

Addition of Uridines to Edited RNAs in Trypanosome Mitochondria Occurs Independently of Transcription*

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RNA editing is a novel RNA processing event of unknown mechanism that results in the introduction of nucleotides not encoded in the DNA into specific RNA molecules. We have examined the post-transcriptional addition of nucleotides into the mitochondrial RNA of *Trypanosoma brucei*. Utilizing an isolated organelle system we have determined that addition of uridines to edited RNAs does not require ongoing transcription. Trypanosome mitochondria incorporate CTP, ATP, and UTP into RNA in the absence of transcription. GTP is incorporated into RNA only as a result of the transcription process. Post-transcriptional CTP and ATP incorporation can be ascribed to known enzymatic activities. CTP is incorporated into tRNAs as a result of synthesis or turnover of their 3' CCA sequences. ATP is incorporated into the 3' CCA of tRNAs and into mitochondrial messenger RNAs due to polyadenylation. In the absence of transcription, UTP is incorporated into transcripts known to undergo editing, and the degree of UTP incorporation is consistent with the degree of editing occurring in these transcripts. Cytochrome *b* mRNAs, which contain a single editing site near their 5' ends, are initially transcribed unedited at that site. Post-transcriptional labeling of cytochrome *b* mRNAs in the organelle with [α - 32 P]UTP results in the addition of uridines near the 5' end of the RNA but not in a 3' region which lacks an editing site. These results indicate that RNA editing is a post-transcriptional process in the mitochondria of trypanosomes.

Recently, RNAs from several sources have been discovered which differ from their genes by the addition, deletion, or modification of nucleotides. These forms of modification have been termed RNA editing. In *Trypanosoma brucei*, RNA editing occurs in a subset of mitochondrial transcripts and involves addition and removal of uridine residues (1, 2). The mechanism of the addition process is unknown.

Trypanosome mitochondria have a uniquely organized mitochondrial genome, as do the other members of the Order Kinetoplastidae. The mitochondrial genome, termed the kinetoplast (kDNA),¹ is a complex DNA network composed of

interlocked 23-kilobase (kb) maxicircles and 1-kb minicircles present at approximately 50 and 10,000 copies, respectively (3-5). While the function of this complex structure and the role of minicircles are unclear, the maxicircles are functionally analogous to other mitochondrial DNAs in that they contain the genes for the mitochondrial rRNAs and several mitochondrial proteins. The minicircles, although they are transcribed (6, 7), have no known coding function. Only transcripts from the maxicircle have been shown to undergo internal uridine addition.

In 1986, Benne and co-workers (8) observed that the transcript for the mitochondrial cytochrome oxidase subunit II (COII) contained four extra uridine residues that were not coded in the maxicircle DNA. The added uridines correct a frameshift in the COII gene and are apparently responsible for the production of a functional COII mRNA. Since no gene corresponding to the altered sequence could be found in either the mitochondrial or nuclear genomes, they concluded that the uridines were introduced as the result of a novel form of RNA processing. This type of RNA modification could conceivably involve either a co-transcriptional or post-transcriptional mechanism.

Several other RNAs, both in *T. brucei* and other trypanosomatids, have been shown to undergo this same form of sequence modification. Primer extension sequencing and cDNA cloning of mitochondrial RNAs has revealed the following. In *T. brucei* the mitochondrial cytochrome *b* (CYb) mRNA as well as the mRNAs for the cytochrome oxidase subunit III (COIII) and for an unidentified protein (mitochondrial unassigned reading frame 2 (MURF 2)) are edited by uridine addition (9-12). Analysis of the 9 and 12 S mitochondrial rRNA has shown that they have defined numbers of uridines added to their 3' ends.² The phenomenon ranges from the modest, four uridines added to the COII mRNA, to the incredible, 347 uridines added at 121 different sites in the sequenced portions of the COIII transcript. In most cases the added uridines have an implied functional role. Editing creates potential AUG codons in the CYb and MURF 2 mRNAs (9, 10, 13) and corrects the frameshifted COII mRNA (8). Almost 60% of the coding region of the COIII mRNA is composed of added uridines (11). Edited regions include both 5'- and 3'-untranslated regions and the coding region. Runs of uridines have also been found in the poly(A) tails of edited mRNAs (11, 14).

Related forms of RNA modification have been found in other organisms. In mammals, tissue-specific expression of different forms of apolipoprotein B involves creation of a stop codon by a C to U conversion in the coding region of apolipoprotein B mRNAs (15). A process more analogous to editing

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¹ The abbreviations used are: kDNA, kinetoplast DNA; kb, kilobase pairs; COII and -III, cytochrome oxidase subunits II and III; CYb, cytochrome *b*; MURF, mitochondrial unassigned reading frame; TREU, trypanosome research Edinburgh University; ND 1, 4, and 5, NADH dehydrogenase subunits 1, 4, and 5; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; bp, base pair(s); [32 P]pCp, cytidine 3',5'-[5'- 32 P]bisphosphate.

² B. Adler, M. E. Harris, K. Bertrand, and S. L. Hajduk, unpublished results.

in kinetoplast mitochondria is found in certain paramyxoviruses. Non-template-encoded G residues have been found inserted into transcripts from the viral P gene. Addition of the G residues allows translation of the 3' half of this mRNA in a different reading frame (16, 17). Multiple C to U conversions, which generate conserved amino acid sequences, have recently been discovered in plant mitochondrial mRNAs (18, 19).

The mechanism by which defined numbers of uridines are inserted at specific sites in trypanosome mitochondrial RNAs is unknown. It has been suggested that editing occurs in a post-transcriptional fashion since unedited and partially edited forms of edited transcripts occur in the steady state RNA population (9, 11, 14, 20), but there is no direct evidence that this is the case. Terminal uridylyl transferase and RNA ligase activities have been detected in mitochondrial preparations from the kinetoplastid *Leishmania*; however, the relationship of these activities to the editing process remains speculative (21). Until now, addition of uridines to edited transcripts in the absence of transcription had not been demonstrated.

We have developed an isolated organelle system to study the biosynthesis of *T. brucei* mitochondrial RNA. Using this system we found that *T. brucei* mitochondria incorporated [α - 32 P]NTPs into RNA as a result of maxicircle and minicircle transcription. In addition, these isolated mitochondria also incorporated CTP, ATP, and UTP into RNA in the absence of transcription. CTP and ATP are added to mitochondrial tRNAs in the formation or turnover of tRNA CCA 3' end sequences. ATP also labels mitochondrial mRNAs, probably as a result of poly(A) addition at their 3' ends. Hybridization of the UTP-labeled RNA to kDNA shows that the uridine addition is specific to transcripts known to undergo RNA editing. The degree of post-transcriptional UTP labeling correlates with the amount of editing known to occur in these transcripts. Analysis of the transcription and editing of the maxicircle-encoded cytochrome *b* gene, which when edited contains 34 added uridines near its 5' end, revealed the following. Newly transcribed CYB mRNAs are unedited. The 5' region of this mRNA containing the editing site incorporates [α - 32 P]UTP in the absence of transcription while a 3' region with no editing site does not. These studies indicate that RNA editing is a post-transcriptional RNA processing event in the mitochondrion of *T. brucei*.

