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T7 RNA polymerase-driven transcription in mitochondria of *Leishmania tarentolae* and *Trypanosoma brucei*

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Abstract

The study of RNA editing and other molecular processes in the trypanosome mitochondrion would benefit greatly from the ability to insert and express exogenous DNA in the organelle. However, even with a method to introduce DNA, the current lack of knowledge about mitochondrial transcription would hinder efforts to obtain expression. To circumvent this problem, *Leishmania tarentolae* promastigotes and *Trypanosoma brucei* procyclic cells have been transfected with bacteriophage T7 RNA polymerase targeted to the mitochondrion. Mitochondria isolated from the transfectants contained active T7 RNA polymerase, as shown by a comigration in density gradients of mitochondrial marker enzymes and T7 polymerase activity. A DNA cassette under T7 control was introduced into isolated mitochondria from the transfectants by electroporation and the DNA was shown to be transcribed. This system should allow the transcription of foreign genes of choice within the mitochondrial matrix either in a transient assay using electroporation of DNA into isolated mitochondria, or in a stable assay using cells transfected with DNA by the biolistic gun method.

Keywords: Kinetoplast DNA; *L. tarentolae*; *T. brucei*; Mitochondria; T7 RNA polymerase

1. Introduction

Techniques that would allow the insertion and expression of exogenous DNA in the mitochondrion of trypanosomatid protozoa would be of great utility for investigating this novel mitochondrial genetic system, including the molecular mechanisms involved in uridine (U) insertion/deletion RNA editing. A major impediment towards achieving this goal is our lack of knowledge

Abbreviations: Deoxyribonuclease I, Dnase; GDH, mitochondrial glutamate dehydrogenase; kDNA, kinetoplast DNA; SDH, succinate dehydrogenase; T7RNAP, bacteriophage T7 RNA polymerase.

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of both transcription and translation in this organelle.

One method to overcome the deficiency of the knowledge of the endogenous transcription system is to construct a heterologous transcription system using T7 RNA polymerase. The T7 RNA polymerase (T7RNAP) is a single 100 kDa polypeptide that recognizes an 18-nucleotide promoter and has been widely used for both in vivo and in vitro expression systems. Stable *Trypanosoma brucei* transfectants expressing T7RNAP have been obtained by electroporation and drug selection, and these cells have been shown to express T7 promoter-controlled transgenes [1]. In addition, the tetracycline repressor has also been expressed in *T. brucei* and a tightly regulated conditional expression system developed for expression of transgenes [2,3].

In yeast, the T7RNAP has been targeted to the mitochondrion by fusing the protein to a COXIV mitochondrial import peptide [4]. A target COX2 gene integrated into the mitochondrial genome was shown to be efficiently transcribed. It was decided to target T7RNAP in *Leishmania tarentolae* and *T. brucei* to the mitochondrion for use in establishing a mitochondrial expression system which would not rely on the endogenous expression apparatus.

2. Materials and methods

2.1. Cell culture and mitochondrial isolation

L. tarentolae (UC strain) cells were grown as described previously to $1.0–1.5 \times 10^8$ cells/ml [5]. *T. brucei* strain 427 cells were grown as procyclic forms at 27°C in SDM medium [6] to a density of $1.0–1.5 \times 10^7$ cells/ml. The mitochondrial fraction was isolated by hypotonic lysis followed by isopycnic centrifugation in Renografin [7] or Percoll [8] gradients. The purified mitochondrial fraction was extensively washed in 0.25 M sucrose, 10 mM Tris–HCl, pH 7.9, 1 mM EDTA, resuspended at a concentration of 100 mg/ml of mitochondria (wet weight) in the same solution containing 50% glycerol and frozen in aliquots at −80°C.

