MOLECULAR AND CELLULAR BIOLOGY, Dec. 1991, p. 5878–5884
0270-7306/91/125878-07$02.00/0

Vol. 11, No. 12

Modification of *Trypanosoma brucei* Mitochondrial rRNA by Posttranscriptional 3′ Polyuridine Tail Formation

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Received 21 May 1991/Accepted 3 September 1991

***Trypanosoma brucei* mitochondrial transcripts can be posttranscriptionally processed by uridine addition or deletion. With editing of mRNAs, uridine addition and deletion create precisely altered reading frames. The addition of nonencoded uridines to mitochondrial guide RNAs results in a less precise modification. Although uridines are specifically added to the 3′ termini, their number varies, which results in heterogeneous oligo(U) tails on guide RNAs. In this paper, we show that the mitochondrial 9S and 12S rRNAs are also modified by uridine addition. These modifications appear to have aspects in common with both RNA editing and oligo(U) tail formation. Metabolic labeling studies with intact mitochondria and [α-^{32}P]UTP, in the absence of transcription, demonstrated the posttranscriptional timing of the event. T_1 RNase comparison analyses of cytidine 3′,5′-[5′-^{32}P]biphosphate 3′-end-labeled and [α-^{32}P]UTP metabolically labeled rRNAs, along with direct RNA sequencing of the 3′ termini, identified the site of uridine addition and revealed the creation of an oligo(U) tail for both rRNAs. 12S and 9S rRNAs hybrid selected from total cell RNA exhibited the same modification, demonstrating the presence of this processing in vivo. Moreover, only 3′-poly(U)-tailed 9S and 12S rRNAs were detected in total cellular and mitochondrial RNAs, which suggests that they are the most abundant and probable mature forms. The 12S and 9S rRNA oligo(U) tails differed significantly from each other, with the 12S having a heterogeneous tail of 2 to 17 uridines and the 9S having a tail of precisely 11 uridines. The mechanism of formation and the function of the rRNA poly(U) tails remain to be determined.**

Within the mitochondrion of *Trypanosoma brucei*, RNA transcripts can undergo the addition of uridine residues which are not encoded or the deletion of those that are. This appears to be an unusual form of RNA processing which is necessary for the ultimate creation of a mature transcript. To date, this process appears to take at least two general forms: RNA editing and heterogeneous uridine 3′ tail formation. Little is known of the actual mechanisms involved in the creation of either one, but there is strong evidence to suggest an important function for both.

The mitochondrion of *T. brucei* contains an unusual DNA structure, the kinetoplast, which consists of two types of circular elements (11). The larger of the two, the maxicircles, are approximately 23 kb in size and are present at 50 to 100 copies per cell. They correspond to the mitochondrial DNA of other species, for they encode mitochondrial proteins and rRNAs. The maxicircles are interlocked with approximately 10,000 (1-kb) minicircles to form a huge catenated network. Recent discoveries in RNA editing provide a functional link for these two DNA elements, in that both maxicircle and minicircle transcripts can be involved in the process.

RNA editing has been defined as a process which produces an RNA that has an altered nucleotide sequence in the protein coding region, compared with that of the DNA template (27). Many of the primary mitochondrial mRNAs are cryptic, lacking information necessary to create a meaningful translation product. Posttranscriptional addition and, occasionally, deletion of specifically placed uridines create new codons, start codons, and frameshifts in the precursor mRNA, resulting in a mature mRNA capable of translation (2, 13, 15, 27). The information for proper modification of the precursor is putatively provided by small, ~70-nucleotide (nt) mitochondrial transcripts called guide RNAs (gRNAs), which contain sequences complementary to the edited regions (when G-U wobble base pairing is allowed) (3). The models to date propose the formation of a complementary duplex between the mRNA and gRNA at the editing site, with the gRNA eventually determining the uridine number and placement within the preedited mRNA (3, 10). The end result of RNA editing is a precisely modified mRNA which is capable of translation and contains the complete, correct information for creating a functional protein.

In contrast to the precision of uridine addition in editing, 3′ poly(U) tails can be quite heterogeneous in length. Trypanosomatid gRNAs of both maxicircle (4) and minicircle (24) origins have nonencoded 3′ uridine termini which range from 5 to 24 residues. Modeling has demonstrated a possible functional role for the tail in stabilizing the initial preedited mRNA and gRNA duplexes (4), but other possibilities have also been proposed (24), including RNA stabilization, protein binding, and interaction with the mRNA poly(A) tail. Another proposal is that the uridine tail might serve as a donor and/or acceptor of the edited uridines (7). Irrespective of these hypotheses, the conservation of poly(U) 3′ tails across kinetoplastid species despite marked differences in transcript sequences argues for an important functional role of the uridine tails of gRNAs.

Uridine addition may also occur within the polyadenylated tails of mitochondrial mRNA (29). However, this appears to be a random process, in that addition occurs diffusely and variably throughout the poly(A) region rather than at specific sites. To date, the only nonrandom posttranscriptional uridine addition that has been described is in preedited mRNA and gRNA, elements which are central to the proposed editing process.

