Developmental Regulation of Trypanosome Mitochondrial Gene Expression*

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Mitochondrial respiratory activities in the protozoan parasite Trypanosoma brucei are developmentally regulated. The trypanosomes in the mammalian bloodstream derive ATP entirely from glycolysis. The trypanosomes found in the midgut of the insect vector or in culture at 26 °C have fully functional mitochondria with cytochrome-mediated respiration. In this paper, we show that the steady state levels of the 9 S and 12 S mitochondrial ribosomal RNAs (rRNAs) are 30-fold lower in an early developmental stage in the mammal, the slender forms, relative to the levels in the stumpy trypanosomes, a later developmental stage in the mammalian infection. Transcripts from three other mitochondrial genes, cytochrome b and subunits I and II of cytochrome oxidase, are undetectable in the slender trypanosomes and increase in the stumpy trypanosomes to levels approaching those in trypanosomes from 26 °C cultures. Transcription of other mitochondrial genes, including NADH-dehydrogenase subunit 5, is unregulated during trypanosome development. These results show that the level of some mitochondrial transcripts is developmentally regulated in bloodstream trypanosomes and suggest that the stumpy bloodstream trypanosomes accumulate mitochondrial transcripts prior to development of a functional mitochondrion. These results also show that the developmental activation of mitochondrial activities at 26 °C is not controlled at the level of mitochondrial transcription.

Trypanosoma brucei is a protozoan parasite which possesses the unique ability to developmentally regulate mitochondrial gene expression. During the complex life cycle of T. brucei, ATP production alternates between being entirely glycolytic in the mammalian bloodstream to being coupled to electron transport and oxidative phosphorylation in the insect vector (1–3). In the mammalian bloodstream, two major developmental stages can be distinguished. Slender, rapidly dividing trypanosomes predominate in the early stages of the infection, while later stages of the bloodstream infection are characterized by a high percentage of nondividing stumpy trypanosomes (4). The stumpy trypanosomes are considered to be preadapted for differentiation to the procyclic developmental stage found in the midgut of the insect vector or in vitro at 26 °C (5, 6).

The mitochondrial DNA of trypanosomes is also unique. The DNA is organized as a complex catenated network, called the kinetoplast, which contains 50 maxicircles (22 kilobase) and 5000 minicircles (1 kilobase) (7, 8). Maxicircles are the equivalent of mitochondrial DNA in other organisms (7–9), while minicircles may have a structural rather than coding function (8, 10). About 70% of the T. brucei maxicircle has been sequenced, and the genes for the mitochondrial rRNAs (11), cytochrome b (12), cytochrome oxidase subunits I and II (13–15), and NADH dehydrogenase subunits 4 and 5 (13, 16) have been identified based upon sequence homology with corresponding genes from other organisms. In this paper, we demonstrate that the level of mitochondrial transcripts is highly regulated in the bloodstream developmental stages of T. brucei. The amount of mitochondrial rRNAs and the amount of cytochrome b and cytochrome oxidase subunits I and II transcripts are low in the slender developmental stage. In contrast to other studies of maxicircle transcription (10, 14, 15, 17, 18), we find that the level of maxicircle transcripts increases at least 30-fold for the rRNAs and several hundredfold for the cytochrome b and cytochrome oxidase transcripts during the differentiation from the slender to the stumpy trypanosomes. We also find that the level of other maxicircle transcripts, such as NADH dehydrogenase subunit 5, is not regulated during development, suggesting that the maxicircle genome has multiple promoters for transcription under differing developmental control. In addition, we have precisely mapped the 5’ ends of the 12 S rRNA and the cytochrome b transcript and have examined flanking sequences for possible promoter sequences.

MATERIALS AND METHODS

Growth of Organisms—T. brucei TREU 667 was used in these studies. Bloodstream infections in mice (female Swiss, 4–6 weeks old) were initiated via intraperitoneal injection of 1 × 10⁷ trypanosomes from frozen stocks stored at −196 °C in 7.5% dimethyl sulfoxide. Trypanosomes were harvested at various times during the infection by cardiac puncture, and parasites were purified by anion-exchange chromatography (19). To isolate stumpy form trypanosomes, mice were immunosuppressed by irradiation with 600 rads prior to infection. Procyclic forms were grown in a semidefined medium (20) and were harvested at a cell concentration of 1 × 10⁷/ml.

