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Trypanosoma brucei mitochondrial ribosomal RNA synthesis, processing and developmentally regulated expression

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The steady-state levels of the mitochondrial ribosomal RNAs of *Trypanosoma brucei* are repressed in the early bloodstream developmental stage of the parasite and accumulate approximately 30-fold during differentiation to the stage found in the midgut of the insect vector. In order to determine the mechanism regulating this developmental process, we have examined the transcription and processing of the 9S and 12S mitochondrial rRNAs of *T. brucei*. A short-lived RNA was detected in pulse labeling experiments which contains the mature 12S and 9S rRNAs and at least 1200 nucleotides of RNA transcribed from upstream of the 12S rRNA gene. This putative processing precursor RNA was identified in both intact cells and in run-on experiments using isolated mitochondria. The transcripts containing the upstream sequences are unstable and reach isotopic equilibrium within 15 min. Mature rRNAs in the insect developmental stage are stable and show no detectable turnover during a 36-h chase. Comparison of rRNA synthesis in bloodstream and insect life-stages indicates that mitochondrial rRNA levels are controlled not at the transcriptional level, but rather by a mechanism which likely modulates the stability of the mature rRNAs. These results suggest that a short-lived rRNA precursor is synthesized and processed at comparable rates in both bloodstream and insect stages of the parasite. Thus, it appears that differential stability of the mature 9S and 12S rRNAs plays a major role in modulating mitochondrial gene expression during the developmental cycle of *T. brucei*.

Key words: *Trypanosoma brucei*; kDNA; Mitochondrial rRNA; Maxicircle transcription and expression

Introduction

Trypanosoma brucei is a protozoan parasite which developmentally regulates mitochondrial activity during its life cycle [1–5]. Procyclic trypanosomes growing in the midgut of the tsetse fly or cultivated in vitro at 26°C have a fully functional mitochondrion. Developmental stages found in the mammalian bloodstream repress mitochondrial activities and derive ATP via glycolysis. Two distinct developmental stages exist in the mammalian bloodstream. Rapidly dividing, long slender trypanosomes predominate in the early bloodstream infection and eventually differentiate into the non-dividing, short stumpy form. Several lines of evidence have suggested that short stumpy, and not long slender, trypanosomes are preadapted for differentiation to the insect developmental stages [5,6]. Other studies have suggested that both slenders and stumpies can differentiate to the insect developmental stages with equal efficiency [7].

The mitochondrial or kinetoplast DNA

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Abbreviations: nt, nucleotide(s); kDNA; kinetoplast DNA.

(kDNA) of trypanosomes is noted for its unique organization, consisting of a catenated network containing about 50 maxicircles and 5000 minicircles [1,2]. *T. brucei* maxicircles are 20 kb in size and encode the mitochondrial rRNAs as well as several components of the electron transport chain [1,2,6,8–11]. The maxicircles are considered to be analogous to the mitochondrial DNA of other organisms.

Previous studies from our laboratory [6] and others [12] have shown that the steady-state levels of some maxicircle transcripts were regulated during the developmental cycle of *T. brucei* while others remained constant. The regulated transcripts are either undetectable (cytochrome *b*, cytochrome oxidase subunits I and II) or highly repressed (12S and 9S rRNAs) in long slender trypanosomes, dramatically increase in short stumpy forms, and have a further but less substantial increase in the procyclic form. Other maxicircle transcripts, such as the mRNA for NADH dehydrogenase subunit IV, are unregulated throughout the life cycle of *T. brucei*. While the steady-state levels of maxicircle transcripts have been determined, nothing is known about the promoters for maxicircle transcription or the pathway for RNA processing in these mitochondria.

The developmental control of maxicircle transcript levels could be accomplished in one of several ways. Transcription initiation frequencies could be reduced for these genes in long slender forms. Alternatively, transcription of these genes might remain unregulated during development but the processing of the maxicircle transcripts might differ between developmental stages. Lack of processing or altered processing could lead to transcripts with a shorter half-lives in the long slender trypanosomes. Finally, transcription and processing might be identical in the different developmental stages, but the mature transcripts in the long slender bloodstream stage could be unstable. To distinguish between these possibilities we have examined the transcription and processing of the mitochondrial rRNAs, in vivo and in isolated mitochondria, by pulse labeling and pulse/chase experiments. Previously we had shown that the steady state amount of the mitochondrial rRNA increased approximately 30-fold as the trypanosomes differentiated from the long slender bloodstream forms to the procyclic stage [6]. We report here that the mitochondrial rRNAs of *T. brucei* are initially transcribed as a precursor which is rapidly processed to the mature 12S and 9S rRNAs. The rRNA transcription rates in the long slender and procyclic developmental stages were examined and found to be identical relative to the synthesis rates of the unregulated β-tubulin mRNA. The rate and the products of processing the ribosomal precursor transcripts also was indistinguishable in the two developmental stages. This suggests that the stability of the mature 9S and 12S mitochondrial rRNAs is developmentally regulated in *T. brucei*.

