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Posttranscriptional Regulation of Cytochrome *c* Expression during the Developmental Cycle of *Trypanosoma brucei*

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We examined the expression of a nucleus-encoded mitochondrial protein, cytochrome *c*, during the life cycle of *Trypanosoma brucei*. The bloodstream forms of *T. brucei*, the long slender and short stumpy trypanosomes, have inactive mitochondria with no detectable cytochrome-mediated respiration. The insect form of *T. brucei*, the procyclic trypanosomes, has fully functional mitochondria. Cytochrome *c* is spectrally undetectable in the bloodstream forms of trypanosomes, but during differentiation to the procyclic form, spectrally detected holo-cytochrome *c* accumulates rapidly. We have purified *T. brucei* cytochrome *c* and raised antibodies that react to both holo- and apo-cytochrome *c*. In addition, we isolated a partial cDNA to trypanosome cytochrome *c*. An examination of protein expression and steady-state mRNA levels in *T. brucei* indicated that bloodstream trypanosomes did not express cytochrome *c* but maintained significant steady-state levels of cytochrome *c* mRNA. The results suggest that in *T. brucei*, cytochrome *c* is developmentally regulated by a posttranscriptional mechanism which prevents either translation or accumulation of cytochrome *c* in the bloodstream trypanosomes.

Trypanosoma brucei is a protozoan parasite with a complex life cycle which encompasses both a mammalian host and a specific insect vector, the tsetse fly (44). In the mammal, the early bloodstream developmental stage, the rapidly dividing long slender trypanosome, differentiates into the nondividing short stumpy trypanosome (13, 44). These short stumpy trypanosomes are preadapted to life in the insect vector, where they differentiate into the early insect developmental stage, the procyclic trypanosomes (29, 45). The bloodstream forms of *T. brucei* have an inactive mitochondrion which lacks cytochrome-mediated respiration (5, 6, 9, 23). The procyclic form of *T. brucei* has a mitochondrion which is larger and has a more expansive network of cristae than is found in the bloodstream trypanosomes. The mitochondria of procyclic trypanosomes are active in cytochrome-mediated respiration. The regulation of mitochondrial biogenesis in *T. brucei* and the adaptations in energy metabolism that accompany this regulation have a profound effect on the expression of both mitochondrion-encoded and nucleus-encoded mitochondrial proteins. To gain insight into this process, an investigation into the regulation of a nucleus-encoded mitochondrial protein, cytochrome *c*, was initiated.

Cytochrome *c* serves as a component in the electron transport chain. The protein is encoded in the nucleus, synthesized in the cytosol off free ribosomes, and transported into the mitochondria, where a heme prosthetic group is attached, forming mature holo-cytochrome *c* (24, 28). Regulation of cytochrome *c* has been best characterized in *Saccharomyces cerevisiae*. In *S. cerevisiae*, cytochrome *c* expression is dependent on the availability of heme. Intracellular heme stimulates production of the heme activation proteins, which bind to upstream activation sites of the cytochrome *c* transcription unit to allow transcription of the gene (18, 19, 35). Catabolite repression reduces heme levels within *S. cerevisiae*, resulting in a reduction of cytochrome *c* mRNA in the cells. Thus, in yeast cells, cytochrome *c* can be regulated transcriptionally. Transcriptional regulation of cytochrome *c* has also been reported for *Neurospora crassa*, *Manduca sexta*, and *Drosophila melanogaster* (22, 41). It has been reported that cytochrome *c* levels can also be subject to posttranscriptional regulation. In rat skeletal muscle, immobilization-induced muscle atrophy results in mild, transient suppression of cytochrome *c* expression during a period when intracellular levels of cytochrome *c* mRNA appear stable (30). However, transcriptional regulation of cytochrome *c* is observed during extended periods of muscular immobilization. Cytochrome *c* mRNA levels decrease in rat skeletal muscle as the cells are allowed to atrophy, resulting in dramatic reductions in cytochrome *c*.

In this paper we present evidence that the developmental regulation of cytochrome *c* in *T. brucei* occurs by a unique mechanism. Although the apo- and holo-proteins are not detected in the bloodstream forms, the transcripts for the protein are present in all life stages. These results suggest that cytochrome *c* regulation in trypanosomes occurs at the translational or posttranslational level.

MATERIALS AND METHODS

Growth and isolation of trypanosomes. Bloodstream forms of *T. brucei* (TREU 667) were grown in female Wistar rats (approx. 200 g) or female CD-1 mice (approx. 5 to 10 weeks old), which were gamma irradiated with 600 rads prior to infection. The animals were infected by interperitoneal injection of blood from a female CD-1 mouse infected for 3 to 4 days from frozen stocks stored at −196°C in 7.5% dimethyl sulfoxide. Long slender trypanosomes were recovered from a 2- to 3-day infection, and short stumpy trypanosomes were recovered from an 8- to 9-day infection. In both cases, trypanosomes were harvested by cardiac puncture and anion-exchange chromatography over a Whatman diethylaminoethyl cellulose column (DE52, pH 8.0) with a phosphate-buffered saline solution containing glucose and heparin

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(PSGH: 0.057 M Na_2HPO_4, 3 mM NaH_2PO_4, 0.044 M NaCl, 10 g of glucose per liter, 10 U of heparin per ml, pH 8.0).