EXPERIMENTAL PROCEDURES

Isolation and Characterization of Mitochondria—Procyclic trypanosomes (TREU 667) were grown in a semi-defined medium (22) supplemented with 10% fetal bovine serum (Hyclone) and 50 μ g/ml gentamicin sulfate. Generally, 4 liters of culture at 1.0 – 1.5×10^7 cells/ml were used for mitochondria isolation.

Mitochondrial vesicles were isolated as follows. Cells were collected by centrifugation and washed once in 200–300 ml of ice-cold 150 mM NaCl, 20 mM glucose, 20 mM NaHPO₄, pH 7.9. Subsequent steps were all performed at 4 °C. Cells were resuspended at 1.2×10^9 cells/ml in 1 mM Tris, pH 8.0, 1 mM EDTA, briefly homogenized (five strokes) in a Dounce homogenizer (Type B pestle), and lysed by passage through a 26-gauge needle at high pressure. 60% sucrose was immediately added to 250 mM. This lysate was centrifuged for 10 min at $15,800 \times g$ (Sorvall SS-34 rotor, 11,500 rpm). The pelleted material was resuspended at one-fifth of the original lysate volume in 250 mM sucrose, 20 mM Tris, pH 8.0, 2 mM MgCl₂ and treated for 30 min with 9 μ g/ml RNase-free DNase (Bethesda Research Laboratories). This material was diluted 5-fold with 250 mM sucrose, 20 mM Tris, pH 8.0, 2 mM EDTA and centrifuged at $15,600 \times g$ as described above. The pelleted material was resolved on a 20–35% Percoll gradient (45 min, Beckman SW-28 rotor, 24,000 rpm), and a mitochondrial vesicle fraction was collected. Mitochondria were washed four times with an excess of 250 mM sucrose, 10 mM Tris, pH 7.5, 1 mM EDTA to remove Percoll. Vesicles could be stored for up to 1 month in 50% glycerol, 250 mM sucrose, 10 mM Tris, pH 7.5, 1 mM EDTA under

liquid nitrogen with little loss of transcriptional and post-transcriptional activities. After this time a decrease in nucleotide incorporating activities was observed.

For fluorescence microscopy vesicles were resuspended at 1 mg of mitochondrial protein/ml in 250 mM sucrose, 10 mM Tris, pH 7.5, 1 mM EDTA, and 0.1 μ g/ml ethidium bromide. Thin films of this material were photographed by phase contrast and fluorescence microscopy using a Nikon Microphot microscope with Kodak Ektachrome 200 film. Transmission electron microscopy of the mitochondrial fraction was performed as described (23).

Metabolic Labeling, Isolation, and Characterization of Mitochondrial RNA—Metabolic labeling of mitochondrial vesicles was carried out in a transcription 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 at a concentration of 1 mg of mitochondrial protein/ml. Endogenous mitochondrial nucleotide pools were depleted by preincubation for 30 min at 27 °C in transcription buffer containing 100 μ M ATP. Mitochondrial RNA was labeled with individual nucleotides at a concentration of 100–500 μ Ci/ml [α - 32 P]NTP (Amersham Corp., 400 Ci/mmol). The remaining non-radioactive nucleotides were at a concentration of 100 μ M. Mitochondria were double-labeled with [α - 32 P]GTP and [5,6- 3 H]UTP (ICN, 40 Ci/mmol) both at 100 μ Ci/ml under the conditions described above.

Incorporation of NTPs into total mitochondrial RNA was measured by pipetting a 50- μ l fraction of the labeling reaction directly into 5 ml of ice-cold 5% trichloroacetic acid, 100 mM sodium pyrophosphate. The precipitation reaction was left on ice for 10 min, and the precipitated material was collected on 24-mm glass fiber filters (Whatman) using a Hofer vacuum manifold. The amount of incorporation was determined by liquid scintillation counting.

Radioactively labeled RNA was prepared from these reactions by the following procedure. The mitochondrial vesicles were collected by centrifugation of the reaction mixture in a Microfuge for 4 min at 4 °C. The pelleted mitochondrial vesicles were resuspended in 500 μ l of 100 mM NaCl, 10 mM Tris, pH 7.5, 5 mM EDTA, 0.5% sodium dodecyl sulfate per ml of original reaction. The solubilized mitochondria were treated with 10 μ g/ml Proteinase K (Sigma) for 15 min at 37 °C. The RNA was extracted with phenol, phenol/chloroform (50:50), and chloroform and precipitated with sodium acetate and isopropyl alcohol. Labeled RNAs were separated from unincorporated nucleotides by chromatography on Sephadex G-50 (Pharmacia LKB Biotechnology Inc.) in 10 mM Tris, pH 7.5, 5 mM EDTA, 0.1% sodium dodecyl sulfate. RNAs were generally run on 1.5% methyl mercury-agarose gels (24) to estimate purity of the mitochondria and to control for RNase degradation during the isolation and transcription protocols. Labeled RNAs were also resolved on 6% denaturing polyacrylamide gels. Total mitochondrial RNA was end-labeled with cytidine 3',5'-[32 P]bisphosphate ([32 P]pCp) (Amersham Corp.) using T4 RNA ligase (Bethesda Research Laboratories) according to the manufacturer's recommendations.

Hybridization Analysis of Mitochondrial RNA—The various maxicircle clones were isolated as previously described (25). Total kDNA from procyclic trypanosomes were isolated according to the procedure of Fairlamb *et al.* (26). Cloned maxicircle fragments and digests of total kDNA were resolved on 1% agarose gels and transferred to GeneScreen Plus according to the manufacturer's recommendations (Du Pont-New England Nuclear). Filters were prehybridized in the following solution: 5 \times SSC (1 \times SSC is 150 mM NaCl and 15 mM sodium citrate), 1% sodium dodecyl sulfate, 5 \times Denhardt's solution, 5% dextran sulfate, 100 μ g/ml denatured salmon sperm DNA. Hybridizations and prehybridizations were carried out at 55 °C. Mitochondrial RNA labeled as described above and in the text were hybridized in the above solution, lacking salmon sperm DNA, at an activity of approximately 1×10^4 cpm/ml at 55 °C for 24–36 h. Following hybridization filters were washed twice with 3 \times SSC, 0.1% sodium dodecyl sulfate at 55 °C for 30 min and twice with 0.1 \times SSC, 0.1% sodium dodecyl sulfate at 65 °C for 30 min. Filters were dried and autoradiographed.

Prior to dot blotting, antisense cytochrome *b* oligonucleotides were first self-ligated to increase the efficiency of binding. Ligation was performed according to Tessier *et al.* (27). Ligated oligonucleotides were dot-blotted onto 0.05- μ m nitrocellulose filters (Schleicher & Schuell) and probed with labeled mitochondrial RNA as described above. Northern blot analysis with these same oligonucleotides was

performed as described (24). The following oligonucleotides were used.

CYb-1 edited 5' CCTGACATTAAGACAACACAAATTCTAAA 3'
 CYb-2 unedited 5' CCTGACATTAAGACCCTTTCTTTTCTC 3'
 CYb-3 nonedited 5' CCTACCCATATATTCAGTATAAACACCTGAC 3'

Cytochrome *b* gene fragments were produced by polymerase chain reaction using the method of Saiki *et al.* (28). 30 cycles of 1 min at 94 °C, 2 min at 37 °C, and 3 min at 72 °C were used for each synthesis. For fragments CYb-A and -B the following primers were used.