We have found that mitochondrial RNAs of another class, 9S and 12S rRNAs, also undergo nonrandom posttranscrip-

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tional uridine addition in the *T. brucei* mitochondrion. The modifications of the rRNAs share some aspects of both mRNA editing and gRNA 3′ tail formation. The 3′ poly(U) tail of 9S rRNA has precisely 11 uridines, whereas the poly(U) tail of the 12S rRNA is heterogeneous, having 2 to 17 nonencoded uridines. rRNAs with poly(U) tails are present in RNA from isolated mitochondria and in RNA hybrid selected from total cellular RNA. Furthermore, our evidence indicates that rRNAs with processed 3′ ends represent the most abundant steady-state form and, therefore, the probable mature and functional rRNA.

MATERIALS AND METHODS

Isolation of mitochondria and mitochondrial RNA. Procyclic *T. brucei* TREU 667 cells were grown at 27°C in semidefined medium (9) supplemented with 10% fetal bovine serum (Whittaker) plus 25 μg of gentamicin sulfate (Sigma) per ml. The cells were harvested in the late log phase of growth (density, 1.0×10^7 to 1.5×10^7 cells per ml) by centrifugation at 6,000 × *g* for 10 min at 4°C. The isolation of mitochondria followed a previously described procedure (5, 25) with osmotic lysis and separation on a Percoll gradient.

The mitochondria were resuspended in a solution containing 10% glycerol, 10 mM Tris (pH 8.0), and 1 mM $CaCl_2$ and were treated with micrococcal nuclease (1 U/3.5 × 10^6 harvested cells) (Pharmacia LKB Biotechnology, Inc.) for 25 min at 22°C. EDTA was then added to bring the final concentration to 10 mM, and the mitochondria were centrifuged at 32,500 × *g* for 15 min at 4°C. The pellet was dissolved in 100 mM NaCl–10 mM Tris (pH 8.0)–5 mM EDTA–0.5% sodium dodecyl sulfate. Proteinase K was added to a final concentration of 10 μg/ml, and the tube was incubated at 37°C for 15 min. The nucleic acid was extracted with phenol, phenol-chloroform, and chloroform and then precipitated with 0.3 M sodium acetate and 1.2 volumes of isopropanol at −70°C.

3′ end labeling of RNA. Total mitochondrial RNA was 3′ end labeled with cytidine 3′,5′-[5′-^{32}P]bisphosphate ([^{32}P]pCp) (New England Nuclear) by using T4 RNA ligase (Bethesda Research Laboratories) according to the manufacturer's recommendations. Labeled RNA was resolved on 6 or 3.5% denaturing polyacrylamide gels and was visualized by autoradiography. 9S and 12S rRNAs were gel purified on 3.5% gels. The rRNA bands were visualized by autoradiography and were excised and eluted in 0.5 M ammonium acetate–0.1% sodium dodecyl sulfate–5 mM EDTA overnight at 37°C. The RNA was extracted with phenol-chloroform and chloroform, and the RNA was precipitated with isopropanol at −70°C.

Metabolic labeling of mitochondrial RNA. Isolated mitochondria from 5×10^9 cells were incubated for 30 min in 1 ml of buffer containing 5 mM HEPES (pH 7.6), 3 mM potassium phosphate (pH 7.7), 125 mM sucrose, 6 mM potassium chloride, 10 mM magnesium chloride, 1 mM EDTA, 2 mM 2-mercaptoethanol, and 100 mM ATP at room temperature. Previous work with this system verified the cessation of transcription during this incubation period (19). The mitochondria were then pelleted by centrifugation in a Microfuge for 5 min and resuspended in the same buffer plus 0.5 μCi of [α-^{32}P]UTP (New England Nuclear) per ml and incubated for an additional 30 min at room temperature. The mitochondria were again collected by centrifugation. Lysis of mitochondria and isolation of RNA proceeded as described above, except no micrococcal nuclease treatment was used. The precipitated nucleic acid was washed twice with 70% ethanol to remove unincorporated label and was then resolved on 3.5% polyacrylamide gels.

RNase T_1 digestion. Gel-purified rRNAs were treated with 1,400 U of RNase T_1 (G specific) (Pharmacia) for 30 min in a buffer containing 0.3 M NaCl, 50 mM Bicine (pH 7.8), and 1 mM EDTA at 37°C to yield complete cleavage. The RNA was extracted with phenol, phenol-chloroform, and chloroform and was precipitated with isopropanol. The labeled fragments were resolved on a 20% polyacrylamide gel. In some cases, the relevant bands were again removed, and the RNA was eluted as described above.