Procyclic trypanosomes (TREU 667) were grown in culture in a semidefined medium (SM medium) containing gentamicin (25 μg/ml) and supplemented with 10% fetal calf serum (FCS) (Hyclone), heat inactivated at 56°C for 30 min (12, 29). The cultures were grown under sterile conditions with continuous shaking at 26°C and harvested at late log phase (approx. 2×10^7 trypanosomes per ml) by sedimentation at 5,000 × *g* for 15 min at 4°C. Differentiation of trypanosomes from the short stumpy bloodstream form to the procyclic culture form was initiated by suspending the short stumpy trypanosomes in SM medium at 26°C at a concentration of 5×10^6/ml. The short stumpy trypanosomes were isolated as above with two exceptions: sterile conditions were maintained throughout the procedure, and 10% FCS was added to the DE52 resin and to PSGH to improve organism viability.

Holo-cytochrome *c* determinations. Trypanosome cell pellets were washed in isotonic buffer (0.1 M NaCl, 0.05 M Tris hydrochloride pH 8.0), resuspended 0.01 volume of 10 mM Tris hydrochloride (pH 7.0), and sonicated with a Heat Systems Ultrasonics Cell Disruptor with a model H-1 microtip set at maximum output. Total cellular disruption was achieved with repeated 1-min sonication cycles performed on ice. The crude cell lysate was then centrifuged at 10,000 × *g* to sediment debris. A partial purification of the crude lysate supernatants was achieved by cation-exchange chromatography over carboxymethyl-Sephadex (CM) columns. The proteins were eluted with 10 mM Tris hydrochloride (pH 7.0)–0.5 M NaCl. The absorption spectra of the partially purified lysate from 300 to 600 nm was determined with a Varian 2290 spectrophotometer. Relative holo-cytochrome *c* concentrations were calculated based on absorption at the Soret peak maximum of 419 nm and corrected for lysate cell number.

Purification and characterization of cytochrome *c*. *T. brucei* cytochrome *c* was isolated from 20 liters of procyclic trypanosomes cultured to late log phase by the procedure outlined by Hennig (20) with several modifications. Cellular debris was sedimented by centrifugation at 10,000 × *g* for 1 h at 4°C prior to the first chromatographic step, and all chromatography was performed in Tris hydrochloride buffers containing the protease inhibitors leupeptin (0.5 μg/ml), pepstatin (1 μg/ml), and phenylmethylsulfonyl fluoride (17.5 μg/ml). The final purification step was high-pressure liquid chromatography (HPLC) size exclusion chromatography on a TSK 3000 column, eluting with 10 mM Tris hydrochloride (pH 7.0). The elution of cytochrome *c* during each purification step was monitored by absorption at 405 nm and assayed by absorption spectra and by electrophoresis (26).

Purified cytochrome *c* was digested with trypsin to generate peptides for protein sequencing. Approx. 8 pmol of TLCK-treated trypsin was added to approx. 800 pmol of trypanosome cytochrome *c* in 100 mM Tris (pH 8.0)–50 mM NaCl and incubated at 37°C for 3 h. The same amount of trypsin was again added to the solution, and incubation was continued overnight. Generated peptide fragments were isolated by HPLC with a reverse-phase C-18 column. Recovered peptides were sequenced with an Applied Biosystems 470A protein sequencer, with the individual amino acids being identified by reverse-phase HPLC.

Apo-cytochrome *c* was prepared by chemically removing the heme prosthetic group from holo-cytochrome *c* as described by Ambler and Wynn (1).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (26) with a 5% acrylamide stacking gel and a 15% acrylamide resolving gel. Proteins were visualized by Coomasie blue staining or silver staining.

Production of antibodies. Antibodies to *T. brucei* cytochrome *c* were prepared by immunization of both New Zealand White rabbits and BALB/c mice with monomeric cytochrome *c* isolated from procyclic trypanosomes as described by Reichlin et al. (36) with several modifications. Inoculations of the rabbits were performed at monthly intervals with 100 to 200 μg of trypanosome cytochrome *c* in an emulsion with Freund adjuvant, distributed along a series of subcutaneous sites. Inoculations of mice were also performed at monthly intervals with 20 to 30 μg of trypanosome cytochrome *c* in an emulsion with Freund adjuvant, injected interperitoneally. In both cases, the initial inoculations were prepared with Freund complete adjuvant, and all subsequent inoculations were prepared with Freund incomplete adjuvant. Rabbit antisera were affinity purified with yeast cytochrome *c* coupled to cyanogen bromide-activated Sepharose as described by Axen et al. (2) and Cuatrecasas et al. (11).

Western blot (immunoblot) analysis. Proteins were electrophoretically transferred from SDS-PAGE gels onto nitrocellulose (Schleicher & Schuell BA85) in 20 mM Tris–150 mM glycine–20% methanol at 200 V for 4 h at 10°C (17, 43). The filters were washed, blocked with gelatin, probed with the *T. brucei* cytochrome *c* antibodies, and visualized by using a second antibody coupled to alkaline phosphatase (Bio-Rad Immun-Blot assay kit).

Cytochrome *c* cDNA synthesis and isolation. RNA was isolated from procyclic trypanosomes by the guanidine isothiocynate procedure of Maniatis et al. (27). Polyadenylated [poly(A)$^+$] mRNA was selected by affinity chromatography over an oligo(dT) column (27). A cDNA library was constructed in λgt11 from this RNA by using the Amersham cDNA synthesis kit and the Amersham cDNA cloning kit. The cDNA library was plated, and duplicate plaque lifts were performed on nitrocellulose (39, 46). The cDNA library was screened for the expression of *T. brucei* cytochrome *c* by using both the rabbit and mouse antibodies against trypanosome cytochrome *c*, and the positive plaques were visualized by using second antibodies conjugated to alkaline phosphatase. The DNA inserts from dual positive plaques were sequenced by primer extension (United States Biochemical Sequenase sequencing kit). The *T. brucei* cytochrome *c* cDNA was released by *Eco*RI digestion, ligated into Bluescript KS$^+$ (Stratagene), and designated pAT31. The pAT32 subclone was prepared by digestion of pAT31 with both *Eco*RI and *Xho*I; the 190-base-pair fragment was then ligated into Bluescript KS$^+$.