2.2. Construction of plasmids and transfection of parasites

To target the T7RNAP to the mitochondria of the parasites expression vectors able to integrate into the chromosome and containing the sequence corresponding to the first 48 amino acids of the *T. brucei* mitochondrial glutamate dehydrogenase μGDH) (accession AF095907 [3]) in front of the *T7RNAP* gene were designed. The GDH open reading frame was PCR-amplified from *T. brucei* genomic DNA using the 5′ oligodeoxynucleotide S2314 (5′-GGTCTAGATGCGCCGTGCTTCT-TACTGC-3′, *Xba* I site underlined) and the 3′ oligodeoxynucleotide S2195 (5′-GGGTCGACT-TAAGAGATGTGCTTGGCCTG-3′, *Sal* I site underlined). After digestion with *Xba* I and *Sal* I, the termini were partially filled-in using the Klenow fragment of the DNA polymerase I, dTTP and dCTP. The resulting DNA fragment was ligated into the expression vector pLew20 [9] digested with *Hin*d III and *Bam*H I and partially filled-in with dGTP and dATP. The *T7RNAP* gene was obtained from the plasmid pHD328 [10] and inserted between the *Hin*d III site present at position 815 of the *GDH* gene and the *Pst* I site present within the aldolase 3′ untranslated region of pLew20 and pHD328. The resulting plasmid, named pT7mito1 (Fig. 1), was integrated into the 18S *rDNA* spacer, a transcriptionally silent locus [9]. Procyclic trypanosomes were electroporated with *Not* I-linearized pT7mito1 and selected in the presence of 2.5 μg/ml of phleomycin as described [1]. The PARP promoter present in the parental plasmid pLew20 drives the expression of the *GDH::T7RNAP* gene fusion.

To construct the *L. tarentolae* expression vector, the ribosomal RNA small subunit gene was PCR-amplified from *L. tarentolae* genomic DNA as described [11]. The PCR product was cloned into the pCR2.1-TOPO vector (Invitrogen) and the resulting plasmid was named pLt18S. In a second step, the *Not* I–*Bam*H I fragment of pLew90 [12] containing the tetracycline repressor gene was replaced for the *Not* I–*Bam*H I fragment of pT7mito1 containing the *GDH::T7RNAP* gene to yield the intermediate plasmid pT7mito2. Finally, the *Pvu* II–*Bgl* II fragment from

pT7mito2 was replaced for the *Eco*R V– *Bam*H I insert of pLt18S, yielding the construct pT7mito3 (Fig. 1). This plasmid was integrated in the *L. tarentolae* rDNA 18S locus. Cultures at a density of 1×10^8 cells/ml were harvested for electroporation using *Nsi* I-linearized pT7mito3 and 1×10^8