Materials and Methods

Growth and isolation of trypanosomes. The long slender bloodstream developmental stage of *T. brucei* (TREU667) was grown in CD-1 mice (5–10 weeks old) following infection by interperitoneal injection with frozen stocks stored at −196°C in 7.5% DMSO. Long slender trypanosomes were harvested from 2–3-day infections by cardiac puncture, and the trypanosomes were separated from blood constituents [13] by diethylaminoethyl cellulose chromatography (Whatman, DE52) in phosphate-buffered saline with glucose and heparin added (PSGH, 0.057 M Na_2HPO_4/3 mM NaH_2PO_4/0.044 M NaCl/0.1 g glucose/10 units of heparin ml^{-1}, pH 8.0).

Procyclic trypanosomes (TREU667) were grown in a semidefined medium (SM) containing gentamicin (Sigma, 25 $\mu g\ ml^{-1}$) and supplemented with 10% heat-inactivated, bovine fetal calf serum (Hyclone) at 26°C [14]. Procyclic cells were routinely harvested by centrifugation at 6000 × *g* for 6 min at 4°C.

5′ and 3′ labeling of purified mitochondrial RNA. Mitochondria were isolated from *T.*

brucei procyclics as described previously [15]. Total mitochondrial RNA was purified following lysis of mitochondria with 0.5% SDS and treatment with proteinase K (100 μg ml^{-1}) for 10 min at 26°C. The lysate was extracted with phenol, phenol/chloroform (1:1) and chloroform and then precipitated with an equal volume of isopropanol. Purified mitochondrial RNA was labeled at the 5′ terminus with either guanylyltransferase and [α-^{32}P]GTP as previously described [16] or with polynucleotide kinase and [γ-^{32}P]ATP [17]. Mitochondrial RNA was 3′-end labeled with RNA ligase and [^{32}P]pCp [18].

In vivo pulse labeling of trypanosomes. Procyclic cells were harvested in the late log phase of growth (1.8 × 10^7 ml^{-1}) by centrifugation at 6000 × *g* for 6 min at room temperature. The pellet was resuspended in 14.5 ml of either phosphate-free SM medium [14] for [^{32}P]-orthophosphate labeling, or complete SM medium for [^{3}H]adenosine labeling, in both cases containing 10% fetal calf serum. To 5.5 ml of culture media, either 20 mCi of carrier-free [^{32}P]orthophosphate (8 mCi ml^{-1}, Amersham), or 1.4 mCi of [^{3}H]adenosine (ICN, 40 Ci mmol^{-1}) was then added. At each time point a fraction of the cells was chilled on ice for 5 min, centrifuged at 4°C at 6000 × *g* for 8 min, and the cell pellet was resuspended in 1 ml of ice-cold NET-10 (0.1 M NaCl/10 mM EDTA/10 mM Tris-HCl, pH 8.0). Cells were then pelleted in a microfuge at 4°C for 3 min. In pulse/chase experiments with [^{3}H]adenosine, the chase was initiated by centrifugation at 6000 × *g* for 6 min at room temperature, and the cells were resuspended in SM medium supplemented with 50 μM adenosine.

Long slender trypanosomes were pulse labeled with [^{3}H]adenosine and [^{32}P]-orthophosphate as described for the procyclic forms, except that the long slender forms were incubated at 37°C with the radioactive precursors in F12 medium (Gibco), containing 10% heat-inactivated fetal bovine serum.

RNA isolation and ribonuclease T1 protection analysis. Total cellular RNA was isolated by LiCl precipitation [6] and then treated with RNase free DNase (BRL) as described elsewhere [15]. In vivo labeled RNAs were quantitated using a RNase T_1 protection assay [6] with the following modifications. In vitro synthesized antisense RNA was annealed to labeled RNA in 50 μl of hybridization buffer (0.6 M NaCl/50 mM bicine, pH 7.8/1 mM EDTA) at 65°C for 12–18 h. The annealed RNA sample was diluted with 50 μl of 50 mM bicine, pH 7.8/1 mM EDTA, and 1 μl of T_1 RNase (1400 units; BRL) was added and allowed to digest at 37°C for 30 min. Proteinase K (1 μl, 2.5 mg ml^{-1}, Bethesda Research Laboratories) was added and the incubation continued at 37°C for another 30 min. Double-stranded RNA was then purified on a Nucleic Acid Chromatography (NACS, Bethesda Research Laboratories) column. For this purpose, the salt concentration was adjusted to 0.5 M with 2 M NaCl/10 mM Tris-HCl, pH 7.2/1 mM EDTA. The RNA samples were then loaded on a NACS column, washed with 800-μl volumes of 0.5 M NaCl/10 mM Tris-HCl, pH 7.2/1 mM EDTA, and double-stranded RNA was eluted with 400 μl of 1.5 M NaCl/10 mM Tris-HCl, pH 7.2/1 mM EDTA. To this sample 10 μg of yeast tRNA carrier and 800 μl ethanol was added. RNA was precipitated by incubation at −70°C for at least 30 min. Samples were heated for 3 min at 100°C prior to loading on 4% sequencing gels which were run at 30 W. Bands were visualized with Kodak XAR5 film with or without a Dupont Lightning Plus intensifying screen at −70°C. Labeling of Riboprobes and quantitation of the steady state amounts of the rRNA was carried out as described [6].