Isolation of total cell RNA and hybrid selection of 12S and 9S rRNAs. Cells were grown and harvested as described above. After being washed with ice-cold 150 mM NaCl–20 mM glucose–20 mM $NaPO_4$ (pH 7.9), the cells were lysed with 100 mM Tris (pH 8.0)–10 mM EDTA–0.5% sodium dodecyl sulfate and were immediately extracted with phenol, phenol-chloroform, and chloroform and were isopropanol precipitated. The pellet was resuspended in a solution containing 10 mM Tris (pH 8.0), 5 mM EDTA, and 0.5% sodium dodecyl sulfate and was treated with proteinase K at a final concentration of 250 μg/ml for 30 min at 37°C. The solution was extracted and precipitated as described above. The pellet was then resuspended in a solution containing 250 mM sucrose, 20 mM Tris (pH 8.0), and 2 mM $MgCl_2$ and was treated with 10 U of RNase-free DNase (Bethesda Research Laboratories) per 10^9 cells for 30 min at 37°C. The solution was extracted with phenol, phenol-chloroform, and chloroform, and the RNAs were precipitated as described above. The RNAs were examined on 1.5% CH_3Hg agarose gels to control for degradation.

A plasmid clone containing the maxicircle 12S and 9S rRNA genes was generated by using *Hin*dIII- and *Bam*HI-digested kinetoplast DNA. Plasmid DNA was purified (21) and linearized with *Eco*RI restriction endonuclease. After denaturation with 0.3 M NaOH for 10 min at 65°C, NH_4Cl was added to achieve a final concentration of 1 M, and the solution was applied to nitrocellulose by using a manifold vacuum (approximately 50 μg of DNA per 1-cm-diameter piece) and then baked at 80°C for 2 h under a vacuum.

Mitochondrial rRNAs were hybrid selected by using 200 μg of total cell RNA per 1-cm-diameter filter in a buffer of 20 mM $NaHPO_4$ (pH 7.5)–2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–2.5 mM EDTA–0.1% sodium dodecyl sulfate at 55°C for 20 h. The filters were subsequently washed at 55°C (twice with hybridization solution containing 1× SSC and once with hybridization solution containing 0.5× SSC), and then they received a final wash of 0.5× SSC hybridization solution at 65°C. Annealed RNA was eluted from the membrane with 10 mM Tris (pH 7.6)–5 mM EDTA–0.1% sodium dodecyl sulfate and was heated to 95°C for 10 min. The RNA was then processed as described above.

Sequencing of RNA. Gel-purified 3′-end-labeled 9S rRNA was sequenced by using U_2 (A-specific), Phy M (A+U-specific), *Bacillus cereus* (C+U-specific), and T_1 (G-specific) RNases according to the instructions of the supplier (Pharmacia). Complete RNase T_1 cleavage of 3′-end-labeled 12S rRNA generated fragments of multiple lengths. They were purified by isolation from a 20% polyacrylamide gel, and the fragments were sequenced individually by the method described above.

The terminal nucleotides were determined by using [^{32}P]pCp-3′-end-labeled RNase T_1-generated and gel-purified fragments of both 9S and 12S rRNAs, which were then completely digested with RNases T_1, A, and T_2 and were

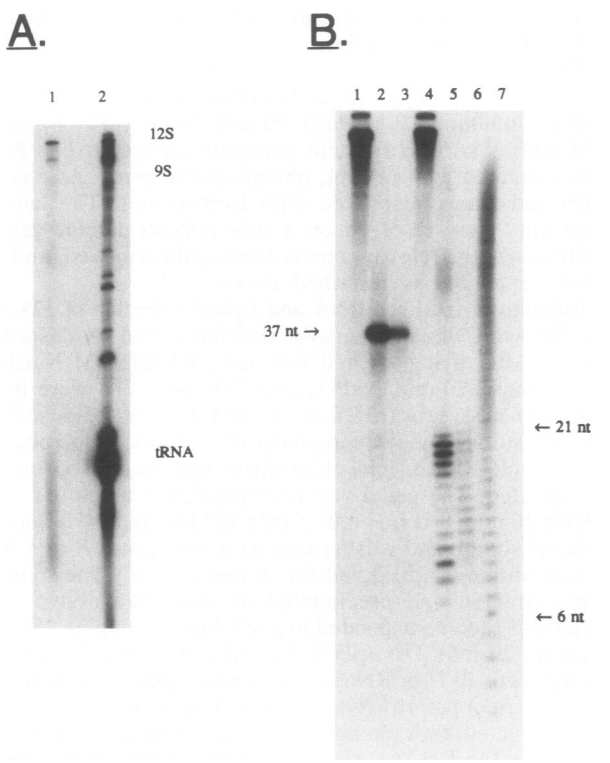


FIG. 1. Metabolic [α-^{32}P]UTP labeling of rRNA. By using isolated mitochondria, RNA was labeled by adding [α-^{32}P]UTP to the system after an initial incubation period during which all transcription ceased. (A) Incorporation of the label was assessed by analyzing the RNA on a 6% denaturing acrylamide gel (lane 1). The [^{32}P]pCp-3′-end-labeled mitochondrial RNA was run as a control (lane 2). (The 9S and 12S rRNAs are 611 and 1150 nt, respectively [28], and the tRNAs average approximately 70 nt). (B) Gel-purified, metabolically [α-^{32}P]UTP-labeled and [^{32}P]pCp-3′-end-labeled 9S and 12S rRNAs were subjected to complete T_1 RNase (G specific) digestion and were evaluated on a 20% denaturing acrylamide gel. Shown are [^{32}P]pCp-3′-end-labeled 9S rRNA without (lane 1) and with (lane 2) T_1 RNase cleavage, T_1 RNase-cleaved [α-^{32}P]UTP metabolically labeled 9S rRNA (lane 3), [^{32}P]pCp-3′-end-labeled 12S rRNA without (lane 4) and with (lane 5) T_1 RNase cleavage, T_1 RNase-cleaved [α-^{32}P]UTP metabolically labeled 12S rRNA (lane 6), and an alkaline hydrolysis ladder (lane 7). Reference nucleotide lengths are given.