RNase T_1 analysis. RNA from long slender, short stumpy, and procyclic forms of *T. brucei* was prepared from total cellular nucleic acid by DNase I (RNase-free) treatment as described by Rohrer et al. (37). The ^{32}P-labeled RNA probes and unlabeled RNA sense strand controls were prepared as described elsewhere (37). The hybridization of riboprobes and all cellular RNA or control RNAs and the RNase T_1 digestions were performed by the method of Mueller et al. (32) with several modifications. The hybridization mixes were incubated overnight in a water bath that was initially 100°C and was allowed to cool slowly to 65°C. The digestions were performed with 2 U of RNase T_1 per μl at 37°C for 1 h. The RNase T_1 products were precipitated and analyzed by electrophoresis through a 6% denaturing polyacrylamide gel.

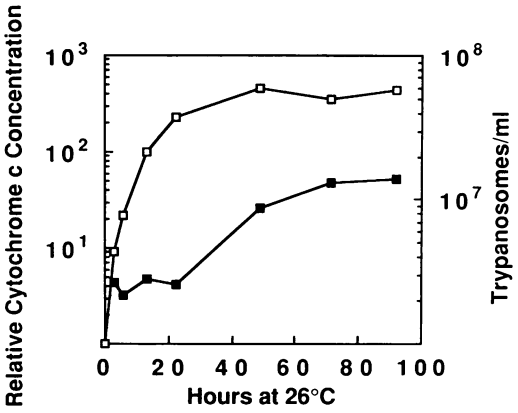

FIG. 1. Holo-cytochrome *c* expression in differentiating trypanosomes. Short stumpy trypanosomes were placed in SM medium at 26°C to initiate differentiation to the procyclic form. At the indicated times, portions were removed and assayed for spectrally detectable holo-cytochrome *c* expression as described in Materials and Methods. The open squares represent the relative concentration of holo-cytochrome *c* in the differentiating trypanosomes based on absorbance at 419 nm and corrected for cell number. The solid squares represent cell growth in the culture, plotted as number of trypanosomes per milliliter. The time course followed the differentiation of trypanosomes starting from the short stumpy form (0 h) and continuing for 96 h into the differentiation process.

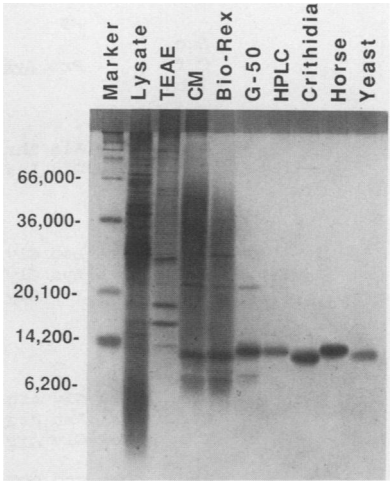

FIG. 2. SDS-PAGE of cytochrome *c* purification from procyclic trypanosomes. Portions from each step in the purification of cytochrome *c* were analyzed on 15% SDS-PAGE and visualized by silver staining of the gel. The samples included the procyclic trypanosome lysate supernatant (Lysate), the flowthrough off the TEAE-cellulose column (TEAE), the eluate from the CM-Sephadex column (CM), the eluate from the Bio-Rex 70 column (Bio-Rex), the pooled fractions collected off the Sephadex G-50 column (G-50), and the pooled fractions off the HPLC TSK-3000 column (HPLC). In addition, cytochrome *c* isolated from *C. fasciculata* (Crithidia), horse, and *S. cerevisiae* (yeast) were also analyzed. Approximately 10 μg of protein from the lysate and 5 μg of protein from the TEAE flowthrough were loaded onto the gel. All other samples were equally loaded onto the gel based on cytochrome *c* content, approximately 2 μg of cytochrome *c* from each.

RESULTS

Expression of spectrally detectable holo-cytochrome *c*. Cytochrome *c* expression during the developmental life cycle of *T. brucei* was examined by absorption spectroscopy. The absorbance spectrum from 350 to 600 nm was determined for the partially purified cell lysates from long slender, short stumpy, and procyclic trypanosomes. Lysates of the bloodstream forms of *T. brucei* do not absorb to any significant extent over this range. Lysates of the procyclic trypanosomes showed absorbance maxima at 419 nm, 523 nm, and 556 nm, which is characteristic of cytochrome *c* (results not shown). To establish the kinetics of cytochrome *c* accumulation, the absorption spectra of partially purified cell lysates prepared from trypanosomes in the process of differentiation were determined (Fig. 1). The short stumpy trypanosomes (0 h) had no spectrally detectable cytochrome *c*; however, holo-cytochrome *c* was detected in the trypanosomes 3 h after the short stumpy forms were shifted into SM medium at 26°C. The holo-cytochrome *c* concentration increased in the differentiating cells, approaching the level in fully differentiated procyclic trypanosomes within 30 h. Cell growth in the differentiating culture experienced a lag which coincided with the time required for cytochrome *c* levels to reach a plateau. These results indicate that cytochrome *c* is developmentally regulated in *T. brucei*.