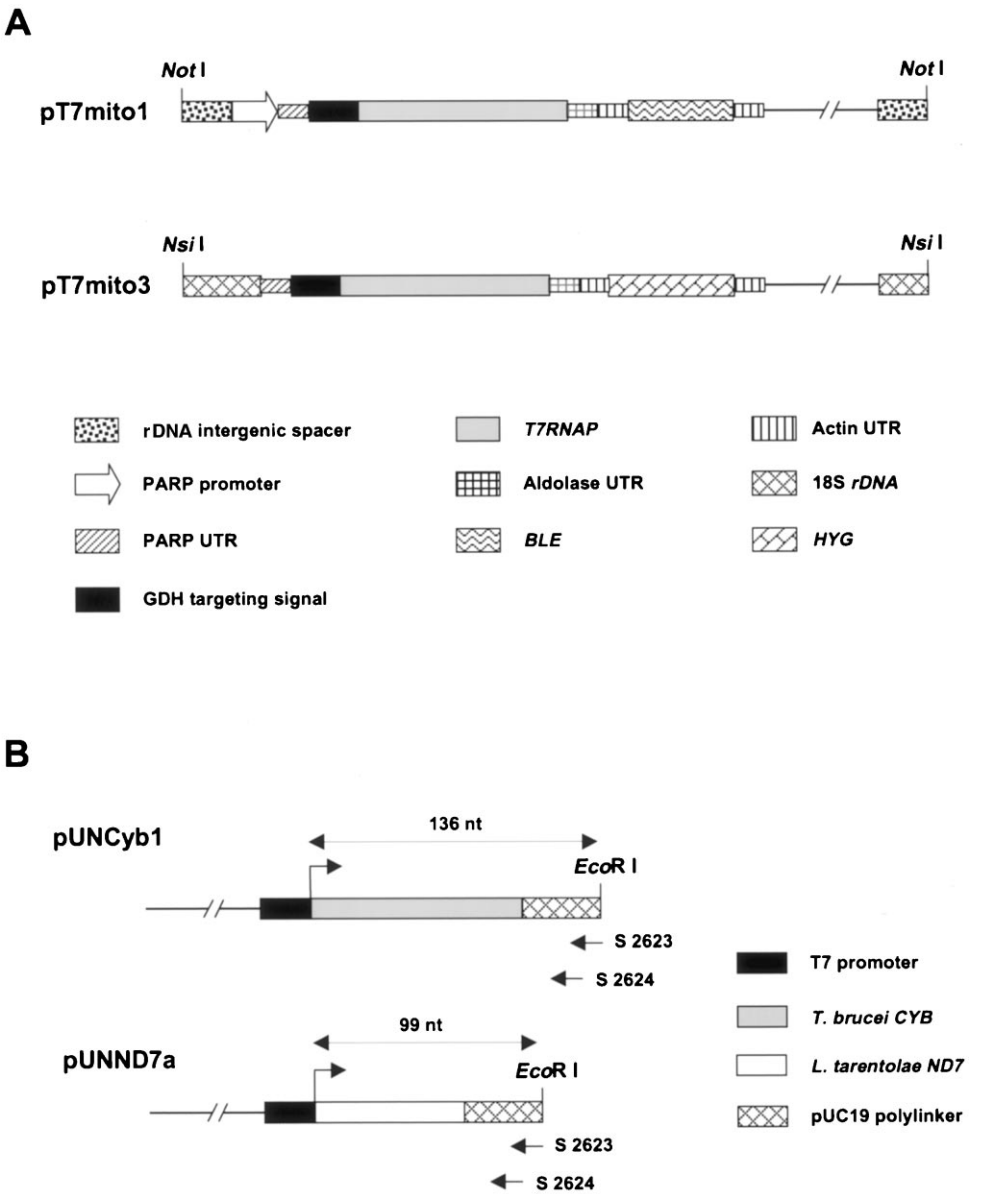

Fig. 1. Plasmid constructs used in this work. (A) plasmids used to integrate the *T7RNAP* gene in the genome of *Trypanosoma brucei* (pT7mito1) and *Leishmania tarentolae* (pT7mito3). The broken lines indicate the vector backbone. The plasmids are shown linearized at the restriction sites used for integration; PARP, procyclic acidic repetitive protein promoter; UTR, untranslated region; *BLE*, phleomycin resistance gene; *HYG*, hygromycin resistance gene. (B) Plasmids used for T7RNAP-driven transcription in isolated mitochondria; the bent arrow indicates the start of transcription by T7RNAP; the broken line represents the vector backbone. The sizes of the expected run-off transcripts are indicated above each construct.

cells per cuvette following the same protocol described for procyclic trypanosomes [1], except that the cells were washed and electroporated in ice-cold Zimmerman post-fusion medium containing glucose [13] and then selected in BHI medium [5] containing 50 μg/ml of hygromicin. The endogenous transcription of the 18S rDNA locus drives the expression of the *GDH::T7RNAP* gene in this case.

The plasmids used in the electroporation of isolated mitochondria, pUNCyb1 and pUNND7a, are also presented in Fig. 1. To make pUNCyb1 we inserted at the *Hin*c II site of pUC19 the 106 first nt corresponding to the mitochondrial *T. brucei CYB* gene preceded by a T7 promoter. Plasmid pUNND7a was constructed using the same strategy, but with the first 65 nucleotides of the mitochondrial *L. tarentolae ND7* gene.

2.3. Electroporation of mitochondria

Mitochondria from *L. tarentolae* and *T. brucei* were isolated by hypotonic lysis and fractionation on Percoll gradients [8]. Aliquots containing 5–10 mg of mitochondria (wet weight) were washed in 0.25 M sucrose, centrifuged at $11\,000 \times g$ for 5 min, resuspended in 50 μl of the same solution and subjected to electroporation in 1 mm-gap cuvettes at 360 Ω and increasing field strengths in the presence of 50 μg/ml of *Eco*R I-linearized plasmids using a BTX electroporator.