Synthesis and processing of rRNA in isolated mitochondria. Mitochondria were isolated from exponentially growing procyclic trypanosomes and the transcripts characterized as described elsewhere [16]. Purified mitochondria were incubated at 26°C in transcription buffer (5 mM Hepes, pH 7.6/3 mM potassium phosphate, pH 7.7/125 mM sucrose/60 mM potassium chloride/6 mM magnesium acetate/1 mM EDTA/2 mM 2-β-mercaptoethanol) at a

concentration of 1 mg of mitochondrial protein ml^{-1}. [α-^{32}P]GTP (Amersham, 400 Ci $mmol^{-1}$) was added to a final concentration of 100 μCi ml^{-1}; unlabeled ATP was added at 500 μM while CTP and UTP were present in the transcription system at 100 μM each. Chase conditions were initiated by dilution of the [α-^{32}P]GTP with a 100-fold excess of unlabeled GTP followed by centrifugation of the mitochondria at 10 000 rev./min for 2 min at 4°C in a microfuge and resuspension of the pelleted mitochondria in transcription buffer containing 100 μM GTP.

Incorporation of GTP into mitochondrial transcripts was measured by liquid scintillation counting of trichloroacetic acid precipitated material collected on glass fiber filters. Isolated mitochondrial RNA was analyzed on 1.5% methyl-mercury hydroxide agarose gel electrophoresis.

Results

Capping of primary mitochondrial transcripts. The 5′ termini of unprocessed, primary mitochondrial transcripts can be identified by labeling with the vaccina enzyme guanylyltransferase which adds [α-^{32}P]GTP to the terminal triphosphate of primary RNA transcripts. Previous studies using mitochondrial RNA from *Leishmania tarentolae* indicated that the 5′ termini of the 9S and 12S mitochondrial rRNAs were triphosphate residues [19]. These results indicate that kinetoplastid mitochondrial rRNA transcription might differ fundamentally from that of vertebrate mitochondrial transcription [20]. In all metazoans examined, mitochondrial rRNAs are transcribed as multicistronic RNAs which are processed to their mature size. This unexpected result suggested that the 9S and 12S rRNA of *L. tarentolae* were synthesized from separate promoters. In order to identify the primary rRNA transcripts in *T. brucei*, total mitochondrial RNA was labeled with guanylyltransferase and [α-^{32}P]GTP and labeled RNAs were separated on 6% sequencing gels (Fig. 1, lanes 3 and 6). The major

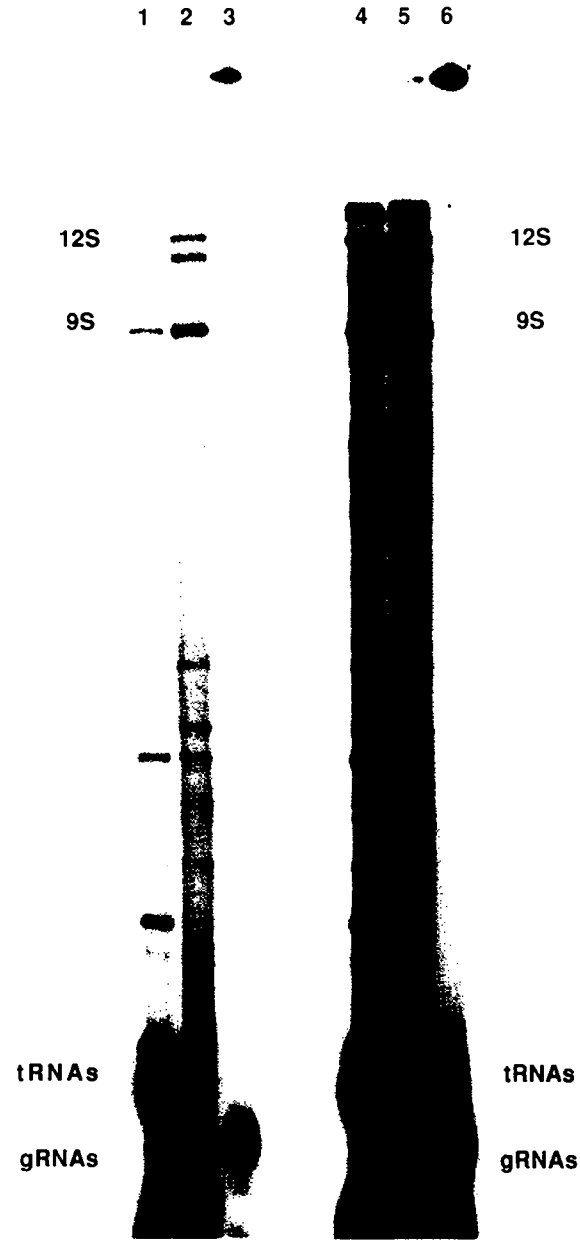

Fig. 1. Labeling of mitochondrial RNA with RNA ligase, polynucleotide kinase and guanylyltransferase. Purified mitochondrial RNAs were labeled in vitro with RNA ligase and [^{32}P]pCp (lanes 1 and 4) (0.005 μg), polynucleotide kinase and [γ-^{32}P]ATP (lanes 2 and 5) (0.005 μg) and guanylyltransferase and [α-^{32}P]GTP (lanes 3 and 6) (5 μg). Lanes 4–6 were overexposed to show the labeling of the rRNAs with guanylyltransferase. Samples were run on 3.5% polyacrylamide 8 M urea sequencing gels at 30 W.

