ATP Synthase Is Responsible for Maintaining Mitochondrial Membrane Potential in Bloodstream Form *Trypanosoma brucei*

Silvia V. Brown, Paul Hosking, Jinlei Li, and Noreen Williams*

*Department of Microbiology and Immunology and Witebsky Center for Microbial Pathogenesis and Immunology, 253 Biomedical Research Building, University at Buffalo, Buffalo, New York 14214*

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The mitochondrion of *Trypanosoma brucei* bloodstream form maintains a membrane potential, although it lacks cytochromes and several Krebs cycle enzymes. At this stage, the ATP synthase is present at reduced, although significant, levels. To test whether the ATP synthase at this stage is important for maintaining the mitochondrial membrane potential, we used RNA interference (RNAi) to knock down the levels of the ATP synthase by targeting the F\(_\text{1}\)-ATPase \(\alpha\) and \(\beta\) subunits. RNAi-induced cells grew significantly slower than uninduced cells but were not morphologically altered. RNAi of the \(\beta\) subunit decreased the mRNA and protein levels for the \(\beta\) subunit, as well as the mRNA and protein levels of the \(\alpha\) subunit. Similarly, RNAi of \(\alpha\) subunit decreased the \(\alpha\) subunit transcript and protein levels, as well as the \(\beta\)-subunit transcript and protein levels.

In contrast, \(\alpha\) and \(\beta\) RNAi knockdown resulted in a 60% increase in the F\(_0\) complex subunit 9 protein levels without a significant change in the steady-state transcript levels of this subunit. The F\(_{\text{F}}\)–32-kDa subunit protein expression, however, remained stable throughout induction of RNAi for \(\alpha\) or \(\beta\) subunits. Oligomycin-sensitive ATP hydrolytic and synthetic activities were decreased by 43 and 44%, respectively. Significantly, the mitochondrial membrane potential of \(\alpha\) and \(\beta\) RNAi cells was decreased compared to wild-type cells, as detected by MitoTracker Red CMXRos fluorescence microscopy and flow cytometry. These results support the role of the ATP synthase in the maintenance of the mitochondrial membrane potential in bloodstream form *T. brucei*.

The mitochondrial ATP synthase couples the electrochemical proton gradient to the synthesis or hydrolysis of ATP (5, 11, 26, 39). The ATP synthase is composed of the soluble F\(_1\) moiety, which contains the catalytic sites and the membrane-bound F\(_0\) moiety, which is involved in proton translocation. The F\(_1\) moiety of the mitochondrial ATP synthase is highly conserved and is composed of five subunits present in a stoichiometry of a\(_5\)b\(_2\)c\(_{10-14}\). The F\(_0\) moiety in *Escherichia coli* is composed of three subunits, a\(_2\)b\(_2\)c\(_{10-14}\), but in eukaryotes its subunit composition increases in complexity to include up to eight additional subunit types (5, 22, 26). The *Trypanosoma brucei* mitochondrial ATP synthase has been isolated and characterized. The molecular composition of the enzyme complex is similar to that of other eukaryotic mitochondrial ATP synthases (43). Both functional assays and analysis of protein levels indicate that the complex is developmentally regulated through the life cycle of the organism (7, 41, 42).

A striking feature of *T. brucei* is its ability to adapt to diverse environments encountered through the stages of its life cycle (21, 34). In the tsetse fly, the mitochondrion of the procyclic trypanosomes is fully developed with many cristae, a complete respiratory chain, Krebs cycle enzymes, and abundant levels of mitochondrial ATP synthase. In contrast, the sparse and tubular mitochondrion of the early (slender) mammalian bloodstream trypanosomes lacks a functional respiratory chain, and energy production at this stage occurs via glycolysis. Although the expression of some mitochondrial components is upregulated in late (stumpy) bloodstream form trypanosomes, oxidative phosphorylation does not occur until the parasite transforms into the procyclic stage in the midgut of the tsetse fly (9, 17, 24, 29) Despite the absence of cytochrome mediated electron transport coupled to ATP production, a mitochondrial membrane potential in bloodstream forms exists and has been found to be comparable to that in mitochondria of procyclic trypanosomes (18, 20, 33). In the bloodstream stage, electrons flow from ubiquinol to the trypanosome alternative oxidase, but this electron flow is not coupled to ATP production and does not generate a membrane potential (8, 20).

