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Developmental Regulation of Mitochondrial Biogenesis in *Trypanosoma brucei*

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The metabolism of *Trypanosoma brucei* undergoes a significant change as the parasite differentiates from the mammalian bloodstream form to the form found in the tse-tse fly vector. Because the mitochondria of bloodstream form cells lack cytochromes and several key citric acid cycle enzymes, the metabolism of these cells is mostly limited to glycolysis. The reducing equivalents generated by this process are passed to oxygen by a plantlike alternative oxidase. As cells differentiate to the insect form, they begin to oxidatively metabolize proline. The mitochondria of insect form cells contain functional, cytochrome-mediated electron transport chains and have complete complements of citric acid cycle enzymes. Although the characterization is far from complete, the nuclear and mitochondrial genes involved in the expression of these mitochondrial functions appear to be developmentally regulated at posttranscriptional and posttranslational levels. This review outlines some of the molecular processes that are associated with the developmental regulation of mitochondrial biogenesis and suggests some possible mechanisms of regulation.

KEY WORDS: Kinetoplastid; organelle; cytochrome; assembly.

INTRODUCTION

The basic functions of the mitochondrion are to compartmentalize various metabolic pathways such as the citric acid cycle and to provide a proton-impermeable membrane environment for the electron transport and ATPase complexes that are necessary for energy production by oxidative phosphorylation. Mitochondria are generally believed to have descended from an endosymbiotic α-purple eubacterial ancestor, and the genome of the mitochondrion is believed to represent a remnant of the ancestral bacterial chromosome (Margulis, 1981; Gray and Doolittle, 1982; John, 1987; Cedergren *et al.*, 1988). The contemporary mitochondrial genome continues to encode several subunits of the mitochondrial electron transport and ATPase complexes as well as the mitochondrial ribosomal RNAs and a limited set of mitochondrial tRNAs, but it can no longer replicate or be expressed in the absence of the nuclear genome (Gray, 1989). A host of nuclear-encoded proteins are required for mitochondrial replication and for the transcription, translation, and maturation of the mitochondrial gene products (Tzagoloff and Myers, 1986). Even the respiratory complexes contain subunit proteins that are encoded in the nucleus. In contrast, the nuclear genome has acquired a gene repertoire that is capable of maintaining both the organelle structure and the nonrespiratory metabolic functions of the mitochondrion in the absence of the mitochondrial genome (ρ° yeast mutants and dyskinetoplastic trypanosomes) (Tzagoloff and Myers, 1986). An estimated 90% by mass of the proteins of the mitochondrion are nuclear encoded (Kagawa and Ohta, 1990).

The spatial separation of these genomes has two consequences. First, proteins that are encoded

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in the nuclear DNA and translated on cytoplasmic ribosomes must be imported across one or both mitochondrial membranes into the appropriate compartments (Hartl *et al.*, 1989; Glick and Schatz, 1991). Second, the mitochondrial and nuclear genes that encode subunits of the electron transport and ATPase complexes must be coordinately regulated to ensure that the required stoichiometric amounts of proteins are available for assembly into functional complexes. This regulation is further complicated in organisms that alter their mitochondrial functions in response to environmental conditions. One of the most dramatic and interesting instances of such mitochondrial regulation can be found in the protozoan parasite, *Trypanosoma brucei.*

GENERAL ASPECTS OF TRYPANOSOME DEVELOPMENT AND MITOCHONDRIAL BIOGENESIS

T. brucei is an extracellular parasite that cycles between the bloodstream of a mammalian host and the digestive tract of an insect vector, the tse-tse fly (*Glossina* spp.). In sub-Saharan and equatorial Africa two human-infective subspecies. *T. b. rhodesiense* and *T. b. gambiense*, are responsible for approximately 50,000 cases of sleeping sickness resulting in 20,000 fatalities annually (ASM, 1990; TDR, 1990). In addition, a third subspecies which is not infective to humans, *T. b. brucei*, is of considerable economic importance since it causes a fatal disease called nagana in the domestic livestock of the area. These three subspecies are morphologically indistinguishable and follow the same developmental cycle (Vickerman, 1965).

The activities of the trypanosome mitochondrion are modulated during the developmental cycle to take advantage of the changing environmental conditions. During early stages of a mammalian bloodstream infection, trypanosomes exist as a rapidly dividing long slender (LS)[3] form. LS form cells satisfy their energy requirements exclusively by glycolysis and release pyruvate as a waste product (Ryley, 1962; Flynn and Bowman, 1973). Most of the enzymes of the glycolytic pathway and an NAD-dependent glycerol-3-phosphate dehydrogenase enzyme are localized in an unusual, non-DNA containing organelle called the glycosome (Fairlamb and Opperdoes, 1986; Hannaert and Michels, this volume). LS mitochondria do not contain spectrally detectable cytochromes, have little or no NADH dehydrogenase, and lack several key enzymes of the citric acid cycle (pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, citrate synthetase, and succinate dehydrogenase) (Ryley, 1956; Vickerman, 1965; Bowman *et al.*, 1972; Brown *et al.*, 1973; Flynn and Bowman, 1973; Bienen *et al.*, 1991). LS mitochondria respire at a very high rate because of a unique terminal oxidase system (Fairlamb and Opperdoes, 1986). Reducing equivalents generated during glycolysis in the glycosome are moved into the mitochondria by a glycerol-3-phosphate/dihydroxyacetone phosphate shuttle system and are then used by the trypanosome alternative oxidase to reduce O_2 to water (Grant and Sargent, 1960; Opperdoes *et al.*, 1977; Fairlamb and Bowman, 1977). The glycerol-3-phosphate oxidase and terminal oxidase enzymes of the inner mitochondrial membrane are linked together by a lipid-soluble pool of Coenzyme Q_9 (Clarkson *et al.*, 1989). The terminal oxidase is not sensitive to cyanide but is inhibited by aromatic hydroxamic acids (Evans and Brown, 1973; Flynn and Bowman, 1973; Clarkson *et al.*, 1981).