CYb-A forward 5' GTAGGAAGTTAAGAATAATGG 3'
 CYb-A reverse 5' GTACACTTCTTATCACAAAACCC 3'
 CYb-B forward 5' GAGTTCTGATGCATTTTGTGATAGG 3'
 CYb-B reverse 5' CCCATAAAGTATCTGGGATTGCC 3'

When the CYb-A forward and reverse primers were used a 246-bp DNA was generated corresponding to nucleotides -4 to 246 of the CYb gene (numbering beginning from the CYb mRNA 5' end). When the CYb-B forward and reverse primers were used a 263-bp DNA was generated which corresponded to nucleotides 617 to 880 in the CYb gene. These gene fragments were resolved on 1.5% agarose gels, blotted, and probed as described above with the following modification. Labeled mitochondrial RNA probes were base-hydrolyzed to fragments 200 nucleotides and smaller to examine labeling of specific portions of the CYb mRNA. The labeled RNA was hydrolyzed by resuspending it at approximately 25 µg of total mitochondrial RNA in 50 µl of 50 mM sodium carbonate and incubation at 50 °C for 60 min. The extent of hydrolysis was monitored by electrophoresis and autoradiography.

RESULTS

Characterization of Isolated *T. brucei* Mitochondria—Mitochondria were isolated from cultures of procyclic trypanosomes as follows. After hypotonic lysis, a rough granular fraction was obtained by centrifugation. This pellet was treated with DNase and a mitochondrial fraction obtained by density gradient centrifugation of this material on a 20–35% Percoll gradient. Fig. 1, A and B, shows phase contrast and ethidium bromide fluorescence microscopy of the mitochondrial fraction. Vesicles were generally uniform in size and free from cell ghosts and flagella. Typically 50–70% of the vesicles contained compact material that stained brightly with ethidium bromide. The ethidium-stained material was localized to the periphery of the vesicles and was probably kinetoplast DNA (Fig. 1B).

Transmission electron microscopy of the vesicle preparations (Fig. 1C) revealed that the majority of the vesicles are mitochondrial in origin. Most of the vesicles have a double membrane and contain cristae, indicative of mitochondrial vesicles. Electron microscopy showed that the major contaminants of these preparations are small groups of densely staining vesicles, which may be glycosomes, and flagella. Kinetoplast DNA was clearly visible in some of the sectioned mitochondria near their periphery. There appears to be some fragmentation of the kDNA and several of the mitochondrial membranes appear disrupted. This is probably due to the rigors of the isolation procedure. Mitochondria prepared from 2×10^{10} cells contained approximately 5 mg of protein. Since this figure is consistent between separate mitochondrial preparations, all labeling reactions are performed containing a constant amount (1 mg/ml) of mitochondrial protein.

Transcriptional and Post-transcriptional Incorporation of Nucleotides into *T. brucei* Mitochondrial RNA—There are three activities in *T. brucei* mitochondria that might be expected to incorporate nucleotides into RNA that are not due directly to the transcription process. First, ATP is incorporated into the poly(A) tails of mRNAs (29). Second, both ATP and CTP might be expected to be incorporated into mitochondrial tRNAs as a result of synthesis and turnover of the 3' CCA sequence (30). And finally, the editing process, if

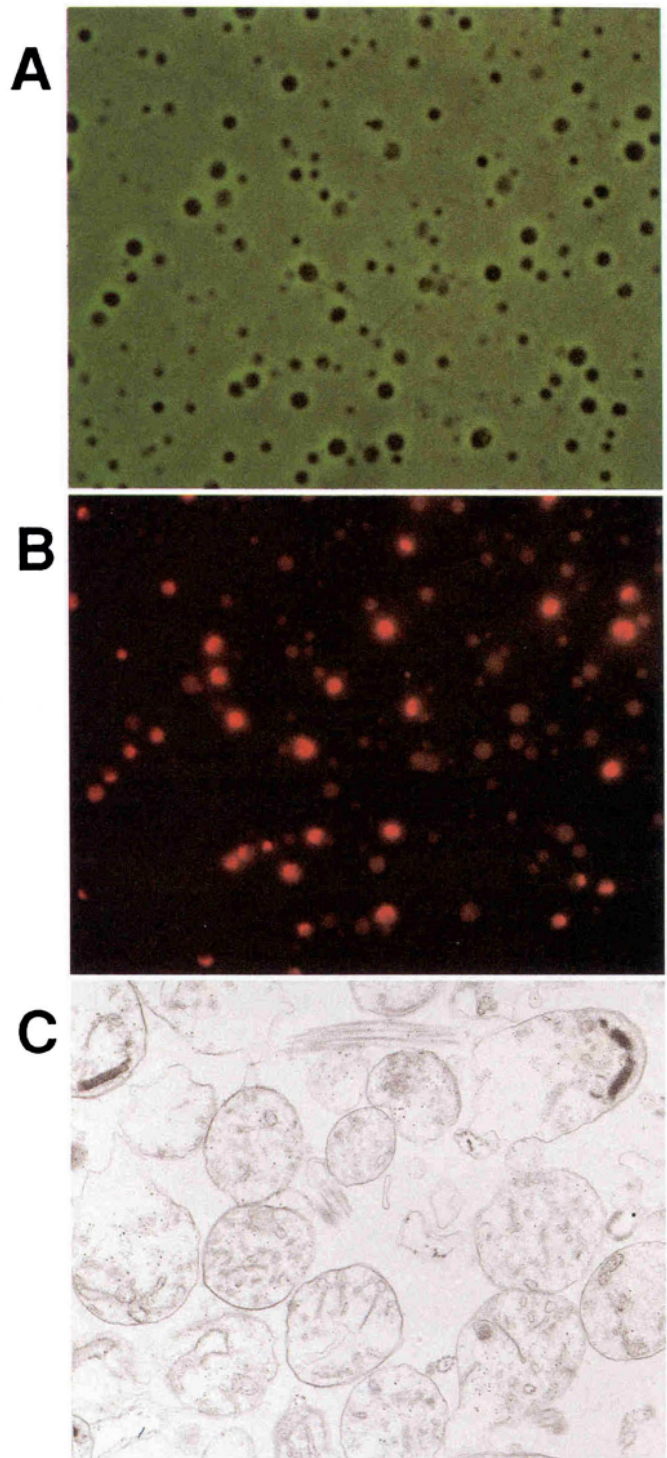


FIG. 1. Microscopic analysis of *T. brucei* mitochondrial vesicles. A, phase contrast microscopy of the vesicle preparations. Vesicles are magnified $\times 2,500$. B, fluorescence imaging of the same field as in A. Vesicles are stained with 0.1 µg/ml ethidium bromide. C, transmission electron microscopy of vesicles from the same preparation as A and B. Magnification is $\times 13,636$.

post-transcriptional, would result in the incorporation of UTP into specific transcripts. UTP should not be incorporated into RNA when transcription is inhibited if the editing process is co-transcriptional or requires ongoing transcription. On the other hand, GTP should be incorporated into mitochondrial RNA only as a result of the transcription process.