The ATP synthase, in contrast to the cytochromes, can be detected in all life cycle stages but is most abundant during the procyclic stage, where it functions in ATP generation through oxidative phosphorylation (2, 42). We and others have hypothesized that the ATP synthase in bloodstream form trypanosomes is responsible for the generation of the mitochondrial membrane potential by hydrolyzing ATP generated by substrate level phosphorylation (2, 20, 42). The mechanism by which this reversible enzyme switches from the ATP synthetic to the ATP hydrolytic activity is still not well understood, but in *E. coli*, yeast, and mammalian cells it has been suggested to be a response to the proton motive force and the ADP/ATP balance. ATP hydrolytic activity, therefore, predominates at high ATP levels and low membrane potential, forcing the reverse pumping of protons to generate a membrane potential (28, 35, 38). The membrane potential of the bloodstream form is presumably essential for the transport of ions and nutrients, as well as for the import of nuclearly encoded proteins required as the bloodstream trypanosomes transform to the procyclic form (19, 20). To test this hypothesis, we have used RNA interference (RNAi) to decrease the levels of the ATP synthase in bloodstream form trypanosomes by targeting the F\(_1\) \(\alpha\) subunit...
or β subunits, which together comprise the catalytic site of the ATP synthase. Our results indicate that the decrease in expression of the α or β subunits is reflected in a significantly decrease in the oligomycin-sensitive activities of this enzyme and results in parallel decrease in the mitochondrial membrane potential of bloodstream trypanosomes. These results support the role of the ATP synthase in the maintenance of the membrane potential in bloodstream trypanosomes.

**MATERIALS AND METHODS**

**pZJM-SB RNAi vector.** A 289-bp fragment from nucleotides 171 to 460 of the β subunit gene was amplified by PCR from the ATP synthase β subunit cDNA clone (4) with the forward primer containing an XhoI site (underlined), 5′-GCC TGG AGG ACT GCC CCT GAG GTT GAC AAA CT-3′, and the reverse primer containing a HindIII site (underlined), 5′-GCA AATC CTG GTG CGC AAG CTT GGG AGC CAC GG-3′. The 289-bp fragment was ligated into the XhoI/HindIII sites between two head to head T7 promoters of the pZJ vector (37) to generate the pZJM-SubunitBeta (pZJM-SB) construct.

**pLewLoop-SA vector.** The pLewLoop-SA vector produced a double-stranded RNA as a stem-loop construct to target the ATP synthase α subunit and was constructed as described by Wang et al. (37). Briefly, a 445-bp fragment from nucleotides 169 to 614 was amplified by PCR from the pLewα subunit cDNA clone (4) by using the primer CAAGTGTCGGAGCGGTGATCGT GCACCTTGGC containing the HindIII and MluI linkers (underlined), respectively, and the reverse primer GGTATCCGATTCAATTCGCTGTC containing the XbaI linker (underlined). This product was first ligated into pCRII (Invitrogen), liberated by XbaI/HindIII digestion, and ligated into the NheI/HindIII sites of pJM352 vector. The construct was then digested with HindIII/XbaI to liberate an ~1,000-bp fragment containing the target gene and the ~550-bp stuffer fragment. The pLew100 vector was then digested with XbaI/MluI to release the luciferase reporter. The same ATPase subunit fragment was amplified by PCR and digested with XbaI/MluI, followed by ligation into the prepared pLew100 vector to generate the pLewLoop-SubunitAlpha (pLewLoop-SA) construct. The resulting stem-loop plasmid contained two copies of the α subunit fragment in opposite orientation separated by the stuffer fragment.

**Cell growth, transfection, and RNAi induction.** Bloodstream *T. brucei* single marker strain, BS15 (a gift from George Cross, Rockefeller University), henceforth referred to as wild type, was grown in HMI-9 medium supplemented with 10% fetal bovine serum (15). This cell line previously engineered to express T7 marker strain, BSSM (a gift from George Cross, Rockefeller University), henceforth referred to as engineered. Cell growth, transfection, and RNAi induction were accomplished by the addition of phleomycin (2.5 μg/ml) to maintain the T7 polymerase and the tetracycline repressor allows the regulatable expression of 10% fetal bovine serum (15). This cell line previously engineered to express T7 marker strain, BSSM (a gift from George Cross, Rockefeller University), henceforth referred to as engineered.

**Western blot analysis of RNAi cells.** Bloodstream RNAi cell cultures were induced and harvested as described above. For protein samples, 5 × 10^6 cells were collected per time point. Whole-cell extract from half the sample (2.5 × 10^6 cells) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, followed by Western blot analysis with antibodies directed against the T. brucei complete F1 complex, β subunit, subunit 9, and the F0 complex as previously described (4, 6, 43). An aliquot of equal cell number was used for Western blot analysis probed with a monoclonal antibody against β-tubulin (Chemicon International) as a loading control. Western blot results for each subunit were compared as described above the method of Pullman and Racker, as previously described (43). To determine the specificity of the ATPase activity, oligomycin (5 μg/ml of protein) was added to the mitochondria and preincubated for 45 min prior to the assay. The ATP synthetic activity was determined spectrophotometrically at 340 nm by the method of Pullman and Racker, as previously described (43).