Later in the course of infection, a nondividing short stumpy (SS) trypanosome form predominates. Like the LS form, SS form cells metabolize glucose to pyruvate and utilize the glycerophosphate oxidase system to regenerate NAD^+. Unlike the LS form, SS form cells are also capable of metabolizing α-ketoglutarate to succinate (Vickerman, 1965; Bowman *et al.*, 1972; Flynn and Bowman, 1973). The mitochondrion of this form contains a more complete citric acid cycle in that pyruvate dehydrogenase and α-ketoglutarate dehydrogenase are present (Bowman *et al.*, 1972; Flynn and Bowman, 1973). Cytochromes are not present in SS form cells, but inhibitor studies suggest the presence of functional NADH dehydrogenase complex (Bowman *et al.*, 1972; Flynn and Bowman, 1973; Bienen *et al.*, 1991). SS form cells seem to be an intermediate between the LS bloodstream form and the insect form, but they are clearly not an obligate intermediate in the developmental cycle (Bass and Wang, 1992).

The transmission of the bloodstream form cells to a tse-tse fly along with a bloodmeal induces the development of a cristate mitochondrion with

[3] Abbreviations: LS, long slender; SS, short stumpy; kDNA, kinetoplast DNA; kb, kilobase; nt, nucleotide; gRNA, guide RNA; bp, basepairs; ND, NADH dehydrogenase; Cyb, cytochrome b; CO, cytochrome oxidase; MURF, mitochondrial unidentified reading frame; A6, ATPase subunit 6; S12, small ribosomal subunit protein 12; VSG, variant surface glycoprotein.

functional cytochrome-mediated electron transport and a complete citric acid cycle and a shift to the use of proline as the preferred energy source (Evans and Brown, 1972; Brown *et al.*, 1973). Proline, the most abundant amino acid in the fly midgut, is apparently used by the insect as an energy source for flight (Vickerman, 1985). As indicated by the increased cyanide sensitivity of this form, insect (procyclic) form cells utilize a conventional cytochrome *c* oxidase (perhaps in addition to the trypanosome alternative oxidase) as the terminal enzyme of electron transport (Hill, 1976; Bienen *et al.*, 1983). Procyclic cells divide rapidly in the fly midgut before migrating to the salivary glands of the fly. The salivary gland stages of the trypanosome are not well studied at the biochemical level, but is it believed that mitochondrial activity is repressed before the organisms become infective for a new mammalian host. This is based on the observation that the mitochondria of the infective metacyclic form have the unbranched, noncristate appearance that is characteristic of the bloodstream form cells (Vickerman, 1985).

The mitochondrial development associated with the transformation of bloodstream form cells to the procyclic form can be mimicked *in vitro*. The triggers for this transformation appear to be a shift in the growth temperature from 37°C to 26°C and the availability of certain citric acid cycle intermediates and proline (Bienen *et al.*, 1981; Czichos *et al.*, 1986; Overath *et al.*, 1986; Bass and Wang, 1991, 1992). Bloodstream form cells, when incubated in a semi-defined medium at 26°C, differentiate to the procyclic form in 48–72 h (Cunningham, 1977; Michelotti and Hajduk, 1987; Torri and Hajduk, 1988; Bass and Wang, 1991; Priest and Hajduk, submitted). The ability to reproducibly induce this transformation *in vitro* makes the trypanosome an inviting system for the study of the nuclear and mitochondrial interactions that are involved in mitochondrial biogenesis.

DEVELOPMENTALLY REGULATED EXPRESSION OF MITOCHONDRIAL GENES

The developmental changes in mitochondrial structure and activity in *T. brucei* suggest that both mitochondrial and nuclear gene expression might be coordinately regulated during the life cycle of the parasite. A number of studies have revealed that mitochondrial gene expression is developmentally regulated both at the level of RNA stability and at the level of posttranscriptional mRNA modification. Somewhat surprisingly, regulation at a transcriptional level does not appear to play a pivotal role in mitochondrial gene expression in trypanosomes.

Mitochondrial Genome, Transcription, and RNA Processing

Like all eukaryotic cells with functional mitochondria, *T. brucei* contains a mitochondrial genome that encodes a few essential proteins and RNAs. However, both the structure of the mitochondrial genome and the mechanisms controlling mitochondrial genome expression are extremely unusual in *T. brucei.* The mitochondrial DNA of these organisms, termed the kinetoplast, contains two classes of circular DNA molecules that are structurally linked by catenation to form a huge network of thousands of DNA molecules (Simpson, 1987). The function of the unique catenated network structure of the kinetoplast is completely unknown (Borst, 1991; Simpson, 1987). The major component of the kinetoplast DNA (kDNA) network of *T. brucei* is the 1-kilobase (kb) minicircle. Minicircles are heterogeneous in sequence and are present at about 5–10,000 copies per trypanosome mitochondrion. Despite their abundance, it is unlikely that minicircles encode proteins. However, minicircle transcripts do appear to have an essential function in trypanosome mitochondrial biogenesis. The minicircle of *T. brucei* contains three gene sequences that code for small RNAs of 55–70 nt, and each of these genes is flanked by inverted 18-bp repeats (Sturm and Simpson, 1990; Pollard *et al.*, 1990) (Fig. 1B). The gene transcripts, which are termed guide RNAs (gRNAs), appear to play a critical role in the maturation of the mitochondrial mRNAs by a process called RNA editing (Stuart, 1991a,b; Hajduk *et al.*, 1993; Sloof *et al.*, this volume). The gRNAs potentially serve both a catalytic and a templating role in the editing of the precursor mRNA (see below).