Purification and characterization of cytochrome *c*. Cytochrome *c* was purified from procyclic forms of *T. brucei* so that the protein could be characterized and used in the production of antibodies. Ion-exchange chromatography of the trypanosome lysate, utilizing TEAE cellulose, CM Sephadex, and Bio Rex 70, was followed by gel filtration chromatography over G-50 Sephadex and HPLC size exclusion on a TSK 3000 column (20). Cytochrome *c* was monitored throughout the purification by absorbance at 405 nm. At various steps during the purification, portions were removed and characterized by SDS-PAGE (Fig. 2). The chromatography resulted in the purification of a 12,500-dalton protein from the procyclic lysate. The *T. brucei* protein was similar in size to purified cytochrome *c* from *Crithidia fasciculata*, horse, and *S. cerevisiae*. Protein migration in the gel was affected by the different salt concentrations in the various samples. Spectral analysis of the isolated *T. brucei* protein revealed an absorbance profile characteristic of cytochrome *c*, with absorbance maxima at 419 nm, 523 nm, and 556 nm (results not shown). The *T. brucei* protein was digested with trypsin, and the peptide fragments were separated by reverse-phase HPLC. The sequence of seven tryptic fragments, containing 60 nonoverlapping amino acids, was determined and compared with the complete *C. fasciculata* and *S. cerevisiae* cytochrome *c* sequences (Fig. 3). The sequenced portion of the *T. brucei* protein showed more than 85% homology with cytochrome *c* from *C. fasciculata* and almost 45% homology with cytochrome *c* from *S cerevisiae*. Thus, the 12,500-dalton protein isolated from procyclic forms of *T. brucei* had the size, spectral properties, and amino acid sequence expected for cytochrome *c*.

Expression of apo-cytochrome *c* during developmental differentiation. The expression of apo-cytochrome *c* during the developmental cycle of *T. brucei* was examined by Western blots probed with affinity-purified rabbit antibodies to *T. brucei* cytochrome *c*. The Western blot assays were performed on partially purified cell lysates of trypanosomes at various stages of differentiation from the short stumpy to the procyclic form. The Western blot results (Fig. 4) indicated that the antibodies detected both apo- and holo-cytochrome *c* from either *T. brucei* (compare procyclic with procyclic-H) or yeast cells (compare yeast holo-cyt c with yeast apo-cyt c). The results also demonstrated that the apo-protein from

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          -9                                          1                   5
S.c.                        Thr Glu Phe Lys Ala Gly Ser Ala Lys Lys
C.f.      Pro Lys Ala Arg Glu Pro Leu Pro Pro Gly Asp Ala Ala Lys
T.b.                        Ala Ala Leu Pro Pro Gly Asp Ala Ala

          6                                                           20
S.c.   Gly Ala Thr Leu Phe Lys Thr Arg Cys Leu Gln Cys His Thr Val
C.f.   Gly Glu Lys Ile Phe Lys Gly Arg Ala Ala Gln Cys His Thr Gly
T.b.                                  Ala Ala Gln

          21                                                          35
S.c.   Glu Leu Gly Gly Pro His Lys Val Gly Pro Asn Leu His Gly Ile
C.f.   Ala Lys Gly Gly Ala Asn Gly Val Gly Pro Asn Leu Phe Gly Ile
T.b.         Gly Gly Ser Asn Gly Val Gly Pro Asn Leu Tyr Gly Ile

cDNA                                                        5'-GA ATT

          36                                                          50
S.c.   Phe Gly Arg His Ser Gly Gln Ala Glu Gly Tyr Ser Tyr Thr Asp
C.f.   Val Asn Arg Gly Ser Gly Thr Val Glu Gly Phe Ala Tyr Ser Lys
T.b.   Val Gly Arg Lys Ser Gly Thr Val Glu Gly Phe Thr Tyr Ser Lys

cDNA   GTT GGC CGT AAA TCC GGA ACT GTT GAG GGT TTT ACG TAC AGC AAA

          51                                                          65
S.c.   Ala Asn Ile Lys Lys Asn Val Leu Trp Asp Glu Asn Asn Met Ser
C.f.   Ala Asn Ala Asp Ser Gly Val Val Trp Thr Pro Glu Val Leu Asp
T.b.   Ala Asn Gln Asp Ser Gly Val Met Trp Thr Pro Gln Val Leu Asp

cDNA   GCC AAT CAA GAT TCC GGT GTT ATG TGG ACT CCG CAG GTA CTT GAC

          76                                                          80
S.c.   Glu Tyr Leu Thr Asn Pro Lys Lys Tyr Ile Pro Gly Thr Leu Met
C.f.   Val Tyr Leu Glu Asn Pro Lys Lys Phe Met Pro Gly Thr Lys Met
T.b.   Val Tyr Leu Glu Asn Pro Lys Lys Phe Met Pro Gly Thr Lys Met

cDNA   GTG TAT TTG GAG AAT CCA AAG AAA TTT ATG CCC GGC ACT AAA ATG

          81                                                          95
S.c.   Ala Phe Gly Gly Leu Lys Lys Glu Lys Asp Arg Asn Asp Leu Ile
C.f.   Ser Phe Ala Gly Ile Lys Lys Pro Gln Glu Arg Ala Asp Leu Ile
T.b.   Ser Phe Ala Gly Leu Lys Lys Pro Gln Glu Arg Ala Asp Leu Ile