**Fluorescent microscopic detection of mitochondrial membrane potential.** The membrane potential-dependent stain MitoTracker Red CMXRos (Molecular Probes) was used to assess the mitochondrial membrane potential in bloodstream form trypanosomes. A total of 5 × 10^6 wild type, day 4-induced β subunit RNAi cell lines, and day 3-induced α subunit RNAi, as previously described (43). The APase activity was determined spectrophotometrically at 340 nm by using a coupled enzyme assay, with 100 μg of protein by the method of Pullman and Racker, as previously described (43). To determine the specificity of the ATPase activity, oligomycin (5 μg/ml of protein) was added to the mitochondria and preincubated for 45 min prior to the assay. The ATP synthetic activity was determined spectrophotometrically at 340 nm by the method of Pullman and Racker, as previously described (43).

**Determination of ATPase and ATP synthase activity.** An enhanced mitochondrial fraction was prepared from 3 to 5 × 10^6 wild type, day 4-induced β subunit RNAi cell lines, and day 3-induced α subunit RNAi, as previously described (43). The APase activity was determined spectrophotometrically at 340 nm by using a coupled enzyme assay, with 100 μg of protein by the method of Pullman and Racker, as previously described (43). To determine the specificity of the ATPase activity, oligomycin (5 μg/ml of protein) was added to the mitochondria and preincubated for 45 min prior to the assay. The ATP synthetic activity was determined spectrophotometrically at 340 nm by the method of Pullman and Racker, as previously described (43).

To induce RNAi expression, cells growing at mid-log phase were diluted to 3 × 10^5 cells/ml, cultured in the medium described above supplemented with 1.0 μg of the NotI-linearized pZJM-SB construct or the EcoRV-linearized pLewLoop-SA vector to target the rRNA gene spacer region by homologous recombination. Transfections were performed in 4-mm cuvettes by homologous recombination. Transfections were performed in 4-mm cuvettes by homologous recombination. Transfections were performed in 4-mm cuvettes by homologous recombination. Transfections were performed in 4-mm cuvettes by homologous recombination. Transfections were performed in 4-mm cuvettes by homologous recombination. Transfections were performed in 4-mm cuvettes by homologous recombination. Transfections were performed in 4-mm cuvettes by homologous recombination. Transfections were performed in 4-mm cuvettes by homologous recombination.
and indicated that a decrease in the levels of the ATP synthase 
Dickinson) was used to analyze the results. Using absorption at 578 nm and emission at 599 nm. CellQuest software (Becton 
detected in 1 ml of PBS. Changes in mitochondrial fluorescence intensity were 
alyzed with a FACSCalibur (Becton Dickinson) analytical flow cytometer 
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RESULTS

Expression of α- and β-subunit dsRNAi in the bloodstream 
stage resulted in a change in cell growth. RNAi was used to 
 knock down the ATP synthase α and β subunits in order to 
study the function of the mitochondrial ATP synthase in the 
bloodstream stages of T. brucei. The RNAi of the ATP synthase 

β subunit was mediated by the dsRNA construct, pZJM-
SB, which contains a 289-bp fragment of the ATP synthase 
β-subunit coding region. This vector allows the regulatable 
expression of dsRNA of the β-subunit fragment from opposing 
T7 promoters in cell lines previously engineered to express the 
T7 polymerase and the tetracycline repressor. The RNAi of the 
ATP synthase α subunit was mediated by a tetracycline-indu-
duced stem-loop construct (pLewLoop-SA), containing a 
445-bp fragment of the α-subunit coding region. Clonal cell 
lines of bloodstream trypanosomes transfected with the α-
subunit stem-loop construct (pLewLoop-SA) were established by 
limiting dilution. Although several attempts were made to de-
velop clonal cell lines of the pZJM-SB expressing β RNAi, no 
viable lines could be maintained. We believe that expression of 
dsRNA without induction in pZJM (30, 37) acted to select 
against the establishment of a cell line capable of a more 
complete knock down. Thus, two nonclonal β-subunit RNAi 
cell lines that showed a substantial decrease in the β-subunit 
protein, relative to the uninduced, were used to monitor effects 
of RNAi expression of the β subunit.