Maxicircles, the other component of the trypanosome mitochondrial genome, are present at about 50 copies per kDNA network and encode both mitochondrial mRNAs and rRNAs (Fig. 1A). The genetic function of this DNA is analogous to other mitochondrial DNAs. The 22-kb maxicircle of *T. brucei* encodes at least 13 proteins and the 9S and 12S mitochondrial rRNAs (Fig. 1A).

Conspicuous in their absence are genes encoding the mitochondrial tRNAs. The genes for the mito-

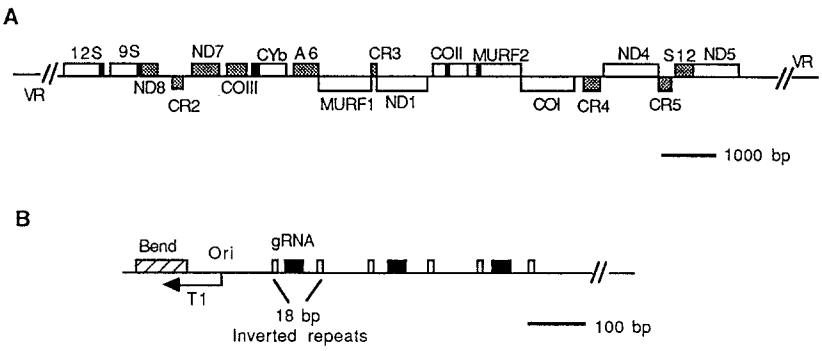

Fig. 1. Transcription maps of the maxicircle and minicircle genomes of *T. brucei*. (A) Linear map of the 22-kb maxicircle of *T. brucei*. The genes on the top of the line are transcribed from left to right, whereas the genes beneath the line are transcribed from right to left. The 9S and 12S rRNA genes have added uridines at their 3′ termini (black box). Transcripts from Cyb, COII, and MURF2 genes have limited internal editing (black box). Shaded boxes indicate genes that are extensively edited. The variable region of the maxicircle is indicated by VR. (B) Linear map of the 1-kb minicircle of *T. brucei*. The bent helical region of the minicircle (hatched box) and the origin of replication (Ori) are within the conserved region of the minicircle. The Transcript T1 is probably a primer for DNA replication. The gRNA genes (black boxes) are flanked by 18-bp inverted repeat sequences. This figure is taken from Hajduk *et al.*, 1993.

chondrial tRNAs of *T. brucei* are located in the nucleus and therefore must be selectively imported into the mitochondrion (Hancock and Hajduk, 1990; Mottram *et al.*, 1991). Although the mechanism of tRNA import is unknown, a class of precursor tRNAs with 5′ leader sequences of 120–150 nts have been identified in *T. brucei* mitochondria (Hancock *et al.*, 1992). These pre-tRNAs might serve as the substrates for export from the nucleus, for targeting to the mitochondrion, and for translocation across the mitochondrial membranes.

Little is known about the transcription of either the minicircle or maxicircle of *T. brucei*. As stated earlier, each minicircle of *T. brucei* contains three gRNA genes flanked by imperfect 18-bp inverted repeat sequences (Jasmer and Stuart, 1986; Pollard *et al.*, 1990). In order to determine the start site for gRNA transcription, *in vitro* studies were undertaken using trypanosome mitochondrial RNA and the Vaccinia capping enzyme guanyly transferase (Pollard *et al.*, 1990; Pollard and Hajduk, 1991). These experiments revealed the presence of a nucleotide triphosphate at the 5′ terminus of the mature gRNAs and indicated that gRNAs are primary transcripts and that transcription is initiated 32–33 bp from the upstream repeat of the gene. Direct enzymatic sequencing of the gRNAs revealed that transcription terminates at a run of Ts in the minicircle and that the mature gRNAs are modified by the addition of a short, 5–15 residue poly(U) tail (Pollard and Hajduk, 1991).

Neither a promoter nor the start site for maxicircle transcription have been identified. Although pulse labelling experiments with intact cells and isolated mitochondria have mapped the initiation of mitochondrial rRNA transcription to a site at least 1.5 kb upstream of the mature 5′ end of the 12S rRNA, the start site has not been identified (Michelotti *et al.*, 1993). Interestingly, it appears that the rRNA promoter may map to a fragment containing a potential origin for maxicircle replication (Myler *et al.*, 1993). Results from several studies indicate that maxicircle transcription may produce polycistronic transcripts which are processed by endonuclease cleavage to form the mature rRNAs and mRNAs (Michelotti *et al.*, 1993; Adler *et al.*, 1991; Read *et al.*, 1992). This is analogous to the transcription and processing of the mammalian mitochondrial transcripts (Clayton, 1991).

The expression of several of the maxicircle genes requires the editing of the mRNAs. Briefly, RNA editing in trypanosome mitochondria is a post-transcriptional process in which uridines are either added to or deleted from internal sites within precursor mRNAs (Hajduk *et al.*, 1992; Adler and Hajduk, in press; Stuart, 1991a,b). Overall it appears that editing progresses along the mRNA in a 3′ to 5′ direction. The extent of RNA editing varies from

Table I. The Extent and Regulation of *T. brucei* mRNA Editing[a,b]

Gene	Number of uridines		Length (nt)	Development stage edited[c]	Reference[d]
	Added	Deleted			
Cyb	34	0	1151	Pro	1
COII	4	0	663	Pro	2
ND7[e]	553	88	1238	5′ All 3′ BS	3
MURF2	26	4	1111	All	4
COIII	547	41	969	All	5
A6	447	28	811	All	6
S12	132	28	325	All	7
ND8	259	46	574	All	8