capped RNA species in these reactions ranged in size from 55 to 70 nt and hybridized exclusively to *T. brucei* minicircles (Fig. 1; see ref. 17). The 9S and 12S rRNAs were also labeled but to a far lesser degree. This was best seen when the autoradiograph was overexposed (Fig. 1, lane 6). While these results suggest that the mature 9S and 12S rRNAs might be primary transcripts and that transcription initiates at their 5′ terminus we were cautious of this interpretation due to the low efficiency of labeling of these major mitochondrial transcripts. The inefficiency of the 5′ labeling with guanylyltransferase could result from RNA secondary structure, leaving the 5′ end of the rRNAs inaccessible to the enzyme.

To examine this possibility total trypanosome mitochondrial RNA was labeled at the 5′ terminus with polynucleotide kinase and [γ-^{32}P]ATP (Fig. 1, lanes 2 and 5). The labeling with kinase was approximately 1000-fold more efficient than the capping reactions; more importantly, the rRNAs and not the minicircle transcripts were the major labeled products. This is consistent with the relative abundance of the minicircle transcripts and the mature rRNAs in *T. brucei* mitochondria. The abundance of the rRNA and the minicircle transcripts was also verified by 3′ end labeling with pCp and RNA ligase (Fig. 1, lanes 1 and 4). An alternative explanation for the low level of 9S and 12S labeling with guanylyltransferase could be the relaxed specificity of the enzyme for triphosphate substrates. This interpretation is supported by the early studies of Martin and Moss [21] which showed that the enzyme could also label RNAs with 5′-monophosphate termini, though with much lesser efficiency than RNAs with 5′-triphosphate.

Since the capping reactions could not conclusively identify the primary rRNA tran-

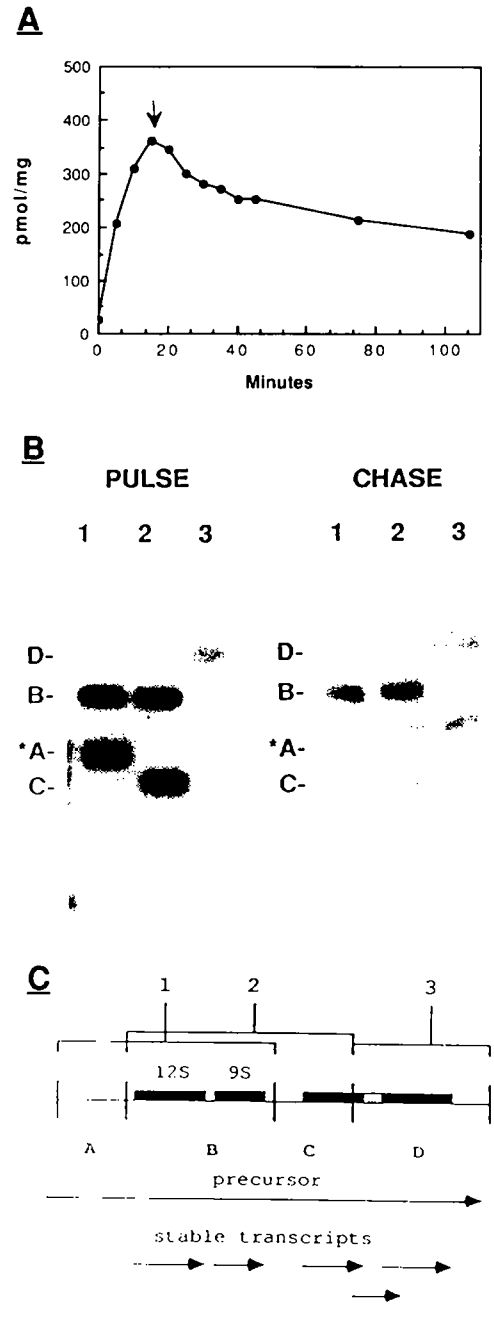

Fig. 2. Synthesis and turnover of mitochondrial rRNA in isolated mitochondria. (A) Incorporation and turnover of [α-^{32}P]GTP into trichloroacetic acid precipitated material. Samples were taken every 5 min during the 15-min pulse and the chase initiated by the addition of unlabeled GTP (arrow). (B) Hybridization of pulse labeled and pulse-chased mitochondrial RNA to Southern blots of cloned maxicircle fragments. Fragment A*, 1.2-kb region upstream of the rRNA genes; fragment B, 2.2-kb mitochondrial rRNA genes; fragment C, 0.9-kb NADH dehydrogenase subunit 7 gene; fragment D, 3.2 kb encoding cytochrome oxidase III and cytochrome *b*. (C) Diagram of the maxicircle indicating the cloned maxicircle fragment (numbers above map) and the position of stable transcripts. The letters below the map correspond to the fragments on the gel shown in (B).

scripts we examined the mechanism of mitochondrial rRNA transcription and processing in pulse labeling and pulse-chase experiments.

