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SELECTIVE KNOCKDOWN OF THE *TRYPANOSOMA BRUCEI* FLA
GENES AND DEVELOPMENT OF A CHEMOTAXIS ASSAY

by

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for eight credits.

<u>DEDICATION</u>

I dedicate this paper to my sister, Angèlica,

for believing in me no matter what.

ACKNOWLEDGMENTS

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<u>ABSTRACT</u>

Sleeping sickness, or African trypanosomiasis, is a disease found in Sub-Saharan Africa caused by the *Trypanosoma brucei* parasite. Trypanosomes move around within their vector, tsetse flies, and mammals by use of their single flagellum. The flagellum is unique in that it is attached down the length of the cell, in what is known as the flagellar attachment zone (FAZ). I have chosen to study two closely related genes, FLA1 and FLA 2, because the FLA1 protein is thought to localize to the FAZ. The first objective of this project is to suppress the product of each FLA gene individually in order to observe and compare the resulting phenotypes to each other, as well as to the results of previous experiments.

Chemotaxis is the process by which an organism senses a chemical gradient and uses it to direct motion either toward or away from the source of the chemical. The parasites are taken up with a blood meal into the midgut of the fly where they must undergo several developmental stages before migrating to the salivary gland of the fly. This process is thought to be controlled by chemotaxis. The second objective of this project is to observe trypanosomes on agar plates in order to better understand how they behave, with the long term goal of being able to develop an assay for chemotaxis.

INTRODUCTION

African Trypanosomiasis

African trypanosomiasis, or sleeping sickness, is a disease caused by the parasite *Trypanosoma brucei*. There are three subspecies of *T. brucei*: *T. brucei rhodisiense* infects only humans, *T. b. gambiense* infects humans and some other mammals, such as cattle, and *T. b. brucei* infects only certain mammals, not including humans (Brun *et al.* 2001). *T. brucei* parasites are transmitted to humans and other mammalian hosts by the tsetse fly. Trypanosomes can also be transmitted mechanically, in rare cases, between mammals if blood to blood contact occurs, and can cross the placental barrier from mother to fetus (Legros *et al.* 2002). In 1998 there were 40,000 cases of Human African Trypanosomiasis (HAT) reported, but the World Health Organization estimates the actual number of cases to have been between 300,000 and 500,000 (Legros *et al.* 2002). Figure 1 shows a map of the distribution of HAT.

African trypanosomiasis is found only in Sub-Saharan Africa, where tsetse flies are found, although inexplicably in some regions inhabited by the flies, trypanosomiasis is not present (Smith *et al.* 1998). The prevalence of animal sleeping sickness, known as *nagana* in cattle, greatly impacts economic growth in

developing countries by limiting grazing land to areas without tsetse flies and

where the disease is not prevalent (Pays *et al.* 1998).

T.b. gambiense

Trypanosomiasis
- No risk
- At risk
- Endemic
- High endemic
- Epidemic

T.b. rhodesiense
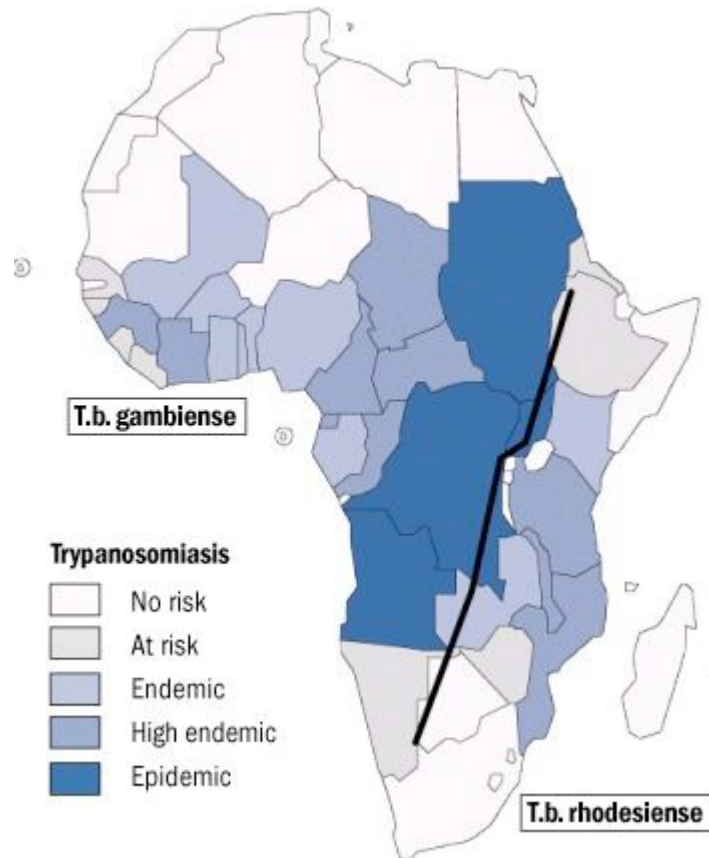
Figure 1: Distribution of Human African Trypanosomiasis by sub-species in Sub-Saharan Africa This map shows the countries affected by different sub-species of Human African Trypanosomiasis as of 1999. Countries to the left of the thick line are at risk for infection by *T.b. gambiense*, and countries to the right of the line are more at risk for infection by *T.b. rhodesiense*. The shading of each country indicates the severity of HAT infection rates. The shades range from white, almost no instances of HAT, to dark, where HAT is considered an epidemic (World Health Organization 2006)

HAT consists of an early and late phase, the hemolymphatic, and the

encephalitic phases respectively (Kennedy et al 2004). The hemolymphatic phase

is characterized by nonspecific symptoms: general malaise, fever and headache.