[a] Table modified from Pollard *et al.*, 1993.
[b] Uncharacterized "C-rich" genes encoding edited mRNAs, CR2, CR3, CR4, and CR5 are not included. Non-edited mRNAs COI, ND1, ND4, ND5, and MURF1 are not included.
[c] Abbreviations: Pro, procyclic; BS, bloodstream form; All, both procyclic and bloodstream forms.
[d] References: 1, Feagin *et al.*, 1987; 2, Benne *et al.*, 1986; 3, Koslowsky *et al.*, 1990; 4, Feagin and Stuart, 1988; 5, Feagin *et al.*, 1988a; 6, Bhat *et al.*, 1990; 7, Read *et al.*, 1992; 8, Souza *et al.*, 1992.
[e] The 3′ and 5′ editing domains in the ND7 mRNA are both edited in bloodstream forms but only the 5′ domain in the procyclic forms.

transcript to transcript. Several *T. brucei* mitochondrial mRNAs are edited at hundreds of sites, other mRNAs are edited only to a very limited extent, while still others are not edited at all (Table I). Regardless of the extent of editing, the process is essential for the formation of functional mitochondrial mRNAs. Interestingly, the editing of mitochondrial mRNAs is developmentally regulated and thus may play an important role in the regulation of mitochondrial biogenesis in *T. brucei.*

Regulated Expression of Mitochondrial rRNAs

Maxicircle gene expression is developmentally controlled at several levels. The steady-state abundance of many of the maxicircle-encoded mRNAs and the 9S and 12S rRNAs changes during the developmental cycle of *T. brucei.* Several mechanisms, including transcription initiation, attenuation of polycistronic transcription, and RNA stability, might be involved in the modulation of transcript abundance.

The steady-state amount of mitochondrial rRNA increases approximately 30-fold as LS bloodstream forms differentiate to the procyclic developmental stage (Michelotti and Hajduk, 1987). The amount of rRNA present in the different life-cycle stages of the parasite does not reflect changes in the rate of transcription initiation. Both *in vivo* and *in vitro* pulse labelling experiments indicated that the rate of transcription initiation was identical in LS and procyclic trypanosomes (Michelotti *et al.*, 1993). Thus, abundance of rRNA in the life-cycle stages of *T. brucei* is controlled at the level of RNA stability.

The mechanism(s) involved in developmentally regulating the stability of the mitochondrial rRNA is(are) unknown. However, the discovery of an RNA modifying activity that selectively adds a number of uridines to the 3′ terminus of the 9S and 12S rRNAs presents an interesting possible mechanism (Adler *et al.*, 1991). The posttranscriptional addition of uridines to the 3′ end of these rRNAs alters their predicted secondary structure and, in the case of the small 9S rRNA, results in the formation of a phylogenetically conserved stem-loop structure (Adler and Hajduk, unpublished data). The structure may mediate the binding of proteins and the stabilization of the rRNAs in the mitochondrial ribosome. Alternatively, the added uridines might form an exoribonuclease resistant structure and thus protect the rRNA from degradation.

Developmental Regulation of Mitochondrial mRNA

The steady-state amount of several of the maxicircle encoded mRNAs vary with the life cycle of *T. brucei* while the amount of other mRNAs remain unchanged (Table II). Thus, the modulation in abundance of mitochondrial rRNA and mRNAs operates in a transcript-specific manner.

As might be expected, the mRNA levels for some cytochrome subunits (COI, COII, and Cyb) are very low or undetectable in the LS trypanosomes lacking active mitochondria (Table II). Other

Table II. Developmental Regulation of Mitochondrial-Encoded Mitochondrial mRNA and rRNAs of *T. brucei*

Mitochondrial gene	Relative level of mature RNA[a]			Reference[b]
	Long slender	Short stumpy	Procyclic	
Ribosomes				
12S rRNA	0.04	1.3	1.0	1
9S rRNA	0.07	1.4	1.0	1
Cytochrome reductase				
Cytochrome *b*	ND[c]	0.5	1.0	1
Cytochrome oxidase				
Subunit I	0.07	0.4	1.0	1
Subunit II	ND	0.5	1.0	1
Subunit III[e]	1.0	—[d]	1.0	2
NADH dehydrogenase				
Subunit 5	0.5	0.8	1.0	1
Subunit 7[e]	≈ 10	—	1.0	3
Subunit 8[e]	≈ 20	—	1.0	4
ATPase				
Subunit 6[e]	1.0	—	1.0	5

[a] The amount of edited RNA present is expressed relative to the levels in established procyclic cells. Procyclic levels are arbitrarily taken as 1.0 in all cases.
[b] References: 1, Michelotti and Hajduk, 1987; 2, Feagin *et al.*, 1988a; 3, Koslowsky *et al.*, 1990; 4, Souza *et al.*, 1992; 5, Bhat *et al.*, 1990.
[c] No transcripts were detected.
[d] The amount of transcript was not determined.
[e] Estimated from Northern blots in the indicated references.

mitochondrial mRNAs are either constitutively expressed throughout development (ATPase 6, ND4, and ND5) or elevated in the bloodstream developmental stages (ND7 and ND8). It is unknown whether differential RNA stability or transcription initiation plays a role in the regulation of mitochondrial mRNA abundance since studies comparing the transcription initiation rates for the maxicircle mRNA genes in the bloodstream and procyclic forms are lacking.

Studies on the transcription of the rRNAs provides some evidence suggesting that the mitochondrial mRNA levels may be developmentally regulated at steps following transcription initiation. The strongest support for this interpretation is the presence of polycistronic transcripts containing rRNA and mRNA sequences within a common precursor (Adler and Hajduk, unpublished data). Since rRNA levels are not influenced by transcription initiation, the downstream ND7 and ND8 genes (Fig. 1 and Table II) are likely to be part of a common, constitutively transcribed precursor, and posttranscriptional events are likely to influence transcript abundance. Regardless of whether the steady-state amount of mitochondrial mRNAs is influenced by transcription initiation rates, it is clear that the expression of these RNAs is at least partially controlled at a posttranscriptional level by RNA editing.