The pZJM-SB cell lines were induced for the expression of 
β-subunit RNAi for up to 6 days and showed a 50% decrease 
and a lag in cell growth compared to wild-type and uninduced 
cells starting at day 3 postinduction (Fig. 1A). Similarly, cell 
lines expressing α-subunit RNAi induced with tetracycline for 
up to 6 days showed a decrease in cell grown of ~50% starting 
at day 3 postinduction compared to wild-type and uninduced 
cells (Fig. 1B). Despite a decrease in the rate of cell growth, 
microscopic analysis of the α and β RNAi induced cell lines did 
not show significant changes in motility or morphology. We 
also obtained an α stem-loop clone (pLewLoop-SA clone 11) in 
which induction with tetracycline resulted in cell death within 
24 h of induction (Fig. 1C). This phenotype, however, could 
not be maintained in continued culture. Both cell lines were 
monitored for effects on the transcript and protein expression 
for 4 days after induction. Induction of the β-subunit RNAi 
resulted in a 50% decrease in the β-subunit transcript (Fig. 
2A) and protein expression (Fig. 3A) on day 4 postinduction. 
Induction of the α-subunit RNAi resulted in a 50% decrease in 
the α-subunit transcript (Fig. 2B) by day 3 and protein (Fig. 
3B) by day 2 postinduction. By day 3 postinduction, the α clone 
showed 90% decrease in protein expression and began to re-
cover by day 4 (Fig. 3B) Thus, in all subsequent experiments 
the pZJM-SB cells were induced for 4 days for β RNAi and the 
pLewLoop-SA cells were induced for 3 days for α RNAi, with 
cell densities maintained at less than 5 × 10^6. These results 
indicated that a decrease in the levels of the ATP synthase α 
and β subunits was limiting for cell proliferation after day 3 of 
tetracycline addition. After this lag in cell growth, the cells 
began to recover, as is often seen in T. brucei RNAi cell lines 
(13, 14, 40), but did not reach wild-type or uninduced levels 
during the days observed (Fig. 1A and B). This suggests that 
the ATP synthase plays an important role in the growth and 
proliferation of the bloodstream form trypanosomes. More-
over, attempts to differentiate the pZJM-SB cells into the 
procyclic stage resulted in multinucleation, aggregation, and 
cell death (data not shown). This latter result suggests that β 
RNAi-induced cells are already weakened, such that they are
unable to keep up with the cellular demands required for differentiation to the procyclic stage. Moreover, since the ATP synthase is required for metabolism in procyclic trypanosomes, even the reduced content of ATP synthase may be too low to allow sufficient energy generation for cell survival.

Expression of \( \alpha \) - and \( \beta \)-subunit RNAi resulted in a significant change of the mRNA and protein steady-state levels of ATP synthase subunits. Cells induced for 4 days for \( \beta \) and 3 days for \( \alpha \) subunit RNAi were analyzed by RT-PCR and Western blot to determine the effect on their respective ATP synthase subunit steady-state transcript and protein levels. RNAi knockdown of the \( \beta \) subunit resulted in a 50% decrease in \( \beta \)-subunit mRNA steady-state levels by day 2 and a 73% decrease by day 4 postinduction relative to the uninduced day 0 levels (Fig. 2A). RNAi knockdown of the \( \beta \) subunit also decreased the steady-state transcript levels of the \( \alpha \) subunit by 65% on day 2 and by 58% on day 4 postinduction. RNAi induction of the \( \alpha \) subunit decreased the \( \alpha \)-subunit mRNA steady-state levels by 50% on day 3 and was further decreased to by 80% on day 4 postinduction relative to the uninduced day 0 levels (Fig. 2B).

RNAi knockdown of the \( \alpha \) subunit also decreased the \( \beta \)-subunit steady-state transcript levels, similar to the effect of \( \beta \) RNAi on the \( \alpha \) subunit. It is not surprising that \( \beta \) RNAi knockdown has an effect on the \( \alpha \) subunit of the ATP synthase and vice versa, since these two subunits are highly homologous. The 289-bp fragment used in the pZJM-SB vector shares 45% identity to the same region of the \( \alpha \) subunit, and the 445-bp fragment used in the pLewloop-SA stem-loop construct shares 45% identity to the same region of the \( \beta \) subunit. To determine whether this effect was due to sequence homology or if this was a global effect on the ATP synthase, we examined other subunits of the complex. The steady-state mRNA levels of the ATPase \( F_0 \) complex subunit 9 (S9) decreased only by 10% on day 2 and by 15% on day 4 postinduction upon \( \beta \) RNAi knockdown. Similarly, steady-state mRNA levels of the ATPase \( F_0 \) complex S9 remained relatively stable upon \( \alpha \) RNAi induction. The actin (Fig. 2) and p34/p37 (data not shown) control levels remained constant throughout induction of either \( \alpha \) or \( \beta \) RNAi (Fig. 2).