When mitochondria were incubated in the presence of an [α - 32 P]NTP and the other three unlabeled NTPs, radioactiv-

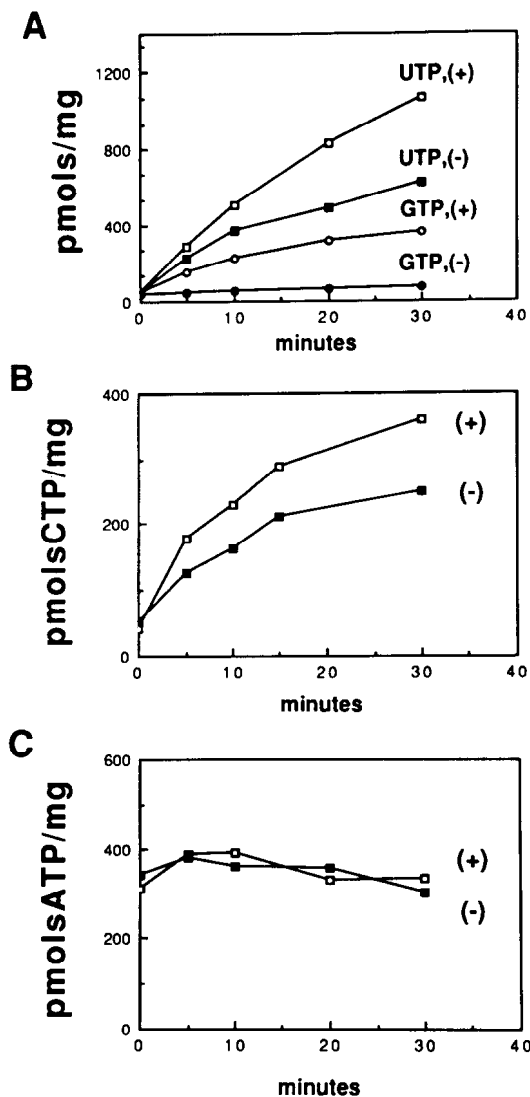


FIG. 2. Transcriptional and post-transcriptional incorporation of nucleotides into trichloroacetic acid-precipitable material. All mitochondria were preincubated at room temperature for 30 min prior to the labeling reaction to exhaust endogenous nucleotide pools. The mitochondria were resuspended in either transcription buffer containing all four nucleotide triphosphates (*open symbols* (+)) or lacking one of the four nucleotides (*filled symbols* (-)). *A*, mitochondria were double-labeled with [α - 32 P]GTP and [3 ,5- 3 H]UTP under transcription and transcription inhibition conditions. *Circles* indicate GTP incorporation; *squares* indicate UTP incorporation. Mitochondria were starved for CTP to block transcription. *B*, mitochondria were labeled with [α - 32 P]CTP under transcription and transcription inhibition conditions. In this case mitochondria were starved for GTP to block transcription. *C*, mitochondria labeled with [α - 32 P]ATP under transcription and transcription inhibition conditions. Mitochondria were starved for CTP to inhibit transcription. Amount of incorporation of the labeled nucleotides was determined by trichloroacetic acid precipitation of aliquots of an individual labeling reaction at the times indicated.

ity was incorporated into trichloroacetic acid-precipitable material (Fig. 2, A–C). If either CTP, UTP, or ATP are omitted from the reaction buffer, transcription, as monitored by incorporation of [α - 32 P]GTP into trichloroacetic acid-precipitable material, continued for 5–10 min and then stopped.³ If the mitochondria were first pre-incubated in transcription buffer in the absence of exogenous nucleotides for 30 min prior to the addition of labeled NTPs, incorporation of [α -

32 P]GTP into trichloroacetic acid-precipitable material was completely inhibited if CTP was omitted from the reaction mixture (Fig. 2A). If exogenous nucleotides were added to the transcription-inhibited mitochondria, transcription resumed and reached levels comparable to transcription in control mitochondria.³ When preincubated mitochondria were supplied with all four NTPs, [α - 32 P]GTP was incorporated with kinetics similar to that in mitochondria that had not undergone preincubation. This means that during the preincubation the endogenous pool of CTP had been exhausted. Preincubated mitochondria and mitochondria that had not undergone preincubation both incorporated [α - 32 P]GTP at a rate of approximately 12 pmol/min/mg of mitochondrial protein under transcription conditions. This shows that the preincubated mitochondria were transcription-competent, lacking only sufficient nucleotide substrates, and that transcription could be inhibited efficiently by depletion of CTP. Similar results were observed when using either UTP or ATP as the limiting nucleotide.

To determine whether the UTP incorporation continued in the absence of transcription, mitochondria were double-labeled with [α - 32 P]GTP and [5 ,6- 3 H]UTP under transcription (all four NTPs present) and transcription inhibition (GTP, UTP, and ATP present) conditions (Fig. 2A). In this way both the extent and rate of transcription and of editing could be measured on the same mitochondrial population using GTP incorporation as an internal measure of transcription. Fig. 2A shows the comparison of GTP and UTP incorporation in transcribing mitochondria and mitochondria in which transcription has been blocked by CTP starvation.

Under transcription conditions GTP was incorporated at a rate of 12 pmol/min/mg, while UTP was incorporated at the distinctly higher rate of 45 pmol/min/mg. When transcription was inhibited by CTP starvation, GTP incorporation was reduced by 95%. In contrast, UTP incorporation continued at a rate of 32 pmol/min/mg. UTP and GTP were incorporated only into RNA since the labeled material was insensitive to Proteinase K and DNase I while it was completely sensitive to RNase A (data not shown). Since transcription had been abolished, this rate reflects total post-transcriptional uridine addition which might include both site specific addition, and addition into poly(A) tails.

Incorporation of [α - 32 P]CTP, in mitochondria in which transcription has been blocked, continued at a different rate than that observed under transcription conditions (Fig. 2B). In this case transcription was blocked by omitting GTP from the transcription mixture. Similar results are observed in mitochondria starved for either UTP or ATP. ATP incorporation could not be measured in this system due to the high background of [α - 32 P]ATP precipitated in all of the samples (Fig. 2C). However, isolation and electrophoresis of RNA from mitochondria labeled with [α - 32 P]ATP reveals that ATP is incorporated into mitochondrial RNA in the absence of transcription (see Fig. 4, lane 9).

Fig. 3, A–C, shows the optimization of transcriptional GTP incorporation and post-transcriptional UTP incorporation. Both activities require magnesium at an optimal concentration of about 6 mM. High concentrations of KCl inhibited both activities. ATP was required for optimal levels of both activities. For these experiments ATP was used at a concentration of 100 μ M.

Characterization of Labeled Mitochondrial RNAs—The RNAs labeled in the presence and absence of transcription (as described in Fig. 2) were resolved on 6% denaturing acrylamide gels (Fig. 4A). To control for degradation during the isolation procedure, RNA extracted from the labeling

³ M. E. Harris and S. L. Hajduk, unpublished results.