cDNA   TCT TTT GCA GGT TTA AAG AAA CCA CAG GAA CGC GCC GAC CTC ATC

          96                        103
S.c.   Thr Tyr Leu Lys Lys Ala Cys Glu
C.f.   Ala Tyr Leu Glu Asn Leu Lys Gly
T.b.   Ala Tyr Leu Glu Thr Leu Lys Asp

cDNA   GCA TAC CTC GAG ACA TTA AAG GAC TAA AAT GAG ATA ATA GAA AAA

       TTT ATA ATA TTT ATA ATA TAA CTA CAT TAT TGA AAA TTT ATG TTA

       TCA TAC ATG TAT TAT TAA AGC AGA GGA TGG AAG GAA GAA AGG AGG

       GGG GGG GGT TAT GTT ATG ATG GCA AGG ATT CCA TTA TTA TTA TTA

       TTA TTA TTT TAC TTT GTG ACT ATC ATT ATC ATT ATT TAT ATT TGT

       TAT TTG GGA CTA TGA GGC GAG CAA GAA AAC GAA GGA AAA CAT AAC

       CAA TAG TTA ATG TTA TTG TTT TTT TTT TAA ATT CTT TTT TGT TTT

       TGA TAA GAG GAA AAA GAA GAA GAG ACG CCA CGA AGG AAA AGA AAA

       GAA GAA CAT AAA TGA ATT ACA AAA AAA AAA AAA A-3'
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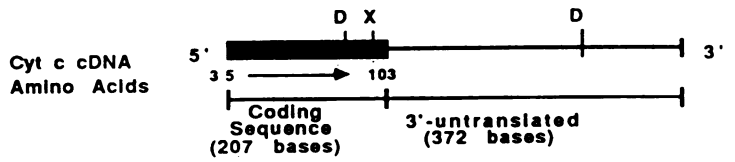


FIG. 3. Comparison of cytochrome *c* sequences. The partial amino acid sequence of *T. brucei* cytochrome *c* (T.b.) is aligned with the amino acid sequence of cytochrome *c* from both *C. fasciculata* (C.f.) (23) and *S. cerevisiae* (S.c.) (38). The sequence of the partial *T. brucei* cytochrome *c* cDNA is aligned by the protein sequence it encodes. *T. brucei* cytochrome *c* amino acid sequence based only on the cDNA sequence data is underlined. The bottom diagram is of the partial *T. brucei* cytochrome *c* cDNA. The size, orientation, and approximate positions of certain restriction sites are indicated (D, *Dra*I sites; X, *Xho*I site). *Eco*RI sites flank the cDNA.

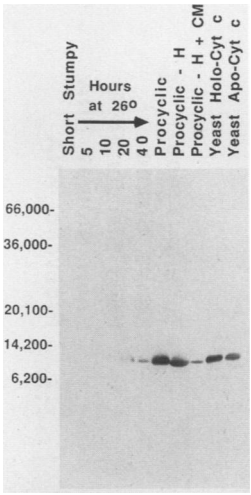


FIG. 4. Analysis of cytochrome *c* expression by Western blot. Trypanosome cell lysates were partially purified by chromatography over CM-Sephadex as described in Materials and Methods. The eluted proteins were fractionated on 15% SDS-PAGE, blotted onto nitrocellulose, and probed with affinity-purified rabbit antibodies against *T. brucei* cytochrome *c*. The antigen-antibody complexes were visualized by using a goat anti-rabbit immunoglobulin antibody conjugated to alkaline phosphatase. The blot includes a cell lysate of 2×10^9 short stumpy trypanosomes, cell lysates of 1×10^9 trypanosomes at specific times after initiating differentiation from the short stumpy to the procyclic form in SM medium at 26°C (5, 10, 20, and 40 h), and a cell lysate of 2×10^9 trypanosomes from an established procyclic culture. The blot also includes a CM-Sephadex-purified lysate of 2×10^9 procyclic trypanosomes which had been chemically treated to remove heme (Procyclic-H) and a similiarly treated lysate of 1×10^9 procyclic trypanosomes which was then rechromatographed on CM-Sephadex (Procyclic-H + CM). The final two lanes contain 1 μg of yeast cytochrome *c* (Yeast Holo-Cyt c) and 1 μg of yeast cytochrome *c* chemically treated to remove the heme prosthetic group (Yeast Apo-Cyt c). Sizes are shown to the left (in daltons).