Posttranscriptional Regulation

Shortly after the discovery of mRNA editing in trypanosomes, it was recognized that editing could play an important role in developmentally regulating mitochondrial gene expression. Studies by Stuart and co-workers (Feagin *et al.*, 1987; Feagin and Stuart, 1988) showed that the amount of edited mRNA from several maxicircle genes varied during the life cycle of *T. brucei* (Table II). Three distinct developmental patterns of mRNA editing have been observed. (1) The mRNAs for COIII, ATPase 6, ribosomal protein S12, and MURF2 appear to be edited constitutively in both bloodstream and procyclic forms of *T. brucei* with little or no difference in the steady-state amounts of these edited mRNAs (Bhat *et al.*, 1990; Feagin *et al.*, 1988a). (2) The levels of edited COII and Cyb mRNAs increase in the procyclic form of the parasite (Feagin and Stuart, 1987). (3) Edited ND7 and ND8 mRNAs are more

abundant in the bloodstream forms of *T. brucei* (Koslowsky *et al.*, 1990).

The elevated levels of edited Cyb and COIII mRNAs in procyclic forms are consistent with the role of the translation products of these mRNAs in the assembly of functional cytochrome complexes. Editing of COII, by addition of 4 uridines, alters the reading frame of the mRNA and removes an in-frame translation termination codon. It is speculated that the unedited form of the COII mRNA would encode a nonfunctional protein that would be truncated at the carboxy terminus and that would be incapable of assembly with the other cytochrome *c* oxidase components to form an active respiratory complex (Benne *et al.*, 1986). The consequence of not editing the Cyb mRNA is even more dramatic. The unedited form of the Cyb mRNA is unlikely to be translated since it lacks the AUG initiation codon which appears to be formed by the editing (Feagin *et al.*, 1988b).

The increased amounts of edited mRNAs for the NADH dehydrogenase subunits in the bloodstream forms was initially difficult to reconcile with the decreased levels of mitochondrial electron transport and oxidative phosphorylation in these trypanosomes. However, recent studies suggest that the mitochondrial NADH dehydrogenase might play a role in energy production in SS form bloodstream trypanosomes (Turrens, 1991; Bienen *et al.*, 1991). The terminal acceptor of electrons in both the procyclic and bloodstream forms of *T. brucei* is oxygen. The bloodstream trypanosomes utilize a plantlike alternative terminal oxidase which oxidizes ubiquinone in the mitochondrion to regenerate NAD^+ from the NADH that is formed during glycolysis (Opperdoes, 1987; Clarkson *et al.*, 1989). The decreased amount of edited ND7 and ND8 mRNA in the procyclic trypanosomes is consistent with the suggestion (Turrens, 1991) that procyclic trypanosomes lack a requirement for the NADH dehydrogenase complex (Complex I). Succinate is the preferred donor of electrons to ubiquinone in procyclic cells, and thus the requirement of a functional Complex I is by-passed. The LS form contains high levels of edited ND7 and ND8, yet no NADH dehydrogenase activity has been detected (Vickerman, 1965; Bienen *et al.*, 1991). The accumulation of edited ND7 and ND8 mRNA in LS forms might either serve as a preadaptation for differentiation to the SS form or it may reflect an alternative function for the ND7 and ND8 gene products in the LS trypanosomes. If the latter is indeed the case, these products must be nonessential to LS survival since dyskinetoplastic strains completely lacking maxicircles can survive in the bloodstream of the mammalian host.

In addition to the regulated editing of mitochondrial mRNAs, it appears that the length of the poly(A) tails on several of the maxicircle transcripts is developmentally modulated (Bhat *et al.*, 1992). The correlation of poly(A) tail length and RNA stability has been shown in both mammals and yeast (Munroe and Jacobson, 1990). It is tempting to speculate that the increased length of the trypanosome mitochondrial mRNA poly(A) tail in procyclic form cells might increase mRNA stability and, potentially, expression.

Currently the mechanisms regulating stage- and transcript-specific mRNA editing in trypanosome mitochondria are unknown. The specificity of the regulation seems to argue against the involvement of a general editing factor. One possiblity is that editing is regulated by the abundance of specific gRNAs. However, recent studies have failed to reveal a correlation between the abundance of gRNAs and the specific edited mRNA (Koslowsky *et al.*, 1992).

DEVELOPMENTALLY REGULATED EXPRESSION OF NUCLEAR GENES ENCODING MITOCHONDRIAL PROTEINS

Much of the attention given to the nuclear-encoded genes of *T. brucei* has been focused on the developmentally expressed surface coat proteins of the bloodstream and procyclic forms, the variant surface glycoprotein (VSG) and procyclin, respectively. Developmental studies of the nuclear-encoded components of the mitochondrion have lagged far behind, in part, because of the difficulties involved in purifying the proteins. Most of the proteins of the mitochondrial electron transport chain are integrated into the mitochondrial membrane as protein complexes and are difficult to solubilize in an unaggregated form suitable for purification. It is no surprise that cytochrome *c*, the only soluble component of the electron transport chain, was the first protein to be purified and cloned and is the best characterized protein in terms of its developmental regulation. Only three subunits of the insoluble electron transport complexes have thus far been cloned: cytochrome c_1 and subunit 4 of the cytochrome *c* reductase complex and an unidentified subunit of the NADH dehydrogenase complex (Priest and Hajduk, 1992; Priest and Hajduk, submitted; Peterson *et al.*, 1993). All three of these

clones were derived from cDNA and two of them (cytochrome c_1 and subunit 4) do not contain the entire coding sequence of the protein. None of the nuclear-encoded subunits of either the bloodstream alternative oxidase or the procyclic cytochrome *c* oxidase have been identified, nor have any of the developmentally expressed citric acid cycle enzymes been cloned. Therefore, one must keep in mind that the current knowledge of the developmental regulation of nuclear-encoded mitochondrial proteins is based on studies of a very limited subset of genes.