Isolation of Nucleic Acids and Maxicircle Cloning—Total cellular nucleic acid for quantitative S1 nuclease analysis was isolated from purified bloodstream and procyclic trypanosomes. Purified trypanosomes were counted with a hemocytometer and 5 × 10⁶ cells were pelleted in a Microfuge for 2 min. The cells were resuspended in 300 μl of 100 mM NaCl, 10 mM Tris, pH 8.0, 100 μM EDTA (NET-100) and 300 μl of NET-100 containing 50 μg/ml heparin and 0.1% sodium dodecyl sulfate was added. Samples were then digested with 60 μg of proteinase K (10 mg/ml stock) at 37 °C for 30 min and extracted with equal volumes of phenol, phenol/chloroform (1:1), and chloroform.
Total nucleic acid was precipitated with an equal volume of isopropyl alcohol.

Kinetoplast DNA (kDNA) was isolated as described by Fairlamb et al. (21). A plasmid library of EcoRI-, HindIII-, and PstI-digested maxicircle DNA was prepared in the riboprobe vector GEM-1 using standard conditions (22, 23).

**S1 Nuclease Analysis**—The 32P-labeled RNA probes were prepared by annealing to 3-30 pg of total cellular RNA and extended with reverse transcriptase as described (25). The reaction mixture contained 50 mM Tris-HCl, pH 7.6, 10 mM ZnSO4, 20 pg/ml denatured calf thymus DNA) containing 50 units of S1 nuclease (Bethesda Research Laboratories) and incubated at 37 °C for 30 min (24). Reaction products were analyzed on 4% sequencing gels run at 950 V and visualized by autoradiography.

**Primer Extension Studies**—Two oligonucleotides were synthesized for the determination of the 5' ends of maxicircle transcripts: 1) a 14-nucleotide probe complementary to the 5' end of the 12 S rRNA transcript. Thirty ng of each oligonucleotide was phosphorylated with polynucleotide kinase (Boehringer Mannheim) for cytochrome

**RESULTS**

**Growth and Differentiation of Trypanosoma brucei**—Study of the developmental regulation of gene expression requires a precisely defined experimental system which mimics events that occur in natural populations. We have therefore established an experimental system optimized for the differentiation of *T. brucei* from the slender to stumpy forms in mice. The *T. brucei* TREU 667 strain was also selected because of its ability to efficiently differentiate from stumpy blood forms to procyclic trypanosomes at 26 °C in a semidefined medium (20). Fig. 1A shows the growth of *T. brucei* 667 in an immuno-suppressed mouse. During the first 4 days of infection, less than 10% of the trypanosomes are stumpy. During the next 3 days, 95% of the trypanosomes differentiate to the stumpy developmental stage (Fig. 1A).

We were interested in determining whether the stumpy trypanosomes have a selective advantage over the slender forms in differentiation to the procyclic forms in *vivo* at 26 °C. Slender day 4 and stumpy day 8 trypanosomes were inoculated separately into semidefined medium at a concentration of 2.5 × 106/ml. Fig. 1B shows that at 26 °C the stumpy trypanosomes rapidly differentiate to procyclics and begin multiplying after a 12-h lag. Cultures inoculated with slender trypanosomes showed no increase in cell number for 130 h. It is possible that slender trypanosomes can differentiate to the procyclic forms, but only after a long lag period. A more likely explanation is that the small number of stumpy trypanosomes present at day 4 of infection initiated these 26 °C cultures. These experiments suggest that differentiation to the stumpy developmental stage is obligatory in the trypanosome life cycle and that the stumpy forms may be preadapted for differentiation in the insect vector.

**Developmental Regulation of Mitochondrial rRNA Levels**—Previous studies have focused on differences in mitochondrial rRNA levels between bloodstream and procyclic trypanosomes (10, 17, 18). To determine whether mitochondrial
rRNA transcription is regulated in other developmental stages of *T. brucei*, we isolated slender trypanosomes from days 2, 3, and 4 of infection, differentiating trypanosomes from day 6 of infection, stumpy trypanosomes from day 8 of infection, and procyclic trypanosomes from established cultures at 26 °C.