To study the effect of electroporation on plasmid internalization and mitochondrial enzymatic activities, the electroporated mitochondria were centrifuged as above and resuspended in 0.25 M sucrose. One-tenth of this resuspension was supplemented with Tris–HCl (pH 8.0) and $MgCl_2$ to 10 and 2 mM, respectively, and treated for 1 h at room temperature with 20 μg/ml of DNase to remove any non-internalized plasmid. DNase was inactivated by EDTA, heat and proteinase K treatments as described [14]. The mitochondria was lysed with 0.5% of SDS and the DNA was extracted with phenol:chloroform, ethanol precipitated, electrophoresed in a 0.8% agarose-TBE gel, transferred to a Zeta-Probe membrane (BioRad) and hybridized with a radiolabeled pUC19-specific probe (a 0.9 kb *Eco* RI–*Sca* I fragment from pUC19).

The remaining mitochondria were either lysed in the presence of 0.3% Triton X-100 for GDH determination or incubated in transcription conditions to assay for GTP-incorporation in intact mitochondria as described below.

2.4. Determination of enzymatic activities.

Succinate dehydrogenase (SDH) was assayed at 24°C as described [15]. One unit of SDH is defined as the amount of enzyme that catalyzes the conversion of 1 μmol of succinate per min. GDH was assayed as described previously [16]. T7RNAP was assayed in vitro in 50 μl of a mixture containing 40 mM Tris–HCl, pH 8.0, 8 mM $MgCl_2$, 2 mM spermidine, 25 mM NaCl, 1 μg of pBluescript SK^+ supercoiled plasmid (Stratagene), 0.5 mM of each NTP, 3 μCi of [α-^{32}P]ATP (800 Ci/mmol) and 5 μl of the sample to be tested. The reactions were incubated for 10 min at 37°C. One unit of T7RNAP is defined as the amount of enzyme that catalyzes the incorporation of 1 nmol of labeled nucleotide into acid-insoluble products per hour.

The endogenous RNA transcription in intact organelles was estimated by the amount of [α-^{32}P]GTP incorporated into acid-insoluble products essentially as described [8]. This assay was done in the absence of added DNA.

The T7RNAP activity was also measured in intact mitochondria. The mitochondrial samples were nucleotide-depleted by incubation in mitochondrial transcription buffer [8] without nucleotides for 30 min at 27°C. After electroporation with linearized pUNCyb1 (*T. brucei* mitochondria) or pUNND7a (*L. tarentolae* mitochondria), the mitochondrial suspension was split into three tubes. DNase was added to one tube at a concentration of 25 U/ml, proteinase K to a second tube at a concentration of 50 μg/ml and the third tube was left untreated. Triton X-100 (0.3% final) was added to half of each sample. After 30 min incubation on ice, nucleotides were added at a concentration of 0.5 mM and the mitochondria were further incubated at 27°C for 10 min. RNA was extracted and T7 transcripts analyzed by primer

extension [3] using a radiolabeled primer specific for the pUC19 polylinker, S2623 (5′-GAGCTCG-GTACCCGGGGATC-3′, Fig. 1). The cDNAs were loaded onto a 6% PAGE-urea gel and the extended products were isolated from the gel and subjected to 5′-anchored PCR [17] by tailing the cDNA with dGTP and terminal deoxynucleotidyl transferase. A first round of PCR was carried out using oligo(dC) as the 5′ primer and S2623 as the 3′ primer. The second round of PCR was performed using 1/100 vol. of the first PCR reaction, oligo(dC) and the 3′ oligodeoxynucleotide S2624 (5′-GGGGATCCTCTAGAGTC-3′, Fig. 1). The PCR products were loaded onto 3% agarose-TBE gels, isolated, cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced.

3. Results

3.1. Targeting of the T7RNAP to the mitochondria of T. brucei and L. tarentolae

The *GDH::T7RNAP* gene fusion was targeted to the *T. brucei* 18S *rDNA* intergenic region or to the *L. tarentolae* 18S *rDNA* locus by electroporation using the plasmids pT7mito1 and pT7mito3, respectively (Fig. 1). The correct integration of these DNA cassettes was confirmed by Southern blot analysis (data not shown).