Transcriptional Regulation

Restriction endonuclease mapping of the genes that encode cytochrome *c* and cytochrome c_1 suggest that they are present in only one copy per haploid genome (Bertrand and Hajduk, unpublished data; Priest and Hajduk, unpublished data). This is in contrast to the genes for such proteins as tubulin, actin, calmodulin, fructose bisphosphate aldolase, and hsp 70 which are present as tandem repeats of two or more genes (Thomashow *et al.*, 1983; Ben Amar *et al.*, 1988; Tschudi *et al.*, 1985; Marchand *et al.*, 1988; Glass *et al.*, 1986). The nuclear organization of the genes encoding the mitochondrial proteins has not yet been examined but is of particular interest given that many protein-coding genes are transcribed as polycistronic precursor RNAs in *T. brucei* (Johnson *et al.*, 1987; Ben Amar *et al.*, 1988; Muhich and Boothroyd, 1988; Huang and Van der Ploeg, 1991). Gene products that are required in stoichiometric amounts (such as the subunits of an electron transport complex) could be clustered at a particular locus under the control of a single promoter and thus could be coordinately regulated at

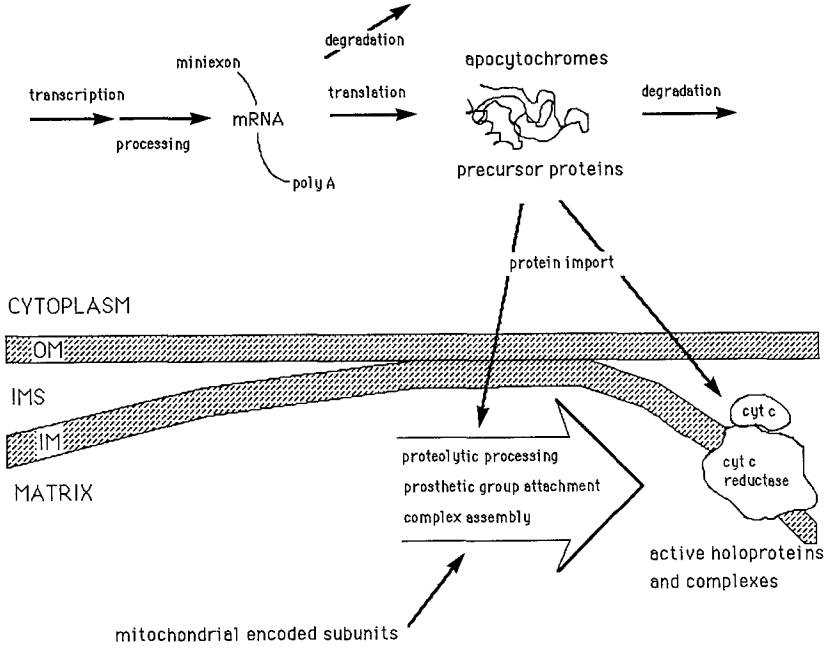

Fig. 2. Proposed expression pathway of the nuclear-encoded mitochondrial proteins of *T. brucei*. Nuclear genes encoding mitochondrial proteins are transcribed, and the transcripts are processed by poly(A) addition and mini-exon *trans* splicing to yield mature mRNAs. These mRNAs can either be degraded or translated. Precursor proteins and apocytochromes are either degraded in the cytoplasm or imported into the mitochondrion. Cytochrome *c* is probably transported only across the outer mitochondrial membrane (OM) into the intermembrane space (IMS). Other proteins may be transported across both the outer and inner mitochondrial (IM) membranes into the mitochondrial matrix where they may be subject to proteolytic processing to a mature form, prosthetic group attachment (for the cytochromes), and complex assembly. Re-export from the matrix to the intermembrane space is also possible (not shown). Several of the subunit proteins of the electron-transport complexes are encoded within the mitochondrion and are translated by mitochondrial ribosomes. These proteins are also subject to processing, prosthetic group attachment, and assembly into complexes.

Table III. Developmental Regulation of Nuclear-Encoded Mitochondrial Proteins of *T. brucei*

Mitochondrial protein	Relative mRNA level[a,b]			Relative protein level[a,c]		Reference[d]
	Long slender	Short stumpy	Procyclic	Short stumpy	Procyclic	
Cytochrome *c*	0.06	0.17	1.0	< 0.01	1.0	1
Cytochrome c_1	0.1[e]	0.17	1.0	< 0.1[f]	1.0	2
Cytochrome *c* reductase Subunit 4	0.08[e]	0.16	1.0	< 0.1	1.0	2
Cytochrome *c* reductase Subunit 2	—[g]	—	—	< 0.05	1.0	2
NADH dehydrogenase Subunit[h]	> 1.0	> 1.0	1.0	—	1.0	3

[a] Levels are expressed per cell equivalent relative to the level found in established procyclic cells (= 1.0).
[b] Determined by RNase T1 protection assays except as noted.
[c] Determined by Western blot analysis of protein except as noted.
[d] References: 1, Torri and Hajduk, 1988; 2, Priest and Hajduk, submitted; 3, Peterson *et al.*, 1993.
[e] Cells were from day 4 of infection and were a mixture of the long slender and intermediate form.
[f] Estimated by spectral analysis of holoproteins in detergent extract.
[g] Level was not determined.
[h] Estimated from a Northern blot in the paper referenced.

the level of transcription. There is currently no evidence of such organization. In fact, the presence of unrelated and differentially expressed mRNAs within the fructose bisphosphate aldolase polycistronic precursor would argue against such genomic organization as a general rule (Vijayasarathy *et al.*, 1990).