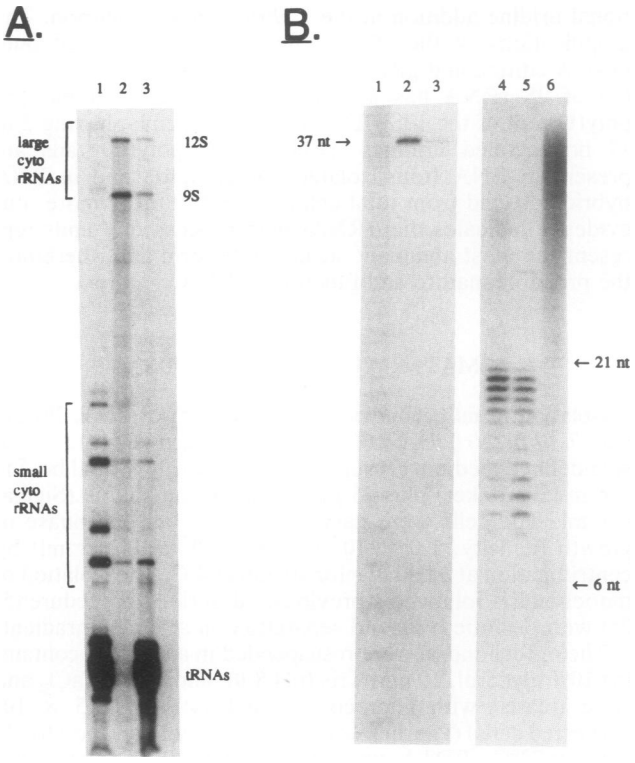


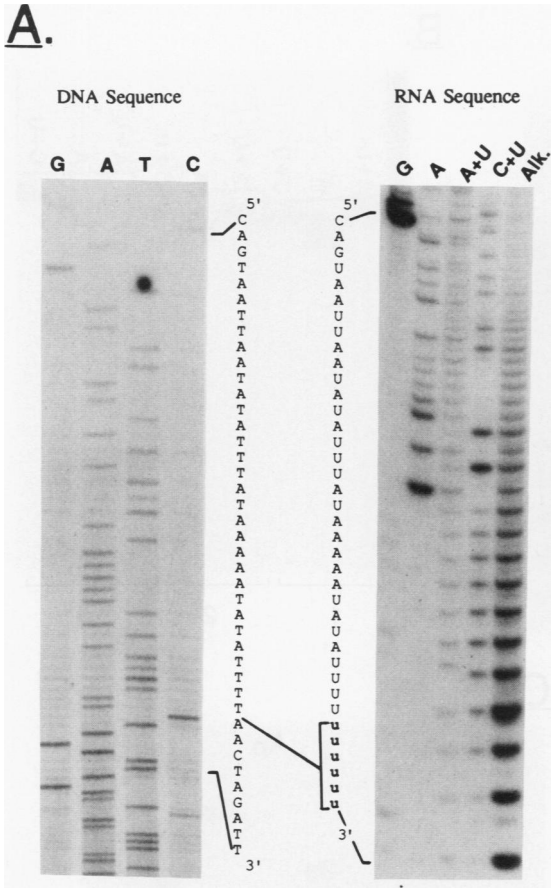
FIG. 2. Analysis of 3′ termini from hybrid-selected 12S and 9S rRNAs. (A) Total cellular RNA was obtained by detergent lysis and rapid extraction of nucleic acids followed by DNase treatment. Lane 1, [^{32}P]pCp-labeled total-cell RNA. The expected cytoplasmic (cyto) rRNAs are indicated (6, 8, 20). The mitochondrial rRNAs were selected by incubation with cloned 12S and 9S rRNA genes fixed to nitrocellulose. Lane 2, RNA eluted and 3′ end labeled with [^{32}P]pCp; lane 3, [^{32}P]pCp-labeled RNA from isolated mitochondria. (B) Hybrid-selected and isolated mitochondrial 9S and 12S rRNAs were gel purified, 3′ end labeled with [^{32}P]pCp, and subjected to complete cleavage with T_1 RNase. The labeled fragments were visualized by autoradiography after separation on a 20% acrylamide gel. Lanes 1 and 6, alkaline hydrolysis ladders; lane 2, 9S rRNA from isolated mitochondria; lane 3, hybrid-selected 9S rRNA; lane 4, 12S rRNA from isolated mitochondria; lane 5, hybrid-selected 12S rRNA. The faint higher-molecular-weight band seen in lane 5 is 37 nt and represents contamination of the 12S rRNA with 9S T_1 fragments.

subsequently analyzed by two-dimensional thin-layer chromatography (23).