Total nucleic acids were prepared from the harvested trypanosomes as described under “Materials and Methods.” The levels of mitochondrial 9 S and 12 S rRNAs were determined by quantitative S1 nuclease protection using a 32P-labeled antisense RNA transcript (Fig. 2A). These kinetic studies show that the levels of mitochondrial rRNAs remain repressed through the first 4 days of infection and that there is not a gradual accumulation during the early portion of the bloodstream infection. The levels of the 9 S and 12 S rRNAs increase 30-fold as the trypanosomes differentiate from the slender to the stumpy forms (Table I). The large increase in mitochondrial rRNA between days 6 and 8 suggests that the trypanosomes have responded to a specific activating event.

**Suppression of Mitochondrial rRNA Transcription by Serial Passaging in Mice**—There are several possible explanations for the dramatic increase in mitochondrial rRNA levels between days 6 and 8 of infection. The parasite could be responding to a host-derived factor released as a result of the infection or to a factor secreted by the parasites which must rise above a threshold concentration in order to act. Alternatively, the parasites could be responding to a biological clock which is initiated upon introduction into the mammalian bloodstream. Suppression of mitochondrial transcription might continue until a critical number of cell divisions had been executed. If this is the activation event, rRNA levels would be identical in trypanosomes maintained in a single mouse for 8 days or in infections passaged at 2-day intervals for a total of 8 days.

The passaging scheme used in this experiment is shown in Fig. 3A. Animals were infected with 1 × 10^7 trypanosomes, and the infections either passaged to new mice at 2–3-day intervals for a total of 7 days or were allowed to proceed in a single mouse for 8 days. Trypanosomes were isolated at each passaging and, following 8 days of continuous infection and total nucleic acids, were prepared. The level of rRNAs in each sample was determined by quantitative S1 protection analysis (Fig. 3B). Mitochondrial rRNA levels in the infections passaged in mice for 8 days remain suppressed relative to bloodstream forms in trypanosomes maintained in one animal for the 8-day period. These results demonstrate that the derepression of mitochondrial rRNA transcription is not influenced by the number of cell divisions the bloodstream trypanosomes undergo.

The increased level of mitochondrial rRNA in the stumpy population is a marker for this developmental stage. To determine whether the stumpy trypanosomes are capable of reverting to the slender forms upon injection into a new host, we analyzed the level of mitochondrial RNA in trypanosomes 2 days after inoculation with 1 × 10^7 stumpy day 8 trypanosomes. Fig. 3B shows that the mitochondrial rRNA levels are reduced to levels nearly identical with those in slender populations (compare lanes 7 and 8 of Fig. 3B). There are several possible interpretations of these data. Mitochondrial rRNA expression may be readily reversible. Alternatively, trypanosomes expressing high mitochondrial rRNA levels are selected against when passaged into a new host. Since mitochondrial rRNA expression may be an early marker of differentiation to the nondividing stumpy form the latter possibility is not unlikely.

**Developmental Regulation of the Level of Cytochrome b, and Cytochrome Oxidase Subunits I and II Transcripts**—To determine whether the amount of other maxicircle transcripts change during the trypanosome life cycle, we have examined

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**TABLE I**

Relative levels of maxicircle transcripts in *T. brucei* developmental stages

<table>
<thead>
<tr>
<th>Mitochondrial gene</th>
<th>Bloodstream infection</th>
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<tbody>
<tr>
<td></td>
<td>Day 2</td>
</tr>
<tr>
<td>12 S rRNA^a</td>
<td>—</td>
</tr>
<tr>
<td>9 S rRNA</td>
<td>—</td>
</tr>
<tr>
<td>Cytochrome b (1120 nt)</td>
<td></td>
</tr>
<tr>
<td>Cytochrome oxidase I</td>
<td>0.98</td>
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<tr>
<td>Cytochrome oxidase II</td>
<td>0.9</td>
</tr>
<tr>
<td>NADH dehydrogenase 5</td>
<td>0.15</td>
</tr>
<tr>
<td>URT1</td>
<td>0.52</td>
</tr>
<tr>
<td>URT2</td>
<td>0.9</td>
</tr>
</tbody>
</table>