The effect of \( \alpha \) and \( \beta \) RNAi induction on the steady protein

![FIG. 2](image-url) RNAi knockdown of the ATP synthase \( \alpha \) and \( \beta \) subunits affects the steady-state transcript expression of ATP synthase complex subunits. RNA extracted from \( 5 \times 10^6 \) cells of RNAi-induced cells from each day 0, day 2, day 3, and day 4 postinduction and used in RT-PCR to analyze the steady-state transcript levels of the \( F_1 \) complex \( \alpha \) and \( \beta \) subunits and the \( F_0 \) complex subunit 9, using the respective internal subunit primers with the miniexon primer. (A) Effect of \( \beta \) RNAi induction on the steady-state transcript levels of ATP synthase subunits \( \beta \), \( \alpha \), and 9. (B) Effect of \( \alpha \) RNAi induction on steady-state transcript levels of ATP synthase subunits \( \alpha \), \( \beta \), and 9. Day 0 was set as the 100% reference point. Actin was used as the control.

![FIG. 3](image-url) RNAi knockdown of the ATP synthase \( \alpha \) and \( \beta \) subunits affects the steady-state protein expression of ATP synthase complex subunits. Steady-state protein levels of the \( F_1 \) complex \( \alpha \) and \( \beta \) subunits and the \( F_0 \) complex subunits 9 and the 32 kDa, were analyzed by using Western blots of total protein from \( 2.5 \times 10^6 \) cells from each day 0, day 2, day 3, and day 4 postinduction. The \( \beta \) subunit was detected with anti-\( \beta \) subunit antibodies, the \( \alpha \) subunit was detected with anti-\( F_1 \) complex antibodies, subunit 9 was detected with anti-subunit 9 antibodies, and the 32-kDa subunit was detected with anti-\( F_0 \) complex antibodies. The levels of \( \beta \)-tubulin expression were used as the loading control. (A) Effect of \( \beta \) RNAi on the steady-state protein levels of ATP synthase \( \alpha \), \( \beta \), S9, and \( F_0 \) complex 32-kDa subunits. (B) Effect of \( \alpha \) RNAi on the steady-state protein levels of ATP synthase \( \alpha \), \( \beta \), S9, and \( F_0 \) complex 32-kDa subunits. Day 0 was set as the 100% reference point. \( \beta \)-Tubulin was used as the control.
levels of the ATP synthase complex subunits was monitored by Western blot analysis with the antibodies specific for *T. brucei* subunits. β RNAi knockdown resulted in a 47% decrease in the steady-state protein level for the F₁ complex β subunit on day 2 and a 54% decrease on day 4 postinduction relative to day 0 (Fig. 3A). The steady-state protein levels of the F₁ complex α subunit were significantly decreased by 63% on day 2 and 84% on day 4 postinduction. Similarly, RNAi knockdown of the α subunit resulted in a 65% decrease in the ATP synthase F₁ complex α subunit and a similar 60% decrease of the β subunit by day 3 postinduction. Surprisingly, knockdown of either the α or β subunits resulted in a significant increase of the steady-state protein of the F₉ complex subunit 9. Upon RNAi of the β subunit, the steady-state protein levels of the F₉ complex subunit 9 was significantly increased by 87% on day 2 and by 61% on day 4 postinduction. These results were paralleled upon RNAi induction of the α subunit (Fig. 3). The inverse effect of F₁ complex α or β subunit knockdown on the F₉ complex subunit 9 was highest on day 2 postinduction (87% increase), suggesting that as α- or β-subunit transcript levels were targeted by RNAi, the subunit 9 transcript stability and/or protein stability increased, leading to an increase in the overall level of expression of this subunit. This effect peaked on day 2 and decreased on day 4 (61% increase) as the cells began to recover (Fig. 1). In contrast, knockdown of the α or β subunits did not significantly change the steady-state levels of the F₉ complex 32-kDa subunit (Fig. 3).