To analyze whether T7RNAP was imported to the mitochondria, crude mitochondrial fractions from both organisms were loaded onto Renografin density gradients. After equilibrium centrifugation, fractions were collected from the bottom and assayed for the mitochondrial specific marker enzyme, SDH, and for T7RNAP (Fig. 2). The migration of both activities is identical, indicating a mitochondrial localization of the T7RNAP in these parasites. Approximately 30% of the total T7RNAP activity measured in a cell extract was localized into the purified mitochondrial fraction.

3.2. Internalization of plasmid DNA into T. brucei and L. tarentolae mitochondria

A method for the introduction of DNA into isolated mammalian mitochondria has been described [14]. This method has been adapted to introduce plasmid DNA into isolated mitochondria from *L. tarentolae* and *T. brucei*. Aliquots of mitochondria from both organisms were subjected to electroporation at different field strengths as described in Section 2. A DNase treatment was then performed to remove any non-internalized DNA. The nuclease-resistant DNA within the

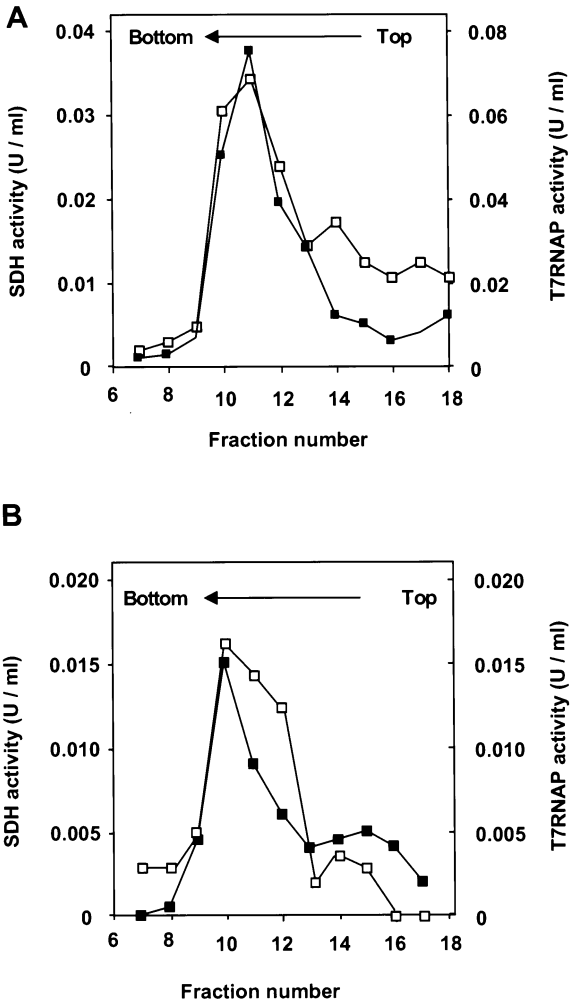

Fig. 2. Comigration of succinate dehydrogenase (SDH) and T7RNAP activities in Renografin density gradients of mitochondrial fractions from *Leishmania tarentolae* (A) and *Trypanosoma brucei* (B). Aliquots of the whole gradient were collected from the bottom, lysed with 0.3% Triton X-100 and assayed for SDH (□), and for T7RNAP (■) as described in Section 2. No T7RNAP activity was detected when a plasmid lacking a T7 promoter was used as a substrate (not shown).

mitochondria was visualized by Southern blot analysis (Fig. 3). The optimal internalization was observed at 1.0 kV for *L. tarentolae* and 1.2 kV for *T. brucei*. No apparent degradation of the plasmid could be detected and no DNA was protected from nuclease digestion in the absence of electroporation.

3.3. Effect of electroporation on the mitochondrial integrity

To analyze the extent of electroporation-induced mitochondrial breakage, the levels of the matrix enzyme, GDH, and the endogenous mitochondrial RNA transcription were determined after electroporation at different field strengths (Fig. 4). The data suggest that approximately 80% of the mitochondria were still intact up to 0.8 kV and then a rapid loss of mitochondrial integrity occurred.