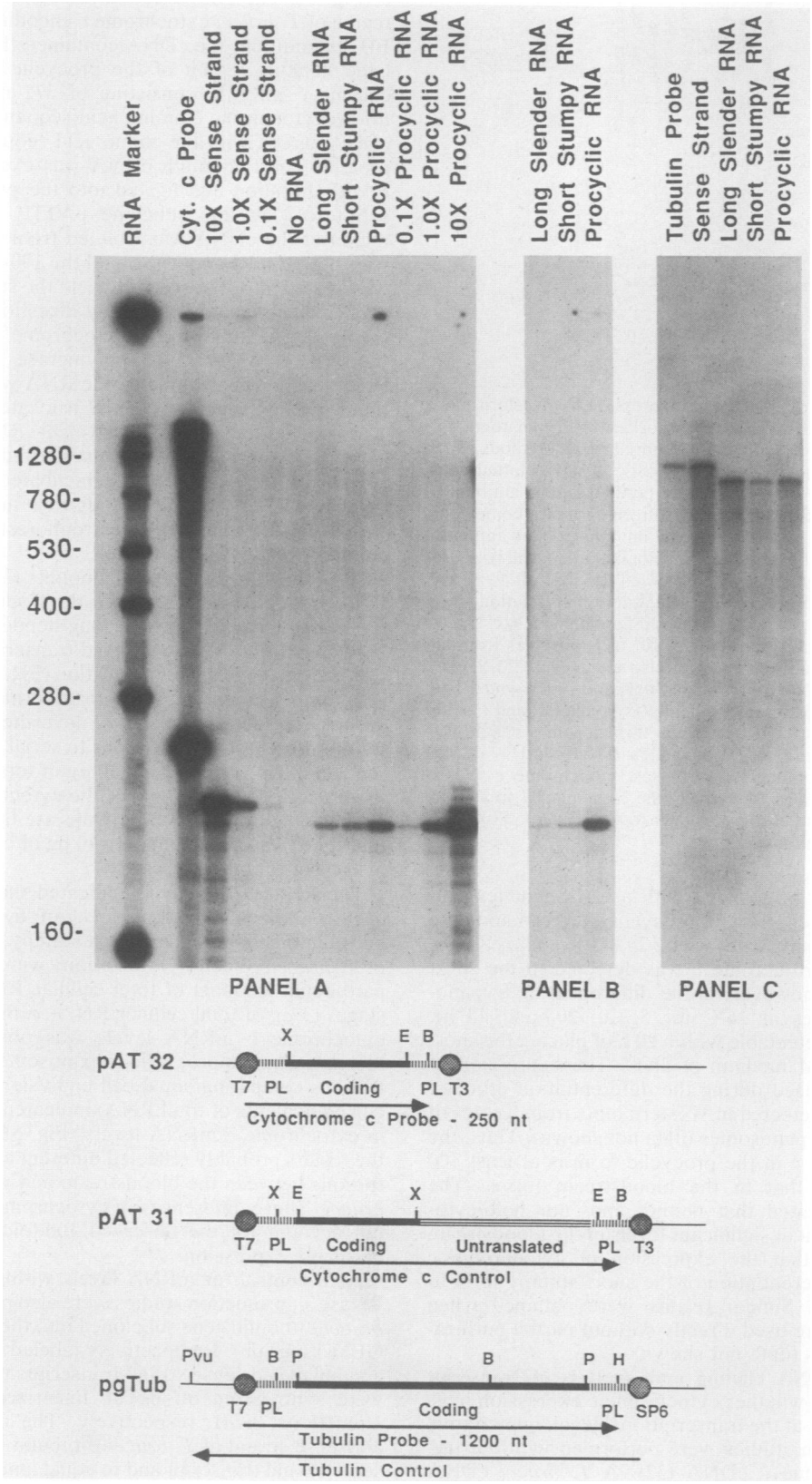
trypanosomes could be isolated with a CM-Sephadex column (compare procyclic-H with procyclic-H + CM and note difference in cell equivalents used, 2×10^9 and 1×10^9, respectively). No cytochrome *c* was detected in the short stumpy cell lysate; however, in the differentiating trypanosomes (trypanosomes at 26°C for 5, 10, 20, and 40 h), cytochrome *c* was detectable within 20 h of placing the short stumpy forms in SM medium at 26°C. The expression of cytochrome *c* increased during the differentiation process. Cytochrome *c* was detected in Western blots from lysates of 2×10^7 procyclic trypanosomes (data not shown). Thus, the level of cytochrome *c* in the procyclic form is at least 100 times greater than that in the bloodstream forms. The Western blots indicated that neither apo- nor holo-cytochrome *c* was present at significant levels in the bloodstream trypanosomes and that the expression of cytochrome *c* occurred during differentiation of the short stumpy form to the procyclic form. Similar results were obtained when total-cell lysates were used directly without partial purification on CM columns (data not shown).

Cytochrome *c* cDNA cloning and analysis of transcript levels. To determine whether cytochrome *c* expression in *T. brucei* was regulated at the transcriptional level, quantitative RNase T_1 protection studies were performed with the trypanosome cytochrome *c* cDNA (32). A *T. brucei* cDNA expression library was constructed in λgt11 from poly(A)$^+$ procyclic RNA. Dual screening of the cDNA library with both rabbit and mouse antibodies against *T. brucei* cytochrome *c* identified a partial cDNA to *T. brucei* cytochrome *c* (Fig. 3). The cDNA contained 207 nucleotides of the 3′ region of *T. brucei* cytochrome *c* encoding amino acids 35 to 103. In addition, the cDNA contained the entire 3′ untranslated flanking region of the procyclic trypanosome cytochrome *c* mRNA, consisting of 372 nucleotides. The *T. brucei* cytochrome *c* amino acid sequence derived from the cDNA agreed with the amino acid sequence of the purified protein. The full-length cDNA was excised from λgt11 by *Eco*RI digestion and ligated into the plasmid vector Bluescript to yield the subclone pAT31. The protein-coding region of the cDNA was isolated from pAT31 by digestion with both *Eco*RI and *Xho*I, and the 190-nucleotide fragment was ligated into Bluescript to yield the subclone pAT32. The pAT32 clone was linearized by digestion with *Bam*HI, and the antisense strand to the cytochrome *c* coding region was transcribed by using T7 polymerase in the presence of [α-^{32}P]GTP. The 250-nucleotide RNA was the T7 transcription product from the *Bam*HI-truncated pAT32 template. The labeled high-molecular-weight RNA smear resulted from run-on transcription of undigested pAT32. The radiolabeled transcripts were then incubated in the presence of unlabeled RNA, and the hybrids were analyzed by digestion with RNase T_1 and gel electrophoresis under denaturing conditions (Fig. 5).