Mitochondrial ribosomal RNA synthesis. The transcription of the mitochondrial rRNAs was initially examined in pulse-chase experiments using isolated mitochondria from *T. brucei* (Fig. 2). The organization of the rRNA coding region is shown in Fig. 2C. Mitochondria isolated from procyclic trypanosomes were pulse labeled with [α-^{32}P]GTP and the kinetics of incorporation into mitochondrial transcripts measured by TCA precipitation (Fig. 2A). The incorporation of [^{32}P]GTP into the mature 9S and 12S rRNAs and also into a smear of RNA ranging in size from approximately 3 kb to 0.6 kb was evident in pulse and chase samples run on methylmercury hydroxide agarose gels (data not shown). Labeled RNAs from the pulse labeled and chased mitochondria were used as hybridization probes to Southern blots of maxicircle clones containing the rRNAs, the 5′ flanking sequence and the 3′ flanking sequences which encode cytochrome oxidase III and cytochrome *b* (Fig. 2B). Pulse labeled mitochondrial RNA hybridized to both the restriction fragment upstream of the rRNAs (band *A) and also to the restriction fragments encoding the rRNA (band B). RNA isolated from pulse-chased mitochondria gave a similar hybridization pattern with the exception that hybridization to the restriction fragment upstream of the rRNA genes was reduced during the chase (band *A). Hybridization to regions encoding other stable transcripts, such as NADH dehydrogenase subunit 7 (Fig. 2, band C) and cytochrome *b* (Fig. 2, band D) indicated that, though reduced in abundance, these transcripts were present following the chase. These results suggested that the 1.2 kb region flanking the 12S rRNA gene might be transcribed as part of a short-lived precursor transcript containing the rRNAs.

In order to study the synthesis of this putative mitochondrial rRNA precursor in more detail, procyclic trypanosomes were labeled in vivo and purified RNA used in ribonuclease T_1 protection experiments. Using

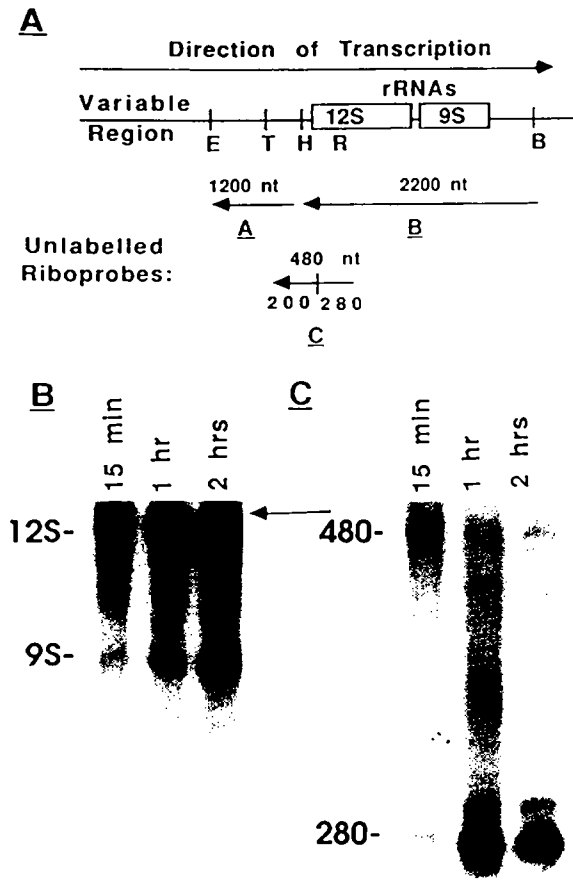

Fig. 3. In vivo labeling of trypanosome mitochondrial rRNAs. (A) Schematic diagram of the region of the maxicircle of *T. brucei* encoding the 9S and 12S mitochondrial rRNAs. Boxes represent the rRNA coding sequences; the direction of transcription is indicated above the map; the uncloned, Variable Region of the maxicircle is indicated and the origin of the 3 antisense Riboprobes A, B and C is shown. (B) Total cellular RNA from *T. brucei* procyclic forms labeled with ^{32}P-orthophosphate for varying times and protected by unlabeled Riboprobes A and B. The positions of the 9S and 12S rRNAs protected with probe B are indicated. The position of the 1.2-kb sequence upstream of the 12S rRNA is indicated by an arrow. (C) RNA from cells treated as in (B) but protected by antisense RNA probe C. The 280-nt protected fragment represents the 5′-end of the mature 12S rRNA. The 480-nt protected RNA is unprocessed precursor containing both 200 nt of 5′ flanking sequence and the 5′ end of the mature 12S rRNA. Restriction sites are *Eco*RI, E; *Taq*I, T; *Hin*dIII, H; *Rsa*I, R; *Bam*HI, B. The size of the RNase T_1-protected fragments was estimated relative to *Hae*-III-digested φX174 DNA fragments (not shown).