Cloning and sequencing of the 3′ terminus of 12S RNA. cDNA was generated by using gel-purified 12S rRNA, avian reverse transcriptase, and an oligonucleotide (5′-CCCTTCG AATTCAAAAAAAAAAAATTC-3′) which included the sequence complementary to the 12S rRNA sequence obtained above. The procedure followed the recommendations of the supplier (Bethesda Research Laboratories). Another oligonucleotide was generated on the basis of the published 12S rRNA sequence (12) from nt 1090 to 1111 (5′-CCGGATCCT CGTTAGTTGGGTTAAAATCG-3′). These two oligonucleotides were used with *Taq* polymerase (Perkin-Elmer Cetus) in the polymerase chain reaction. Samples were denatured at 94°C for 1 min, annealed at 56°C for 2 min, and extended at 72°C for 3 min for a total of 30 cycles. The products of this reaction were resolved on an 8% nondenaturing polyacrylamide gel and were visualized by ethidium bromide staining, and the desired fragment was purified by electroelution. The fragment was then cloned into a Bluescript vector, and the clones were screened for insert size and then sequenced by the dideoxy method (21).

RESULTS

Metabolic-labeling studies with intact *T. brucei* mitochondria provided evidence of posttranscriptional uridine addition to rRNA. This system, which has been described in detail elsewhere (19), permitted the separation of transcriptional and posttranscriptional events. Mitochondria isolated on a Percoll gradient exhausted their endogenous pools of nucleoside triphosphates during an initial incubation period, and transcription ceased. However, following the addition of UTP to the system, uridine was added to RNA in the absence of transcription. When this system was used with [α-^{32}P]UTP, there was evidence of uridine incorporation into mitochondrial rRNA, as defined by subsequent analysis with



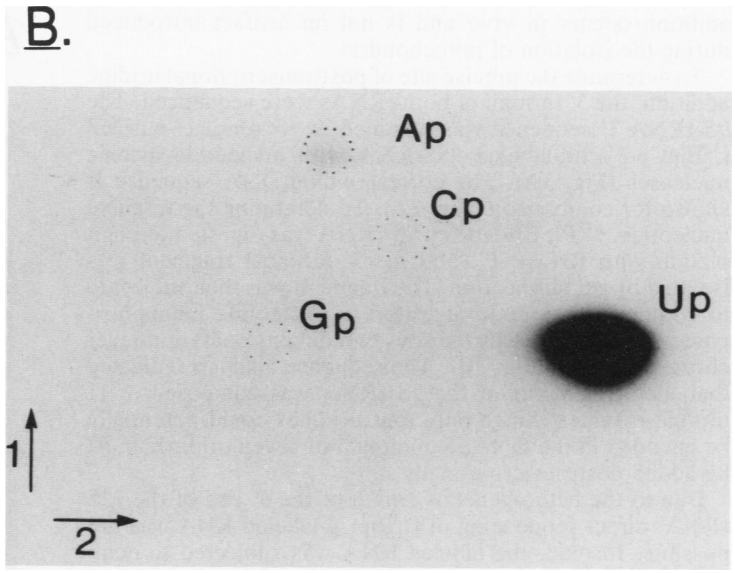


FIG. 3. 3′ nucleotide sequence of 9S rRNA. (A) [^{32}P]pCp-3′-end-labeled 9S rRNA was gel purified and sequenced by nucleotide-specific enzymatic degradation. The 9S rRNA maxicircle gene was cloned into a plasmid vector and sequenced by the dideoxy method, and it is shown for comparison. (B) The terminal nucleotide was determined by enzymatic degradation of [^{32}P]pCp-3′-end-labeled 9S rRNA to the indicated nucleotide monophosphates followed by two-dimensional thin-layer chromatography analysis. Alk, alkaline hydrolysis ladder.

denaturing acrylamide gels (Fig. 1A). Total mitochondrial RNA 3′ end labeled with [^{32}P]pCp is shown for comparison (Fig. 1A, lane 2). It should be noted that the two types of labeling gave different results in terms of the overall labeling of the mitochondrial RNAs. The metabolic [α-^{32}P]UTP labeling defined a relatively limited number of RNAs which included the minicircle transcript region at the bottom of the gel (55 to 70 nt), some less abundant, diffuse higher-molecular-weight RNAs (presumably mRNAs), and the rRNAs. Corresponding RNAs for each of these were found in the 3′-end-labeled RNA (Fig. 1A, lane 2). In addition, the 3′-end-labeled population contained several RNAs which were not metabolically labeled with [α-^{32}P]UTP in the isolated organelle system. These included nuclear-encoded tRNAs (17) and contaminating cytosolic rRNAs. This suggested that the process of [α-^{32}P]UTP addition has specificity, since it occurs only with a subpopulation of mitochondrial RNAs.