3.4. In organello T7RNAP transcription

To test the functionality of the T7RNAP within the mitochondria, *Eco*R I-linearized DNA cassettes pUNCyb1 and pUNND7a (Fig. 1) were introduced in the isolated organelles by electroporation as described in Section 2. The organelles were previously depleted of nucleotides by incubation in mitochondrial transcription buffer without nucleotides [8] to avoid any T7RNAP-driven transcription before the proteinase K or DNase treatments. The proteinase K treatment should digest the T7RNAP present outside the mitochondria as the result of mitochondrial breakage. Similarly, the DNase treatment should remove any non-internalized DNA that would act as a template for T7RNAP transcription. The presence of primer-extended products after DNase or proteinase K treatments in Fig. 5 suggests that T7RNAP is functional within the mitochondrial matrix. The presence of heparin, a large charged molecule which does not penetrate intact mitochondrial membranes and is a known inhibitor of T7RNAP [18] also had no effect on this residual transcription activity (data not shown). No extension products were detected when the exogenous DNA was not included in the electroporation, when the same plasmids without a T7 promoter were used, or when plasmids containing a T7 promoter were electroporated into mitochondria from wild-type cells (data not shown). The absence of a signal in the Triton X-100 treated samples rules out incomplete DNase or proteinase K digestions. The primer extension products were gel-isolated, subjected to 5′-anchored PCR, sequenced and shown to be derived from T7RNAP transcription of the electroporated plasmids (data not shown).

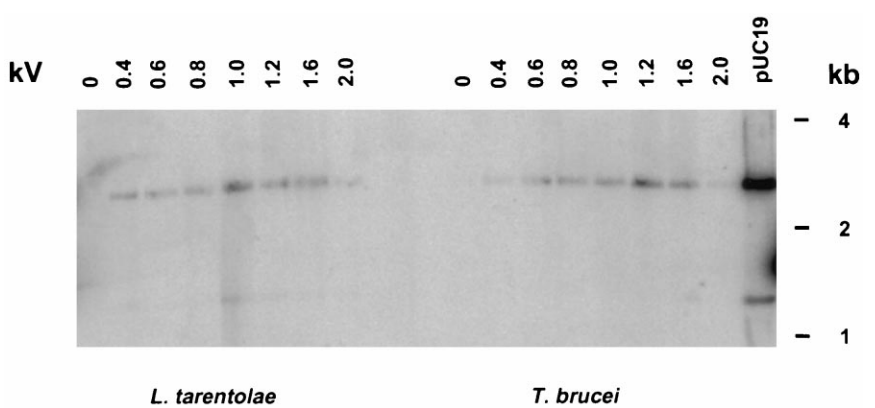

Fig. 3. Southern blot analysis of plasmid DNA introduced into *Leishmania tarentolae* and *Trypanosoma brucei* mitochondria by electroporation. Identical aliquots of mitochondria were subjected to electroporation at increasing field strengths (kV) in the presence of 50 μg/ml of *Eco*R I-linearized pUC19 plasmid as described in Section 2. A 5 ng sample of *Eco*R I-linearized pUC19 was included as a control and run in a parallel lane of the gel.