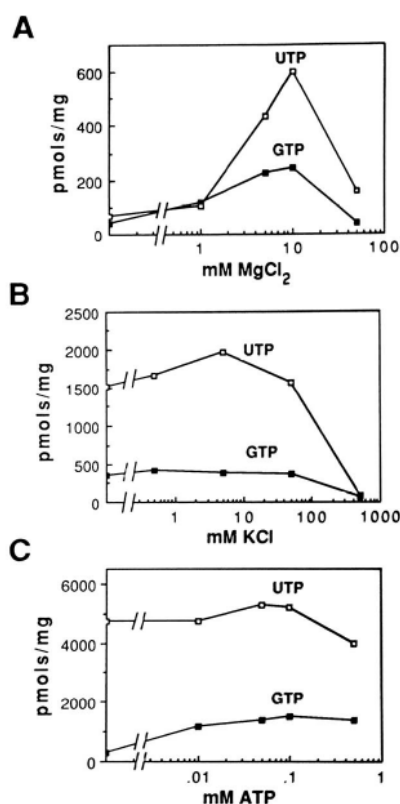


FIG. 3. Optimization of transcriptional GTP incorporation and post-transcriptional UTP incorporation. Preincubated mitochondria were double-labeled with [α -³²P]GTP and [$3,5$ -³H]UTP under transcription and transcription inhibition conditions as indicated. Points represent the amount of incorporation after 15 min of [α -³²P]GTP under transcription conditions (filled symbols) and [$3,5$ -³H]UTP under transcription inhibition conditions (open symbols). Only background levels of [α -³²P]GTP incorporation were observed in the transcription-inhibited reactions (data not shown).

reaction was also resolved on 1.5% methyl mercury-agarose gels (Fig. 4B). Total mitochondrial RNA, 3' end-labeled with [³²P]pCp and T4 RNA ligase, was run as a marker to identify the location of known RNA species (Fig. 4A, lanes 1 and 10). The 9 and 12 S rRNAs, running at approximately 1100 and 600 nucleotides, are present as well as a series of bands, identified as mitochondrial tRNAs, running at approximately 70 nucleotides.⁴ RNA labeled during transcription with [α -³²P]GTP ran as a smear from >2000 nucleotides to 100 nucleotides. No tRNAs were labeled under these conditions (Fig. 4A, lanes 2 and 3). The absence of defined 9 and 12 S rRNA bands in the [α -³²P]GTP-labeled RNA and in the other samples labeled under transcription conditions may mean that these RNAs are inefficiently or incompletely processed in this system.

RNA labeled with [α -³²P]CTP during transcription displayed both similarities and differences to that labeled with GTP (Fig. 4A, lane 6). The same smear of RNAs from 2000 to 100 nucleotides appeared. A series of bands around 70 nucleotides in length that co-migrated with the [³²P]pCp-labeled mitochondrial tRNAs also incorporated CTP. Metabolic labeling of these RNAs was most likely the result of the synthesis and turnover of the 3' CCA sequences of the mitochondrial tRNAs. A series of CTP-labeled RNAs running at 300–400 nucleotides was also observed (labeled 300 nt C-RNA in Fig. 4). The identity of these RNAs is unknown. Preliminary studies suggest that these transcripts are encoded by the

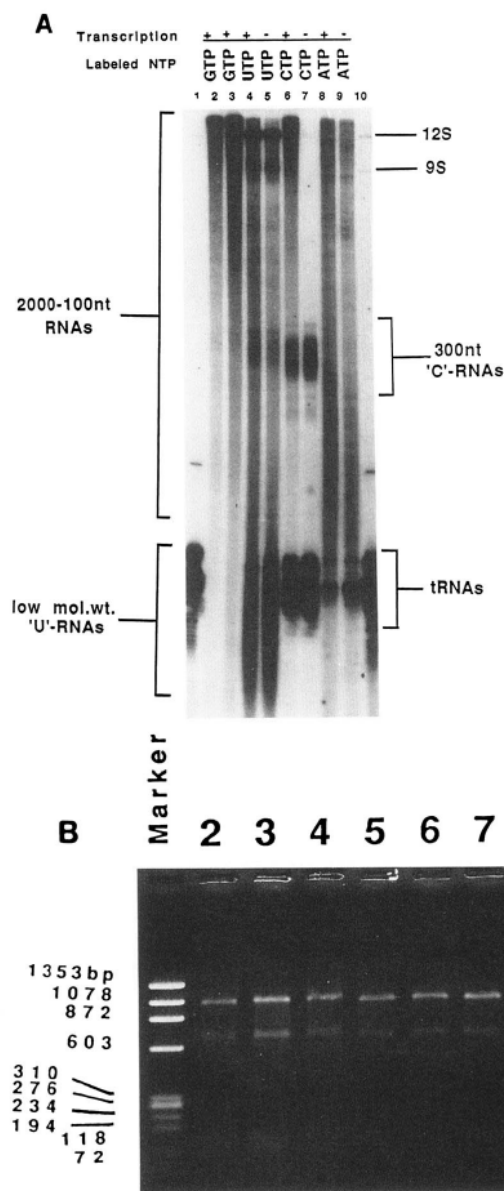


FIG. 4. Electrophoresis of labeled mitochondrial RNAs. A: lanes 1 and 10, pCp end-labeled mitochondrial RNA run as markers; lanes 2 and 3, RNA labeled with [α -³²P]GTP under transcription conditions for 15 min (lane 2) and 30 min (lane 3); lanes 4–9, RNAs labeled under the conditions described in Fig. 2, A–C; lanes 4 and 5, RNA labeled with [α -³²P]UTP in the presence (lane 4) and absence (lane 5) of transcription; lanes 6 and 7, RNAs labeled with [α -³²P]CTP in the presence (lane 6) and absence (lane 7) of transcription; lanes 8 and 9, RNAs labeled under transcription (lane 8) and transcription inhibition (lane 9) conditions. These RNAs were resolved on 6% sequencing gels and detected by autoradiography. The positions of the 12 and 9 S rRNAs and tRNAs are indicated. B, RNA samples used in A were electrophoresed on a 1.5% methyl mercury-agarose gel. RNAs were visualized by ethidium bromide staining. Lanes 2–9 correspond to lanes 2–9 in panel A.

maxicircle.³ When mitochondrial RNA was labeled with [α -³²P]CTP under conditions of transcription inhibition (Fig. 4A, lane 7) only the mitochondrial tRNAs and the C-RNA bands running at 300–400 nucleotides remained.

We also labeled mitochondrial RNA with [α -³²P]ATP under transcription and transcription inhibition conditions (Fig. 4A, lanes 8 and 9). The pattern seen under both conditions is very similar. RNAs from 2000 to 100 nucleotides were labeled. No [α -³²P]ATP was incorporated into the 9 and 12 S rRNAs when transcription was inhibited as expected if the ATP

⁴ K. Hancock and S. L. Hajduk, unpublished results.

incorporation was due to polyadenylation of mRNAs only. Also, some incorporation occurs in tRNAs due to CCA turnover.