[^{32}P]orthophosphate, a small number of counts were incorporated into the trypanosome mitochondrial RNA under pulse conditions. The low level of incorporation into the total trypanosome RNA was likely due to the large intracellular polyphosphate pool in trypanosomes [22]. Under continuous labeling conditions, we expected a precursor to the mitochondrial rRNAs to reach isotopic equilibrium rapidly and to remain constant throughout the remainder of the labeling period. Mature 9S and 12S rRNAs should accumulate throughout the labeling period if their turnover rates are slow. If the rate of synthesis remains constant throughout such an experiment, the amount of time required for a transcript to reach isotopic equilibrium is a direct reflection of its half-life.

Incorporation into mature 12S and 9S rRNAs was quantitated using a 2.2-kb antisense RNA (Fig. 3A, Riboprobe B) while upstream sequences were quantitated using a 1.2-kb antisense RNA (Fig. 3A, Riboprobe A). Intact cells were pulsed for 15 min, 1 h and 2 h. Equal amounts of RNA from each of the time points were annealed to the 2 antisense Riboprobes A and B and treated with RNase T_1. In all T_1 RNase protection assays, the antisense RNAs were in >100-fold molar excess over the complementary in vivo labeled RNAs. Control samples containing equal amounts of labeled cellular RNA but without antisense Riboprobes were digested with RNase T_1 under identical conditions. No T_1-resistant RNA fragments greater than approximately 100 nts in length were present in these samples (data not shown).

The mature 12S and 9S rRNAs accumulated during the 2 h of labeling (Fig. 3B). In contrast, the 1.2-kb sequence upstream from the 12S rRNA reaches isotopic equilibrium within 15 min (Fig. 3B). The signal-to-noise ratio for 15-min pulse labeled samples was reproducibly lower than for longer pulse times. This is probably because the RNA isolated at the shorter pulse labeling times had a higher ratio of nascent to completed transcripts. The nascent transcripts appear as a smear of preterminated RNAs in the T_1 RNase analysis. The protection of a full length 1.2-kb transcript from RNase T_1 digestion by Riboprobe A indicates that transcription initiates further upstream.

To confirm that the 1.2 kb protected RNA was part of the rRNA precursor we next characterized the processing of the rRNA precursor in the region spanning the 5′ end of the mature 12S rRNA. RNase T_1 analysis using probe C (Fig. 3A) protected either the 280 nucleotides at the 5′ terminus of the mature 12S rRNA or 480 nucleotides of the rRNA precursor. Through 2 h of continuous labeling, the specific activity of the 280 nt 5′ end of the mature 12S rRNA continued to accumulate (Fig. 3C), while the 480 nt protected precursor RNA fragment reached isotopic equilibrium within 15 min (Fig. 3C). A second small fragment of unknown origin also accumulated during the pulse. This might represent an alternative processing site at the 5′ end of the 12S rRNA. Since no 200-nt protected fragment was observed in these experiments (data not shown), the 5′ flanking sequence is probably rapidly degraded following processing to form the mature 5′ terminus of the 12S rRNA.

In order to directly demonstrate the high turn-over rate for the rRNA precursor and to eliminate the possibility of artifacts resulting from either the T_1 RNase analysis or an anomaly of phosphate metabolism in trypanosomes, we measured the half-life of the rRNA precursor using [^{3}H]adenosine as a label. Since trypanosomes are auxotropic for purines the intracellular adenosine concentration is sufficiently small for pool equilibration to occur rapidly [22]. Antisense RNA probes A and B were added to RNA isolated from procyclic *T. brucei* pulsed for 30 min with [^{3}H]adenosine and chased for 15 min, 1 h, 2 h and 4 h. β-tubulin antisense RNA was added to each sample as an internal control. The incorporation of [^{3}H]adenosine into precursor sequences upstream of the mature 12S rRNA was approximately equal to that of the mature rRNAs, indicating that some processing had occurred during the radiolabel pulse (Fig. 4A). This region of the precursor was highly