A complete T_1 RNase (G specific) digestion of the metabolically labeled rRNA was done to assess the distribution of the added uridine(s) within the 9S and 12S rRNAs. Gel-purified [^{32}P]pCp-3′-end-labeled rRNAs or [α-^{32}P]UTP metabolically labeled rRNAs were completely cleaved with T_1 RNase and were examined on denaturing acrylamide gels (Fig. 1B). Three conclusions were drawn from these data. First, the fragments generated by the two labeling procedures were the same size, taking into account the extra base which occurs with [^{32}P]pCp labeling, which therefore suggested that they were the same 3′-terminal fragment of rRNA. Second, the mature form of the rRNAs contained 3′ added uridine(s). This conclusion followed from the assumption that [^{32}P]pCp labeling of rRNA reflected the relative abundance, at steady state, of each 3′ terminus. Although exact quantification was not done, it was clear that each [^{32}P]pCp-labeled rRNA fragment had a corresponding metabolic, posttranscriptionally labeled one (Fig. 1B, lanes 2 and 3 and lanes 5 and 6). Therefore, the vast majority, if not all, of the 3′-end-labeled rRNA had undergone prior uridine addition. Third, the nature of the 3′ ends differed between the 12S and 9S rRNAs, with the former having a heterogeneous and the latter having a homogeneous terminus (Fig. 1B, lanes 5 and 2, respectively). The 12S 3′ end ranged from 6 to 21 nt in length beyond the 3′-most guanidine, whereas all 9S rRNAs extended exactly 37 nt from the 3′-most guanidine. The faint smear of higher-molecular-weight RNA in Fig. 1B (lanes 2 and 5) represents the terminal fragments with the polyadenylated tails of the mRNAs which were copurified with the rRNAs.

Although it was unlikely, considering the homogeneity of the 9S rRNA, it was possible that the uridine addition to the mitochondrial rRNAs could have been a nonspecific process which occurred during mitochondrial isolation because of a terminal uridine transferase activity (1). To address this possibility, total RNA was isolated from trypanosomes, following rapid lysis with detergent and extraction of the nucleic acid. The 9S and 12S rRNAs were hybrid selected by using cloned 9S and 12S rRNA maxicircle genes fixed onto nitrocellulose support. Hybrid-selected rRNAs were eluted and 3′ end labeled with [^{32}P]pCp. Analysis on an acrylamide gel demonstrated good selectivity without significant degradation (Fig. 2A). This RNA was gel purified and subjected to T_1 RNase cleavage. The resulting RNA was analyzed on a denaturing gel alongside gel-purified T_1 RNase-treated mitochondrial RNA (Fig. 2B). The RNA from both sources gave fragments of the same length, indicating that the uridine

addition occurs in vivo and is not an artifact introduced during the isolation of mitochondria.

To determine the precise site of posttranscriptional uridine addition, the 3′ termini of both rRNAs were sequenced. The 9S rRNA 3′ sequence was obtained by treating gel-purified [^{32}P]pCp-3′-end-labeled 9S rRNA with nucleotide-specific nucleases (Fig. 3A). The corresponding DNA sequence is shown for comparison (Fig. 3A). To determine the terminal nucleotide, [^{32}P]pCp-labeled 9S rRNA was digested to completion with RNase T_1, and the 3′ terminal fragment was isolated by gel purification. The fragment was then subjected to complete enzymatic digestion to nucleotide monophosphates and was analyzed by two-dimensional thin-layer chromatography (Fig. 3B). The sequence analysis indicated that the 3′ terminus of the 9S rRNA was composed of 11 uridine residues. Since only four uridines could potentially be encoded in the DNA, a minimum of seven uridines must be added posttranscriptionally.

Due to the heterogeneous length of the 3′ end of the 12S rRNA, direct sequencing of [^{32}P]pCp-labeled RNA was not possible. Instead, the labeled RNA was subjected to complete T_1 RNase cleavage, and the 3′-labeled fragments were gel purified individually (Fig. 4A). These fragments were then sequenced separately (Fig. 4B) or were subjected to complete enzymatic degradation, and the terminal nucleotide was determined as described above for the 9S rRNA (Fig. 4C). The sequencing demonstrated that the 12S rRNA also had a 3′ uridine tail and that the 3′ heterogeneity was generated by the variable number (2 to 17) of added uridines.

Each T_1 fragment began with the sequence 5′-GAA (Fig. 4B). Since these were the only non-U residues found in the T_1 fragments, not enough sequence was available to unambiguously determine either the site of the 3′ terminus of the 12S rRNA gene or the minimum number of added uridines. This cDNA was used with the polymerase chain reaction to generate double-stranded DNA containing the 3′-terminal fragment of the 12S gene. The primers for the reaction included the above-described oligonucleotide and a 28-mer oligonucleotide with the sequence of an upstream segment of the 12S rRNA gene. This fragment of DNA was cloned into a Bluescript vector and sequenced (Fig. 5). The cDNA sequence revealed that the 3′ terminus of the encoded 12S transcript was downstream of the published site by 11 bases (28) and that none of the uridines in the tail of the 12S rRNA could have been encoded (Fig. 6).