When mitochondrial RNA was labeled with [α - 32 P]UTP under conditions of transcription and transcription inhibition (Fig. 4A, lanes 4 and 5) the migration of the labeled RNA on sequencing gels was distinct from that observed for GTP, CTP, or ATP-labeled RNAs. Both the 9 and 12 S rRNAs were labeled and also fine banding patterns were observed from 1500 to 100 nucleotides. Interestingly, a series of small RNAs (<100 nucleotides) are also labeled (low molecular weight U-RNAs, Fig. 4). The nature of this RNA was not determined; however, hybridization experiments suggest that these RNAs are minicircle-encoded.³

Transcript-specific RNA Editing—The post-transcriptionally UTP-labeled RNA was hybridized to cloned maxicircle fragments to determine whether specific maxicircle transcripts were UTP-labeled in the isolated mitochondria. Hybridization of RNA labeled with UTP as a result of editing will depend on the degree of editing and the steady state abundance of the edited RNA. If the UTP incorporation was due to specific internal additions then the greatest degree of hybridization of post-transcriptionally UTP-labeled RNA should be observed to the genes encoding the COIII transcript, since this appears to be the most highly edited mitochondrial RNA (1, 11). We also compared the hybridization of post-transcriptionally UTP-labeled RNA to the hybridization of RNA labeled with GTP under transcription conditions.

Fig. 5, B and D, shows the ethidium-stained gels of DNA fragments containing all of the known maxicircle genes as well as nuclear DNA and total kDNA. Cloned fragments of the maxicircle are arranged in the same order shown on the maxicircle map shown in Fig. 5A. When a Southern blot of this gel was hybridized with mitochondrial RNA pulse-labeled for 15 min with [α - 32 P]GTP under transcription conditions, the greatest degree of hybridization was seen to the fragment containing the genes for the 9 and 12 S rRNAs, the most abundant mitochondrial RNAs (Fig. 5C, lanes 2 and 3). In addition, the 1.2-kb fragment upstream of the 12 S gene also showed extensive hybridization (Fig. 5C, lane 2). This portion of the maxicircle is transcribed as part of the rRNA precursor.⁵ These results were consistent with a high rate of transcription of the mitochondrial rRNAs. The extent of hybridization of the transcribed RNA to maxicircle clone VI (Fig. 5C, lane 7) is also high, consistent with this fragment having a higher gene dosage. It contains portions of three maxicircle genes, COI, and the NADH dehydrogenase subunits 4 and 5 (ND 4 and 5). An alternative explanation might be that promoter sequences for the strand encoding COI, ND 1, and MURF 1 are contained in this fragment. The remaining fragments showed a lesser degree of labeling, which was consistent with their lower gene dosage or transcription rate. The hybridization to the *EcoRI* digest of kDNA (Fig. 5C, lane 10) indicates that both the variable region and the rRNA genes are extensively transcribed. Minicircles are also transcribed in this system as noted previously (6). Hybridization to the nuclear DNA sample was due to contaminating kDNA fragments (Fig. 5C, lane 9).

When a similar blot was probed with mitochondrial RNA labeled with [α - 32 P]UTP in the absence of transcription a very different pattern of hybridization is observed. The fragment containing the 9 and 12 S rRNA genes hybridized, but the upstream fragment containing only precursor sequences did not (Fig. 5E, lanes 2 and 3). In contrast to the results observed when transcriptionally labeled RNA was used as a

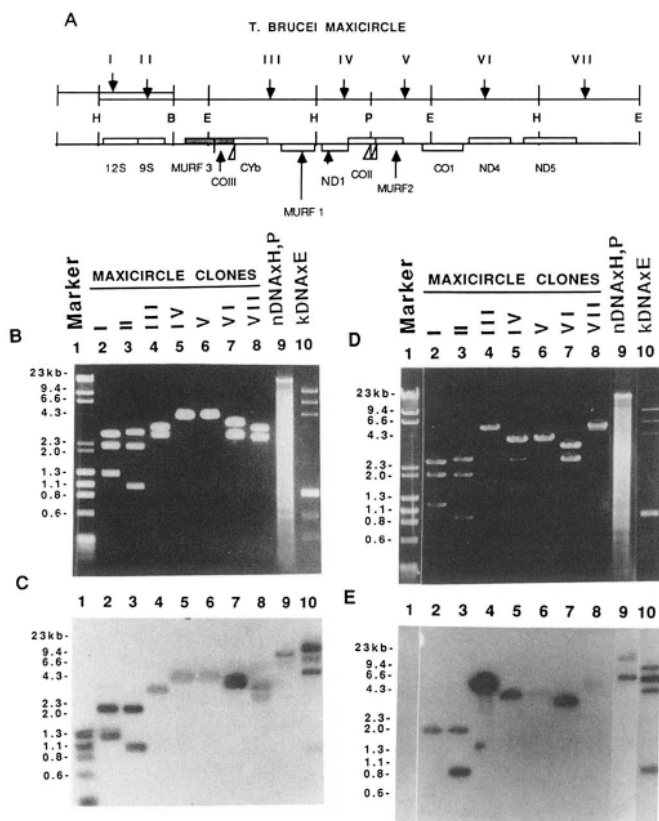


FIG. 5. Hybridization of RNA labeled with GTP under transcription conditions and RNA labeled with UTP under conditions of transcription inhibition to trypanosome DNA. RNA labeled with [α - 32 P]GTP under transcription conditions and RNA labeled with [α - 32 P]UTP under transcription-inhibited conditions was used to probe cloned kDNA fragments and digests of total nuclear and kDNA. **Panel A**, a map of the *T. brucei* maxicircle. Positions of the various cloned fragments (I–VII) and the genes they contain are indicated. Abbreviations are: *E*, *EcoRI*; *H*, *HindIII*; *B*, *BamHI*; and *P*, *PstI*. Δ indicates the position of known editing sites. Highly edited genes are represented by filled boxes. **Panels B and D**, ethidium-stained gels containing cloned kDNA fragments, nDNA, and total kDNA. **Panels C and E**, autoradiographs of Southern blots of the gels pictured in panels B and D, probed with GTP-labeled RNA during transcription (panel C) and UTP-labeled RNA during transcription inhibition (panel E). **Panel B**: lane 1, λ cut with *HindIII* and ϕ X174 cut with *HaeIII* as markers; in this case the ϕ X174 markers were radioactive; lane 2, clone I cut with *EcoRI*, *HindIII*, and *BamHI*; lane 3, clone II cut with *EcoRI*, *HindIII*, and *BamHI*; lane 4, clone III cut with *EcoRI* and *HindIII*; lane 5, linearized clone IV; lane 6, linearized clone V; lane 7, clone VI cut with *HindIII* and *EcoRI*; lane 8, clone VII cut with *EcoRI* and *PvuII*. This digestion yields two fragments both containing a maxicircle sequence; lane 9, *T. brucei* nuclear DNA cut with *HindIII* and *PstI*; lane 10, *EcoRI* digest of kDNA. **Panel D**: lane 2, clone I cut with *EcoRI*, *HindIII*, and *BamHI*; lane 3, clone II cut with *EcoRI*, *HindIII*, and *BamHI*; lane 4, linearized clone III; lane 5, linearized clone IV; lane 6, linearized clone V; lane 7, clone VI cut with *EcoRI* and *HindIII*; lane 8, linearized clone VII. **Panel C**, autoradiograph of a Southern blot of the gel shown in panel B probed with RNA labeled with GTP during transcription. **Panel E**, autoradiograph of a Southern blot of the gel shown in panel D probed with RNA labeled with UTP during transcription inhibition.

hybridization probe, the greatest degree of hybridization was seen to the fragment containing the gene for CYb and the majority of the gene for the highly edited COIII transcript (Fig. 5E, lane 4). The difference in the size of this fragment compared with the size of the fragment hybridizing in Fig. 5C, lane 4, is due to the fact that in this instance the clone was only linearized. Some hybridization was also seen to the small 1-kb fragment in Fig. 5E, lane 3, containing the MURF

⁵ E. F. Michelotti and S. L. Hajduk, unpublished results.