As shown in Fig. 2, the nuclear-encoded genes of mitochondrial proteins can be developmentally regulated at numerous points in the expression pathway. The first potential point of control, regulation at the level of transcription, does not appear to play as pivotal a role in *T. brucei* mitochondrial biogenesis as it does in yeast. In yeast the iso-1-cytochrome *c* and cytochrome c_1 genes are not transcribed in significant amounts when the cells are grown under conditions that repress mitochondrial function (Guarente and Mason, 1983; Schneider and Guarente, 1991). In contrast, LS form *T. brucei* cells do have significant steady-state levels of cytochrome *c*, cytochrome c_1, and reductase subunit 4 mRNAs even though the cytochromes and reductase subunit protein are not detectable (Table III). The presence of these transcripts in the steady-state RNA pools of LS cells suggests that the promoters of these genes are constitutively active and that the genes are regulated by posttranscriptional mechanisms.

Constitutive transcription of developmentally expressed nuclear genes is a fairly common feature of trypanosomes. The developmentally expressed genes that have been examined, the VSG and procyclin surface coat genes (Pays *et al.*, 1990), the glycosomal and cytoplasmic phosphoglycerate kinase genes (Gibson *et al.*, 1988), and the fructose bisphosphate aldolase genes (Vijayasarathy *et al.*, 1990), are constitutively transcribed (or at least initiated) at about the same levels throughout the life cycle and are post-transcriptionally regulated. The relative levels of transcriptional activity in LS and procyclic form cells have not yet been determined for any nuclear-encoded mitochondrial protein gene, but, if other developmentally regulated genes are representative, these genes are expected to be active at the same level throughout the life cycle.

Posttranscriptional Regulation

The second potential point of control is at the level of RNA processing. As mentioned earlier, most trypanosome genes are transcribed as polycistronic precursors. Mature 3′ ends are created by cleavage and poly(A) addition (Huang and Van der Ploeg, 1991; Ullu *et al.*, 1993). Mature 5′ ends are formed by a process of *trans* splicing in which a capped, 39-nucleotide mini-exon is spliced from discontinuously transcribed donor RNAs to the 5′ ends of all nuclear-encoded mRNAs (Walder *et al.*, 1986; Cornelissen *et al.*, 1986; Kooter *et al.*, 1984; Perry *et al.*, 1987). So far there is no evidence for developmental regulation at this level. All of the cytochrome c_1 and reductase subunit 4 mRNAs that are present in the steady-state RNA pools

of bloodstream and procyclic cells are polyadenylated (Priest and Hajduk, submitted). In addition, all of the cytochrome *c* mRNAs of bloodstream and procyclic cells are *trans* spliced at approximately the same 5′ position (Bertrand and Hajduk, unpublished data). Regulation at this level cannot be ruled out, but is unlikely given the rapidity with which other transcripts are *trans* spliced and polyadenylated (Ullu *et al.*, 1993; Huang and Van der Ploeg, 1991).

Convincing evidence of developmental regulation at the level of transcript degradation is available for only two nuclear-encoded genes, phosphoglycerate kinase and procyclin (Gibson *et al.*, 1988; Pays *et al.*, 1990). VSG regulation is believed to occur by premature transcript termination as a consequence of the 45-kb distance between the promoter and the gene (Pays *et al.*, 1990). The stability of the mRNA transcripts of the nuclear-encoded mitochondrial protein genes has not been addressed. What is obvious from the steady-state mRNA amounts of cytochrome *c*, cytochrome c_1, and reductase subunit 4 given in Table III is that either the rate of gene transcription increases or the rate of mRNA degradation decreases as cells differentiate from LS to SS to procyclic form. Given that the gene promoters are probably constitutively active at equivalent levels during the life cycle, the latter seems more likely. Cytochrome *c*, cytochrome c_1, and reductase subunit 4 mRNAs are probably degraded at higher rates in the bloodstream cells. The mechanism whereby these transcripts could be tagged for preferential degradation has not yet been elucidated in trypanosomes, but, based on the studies of the phosphoglycerate kinase and procyclin mRNAs, such a mechanism is clearly operational.

In contrast to the cytochrome *c*, cytochrome c_1, and reductase subunit 4 mRNAs just described, the nuclear-encoded NADH dehydrogenase subunit gene does not appear to be regulated at the RNA level. Steady-state levels of this transcript are essentially constant during trypanosome development (Table III). A similar pattern of expression was described earlier for the mitochondrially encoded subunits (ND5, ND7, ND8) of the NADH dehydrogenase complex (Table II). Transcripts of the mitochondrially encoded ND subunit genes are present in the steady-state RNA pools of bloodstream cell mitochondria long before transcripts of the cytochrome *b* gene are detectable. Recall that cytochrome *b* is the only mitochondrially encoded subunit of the cytochrome *c* reductase complex. The differences observed at the RNA level are reflected by differences at the activity level. NADH dehydrogenase activity is elaborated in the bloodstream as cells differentiate from the LS to the SS form (Vickerman, 1965; Bienen *et al.*, 1991) while the cytochrome *c* reductase proteins are not expressed until 6 h after cells have begun to differentiate to the procyclic form (Priest and Hajduk, submitted). The nature of this coordinate regulation between nuclear and mitochondrial genes is unknown.

Translational and Posttranslational Regulation

Table III shows that SS form cells do not contain detectable amounts of cytochrome *c*, cytochrome c_1, or reductase subunit 4 proteins even though the mRNAs encoding these proteins are present at 16–17% of the procyclic levels. The same is probably true for reductase subunit 2. In the case of cytochrome *c* this amounts to at lease 17-fold regulation at the translational or posttranslational level. Regulation at the level of translation has been convincingly ruled out. When poly(A)$^+$-selected bloodstream and procyclic mRNAs were translated *in vitro*, equivalent amounts of cytochrome *c* mRNA produced equivalent amounts of apocytochrome *c* protein (Torri *et al.*, 1993). *In vivo* labelling studies have also shown that cytochrome *c* is translated at nearly identical rates in bloodstream and procyclic cells (Torri *et al.*, 1993). The observed regulation must therefore occur posttranslationally. *In vivo* pulse/chase studies confirmed that the cytochrome *c* protein is degraded at a high rate in the bloodstream form cells. The calculated half-life of the protein in the bloodstream cells was 1 h or less, while no degradation was apparent in procyclic cells (Torri *et al.*, 1993). The regulation of developmental expression at the level of degradation of proteins (and mRNAs) would seem to be an energetically wasteful process, but energy considerations may not be a factor to a cell awash in the glucose of the mammalian bloodstream.