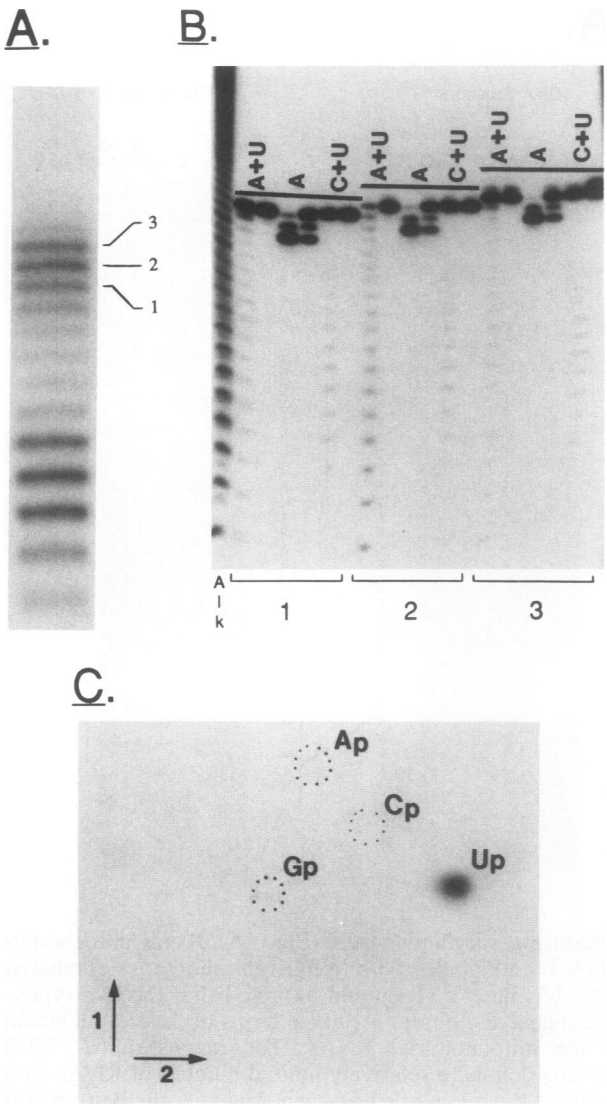


FIG. 4. 12S rRNA 3′ end sequence. (A) 12S rRNA was gel purified, [^{32}P]pCp end labeled, and subjected to complete T_1 RNase degradation. The fragments generated from enzymatic treatment were separated on 20% polyacrylamide gels and purified. (B) Each fragment was sequenced by using nucleotide-specific nucleases. The sequences shown are from the three fragments similarly labeled in panel A. An alkaline hydrolysis ladder is shown (Alk). (C) The 12S rRNA terminal nucleotide was determined by pooling purified [^{32}P]pCp-3′-end-labeled T_1 RNase-generated fragments and enzymatically degrading them to the indicated nucleotide monophosphates and then using two-dimensional thin-layer chromatography.

DISCUSSION

This work demonstrates that, in addition to mRNA and guide RNA, another class of RNA, rRNA, undergoes posttranscriptional uridine addition in the mitochondrion of *T. brucei*. The metabolic labeling results (in the isolated organelle system) verified the posttranscriptional timing of the event, since incorporation of uridine into rRNA continued despite the absence of transcription. The process appeared to be neither indiscriminant nor random. In comparison with [^{32}P]pCp-end-labeled total mitochondrial RNA, only a select population undergoes posttranscriptional metabolic labeling with [^{32}P]UTP (Fig. 1A). Furthermore, specificity of the process is implied by both the precise number of uridines which are added to the 9S rRNA and the defined-length heterogeneity of the 3′ poly(U) tails on the 12S rRNA.

The processed forms of the rRNA found in the isolated mitochondria were the only forms found in vivo. The addition of a poly(U) tail seems to be part of the mature rRNA. Sequencing of the 9S and 12S rRNAs definitively localized the uridine additions to the 3′ termini and demonstrated the presence of a uridine tail in both (Fig. 6). In addition, the 12S cDNA sequence unequivocally identified the transcribed nucleotides found in the 3′ terminus of the mature rRNA and revealed that all the uridines in the tail were part of the posttranscriptional modification.

The mechanism of 3′-end formation is not known. A terminal uridyltransferase in whole-cell extracts from trypanosomes (30) and in the mitochondria of trypanosomes (18) and leishmania (1) has been described, and it may well be involved in this processing of the rRNA. Formation of the heterogeneous 12S rRNA 3′ terminus may require little else