RNAi of the α or β subunit resulted in a decrease in oligomycin-sensitive ATP hydrolytic and ATP synthetic activities. Previous work from this laboratory measured ATP hydrolytic and ATP synthetic activities in an enhanced mitochondrial fraction from bloodstream cells. In the results reported here, 42% of the ATP hydrolytic and 49% of the ATP synthetic activity of wild-type mitochondria was sensitive to inhibition by oligomycin, similar to that previously reported (43). The mitochondrial fraction from cells induced for β RNAi showed a 43% decrease in ATP hydrolytic activity and a 44% decrease in ATP synthetic activity, relative to the wild-type controls. The sensitivity to oligomycin from β RNAi-induced cells was 54% for ATP hydrolytic activity (Table 1) and 40% for ATP synthetic activity (Table 2) compared to RNAi cells. ATP hydrolytic and synthetic activities were similarly affected by RNAi knockdown of the ATPase α subunit (data not shown). This suggests that the ATP synthase complexes that form after α or β RNAi knockdown are fully functional and show similar oligomycin sensitivity to that of wild type.

α- or β-subunit RNAi knockdown affects the mitochondrial membrane potential of bloodstream *T. brucei*. To determine whether knock down of the α or β subunits affects the mitochondrial membrane potential, we used the mitochondrial membrane potential-sensitive dye MitoTracker Red CMXRos to stain the mitochondria of wild type and induced cells with or without treatment with the respiratory uncoupler CCCP (32). The uptake of the MitoTracker Red CMXRos stain is dependent on mitochondrial membrane potential (23); in *T. brucei* cells a single MitoTracker-stained mitochondrion spans the length of the entire cell (10). Fluorescence microscopic analysis of the β RNAi-induced cells showed a population of cells showing different degrees of intensity of the MitoTracker fluorescent staining compared to wild-type cells (Fig. 4A and C). These cells were largely of two groups showing intense MitoTracker staining similar to those in the wild-type cells (Fig. 4A and C) or showing decreased staining similar to wild-type or β RNAi cells pretreated with the uncoupler CCCP (Fig. 4C, arrowhead). Cells treated with the uncoupler CCCP before or after staining with the MitoTracker Red showed equal intensity of staining. In cells induced for β RNAi, 60% of the cell population showed low fluorescence intensity, whereas 40% showed fluorescent intensity similar to wild type (Fig. 4G), indicating that β RNAi in this nonclonal cell line leads to a decrease in the mitochondrial membrane potential of the RNAi targeted cells within this cell population. Cells induced for α RNAi expression showed a decrease in fluorescence similar to that seen for cells expressing β RNAi. For cells expressing α RNAi, two cell lines were followed; clone 8 showed a mixed population of cells showing various degrees of fluorescent intensity similar to what was seen in the β RNAi nonclonal cell population (Fig. 4E, the arrowhead indicates a cell of lower intensity). Treatment with the uncoupler CCCP also resulted in a decrease in the mitochondrial membrane potential of this cell population (Fig. 4F).

**Quantitative analysis of the α and β RNAi effect on the mitochondrial membrane potential by FACS.** To further quantify the effect of α and β RNAi knockdown on the mitochondrial membrane potential, uptake of the MitoTracker Red CMXRos dye was analyzed by flow cytometry. Flow cytometry of the β RNAi-induced cells showed a consistent decrease in fluorescent intensity compared to the wild-type cells high threshold, as indicated by a leftward shift in the fluorescence intensity of the RNAi cells (Fig. 5A, B, C, and D, red trace) (16). Significantly, the fluorescent trace of the β RNAi cells showed a double peak, one of higher fluorescent intensity (Fig. 5B, peak 1, red trace) and one of lower fluorescent intensity which predominantly overlapped with the CCCP-treated peak (Fig. 5B, peak 2 of red trace and green trace). Each of the double peaks represents 50% of the cell population based on the area under the curve. All of the cells treated with the uncoupler CCCP lose their mitochondrial membrane poten-

### Table 1. Mitochondrial ATP hydrolysis activity

| T. brucei bloodstream form | % Activity relative to WT ± SD | % Inhibition relative to WT ± SD |
|---------------------------|-------------------------------|---------------------------------|
| WT                        | 100                           |                                 |
| WT + oligomycin           | 42.0 ± 9.7                    |                                 |
| pZJMβ-RNAi                | 57.0 ± 7.2                    | 49.2 ± 9.7                      |
| pZJMβ-RNAi + oligomycin   | 53.7 ± 7.0                    |                                 |

* Mitochondrion samples (100 µg) were assayed for ATP hydrolytic activity with or without treatment with oligomycin (5 µg/mg of protein). WT, wild type.