^a PCF, procyclic forms from established 26 °C cultures.
^b Amount of 12 S and 9 S rRNA determined by counting gel slices from quantitative S1 analysis.
^c — indicates sample not analyzed.
^d Values are normalized to 1 for the procyclic sample.
^e Cytochrome b, cytochrome oxidases I and II, NADH 5, and URT1 and -2 transcripts were quantitated by densitometry of films from S1 analyses.
^f Size of S1 protected fragment.
^g ND indicates transcripts not detectable.
Trypanosome Mitochondrial Transcripts

Fig. 3. Suppression of mitochondrial rRNA transcription by serial passaging in mice. A, the passaging scheme used in this experiment. Trypanosomes were purified from mice after uninterrupted growth in a single immunosuppressed mouse for 8 days (P8) and after a 2-day passage (P8-2) of $1 \times 10^7$ trypanosomes from the P8 infection in an uninfected mouse. Trypanosomes were also isolated from a mouse infected for 3 days (P3) and from two successive passages of these trypanosomes into uninfected mice for 5 days (P3-2, P3-2-2). B, S1 protection analysis of total cellular RNA from $1 \times 10^6$ trypanosomes from P3 (lanes 1 and 2), P3-2 (lanes 3 and 4), P3-2-2 (lanes 5 and 6), P8-2 (lane 7), P8 (lane 8), procyclic (lane 9). The $^{32}$P-labeled riboprobe was prepared as described in Fig. 2.

The steady state levels of cytochrome b, cytochrome oxidase subunits I and II and NADH dehydrogenase subunits 4 and 5 transcripts, and two unassigned transcripts, were determined for two reasons: 1) to establish whether the multiple bands seen in S1 nuclease protection analysis of cytochrome b transcripts were due to differences at the 5' ends of these transcripts, and 2) so that we could search sequences upstream of the 5' ends of the developmentally regulated maxicircle genes to define necessary features of a trypanosome mitochondrial promoter.

Mapping of the 5' terminus of the cytochrome b and the 12 S mitochondrial rRNA transcripts is shown in Fig. 5. To determine the 5' end of the 12 S transcript, we annealed a 14-base synthetic oligonucleotide primer, complementary to the expected 5' terminus of the 12 S rRNA transcript, to 3 µg of total cellular RNA. The primer was extended with reverse transcriptase, and the products of the primer extension were sized on sequencing gels with appropriate markers (Fig. 5A). A single 272-nt product was made by the reverse transcriptase. For the analysis of the cytochrome b transcripts, we also used a 14-base synthetic oligonucleotide primer. The major products made were of 96 nt and 132 nt (Fig. 5A). This is in agreement with the S1 protection experiments suggesting that the major cytochrome b transcripts differ in length by 36 nt. Also in agreement with the S1 protection experiments is the stoichiometry of the transcripts in the stumpy and procyclic forms. We do not know if the stoichiometry of the two transcripts has any effect on translation of the transcripts.

The DNA sequences flanking the putative transcription start sites for the cytochrome b transcripts and the 12 S mitochondrial rRNA were determined and are presented in Fig. 5C. No obvious consensus sequence was identified.

Discussion

We have studied the role of mitochondrial gene expression in the developmental control of mitochondrial biogenesis in African trypanosomes. Using a well defined biological system, we have shown that the level of several mitochondrial transcripts varies with T. brucei development in the mammalian host. Slender trypanosomes, from early bloodstream infections, display repressed levels of 9 S and 12 S rRNA, cytochrome b, and cytochrome oxidase subunits I and II transcripts. Control of the level of these mitochondrial transcripts appears to involve a common mechanism. The amount of other maxicircle transcripts, such as NADH dehydrogenase subunit 5, does not follow the same pattern of regulation, suggesting that these genes are under independent control.

The increased level of maxicircle transcripts coincides with the morphological change from the slender to the stumpy form in the mammalian host. The stumpy forms have long been considered preadapted for survival in the insect vector (5, 6), and our results present evidence that changes are occurring at a molecular level.