3 gene, which is also highly edited. The fragments containing the genes for COII and ND 1 also hybridized to the UTP-labeled RNA (Fig. 5E, lanes 5 and 6). The COII transcript has four added uridines while the ND 1 gene lacks an AUG initiation codon and therefore may undergo editing near its 5' end (13). Still, hybridization to the COII and ND 1 genes was less than to the fragment containing the CYb and highly edited COIII transcripts (Fig. 5E, compare lanes 4 and 5). Hybridization to fragment VI that contains the MURF 2 gene, which is edited at the 5' end, and a portion of the COII gene was less than to fragment V. Differential amounts of edited RNAs and addition to as yet unidentified edited transcripts may be responsible for these effects.

Some hybridization also occurs in the fragment containing portions of the COI and ND 4 and 5 genes (Fig. 5E, lane 7). It is possible that this is due to labeling in the poly(A) tails of these transcripts or to improper addition. Again, unidentified edited transcripts may also be present in this region. The lesser degree of hybridization seen to the fragment in lane 8 of Fig. 5E which contains a portion of the ND 5 gene is expected, since this transcript is not edited (1, 13).

The fragments of total kDNA hybridizing with [α - 32 P]UTP-labeled RNA (Fig. 5E, lane 10) were consistent with the degree of hybridization seen to the maxicircle clones (lanes 2–8). Only contaminating kDNA in the nuclear DNA sample hybridized to the UTP-labeled RNA probe as was the case with the [α - 32 P]GTP-labeled RNA. Interestingly, minicircles also hybridize with [α - 32 P]UTP-labeled RNA, suggesting that minicircle transcripts may also undergo editing. Clearly, UTP is added post-transcriptionally to a distinct subset of mitochondrial RNAs and not nonspecifically to the most abundant mitochondrial RNAs. This data is consistent with the post-transcriptional editing of specific mitochondrial transcripts

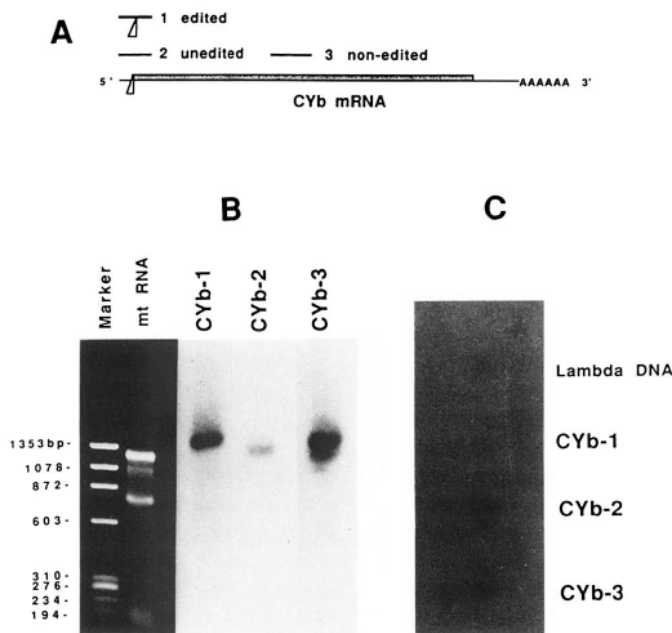


FIG. 6. Characterization of steady state and newly synthesized CYb mRNA. A, location of oligonucleotide probes to the CYb mRNA. CYb-1 is complementary to the editing site in its unedited form while CYb-2 is complementary to the same site in its edited form. CYb-3 is complementary to a region of the CYb mRNA that is unaffected by editing. B, Northern blot of mitochondrial RNA using CYb-1, CYb-2, and CYb-3 oligonucleotide probes. C, dot blot of CYb-1, -2, and -3 oligonucleotides probed with mitochondrial (mt) RNA pulse-labeled with [α - 32 P]GTP for 15 min under transcription conditions.

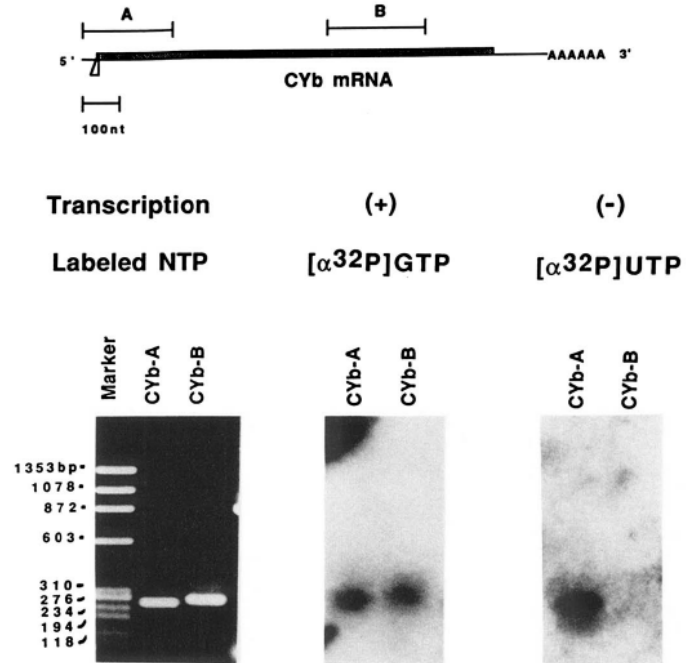


FIG. 7. Addition of uridines near the 5' end of CYb mRNAs. Identical Southern blots of CYb-A and CYb-B gene fragments were probed with either [α - 32 P]GTP-labeled RNA or RNA labeled post-transcriptionally with [α - 32 P]UTP. The CYb-A and CYb-B fragments are separated by 371 nucleotides (nt) in the CYb gene. RNA probes were hydrolyzed to fragments of 200 nucleotides and smaller to eliminate cross-hybridization.

occurring to approximately the degree observed in cDNAs of edited transcripts.

The Editing Site in the Cytochrome b Gene Is Transcribed in Its Unedited Form—The majority of CYb mRNAs in procyclic *T. brucei* mitochondria contain 34 uridines, which are not encoded in the maxicircle gene for CYb, added to 12 sites near the 5' end (9). If addition of these uridines is post-transcriptional then the editing site should be transcribed initially in its unedited form, while a co-transcriptional mechanism would generate CYb mRNAs with uridines already added to the editing site. The following approach was used to distinguish between these two alternatives. RNA was labeled with [α - 32 P]GTP under transcription conditions. This RNA was hybridized to different oligonucleotide probes which detect edited cytochrome *b* mRNA, unedited cytochrome *b* mRNA, and an oligo which detects both forms (Ref. 9 and Fig. 6A). To demonstrate the specificity of these oligos a Northern blot of purified mitochondrial RNA was probed with each of the oligonucleotides (Fig. 6B). When the oligo complementary to the 5'-editing site in its edited form, CYb-1, was used as a probe (Fig. 6B, lane 1) a band of approximately 1300 nucleotides was detected, corresponding to the edited CYb mRNA (9). When the oligonucleotide complementary to the editing site in its unedited form, CYb-2, was used as a probe a 1200-nucleotide mRNA was detected which corresponded to the unedited CYb mRNA (Fig. 6B, lane 2) (9). When an oligo complementary to a region which is unaffected by uridine addition located downstream of the editing site, CYb-3, was used, both mRNAs were detected (Fig. 6B, lane 3). Transcriptionally [α - 32 P]GTP-labeled RNA was hybridized to dot blots of these oligonucleotides (Fig. 6C). The transcribed RNA hybridized only to the CYb-2-unedited and CYb-3-nonedited oligos; no hybridization was seen to the CYb-1-edited oligo. This shows that newly synthesized CYb transcripts are unedited at the 5'-editing site.

