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Mechanism of RNA Synthesis in the Mitochondria of *Trypanosoma Brucei*

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Introduction

The parasite *Trypanosoma brucei* is the causative agent of African sleeping sickness and belongs to a class of organisms known as the kinetoplastids which contain a unique mitochondrial genome comprising a catenated network of minicircles and maxicircles. In contrast to other eukaryotes, the mechanisms involved in the transcription of this complex mitochondrial genome are poorly understood. The objective of this project is to better understand these mechanisms with the long term goal of developing therapeutic approaches.

The World Health Organization defines Human African trypanosomiasis, also known as African sleeping sickness, as a Neglected Tropical Disease. These diseases typically affect large areas of the population in low-income parts of the world. Unfortunately, the funding for the research and treatment of these diseases is low because of the low financial return. The WHO currently treats about 10,000 cases per year and estimates that there are 30,000 cases a year. However, the WHO maintains that the scales could be tipped at any time due to the health and financial instability of the countries in which this disease typically occurs. To date, only relatively ineffective and costly treatments are available (X). Nagana, animal trypanosomiasis, is also a large problem as cattle become infected and die if untreated. This hinders the socio-economic growth of these areas because there are less animals available to plow fields and which leads to low crop yields and little meat available. Importantly, however, the causative parasite *Trypanosoma brucei* has a unique structure for its mitochondrial DNA, specifically a catenated network of maxicircles and minicircles known as the kinetoplast. Since this arrangement of the mitochondrial genome is dramatically different than that of humans, it is possible that the mechanism of mitochondrial transcription in *T. brucei* involves unique features that could be targeted for drug therapy with little or no side effects to humans.

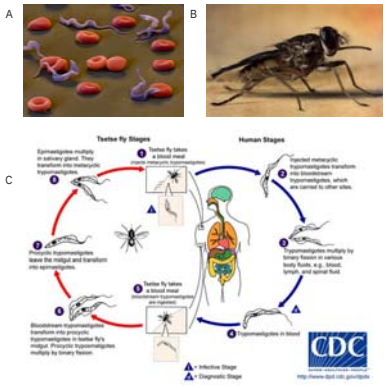

Figure 1. Agents in African Sleeping Sickness (A) *T. brucei*, the causative agent of sleeping sickness (trypanosomiasis) in humans and nagana in animals. (B) Insect vector: tsetse fly of the genus Glossina. (C) *T. brucei* life cycle.

Utilizing homology searches of the *T. brucei* database, we have identified and cloned the genes encoding the *T. brucei* mitochondrial RNA polymerase (MTPol), a putative required accessory factor (TFb2M), and a number of proposed cis-acting minicircle promoter DNA sequences. Our results highlight the progress in the production and purification of recombinant MTPol and TFb2M proteins, the development of a *T. brucei in vitro* mitochondrial transcription system, and the development of an *in vivo* system using the yeast *S. cerevisiae* as a surrogate host for transcription by MTPol and TFb2M. The long term goal will be to use the *S. cerevisiae* surrogate system in high-throughput screening of small drug molecules to potentially identify those that specifically inhibit transcription by the *T. brucei* mitochondrial RNA polymerase.

Experimental Design / Results

I. Identification and characterization of the *T. brucei* mitochondrial transcription factors MTPol and TFb2M

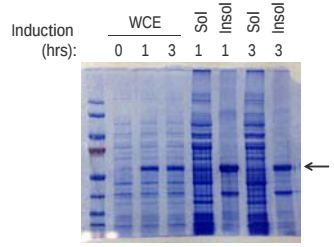

Figure2. Recombinant MTPol and TFb2M proteins expressed in *E. coli* are insoluble. Plasmids containing the MTPol and TFb2M coding regions in expression vector pET15b were transformed into Rosetta *E. coli* cells and protein expression induced with IPTG. Shown is an 8% SDS polyacrylamide gel for the analysis of TFb2Mp; similar results were obtained for the analysis of recombinant MTPol (data not shown).