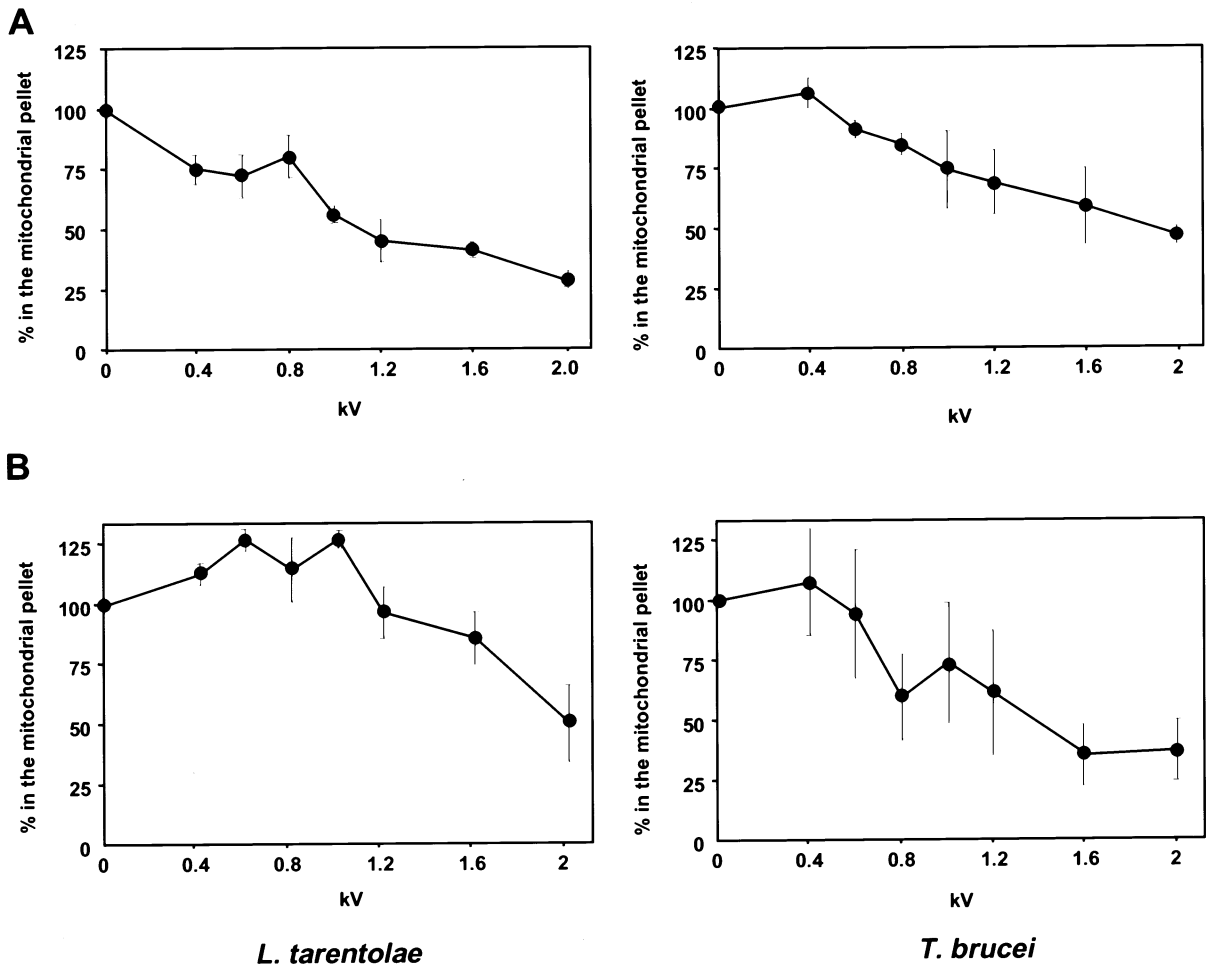

Fig. 4. Effect of electroporation on mitochondrial glutamate dehydrogenase (GDH) activity (A) and mitochondrial transcription (B). The levels of the matrix enzyme GDH and the RNA transcription were determined after electroporation at the indicated field strengths. The samples were then centrifuged and both activities assayed in the pellet as described in Section 2. The amounts were represented as the percentage relative to non-electroporated mitochondria. Mean values were calculated from three independent experiments carried out using different mitochondrial isolations.

4. Discussion

The ability to stably transfect trypanosomatids with the T7RNAP gene and to target the gene product to the kinetoplast-mitochondrion using an endogenous mitochondrial targeting signal should prove extremely useful for studies which require the expression of foreign DNA in this organelle. The presence of this RNA polymerase within the mitochondrion allows the use of the robust and well understood T7 transcription system [19] for expression and circumvents the lack of knowledge of the endogenous transcription system.

The introduction of foreign DNA into the mitochondrion both in vivo and in vitro presents additional problems. It is shown in this paper that electroporation can be used to insert DNA containing a T7 promoter into isolated mitochondria from both *L. tarentolae* and *T. brucei*, and that this DNA is expressed using the targeted T7RNAP. The effect of different electroporation

field strengths on mitochondrial integrity was assessed by studying the loss of mitochondrial matrix enzymatic activities. There is a field strength-dependent loss of GDH and endogenous RNA transcription activities, suggesting that the electroporation induces mitochondrial disruption without directly affecting the enzymatic activities, as was previously reported for mammalian mitochondria [14].