### Table 2. Mitochondrial ATP synthesis activity

| T. brucei bloodstream form | % Activity relative to WT ± SD | % Inhibition relative to WT ± SD |
|---------------------------|-------------------------------|---------------------------------|
| WT                        | 100                           |                                 |
| WT + oligomycin           | 49.2 ± 9.7                    |                                 |
| pZJMβ-RNAi                | 55.6 ± 9.4                    |                                 |
| pZJMβ-RNAi + oligomycin   | 39.8 ± 9.7                    |                                 |

* Mitochondrion samples (100 µg) were assayed for ATP synthesis activity with or without treatment with oligomycin (5 µg/mg of protein). WT, wild type.
FIG. 4. RNAi knockdown of the ATP synthase α and β subunit affects the mitochondrial membrane potential. For each cell line, $5 \times 10^6$ cells were stained with 0.1 μM MitoTracker Red CMXRos. Cells treated with CCCP were either treated with 50 mM CCCP for 15 min before or after staining with MitoTracker Red CMXRos, fixed with 4% paraformaldehyde, and analyzed by using confocal microscopy. (A) Wild-type bloodstream form cells; (B) wild-type cells treated with CCCP; (C) β RNAi-induced cells (the arrowhead shows cells with low fluorescent intensity); (D) β RNAi-induced cells treated with CCCP; (E) α RNAi-induced cells stained with MitoTracker; (F) α RNAi-induced cells treated with CCCP. (G) Quantitative analysis of β RNAi-induced cells scored on the basis of relative fluorescence intensity.
tial and thus show a decrease in the fluorescence intensity as seen in both CCCP-treated wild-type and β RNAi cells (Fig. 5A to D, green trace). In all cell lines, after treatment with CCCP the cells retained a low level of nonspecific fluorescence higher than the unstained background levels (Fig. 5A to D, blue shaded peak), parallel to the results obtained by fluorescence microscopy (Fig. 4B, D, and F). These results confirmed the results seen with fluorescence microscopy (Fig. 4E), which showed that 60% of the RNAi induced cell population has lower fluorescence intensity due to a decrease in the mitochondrial membrane potential. Flow cytometry of α-subunit RNAi induced cell lines also showed a consistent decrease in the relative fluorescence intensity. The pLewloop-SA clone 8 showed a shifted peak of decreased fluorescence intensity (Fig. 5C). Flow cytometry of clone 11, which showed the most severe growth phenotype, showed a single peak of decreased fluorescence intensity (Fig. 5D). In both cases, treatment with CCCP resulted in the further decrease in fluorescence intensity, reflecting the decrease in mitochondrial membrane potential.

DISCUSSION

The mitochondrial ATP synthase is a reversible enzyme with both hydrolytic and synthetic activities. In most organisms and in procyclic trypanosomes, the proton motive force generated by the electron transport chain is coupled to the synthesis of ATP via the mitochondrial ATP synthase (oxidative phosphorylation). Changes in the T. brucei mitochondrial structure and function throughout the stages of its life cycle have been well documented (9, 24, 29, 41, 42). The mitochondrion of the procyclic form trypanosome is fully developed with many tubular cristae. A fully functional respiratory chain is present, the ATP synthase is abundant, and ATP is generated by oxidative phosphorylation. The mitochondrion of the early bloodstream form, however, is tubular and contains few cristae, and the electron transport chain and several Krebs cycle enzymes are missing. The levels of ATP synthase are also substantially reduced at this stage, and ATP synthesis is achieved solely via glycolysis.

Differentiation into the late bloodstream form is accompanied by the upregulation of these mitochondrial components, pre-adapting the trypanosome to transition to the tsetse fly. Interestingly, in early bloodstream form trypanosomes where the cytochromes are completely absent, the ATP synthase is still present, although at somewhat decreased levels. In fact, the ATP synthase can be detected in all stages of the life cycle of T. brucei (3, 42), indicating that it may have a unique role in this organism’s ability to function in the bloodstream of its host. Despite the changes that have been observed in the mitochondrion of the bloodstream forms, the membrane potential is maintained at levels comparable to those found in the procyclic form (20). In the bloodstream form, electron flow is accomplished via the glycerol-3-phosphate shuttle to the trypanosome alternative oxidase but is not coupled to ATP production and does not generate a membrane potential (2, 8, 20). Therefore, it has been hypothesized that the ATP synthase in the bloodstream form acts in reverse as an ATP hydrolase to generate the proton motive force at the expense of ATP derived through substrate level phosphorylation (19, 20).