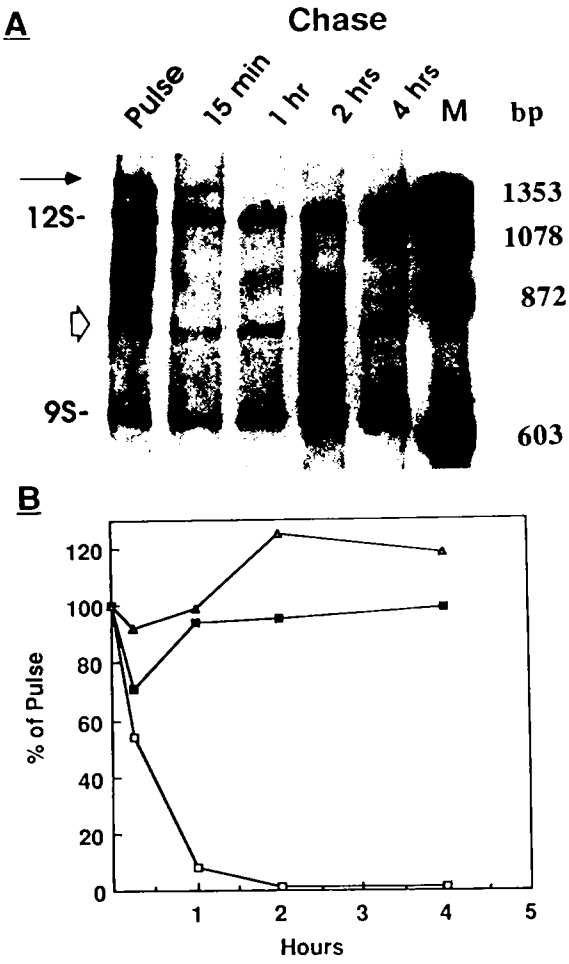

Fig. 4. Pulse chase analysis of mitochondrial rRNA. (A) Quantitative T_1 RNase protection analysis of total cellular RNA from procyclic trypanosomes which had been pulse labeled with [^{3}H]adenosine for 30 min and chased for 15 min, 1 h, 2 h and 4 h. Unlabeled antisense Riboprobes A and B were added to protect the 5′-flanking sequence and the 9 and 12S rRNAs, respectively. The positions of the mature 12S and 9S mitochondrial rRNAs are indicated. The 1.2-kb 5′ flanking sequence is indicated by a solid arrow, that of the β-tubulin protected fragment by an open arrow. (B) The precursor and mature rRNA levels during the pulse and chase were estimated by densitometry of autoradiographs. The quantity of RNA present was expressed relative to the amount of RNA labeled in the pulse sample. The 1.2-kb 5′-flanking sequence is indicated by open squares, the mature 12S rRNA by closed squares and the mature 9S rRNA by open triangles.

unstable and had a half-life of approximately 15 min (Fig. 4B). The mature rRNAs were more stable and showed no turn-over during the 4-h chase (Fig. 4A and B). The 9S and 12S rRNAs are, in fact, extremely stable, and showed no turnover even after a 36-h chase (Michelotti and Hajduk, unpublished results). Quantitation of the amount of radioactivity incorporated into the 1.2-kb upstream region allowed us to estimate the half-life of the precursor rRNA to be 15 minutes (Fig. 4B).

The results presented in Figs. 2–4 showed that the mitochondrial rRNAs of *T. brucei* were transcribed as precursors and that the promoter was located in the variable region of the maxicircle at least 1.2 kb upstream of the 5′ end of the 12S rRNA. The portion of the rRNA precursor located upstream of the mature 12S rRNA had a very short half-life. These studies also established the conditions for pulse labeling trypanosomes and some of the details of mitochondrial rRNA processing.

Mitochondrial rRNA stability. The steady-state levels of the 12S and 9S mitochondrial rRNAs increase 30–60-fold as *T. brucei* differentiates from the long slender bloodstream form to the procyclic developmental stage. The reduced amount of mitochondrial rRNA in the long slender developmental stage could be due to either a lower transcription initiation frequency, or a higher rRNA turnover rate. To distinguish between these possibilities the relative transcription rates of the mitochondrial rRNAs in these two developmental stages was determined (Fig. 5A). The amount of incorporation into the 9S and 12S rRNAs was measured relative to the transcription rate of the unregulated trypanosome gene β-tubulin (Fig. 5B). RNA was isolated from long slender and procyclic trypanosomes pulse labeled for 30 min with [^{3}H]adenosine. The counts incorporated into the rRNAs and β-tubulin were quantitated by T_1 RNase analysis using probe B (see Fig. 1A) and a β-tubulin specific probe. In a separate experiment cells were pulse labeled with [^{32}P]orthophosphate to eliminate possible artifacts due to developmental changes in nucleotide pool sizes (results not shown). The rate of incorporation of both [^{32}P]orthophosphate and [^{3}H]adenosine into the mature 12S and 9S rRNAs was nearly identical, when normalized by densitometry to

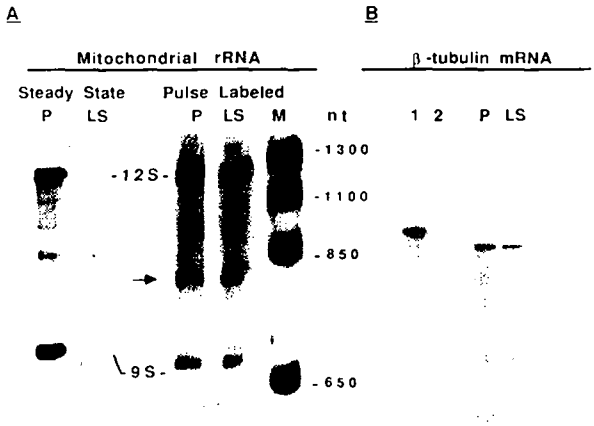

Fig. 5. Transcription rates of the mitochondrial rRNAs in long slender and procyclic *T. brucei*. (A) Long slender (LS) and procyclic (P) trypanosomes were pulse labeled with [^{3}H]adenosine for 30 min and the transcription rates of the mature rRNAs and β-tubulin (arrow) were quantitated by T_1 RNase analysis using unlabeled β-tubulin antisense RNA, and Riboprobe B (see Fig. 1A). Steady-state levels of the 12S and 9S rRNAs were quantitated by RNase T_1 analysis with ^{32}P-labeled Riboprobe B (see Fig. 1A). (B) The steady-state levels of β-tubulin mRNA were quantitated by RNase T_1 analysis with ^{32}P-labeled antisense Riboprobe to *T. brucei* β-tubulin. Lanes 1 and 2, undigested β-tubulin Riboprobe (lane 1) and T_1-digested tubulin Riboprobe without added *T. brucei* cellular RNA.