Possible Role of Heme in Posttranslational Regulation

Is cytochrome *c* degraded in the bloodstream form cells because it is not imported into the mitochondrion, or is it not imported because it is so rapidly degraded? The key to the answer may be found in the availability and function of heme. In yeast and *Neurospora crassa* the covalent attachment of heme to the apocytochrome *c* protein by cytochrome *c* heme lyase enzyme is required for import into the mitochondrion (Nicholson and Neupert,

1989; Nargang *et al.*, 1988). The absence of the heme lyase enzyme prevents import and results in the rapid degradation of the apocytochrome *c* protein in the cytoplasm (Nargang *et al.*, 1988). One would predict that the inability to synthesize or to scavenge heme would lead to similar results. This may be the case in bloodstream *T. brucei* cells. Bloodstream trypanosomes are incapable of synthesizing heme *de novo* and must acquire it from an exogenous source (Chang *et al.*, 1975). However, heme is difficult to obtain from the mammalian bloodstream because of the abundance of serum binding proteins (Meshnick *et al.*, 1977). Thus, apocytochrome *c* protein is probably degraded in the cytoplasm of bloodstream *T. brucei* cells because it cannot be imported into the mitochondrion in the absence of heme.

Heme may play a similar role in the mitochondrial import of cytochrome c_1. In yeast the covalent attachment of heme to the cytochrome c_1 protein is not required for import or sorting but is required for processing of the amino terminus (Nicholson *et al.*, 1989; Ohashi, *et al.*, 1982). Import is along a mitochondrial sorting pathway that requires an amino-terminal targeting sequence (van Loon *et al.*, 1987). The absence of a typical mitochondrial targeting presequence on the c_1 cytochromes of two closely related kinetoplastid species, *Crithidia fasciculata* and *Bodo caudatus*, suggests that the *T. brucei* cytochrome c_1 protein is imported along a different pathway than the one characterized in yeast (Priest *et al.*, 1993). We are currently attempting to determine if cytochrome c_1 enters the trypanosome mitochondrion along a cytochrome *c* type pathway that requires heme and a heme lyase enzyme.

Beyond the direct role of heme in the formation of holocytochromes, and the proposed role in cytochrome protein import, heme may also play an indirect role in the assembly of complexes in the mitochondrial membrane. The c_1 and *b* holocytochromes of yeast cytochrome *c* reductase are the first subunits to be assembled into the complex (Sidhu and Beattie, 1983). The presence of cytochrome *b* in the complex affects the processing and stability of several of the other subunit proteins (Sen and Beattie, 1986, 1985). If the same mechanisms are operative in *T. brucei*, it is possible that complex assembly is regulated by the availability of the holocytochromes. The other subunits would be translated at equivalent levels in bloodstream and procyclic cells, but they would be preferentially degraded in the bloodstream because they could not be assembled into the membrane.

The proposed global role for heme in the regulation of the cytochromes and cytochrome-containing complexes is probably overly simplistic, but there is a precedent for such a role in the yeast system (Forsburg and Guarente, 1989). Testing this hypothesis in the *T. brucei* system may prove more difficult than it has in yeast. Because bloodstream form cells lack catalase (a heme-containing protein), they contain significant levels of hydrogen peroxide and are susceptible to hydroxyl radical-induced damage in the presence of free heme (Meshnick *et al.*, 1977). Addition of free heme to bloodstream cells results in cell death. How differentiating cells are able to acquire heme and yet avoid such damage is only one of the questions that remain to be answered.

CONCLUSIONS AND FUTURE DIRECTIONS

The life cycle of *T. brucei* exposes the parasite to challenging immunological and physiological conditions. In order to survive in the bloodstream of the mammalian host, trypanosomes have evolved the process of antigenic variation which keeps them just a step ahead of the hosts' immunological responses. The physiological demands placed on the organism as a consequence of alternating between an insect vector and the mammalian bloodstream are also immense. Changes in environmental temperature, oxygen tension, pH, and carbohydrate source require the trypanosome to evoke complex regulatory schemes to facilitate altered pathways for energy production. While the basic biochemistry of the metabolic pathways in the bloodstream and procyclic trypanosomes have been resolved to some degree, there is no obvious explanation as to why bloodstream trypanosomes suppress mitochondrial electron transport and oxidative phosphorylation and why they rely exclusively on substrate-level phosphorylation for ATP production. The bloodstream forms have obviously developed the basic machinery necessary for survival utilizing only glycolysis for ATP production. Ample glucose is present in the bloodstream to satisfy the demands of this process for energy production. A more difficult question is why the African trypanosomes suppress mitochondrial activities. We have proposed that development of an extremely efficient glycolytic pathway within the bloodstream trypanosomes might have been necessitated because of the trypanosome's inability to synthesize heme *de novo* or to scavenge free heme or liberated hemoglobin from the blood.

Alternatively, does glycolysis offer a selective advantage to the bloodstream parasite?

Throughout this article we have attempted to suggest possible molecular mechanisms underlying the regulatory events described. While some progress has certainly been made in the molecular characterization of mitochondrial biogenesis in *T. brucei*, very little is known about the precise mechanisms involved. Many of these studies await a better understanding of how trypanosomes transcribe genes, process RNAs, and target proteins and RNAs throughout the cell. Trypanosomes seem to be masters of the unusual. It will be interesting to see the roles that such processes as protein and RNA trafficking and RNA editing have in the regulated expression of the trypanosome mitochondrion.

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