To test this hypothesis, we used RNAi to knock down the ATP synthase by targeting the expression of the α and β subunits which comprise the catalytic site of the ATP synthase. Nonclonal cell lines expressing β RNAi were recovered after phleomycin selection and subsequently characterized for the reduction of the mRNA and protein expression for the β subunit by using RT-PCR and Western blot analyses, respectively. Knockdown of the catalytic β subunit also significantly reduced the levels of the F1,ATPase α-subunit transcript and protein levels (Fig. 2 and 3). Similarly, when the ATPase α...
subunit is targeted by RNAi, the steady-state transcript and protein levels of the β subunit are also decreased. RNAi knockdown of the α or β subunit may directly knock down the expression of the β or α subunit, respectively, due to the high degree of homology shared between these two genes. The nucleic acid sequences of α and β subunits are 50% identical overall; the region used to construct the RNAi vectors shows 45% identity to the same region of the other subunit. It is also possible that the decrease in the β or α subunits may be due to differences in stability of the subunit protein due to the loss of the partner subunit to form the α3β3 catalytic core of the enzyme.

RNAi knockdown of the α or β subunit did not affect the steady-state transcript levels of the F₀ complex subunit 9. Surprisingly, the steady-state protein levels of this subunit are increased in induced cells, suggesting a possible effect on the posttranscriptional regulation of this subunit. The results shown here support our previous results in which the difference in stability of subunit 9 transcript was not proportional to the steady-state protein level, suggesting further posttranscriptional regulation of this protein (3). However, not all ATP synthase subunits were affected since the protein levels of the T. brucei F₀–32-kDa subunit did not change when either the α or β subunit were knocked down. Taken together, these results suggest that a common transacting regulatory factor of some of the ATP synthase genes may help to enhance stability of some but not all ATP synthase subunits at the transcriptional level, thus contributing to the coordination of gene expression. When the levels of the α and β transcripts are decreased, as is the case in both the α and β RNAi cells, this common regulatory factor would presumably be available in higher amounts to further enhance the stability of the subunit 9 transcript and or protein, leading to increased abundance of this protein. We are continuing to examine the mechanism of gene expression that controls the expression of the ATP synthase complex. Thus far, we have identified a common 13-nucleotide AG-rich element found in the 3′ untranslated region of these three genes which may help direct regulation of gene expression of this complex (3).

RNAi knockdown of the α or β subunit resulted in a 50% decrease in catalytically active ATP synthase complex formed (Tables 1 and 2). The ATP synthase complex that assembled after α or β RNAi induction functioned similarly to the wild type, showing ATP hydrolytic and synthetic oligomycin-sensitive activities similar to those previously reported (43). The levels of catalytically active complexes formed paralleled the amount of either subunit present after RNAi knockdown. In yeast and mammalian cells, assembly of the ATP synthase complex has been shown to be dependent on the concentration of either α or β subunit; therefore, a decrease in the α or β subunit leads to a decrease in overall complex formation. In contrast, the loss of other subunits (γ, δ, and ε) results in the formation of partial complexes. The F₁ and F₀ complexes appear to form independently of each other. However, if the F₀ complex forms in the absence of F₁, it does not appear to be protease permeable (18). This may account for our observation that in the α and β RNAi-induced cell lines, the F₀ proteins are either unaffected (32-kDa protein) or are increased, as was seen for subunit 9.

The effect of ATP synthase depletion on the mitochondrial membrane potential was determined by using MitoTracker Red CMXRos. The MitoTracker probes are similar to rhodamine-123 in their selective sequestration in active mitochondria. Rhodamine-123 has been previously used to stain T. brucei mitochondria; however, rhodamine-123-stained cells cannot be fixed, making it more difficult to obtain microscopic images (1, 12). The MitoTracker probes have the advantage of being retained in the mitochondria after fixation and permeabilization of the cells (32). Moreover, the MitoTracker Red CMXRos probe has also been shown to be selectively retained based on mitochondrial membrane potential (23). The membrane potential of the RNAi-induced cells was substantially diminished when the α- or β-subunit expression was decreased, similar to what was seen in cells treated with the uncoupler CCCP. Knockdowns of other mitochondrial complex subunits in the procyclic stage have shown a similar decrease in the mitochondrial membrane potential (16). These effects resulted in a decrease in the growth rate compared to the uninduced and wild-type controls. In T. brucei, as in other organisms, a proton-motive force has been shown to be required for the import of nuclearly encoded mitochondrial proteins and tRNA (25). In these α and β RNAi cells where reduced levels of the ATP synthase resulted in a decrease in membrane potential, the cells were likely decreased in the ability maintain functional requirements for differentiation, leading to a decrease in growth rate. Cellular differentiation requires upregulation of mitochondrial function and hence increased import of the nuclear encoded components into the mitochondrion. Thus, the failure of these cells to be transformed to the procyclic stage was likely due to the inability of these cells to keep up with the cellular requirements for differentiation due to reduced functional mitochondria. These results support the hypothesis that the ATP synthase is responsible in part or in whole for the maintenance of membrane potential in bloodstream form trypanosomes.

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