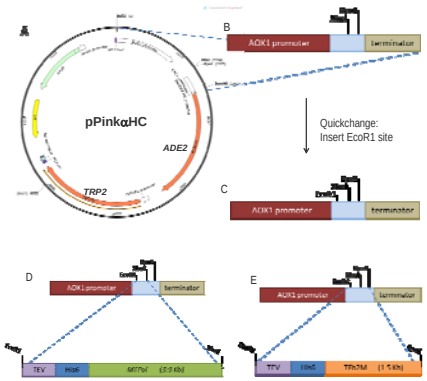

Figure 3. Modified approach for production of recombinant MTPol and TFb2M proteins using inducible expression in the yeast *Pichia pastoris*. (A) Plasmid map of starting plasmid pPinkαHC (Invitrogen) for inducible expression and secretion of recombinant proteins (fused at N-terminus to secreted α-factor). (B) Region containing the methanol-inducible *AOX1* promoter, cloning sites and transcription termination sequence. (C) Introduction of a unique EcoRI site near the end of the α-factor protein sequence using Quickchange site-directed mutagenesis. (D) Final constructs containing MTPol or TFb2M preceded by a TEV protease recognition site and a hexahistidine tag. The hexahistine tag allows for nickel-agarose purification of the secreted recombinant proteins from the growth medium. Treatment with TEV protease removes the N-terminal α-factor sequence from the recombinant proteins.

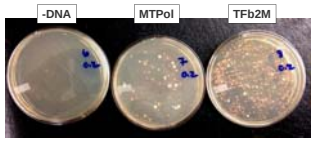

Figure 4. Integration of MTPol and TFb2M expression constructs into the *P. pastoris* genome. *P. pastoris* (relevant genotype *ade2, prb1, pep4*) was transformed by electroporation with the indicated plasmid linearized with *AflII* in the *TRP2* locus to direct chromosomal integration. Stable transformants were selected by complementation on minimal medium lacking adenine (PAD); pink colonies contain at least one copy of the integrated plasmid whereas white colonies (desired) contain multiple integrated copies.

II. Identification and characterization of cis-acting promoter sequences from *T. brucei* minicircles

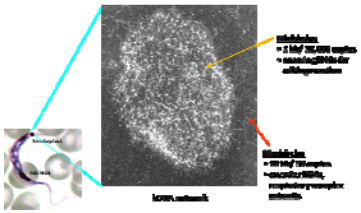

Figure 5. T. brucei kinetoplast DNA (kDNA).

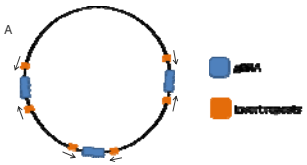

Figure 6. *T. brucei* minicircle structure. (A) *T. brucei* minicircles typically contain 3 to 5 gRNA genes that are flanked by inverted 18-bp imperfect repeats. (B) Consensus sequences of the G-type (guanine) and A-type (adenine) repeats. The hypothesis is that these 18-base pair repeats act to recruit or promote MTPol and/or TFb2M binding for transcription of the gRNAs.

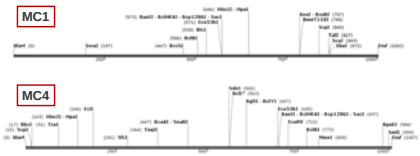

Figure 7. Cloning of *T. brucei* minicircles for *in vitro* assays. Kinetoplast DNA was isolated from *T. brucei* mitochondria and minicircles liberated from the network by digestion with HindIII. Approximately 1 kb linear DNAs were gel purified and cloned into plasmid pSP73. Sequencing of 16 clones demonstrated the presence of 7 distinct cloned minicircles. Shown above are representative structures for 2 minicicle inserts.

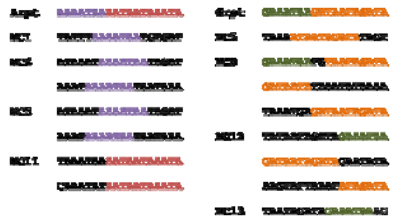

Figure 8. Representative 18 bp repeats from 7 novel minicircles. Above sequences are a representation of some of the identified 18 base pair repeats in the minicircle clones.

Future Directions

- Inductions, purifications of recombinant MTPol and TFb2M from *P. pastoris* integrative transformants.
- Electrophoretic mobility shift assays to identify potential TFb2M binding sites in the vicinity of minicircles 18 bp motifs.
- In vitro transcription assays utilizing minicircle templates and purified MTPol and TFb2M proteins.
- Development of an *in vivo* system using the yeast *S. cerevisiae* as a surrogate host for transcription by MTPol and TFb2M.
- Utilize the *S. cerevisiae* system for high-throughput screening of small drug molecules to potentially identify those that specifically inhibit the T. brucei mitochondrial RNA polymerase.

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