Optimization of the field strength for plasmid internalization represents a compromise with the extent of mitochondrial damage as previously observed for electroporation of mammalian mitochondria [14]. At 1.0–1.2 kV, which represents the optimal conditions for plasmid internalization, approximately 40% of the mitochondrial enzymatic activities were lost. A field strength of 0.6–0.8 kV proved sufficient to introduce significant amounts of plasmid DNA with minimal organelle damage and was adopted for the electroporation of *L. tarentolae* and *T. brucei* mitochondria.

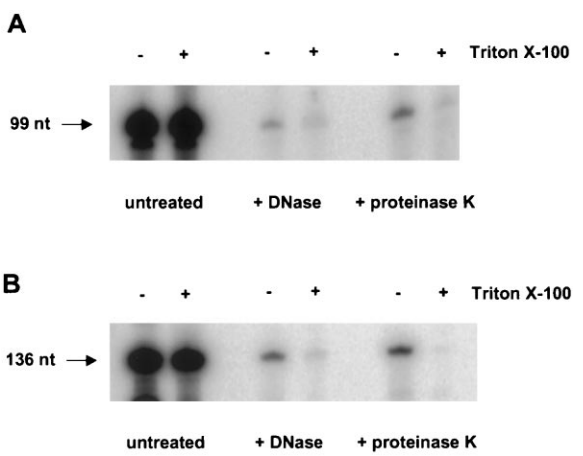

Fig. 5. In organello bacteriophage T7 RNA polymerase (T7RNAP) activity after proteinase K or DNase treatments. Nucleotide-depleted mitochondria isolated from *Leishmania tarentolae* (A) and *Trypanosoma brucei* (B) were electroporated at 0.8 kV and treated with proteinase K or DNase in intact (−) or lysed (+) mitochondria as described in Section 2. An aliquot of the electroporated mitochondria was left untreated. After incubation in mitochondrial transcription buffer [8], the RNA was isolated and subjected to primer extensions analysis using the primer S2623 (Fig. 1).

The evidence for targeting of the T7RNAP to the mitochondrion in both *L. tarentolae* and *T. brucei* includes the comigration of mitochondrial markers enzymes with T7RNAP activity in isopycnic centrifugation of isolated mitochondria, and the presence of DNase- and protease-resistance transcription of electroporated plasmid DNAs in isolated mitochondria. The observed high level of transcription in the mitochondria not treated with DNase or proteinase K is probably due to a breakdown in membrane permeability induced by the electroporation itself and/or to the known mitochondrial damage produced by the hypotonic cell rupture procedure employed [7,20,21]. Nevertheless, the presence of primer-extended products after DNase or proteinase K treatments strongly supports the conclusion that T7RNAP-driven transcription occurs within the mitochondrial matrix.

The fact that only 30% of total T7 RNA polymerase activity could be recovered in the purified mitochondrial fraction could be explained by non-quantitative recovery of mitochondria, in vivo saturation of the mitochondrial protein importation system or an inefficiency of targeting due to the use of a heterologous signal sequence. For in vivo studies of mitochondrial translation, the presence of T7 polymerase in the cytosol or nucleus could potentially pose a problem. However, introduction of the mitochondrial UGA tryptophan codon (normally a stop codon in the nucleus) in the gene designed to be expressed within the organelle should circumvent the problem of cytosolic expression. In this regard, it has been shown that the presence of the mitochondrial UGA-tryptophan codon in a CAT reporter gene reduces translation in the cytosol to 0.2% of normal levels (Alfonzo, J.D., Blanc, V., Estévez, A.M., Rubio, M.A.T., Simpson, L., unpublished results).

The introduction of T7-controlled DNA into the kinetoplast-mitochondrion in vivo should be possible using the biolistic gun technology which has proved successful for mitochondrial [22] and plastid [23,24] transformation in yeast, plants and *Chlamydomonas*. The major impediment to achieving stable mitochondrial transfectants is now the lack of knowledge of the mitochondrial

translation system which must be utilized for expression of a selectable marker. However, the ability to transcribe inserted DNA under T7 control makes this a more approachable problem.

Acknowledgements

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