β-tubulin, in the two life-cycle stages. Incorporation into the rRNA and β-tubulin transcripts was quantified by densitometry of autoradiographs. Quantitative T_1 nuclease analysis using a ^{32}P-labeled antisense RNA probe confirmed that the steady state levels of rRNA in the long slender trypanosomes used in the pulse labeling experiment was >30-fold less than in the procyclic trypanosomes (Fig. 5A). Since the apparent rate of mitochondrial rRNA synthesis does not change during life-cycle of *T. brucei* and we conclude that the steady-state amount of rRNA is modulated by differential RNA degradation rates.

Discussion

The developmental control of *T. brucei* mitochondrial activities requires a complex array of regulatory mechanisms. Not only mitochondrial genes are regulated, but also a wide range of nuclear genes. The expression of the nuclear encoded mitochondrial protein cytochrome *c* is regulated at a post-translational step which results in differential stability of apocytochrome *c* in bloodstream and procyclic forms (ref. 23; Torri et al., unpublished results). The repressed state of the mitochondria in bloodstream forms of *T. brucei* requires high rates of glycolysis to meet the ATP requirements of these cells. This is accomplished in part by the assembly of a unique peroxisome-like organelle, the glycosome, which contains the majority of the glycolytic enzymes of the bloodstream trypanosome [24]. Activation of the mitochondrion in procyclic trypanosomes correlates with a decrease in the amount of several glycolytic enzymes which are post-transcriptionally regulated [25]. Developmental regulation of most trypanosome genes studied, both mitochondrial and nuclear, appears to occur at a post-transcriptional level [26]. An exception appears to be the expression of the glyceraldehyde-3-phosphate dehydrogenase gene which is transcriptionally regulated [27]. The results presented here are consistent with the expression of the mitochondrial rRNAs also being modulated at a post-transcriptional level. It appears that although the rates of rRNA synthesis are very similar in long slender bloodstream forms and procyclic forms that the steady state levels may differ by as much as 30-fold. This means that the stability of the mitochondrial rRNAs differs in the long slender and procyclic trypanosomes.

The mitochondrial rRNA not only appears to be synthesized at comparable rates but the rate of processing of the precursor 9S and 12S rRNAs is similar (Fig. 5). Based on these observations we propose the following pathway for mitochondrial rRNA synthesis, processing and expression during *T. brucei* development. Maxicircle encoded rRNA genes are transcribed in both long slender bloodstream forms and in procyclic forms to produce a multi-cistronic precursor which is rapidly processed in both developmental stages to 9S and 12S rRNAs. The 9S and 12S rRNAs are extremely stable in procyclic forms (half-

life of at least 36 h) but are much less stable in the long slender forms.

The turnover rates for the mitochondrial rRNA could not be directly measured in bloodstream long slender trypanosomes due to the limited viability of the TREU667 strain in bloodstream in vitro cultures (Hajduk, unpublished). Thus, our conclusion that the differential stability of the rRNAs modulates the amount of mitochondrial rRNA is based on indirect evidence. However, the 30–60 fold increase in the steady-state levels of rRNAs in the procyclic trypanosomes, without a detectable increase in the apparent rate of rRNA transcription, can only be explained by increased stability of the rRNA in this developmental stage.

There are several possible mechanisms which might developmentally regulate mitochondrial rRNA stability. Perhaps the simplest would be increased expression of a mitochondrial ribonuclease activity in the long slender forms. This would explain the decreased stability of the rRNAs and other developmentally regulated mRNAs such as cytochrome *b* and cytochrome oxidase II [6]. Alternative mechanisms might involve proteins binding to the rRNA or modification of the rRNA sequence or structure.

The discovery of a novel type of RNA processing called RNA editing [28,29] in trypanosome mitochondria makes it tempting to speculate that modification of the rRNA sequences might play an important role in rRNA stability. RNA editing involves the addition or deletion of uridines generally within the coding sequences of trypanosome mitochondrial mRNAs. Uridines can also be added within the poly(A) tail of mRNAs [30] and at the 3′ terminus of small minicircle and maxicircle transcripts called guide RNAs [17,31]. In addition, uridines are added to the 3′ terminus of the 9S and 12S mitochondrial rRNAs [32]. The added uridines might modulate the stability of the rRNA by mediating the formation of stable rRNA structures in the procyclic trypanosomes. The mechanism of addition and function of these added uridines is currently under investigation.

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