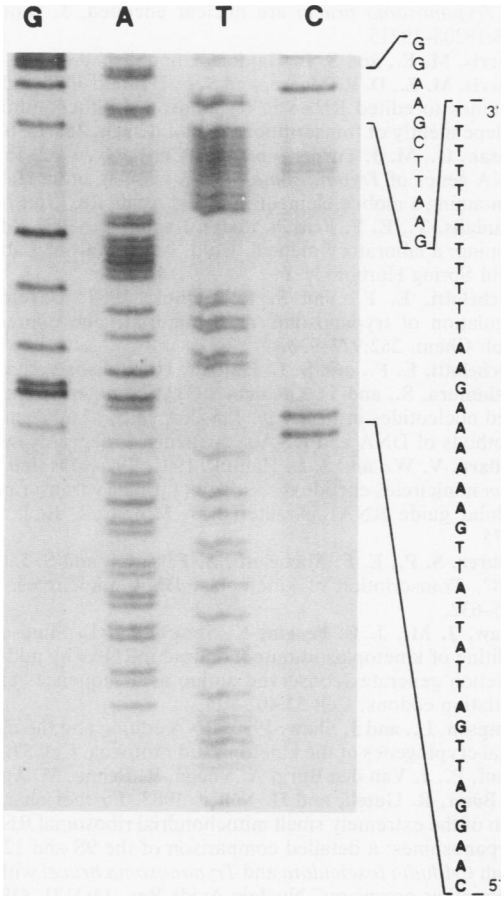


FIG. 5. 12S rRNA 3'-terminal cDNA sequence. First-strand cDNA was synthesized by using gel-purified 12S rRNA, reverse transcriptase, and an oligonucleotide complementary to the poly(U) tail (5'-CCCTTCGAATTCAAAAAAAAAAAATTC-3'). The same oligonucleotide, together with another one containing a segment of published (12) upstream gene sequence (5'-CCGGATCCTCGTTAG TTGGGTTAAAATCG-3'), was used with the polymerase chain reaction to generate double-stranded DNA, which was cloned into Bluescript vector and sequenced by the dideoxy method. The outer column gives the sequence for the 3' end of the 12S rRNA, with the polarity as indicated.

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12S  DNA  5'...CGCAAGGATTGATTATTGAAAAAAGAAAGAAGAATAT...3'
     RNA  5'...CGCAAGGAUUGAUUAUUGAAAAAAGAAu(2-17)  3'

9S   DNA  5'...GTAATTAATATATTTATAAAAATATATTTTAGCTAAA...3'
     RNA  5'...GUAAUUAAUAUAUUUAUAAAAAUAUAUUUUuuuuuuu  3'
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FIG. 6. Comparison of the 12S and 9S rRNA 3'-terminal sequences and the maxicircle DNA sequences. Lowercase letters in the RNA sequences indicate nonencoded bases.

besides this enzymatic activity. However, the precision of the 9S modification implies a different mechanism, perhaps a process with at least some features of RNA editing. Uridine addition might occur internally, within a precursor rRNA, with the number of uridines being directed by a 9S-rRNA-specific gRNA. The uridine addition to the precursor could then define an endonuclease cleavage site to create the 3' terminus of the mature 9S rRNA. Alternatively, the formation of a specific 9S rRNA poly(U) tail length may be a separate step which occurs subsequent to the addition process.

A number of possibilities for the functional significance of the uridine tails exist. The uridines may participate in the secondary or tertiary structure of the rRNA. An important structure of rRNA may be conserved as a result of the modification. In this regard, the trypanosome mitochondrial rRNAs are peculiar because of their small size and absence of sequence homology with other rRNAs (12). Nonetheless, the 9S and 12S rRNAs correspond to the 16S and 23S rRNAs, respectively, of *Escherichia coli*. Although some domains are lacking, many are conserved in trypanosome rRNAs despite their unusual primary structure (28). The few added uridines in the trypanosomes may be essential for the creation of another domain. Alternatively, a protective stem-loop may be formed by poly(U) addition, preventing exonucleolytic degradation of the RNA.

The modification may also be significant for intermolecular interaction. Modeling with gRNAs (4) which contain 3' nonencoded uridines resembling the 12S rRNA provides some precedence for this. In this model, the tail is involved with stabilizing the mRNA-gRNA duplex. The rRNA tail could similarly participate in interactions with other RNAs or proteins. Binding between the tails of polyuridylated rRNA and polyadenylated mRNA is one such possibility. Furthermore, if the uridine tail is involved in stabilization or maturation of the rRNA, then the processing would provide a means of regulating expression. RNA editing in *T. brucei* is known to be a developmentally regulated process (14, 16), with some edited transcripts being absent in long, slender bloodstream forms despite the presence of the unedited transcripts (16). rRNA processing may undergo similar developmental variation. This could explain the 30-fold difference in steady-state levels of rRNA observed in the different developmental forms despite similar rates of transcription (22, 22a).

The demonstration of this unusual RNA processing of the *T. brucei* mitochondrial rRNA presents many interesting possibilities of mechanism and function. The modification has similarities to other known posttranscriptional changes which occur to RNA involved with editing. Should the mechanisms of editing and uridine tail formation in rRNA ultimately prove to be similar, a broader, mechanistic definition of RNA editing may be desirable.

ACKNOWLEDGMENTS

We thank Kathy Hancock for helpful comments on the manuscript.

B.K.A. was supported by NIH Hematology Training grant 5T32DK07488. S.L.H. is a Burroughs Wellcome Fund Scholar in Molecular Parasitology. This work was supported by PHS grant AI 21401 to S.L.H.

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