Post-transcriptional Addition of Uridines to the Edited Region of Cytochrome b mRNAs—If addition of uridines to the 5'-edited region of the CYb mRNA is occurring post-transcriptionally then this portion of the RNA molecule should incorporate [α - 32 P]UTP in the absence of transcription. Conversely, downstream regions of the RNA molecule which are unaffected by editing should not incorporate UTP. To examine this question a 246-bp fragment containing the 5'-editing site, CYb-A, and a 263-bp fragment generated from a downstream region of the CYb gene which is unaffected by editing, CYb-B, were generated (Fig. 7). RNA labeled with GTP during transcription and RNA labeled with UTP in the absence of transcription was used to probe a Southern blot of these CYb gene fragments. The labeled RNAs were first partially hydrolyzed to fragments of approximately 200 nucleotides to eliminate cross-hybridization to the two fragments. The CYb-A and -B DNAs are each approximately 250 nucleotides in size and are separated by 371 bp in the CYb gene. Labeled RNAs of 200 nucleotides or less should hybridize to either the upstream fragment containing the editing site or the downstream fragment but not both. If editing of this region is occurring then [α - 32 P]UTP-labeled RNA should hybridize preferentially to the upstream fragment containing the editing site. RNA labeled with [α - 32 P]GTP during transcription should hybridize to both. Fig. 7 shows that when [α - 32 P]GTP-labeled RNA was used to probe a Southern blot of CYb-A and CYb-B DNAs hybridization to both fragments was seen. In contrast, when [α - 32 P]UTP-labeled RNA was used as a probe, hybridization only to CYb-A was observed. This shows that RNA containing the editing sites incorporates UTP but does not indicate the exact number or location of the uridines added. Exclusive labeling of the 5' region of CYb mRNAs does demonstrate that uridine addition is occurring in the vicinity of an internal editing site and not nonspecifically to other regions of the RNA molecule. The precise structure of the RNAs containing the added uridines is the subject of current studies.

DISCUSSION

We have developed an isolated organelle system to examine *T. brucei* mitochondrial RNA processing. Similar systems have been used to examine transcription and processing of mitochondrial RNAs from both HeLa and yeast cells (31–34). RNA editing represents a novel form of RNA processing that occurs in *T. brucei* mitochondria. We have used this isolated mitochondrial transcription and processing system to examine RNA editing and compare it with various other forms of nucleotide addition to RNA.

Post-transcriptional addition of CTP and ATP to RNA occurs in these organelles. The fact that UTP is incorporated in the absence of transcription, in a fashion analogous to post-transcriptional CTP and ATP incorporation, supports a post-transcriptional editing process. The rate of post-transcriptional uridine addition has been determined and is distinct from the rate associated with transcription.

When post-transcriptionally UTP-labeled RNA was examined by electrophoresis, discrete labeled bands were observed. The 9 and 12 S rRNAs are labeled as well as RNAs of greater and lesser size. It is likely that the UTP-labeled RNAs in the 2000–100-nucleotide range are edited mRNAs or editing intermediates. The identity of the low molecular weight UTP-labeled RNAs in Fig. 4, lane 5, is unknown. Preliminary experiments indicate these RNAs are of minicircle origin.³ Similar minicircle RNAs are modified by a terminal uridylyl transferase in *Leishmania* (21).

Interestingly, post-transcriptionally CTP-labeled RNAs

other than tRNAs are present in trypanosome mitochondria (Fig. 4A, lane 7). It is unlikely that these 300-nucleotide post-transcriptionally [α - 32 P]CTP-labeled RNAs are tRNA precursors. Such a precursor would have to have an unusually long 5' extension as well as a unique processing pattern, since CCA addition is usually the final processing event in the maturation of tRNAs (30). Initial experiments aimed at identifying these RNAs have shown that they are of maxicircle origin.³

We have also shown that the post-transcriptional addition of UTP is specific to known edited transcripts and that the degree of addition seen in the isolated organelles agrees with the known amount of editing occurring in different mitochondrial transcripts (Fig. 5). Those transcripts which are most highly edited incorporated more uridine than those transcripts to which only a few uridines are added. Transcripts known not to undergo RNA editing incorporated very little UTP in the absence of transcription. This experiment does not show, however, that the uridines are added internally, only that the most highly edited RNAs incorporate UTP preferentially. Labeling of specific transcripts with UTP is consistent with modification of these transcripts occurring in a manner analogous to that found *in vivo*. Since the labeling is done under conditions of transcription inhibition the modified RNAs must pre-exist in the organelles prior to labeling. This observation is also consistent with the existence of unedited and partially edited transcripts in the RNA population (9, 11, 14, 20). Alternatively, nascent transcripts in which editing sites have been transcribed might be edited, but ongoing transcription is not required.

The editing site in CYb mRNAs is initially transcribed in an unedited form. RNA labeled during transcription hybridized equally to the unedited probe and to a downstream probe to a region unaffected by editing. Hybridization to the editing site probe and to the downstream probe was equivalent, meaning that both areas of the CYb gene are transcribed at equivalent rates. A co-transcriptional mechanism involving transcriptional pausing at the editing site would not have given this result. This indicates that both regions were transcribed with equal efficiency and that the transcripts were unedited. Whether these transcripts go on to become edited is the focus of current studies.

We have also shown that a region of the CYb mRNA containing the editing site can be labeled with [α - 32 P]UTP in the absence of transcription. A downstream region of the same RNA which does not contain an editing site incorporates no UTP. The exact structure of the labeled CYb mRNAs remains to be determined. It may be that only portions of a multistep editing reaction are occurring. While this does not demonstrate internal addition it is consistent with site-specific addition at the editing site.

The following observations concerning editing have been made. 1) UTP incorporation into mitochondrial RNA continues in the absence of transcription. The rate of UTP incorporation during transcription is distinct from that of UTP incorporation when transcription was abolished. 2) UTP is incorporated into a distinct subset of mitochondrial RNAs. 3) In general, UTP is incorporated into RNAs known to undergo editing, and the amount of addition is consistent with the degree of editing these transcripts are known to undergo. 4) Newly synthesized cytochrome *b* mRNA is unedited at its 5'-editing site. 5) The 5' region of this transcript which contains the editing site is labeled with [α - 32 P]UTP in the absence of transcription while another region of the same RNA is not. We conclude that RNA editing is a post-transcriptional proc-

ess and involves the modification of pre-existing RNAs in trypanosome mitochondria.

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Addition of uridines to edited RNAs in trypanosome mitochondria occurs independently of transcription.

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