Several points can be made concerning the factor(s) which induce the slender to stumpy transformation in the mammalian host. Morphological differentiation to the stumpy forms is suppressed in trypanosome populations passaged for ex-

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1 E. F. Michelotti, and S. L. Hajduk, unpublished data.
2 The abbreviation used is: nt, nucleotide.
FIG. 4. Developmental regulation of levels of nonribosomal mitochondrial transcripts. A, S1 protection analysis of total cellular RNA from 1 x 10^6 trypanosomes collected on day 2 (lane 1), day 3 (lane 2), day 4 (lane 3), day 6 (lane 4), and day 8 (lane 5) of infection in mice and from established procyclic cultures (lane 6). B, antisense ^32P-labeled riboprobe transcripts were prepared as described under “Materials and Methods,” and the S1-resistant RNAs were run on 4% sequencing gels.

FIG. 5. Primer run-off analysis to determine the 5′ ends of the 12 S rRNA and the cytochrome b transcript. A, primer run-off products for the 12 S rRNA. Size markers are end-labeled Sau3A-digested pSP64 fragments (lane 1) and end-labeled Sau3A-digested pBR322 fragments (lane 3). The 12 S rRNA run-off product (lane 2) is indicated with an arrow. B, primer run-off products for cytochrome b transcripts. The major cytochrome b primer run-off products from the bloodstream (lane 1) and procyclic (lane 2) RNAs are indicated with arrows. C, DNA sequences flanking the 5′ ends of apocytochrome b mRNA and 12 S rRNA. Arrows indicate the direction of transcription and the 5′ ends of the RNAs mapped by primer extension.

tended periods of time in mice. The level of mitochondrial rRNAs also remains suppressed by approximately 20-fold relative to stumpy populations maintained in a single animal for an equivalent period of time. This indicates that the increase in mitochondrial rRNA levels and the morphological differentiation to the stumpy form is not due to a biological clock which initiates upon introduction of the trypanosome into the animal. More importantly, these results demonstrate that trypanosomes have developed a means for monitoring a factor which changes in concentration during the course of the bloodstream infection and respond to it appropriately.

The nature of the factor(s) initiating the morphological and molecular differentiation events is unknown. It is possible that the infection induces the synthesis of a positively acting factor which activates the differentiation. Alternatively, it is possible that a negative acting factor maintains the trypanosome in the slender form and is eventually depleted as a result of the infection.

The constant level of NADH dehydrogenase subunit 5 and two other mitochondrial RNAs demonstrates that the necessary transcription machinery is present even in slender trypanosomes when cytochrome b and cytochrome oxidase I and
II transcripts are undetectable. The mitochondrial rRNAs in the slender trypanosomes are readily detectable and may be present so that the unregulated transcripts may be translated. This suggests that even when the mitochondrion is inoperative, translation of these unregulated transcripts would be beneficial to the parasite. It would not, however, be necessary for survival of the organism since dyskinetoplastic trypanosomes (26), mutants completely deficient in kDNA, survive perfectly well in the bloodstream (7–9).

The regulation of mitochondrial activity in the mammalian bloodstream superficially resembles the mitochondrial changes of *Saccharomyces cerevisiae* during glucose-repressed growth (27–29). There are, however, several major differences between the mitochondria of glucose-repressed yeast and the slender trypanosome mitochondrion. 1) Spectrally detectable cytochromes are present in the mitochondria of glucose-repressed yeast, while no cytochromes are detectable in bloodstream trypanosomes (30). 2) The reduction in the steady state levels of mitochondrial transcripts in glucose-repressed yeast (28, 29) is slight when compared to the suppression of cytochrome b and cytochrome oxidase transcripts in slender trypanosomes. 3) Changes in the abundance of the mitochondrial rRNAs have not been reported for glucose-repressed yeast.

A striking result of the primer run-off experiments with the 12 S rRNA and the cytochrome b transcripts is the lack of homology upstream of these genes. While it is not known whether the 5' ends we have mapped result from transcription initiation or RNA processing events, guanylyl transferase experiments of *Leishmania tarentolae* mitochondrial RNA have shown that multiple transcription initiation sites are present in the *Leishmania* maxicircle (31). Most yeast mitochondrial genes share the consensus sequence A-A-T-A-T-A-G-T-A immediately upstream of transcription initiation. The lack of a consensus sequence upstream of trypanosome mitochondrial genes raises the possibility that a DNA structure and not a specific DNA sequence may play the more dominant role in transcription of the trypanosome maxicircle.

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**REFERENCES**


