general, the existence of this complex in trypanosomatids is controversial and its importance unclear (Hernandez and Turens, 1998; Allemann and Schneider, 2000; Opperdoes and Michels, 2008). It has been proposed that, when present, the trypanosomatid complex does not perform proton-pumping across the inner membrane but is only involved in regeneration of mitochondrial NAD<sup>+</sup> (Opperdoes and Michels, 2008). An enzymatically active NADH dehydrogenase complex has been detected in *T. brucei* (Fang et al., 2001) and *Phytomonas serpens* (Čermáková et al., 2007; González-Halphen and Maslov, 2004), organisms that either temporarily (bloodstream trypanosomes) (Vickerman, 1994) or permanently (*Phytomonas* spp.) (Nawathean and Maslov, 2000) lack a cytochrome-mediated electron transport chain. In these organisms, complex I might be required only at specific stages of the natural life cycle, as proposed during the transitional metabolism in short stumpy trypanosomes (Bienen et al., 1991). At other stages or in culture this complex might be fully dispensable.

The repression of cytochrome-containing complexes or the existence of viable natural mutants similar to dyskinetoplastic trypanosomes are not known for *Leishmania* (Hannaert et al., 2003; Opperdoes and Coombs, 2007), and the role of complex I is even less clear. Earlier inhibition studies indicated that this complex is absent in *L. donovani* (Santhamma and Bhaduri, 1995), albeit present in *L. mexicana* (Bermúdez et al., 1997). Yet, the nuclear-encoded homologues of complex I subunits are present in the three *Leishmania* genomes (*L. major*, *L. infantum*, *L. braziliensis*) investigated (Peacock et al., 2007). This evolutionary conservation strongly indicates that this complex is functional at least during some stages of the *Leishmania* life cycle.

It must be mentioned that the *Leishmania* natural life cycle includes a succession of three replicating stages (amastigotes, procyclic promastigotes, leptomonad promastigotes) and two non-replicating stages (nectomonad and metacyclic promastigotes) (Sacks, 2001; Gossage et al., 2003). To date, only the first two of these stages in *L. donovani* are amenable to biochemical analysis by availability in culture (Goyard et al., 2003; Debrabant et al., 2004). The Blue Native gel results presented in this paper suggest that complex I, if present in cultured promastigotes and amastigotes, must be in present in a relatively low amount compared with respiratory complexes III–V. Moreover, expression of some of its subunits seems to be impaired in the 1S LdBob strain of *L. donovani*: a minicircle insert interrupts the reading frame of ND1, and no productively edited mRNAs could be found for the ND3, ND8 and ND9 subunits. Taken together, these results indicate that complex I in *Leishmania* is fully dispensable in culture. This suggests that the lack of any selective pressure to preserve the integrity of editing cascades for the pan-edited cryptogenes, ND3, ND8, ND9 and also possibly G3 and G4, encoding components of complex I, is then responsible for the absence of the respective fully edited mRNAs in the 1S LdBob strain of *L. donovani*. This case may be analogous to the disruption of productive editing for the same set of cryptogenes that was observed in *L. tarentolae* UC (Thiemann et al., 1994) and for the ND8 cryptogene in *P. serpens* 1G (Maslov et al., 1998). It was shown that at least in *L. tarentolae* UC, these defects were caused by the loss of minicircle-encoded guide RNAs that resulted from an intrinsic randomness of minicircle segregation in the absence of a selective pressure (Simpson et al., 2000). Characteristically, more recently isolated strains of *L. tarentolae* and *P. serpens* did show productive editing of the respective mRNAs (Nawathean and Maslov, 2000; Gao et al., 2001) suggesting that the random loss of minicircle classes has not yet proceeded as far as it did in the older laboratory strains. It is remarkable that the ND7 mRNA is still productively edited with the aid of two cognate maxicircle-encoded guide RNAs. Moreover, the first block of editing in the ND3 mRNA is fully completed by a cognate maxicircle-encoded guide RNA. These results show that the potential of the enzymatic machinery to process mitochondrial-encoded complex I mRNA is intact, and when guide RNAs are still present (because they are encoded by a maxicircle) there is productive editing. Therefore, in cases when no complex I mRNA editing is found it must be due to the lack of the respective guide RNAs, as in *L. tarentolae* UC. To verify whether minicircle loss did occur in *L. donovani* strain 1S LdBob, the minicircle content of this strain needs to be investigated and compared with that of a recently isolated strain, as well as the extent of RNA editing in the latter.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpara.2008.10.015.

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