Hybridizations with equal amounts (3 μg) of total cellular RNA isolated from the three developmental stages of *T. brucei* demonstrated that the long slender, short stumpy, and procyclic form RNAs contained transcripts which protected the probe from RNase T_1 digestion (Fig. 5A). This surprising result implies that the bloodstream forms of *T. brucei*, which do not express cytochrome *c*, nevertheless possess mRNA for the protein. Hybridizations to serial dilutions of both the procyclic RNA (0.3, 3, and 30 μg of total cellular RNA) and the unlabeled sense strand of the cytochrome *c* cDNA (500, 50, and 5 pg of pAT31 T7 polymerase transcripts) suggested that there was approximately 10 pg of cytochrome *c* mRNA in 1 μg of procyclic RNA.

The RNase T_1 analysis indicated that there were differences in the steady-state level of cytochrome *c* mRNA between the long slender, short stumpy, and procyclic forms of *T. brucei*. When hybridizations were conducted to compare equal amounts of total cellular RNA between the life stages (3 μg of total cellular RNA), a threefold difference in cytochrome *c* mRNA levels was observed between the bloodstream and procyclic trypanosomes (Fig. 5A). Hybridizations comparing equal cell equivalents of RNA (5×10^6 cell equivalents of total RNA) indicated a fivefold difference in cytochrome *c* mRNA levels (Fig. 5B). The variations in the results probably reflected different cellular RNA concentrations between the bloodstream and procyclic forms of *T. brucei*. These differences in cytochrome *c* mRNA levels do not account for the observed 100-fold regulation in cytochrome *c* expression.

As a control for mRNA levels within the isolated RNA, RNase T_1 protection studies were also performed with the *T. brucei* β-tubulin gene subcloned into the transcription vector GEM-I (pgTub). Radioactively labeled antisense transcripts and unlabeled sense strand transcripts to *T. brucei* β-tubulin were synthesized off pgTub linearized by digestion with *Hin*dIII or *Pvu*II, respectively. The radioactively labeled antisense strand of *T. brucei* β-tubulin was hybridized to its sense strand transcript and to equal amounts of total cellular RNA (2 μg) isolated from the long slender, short stumpy, and procyclic forms of *T. brucei*. The hybrids were analyzed


RNA Marker
Cyt. c Probe
10X Sense Strand
1.0X Sense Strand
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No RNA
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Procyclic RNA
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1.0X Procyclic RNA
10X Procyclic RNA
Long Slender RNA
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Tubulin Probe
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Short Stumpy RNA
Procyclic RNA
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780-
530-
400-
280-
160-
PANEL A
PANEL B
PANEL C
pAT 32
X
E B
T7 PL Coding PL T3
Cytochrome c Probe - 250 nt
pAT 31
X E
X
E B
T7 PL Coding Untranslated PL T3
Cytochrome c Control
pgTub
Pvu
B P
B
P H
T7 PL Coding PL SP6
Tubulin Probe - 1200 nt
Tubulin Control

as before (Fig. 5C). Dividing long slender and procyclic trypanosomes had equivalent β-tubulin transcript levels, while the nondividing, short stumpy trypanosomes had slightly lower steady-state levels of β-tubulin mRNA. The results indicated that there was no selective loss of mRNA during the bloodstream trypanosome RNA isolation procedure which would account for the signal variations observed with the cytochrome *c* probe.

DISCUSSION

Previous studies on *Trypanosoma brucei* have demonstrated that mitochondrial biogenesis is regulated during different developmental stages of the parasite (5, 6, 29). In particular, investigations done with respiratory inhibitors, such as cyanide, antimycin, and carbon monoxide, have shown that bloodstream forms of *T. brucei* lack the intact cytochrome-mediated respiratory chain present in the procyclic form (8, 23). The absence of the spectrally detectable holo-cytochrome *c* in the bloodstream forms of *T. brucei* was previously observed, but the level at which this regulation occurs and the mechanism of its developmental control were unknown (5). Our results indicate that cytochrome *c* expression is developmentally regulated by a novel posttranscriptional mechanism in *T. brucei*.

T. brucei cytochrome *c* was isolated from procyclic trypanosomes and characterized by SDS-PAGE, absorption spectroscopy, and amino acid sequence analysis. The trypanosome cytochrome *c* has atypical characteristics similar to those observed for cytochrome *c* isolated from other members of *Kinetoplastida* (23). *T. brucei* cytochrome *c* lacks a cysteine residue at position 14, which is very highly conserved in cytochrome *c* isolated from most eucaryotic cells. The substitution at this position is observed in all *Kinetoplastida* forms of cytochrome *c* and restricts the protein to a single thioether linkage to the heme prosthetic group. This unusual heme attachment results in a red-shifted absorption spectrum for *Kinetoplastida* cytochrome *c*. The absorbance maxima of the reduced form of *T. brucei* cytochrome *c* are 556 nm, 523 nm, and 419 nm. This compares with 555 nm, 525 nm, and 420 nm reported for *C. fasciculata* and 556 nm, 524 nm, and 420.5 nm reported for *T. rhodesiense*.

Antibodies raised in both rabbits and mice to the *T. brucei* cytochrome *c* were shown to recognize the apo- as well as the holo-protein. Since spectral analysis can only detect the holo-protein, the antibodies were used as probes to demonstrate that bloodstream forms of trypanosomes do not possess the apo-cytochrome *c* (Fig. 4). Analysis of cells differentiating from the short stumpy to procyclic form indicate that the production of cytochrome *c* is an early step in the differentiation process and that the synthesis of the apoprotein is coincidental with the appearance of the holoprotein (Fig. 1 and 4). RNase T_1 analysis of steady-state cytochrome *c* mRNA levels in the long slender, short stumpy, and procyclic forms of *T. brucei* indicates that cytochrome *c* is not transcriptionally regulated. Although there is a three- to fivefold difference in the level of steady-state cytochrome *c* mRNA between bloodstream and procyclic trypanosomes, it is not great enough to account for the difference in regulation of cytochrome *c* expression. These results argue against cytochrome *c* expression being regulated either at the level of transcription initiation or by mRNA stability in *T. brucei*.

The mechanism by which developmental regulation of cytochrome *c* occurs in *T. brucei* is presently under investigation in this laboratory. It is possible that the apo-protein is synthesized but then is rapidly degraded; however, the unique biology of *T. brucei* allows for possible mRNA modifications which could interfere with mRNA translation. Most, if not all, translatable mRNAs in trypanosomes contain a unique 5′ miniexon (7, 10, 25). This 39-mer, which is *trans*-spliced onto mRNAs, may be involved in the recognition of translatable mRNAs. The cytochrome *c* mRNA in the bloodstream forms of *T. brucei* may lack this 39-mer and thus be untranslatable.

Alternatively, the translatability of the cytochrome *c* mRNA may involve the secondary structure of the RNA molecule. A temperature-sensitive mutant of *S. cerevisiae* has been reported in which an alteration in the secondary structure of the *oli1* mRNA results in a defect in translation at the higher temperature (33). This translational block in the yeast mutant requires only an 8°C temperature shift, from 28 to 36°C. The 11°C temperature difference between the procyclic and bloodstream trypanosomes could also affect the secondary structure of the cytochrome *c* mRNA and prevent translation of the protein in the bloodstream forms. The effect of mRNA structure on translation of cytochrome *c* has already been demonstrated in *S. cerevisiae* (3). The introduction of stable hairpin structures in the cytochrome *c* mRNA immediately 5′ to the initiating AUG codon inhibits translation of the protein.

Finally, translatability of the *T. brucei* cytochrome *c* mRNA may be dependent on internal uridine additions. *T. brucei* has recently been discovered to possess a unique RNA modification activity which allows internal additions of uridine to mitochondrial transcripts (4, 14–16). This activity

FIG. 5. RNase T_1 protection analysis of trypanosome RNA. Radioactively labeled riboprobes were hybridized to RNA, and the hybrids were digested with RNase T_1. The digestion products were analyzed on a 6% denaturing gel and visualized by autoradiography. Sizes are indicated in nucleotides. (A) Undigested pAT32 T7 transcript (Cyt. c Probe), and the RNase T_1 digestion products of the hybridizations between the pAT32 transcript and various RNAs. Hybridizations to 500 pg (10× Sense Strand), 50 pg (1.0× Sense Strand), 5 pg (0.1× Sense Strand), and 0 pg (No RNA) of pAT31 T7 transcript; equal amounts (3 μg) of total cellular RNA isolated from each of the three developmental stages (Long Slender RNA, Short Stumpy RNA, and Procyclic RNA); and 0.3 μg (0.1× Procyclic RNA), 3 μg (1.0× Procyclic RNA), and 30 μg (10× Procyclic RNA) of total procyclic RNA. (B) The pAT32 transcript was hybridized to equal cell equivalents (5×10^6 trypanosomes) of total cellular RNA from each of the three developmental stages (Long Slender RNA, Short Stumpy RNA, and Procyclic RNA). (C) Undigested pgTub T7 transcript (Tubulin Probe) and the RNase T_1 digestion products from hybridizations of the pgTub T7 transcript to RNA. Hybridizations to the pgTub SP6 transcript (Sense Strand) and equal amounts (2 μg) of total cellular RNA from each life stage (Long Slender, Short Stumpy, and Procyclic). The plasmid constructs used to prepare the riboprobes and the control transcripts are shown at the bottom. The solid lines represent the coding regions of the respective mRNAs (Coding), the horizontally lined area is the noncoding flanking region of the cytochrome c cDNA (Untranslated), the vertically lined areas are the plasmid polylinkers (PL), and the cross-hatched circles are the designated polymerase promoters (T7, T3, and SP6). The transcribed regions and the direction of transcription are indicated by the labeled arrows (Cytochrome c Probe, Cytochrome c Control, Tubulin Probe, and Tubulin Control), and the pertinent restriction sites are designated (X, *Xho*I; E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; Pvu, *Pvu*II). nt, Nucleotides.

appears to be developmentally regulated, is very specific, and has been found to occur in both the coding and noncoding regions of transcripts. It is possible that such an RNA modification activity may also affect nucleus-encoded transcripts and that cytochrome *c* mRNA in *T. brucei* is subject to these uridine additions. Developmentally regulated uridine additions to the cytochrome *c* mRNA may alter the sequence required for translation in the bloodstream forms.

The developmental regulation of cytochrome *c* expression by a posttranscriptional mechanism is unique. Other developmentally regulated genes which have been examined in trypanosomes are regulated at the transcriptional level (31, 34). The possible involvement of mRNA modification by uridine addition might provide a common mechanism for the regulation of both mitochondrion- and nucleus-encoded mitochondrial proteins in trypanosomes.

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