This phase can last for a prolonged period of months to years in *T. b. gambiense* infections, and can often go undiagnosed until the disease progresses. However, in *T. b. rhodesiense* infections, the first phase is very rapid, generally spanning only several weeks (Kennedy et al 2004). When the parasites cross the blood-brain barrier and invade the central nervous system, the disease is considered to be in the late encephalitic phase. This phase is characterized by psychiatric and motor disturbances, sensory abnormalities and sleep disruption (Kennedy et al 2004). Untreated, encephalitic stage HAT leads to seizures, coma, organ failure and inevitably death (Kennedy et al 2004).

There are several drugs used to treat HAT, all of which cause undesirable side effects. Pentamidine is used to treat an early *T. b. gambiense* infection, its side effects include hypotension, hypoglycaemia, or hyperglycaemia (Kennedy et al 2004). Suramin is used to treat early stage *T. b. rhodesiense* infections, and can cause anaphylactic shock, skin lesions, neurological effects, and renal failure. Melarsoprol is the primary drug used to treat late stage infections of either sub-species, but can cause cardiac arrhythmias, dermatitis, and agranulocytosis (Kennedy et al 2004). Recently, Eflornithine has been used to treat late stage *T. b. gambiense* infection, its side effects include: bone marrow toxicity, gastrointestinal effects, alopecia, and seizures (Kennedy at al 2004). In addition to the numerous detrimental side effects, these drugs can be ineffective due to developing parasite drug resistance. However, HAT is 100% fatal if untreated (Kennedy et al 2004, Brun *et al.* 2001).

The Life Cycle of *T. brucei*

When a tsetse fly infected *T. brucei* parasites, here referred to as trypanosomes, feeds on a mammal, metacyclic, trypanosomes are transferred from the salivary gland of the fly into the mammalian bloodstream (Matthews *et al.* 2004). This stage is a form of the parasite, adapted to survive in the salivary gland of the tsetse, but also to be able to quickly resume the cell cycle in the event of injection into the bloodstream of a mammal (Matthews *et al.* 2004). These non-dividing metacyclic parasites can choose between only a small number of variant surface glycoproteins (VSGs) that can appear on their surface (Matthews *et al.* 2004). Upon entering a bloodstream, however, the parasites immediately begin dividing and exchange the small metacyclic library of VSGs for the enormous repertoire characteristic of the bloodstream form of the parasite (Matthews *et al.* 2004).

VSG molecules are essential for the survival of trypanosomes in the mammalian bloodstream. The trypanosome genome contains more than one thousand distinct VSG genes, each of which presents completely different epitopes to the host immune system (Vanhamme *et al.* 2001). Only one of the myriad VSG genes available is expressed on the cell surface at a time (Vanhamme *et al.* 2001). In order to be expressed, a VSG gene must either be present in one of the estimated 20-40 telomeric bloodstream expression sites present in the trypanosome genome, or be transposed there by recombination (Borst and Rudenko 1994). Since only one VSG can be expressed at a time, the cell must switch expression to another site in order to activate a new VSG gene.

There are separate metacyclic expression sites, which are used exclusively with a limited number of VSG genes that are associated with the metacyclic stage of the parasite's life cycle (Donelson 2003). Trypanosomes in the wild switch their surface antigens at an estimated rate of $10^{-2} - 10^{-5}$ times per cell per generation, however, laboratory strains tend to switch much more slowly (Vanhamme *et al.* 2001).

Once established in the bloodstream, the parasitic population divides into two distinct morphologies. The slender morphology continues proliferation within the bloodstream, but as the density of the population increases stumpy forms become increasingly prevalent (Matthews 2005). The stumpy form is characteristically arrested at the G1 phase of the cell cycle, in preparation for uptake by a tsetse fly. The stumpy form is non proliferative and, if not ingested by a fly with a blood meal, is eventually overwhelmed by the host's immune response. These cells are eventually recognized by the immune system because the parasites are only able to switch their active VSG during cell division (Matthews 2005).

When an uninfected tsetse fly draws a bloodmeal from an infected mammal, stumpy form trypanosomes are transferred to the midgut of the fly. Within two hours of this event, the surface molecules consistent with the insect, or procyclic, form of the parasites appear on the surface of the cell. These molecules, called procyclins, come in two types, EP and GPEET, named for repeats of amino acids in their sequence (Matthews *et al.* 2004).

In order to fully differentiate into procyclic form cells, the parasites then undergo further development involving cytoskeletal remodeling and organelle re-positioning (Matthews *et al.* 2004). Their main metabolic pathway also changes from glycolysis, which takes advantage of the high levels of glucose in the mammalian bloodstream, to the Krebs cycle, in which proline is the primary metabolite (Matthews *et al.* 2004; Lamour *et al.* 2005). The procyclic form parasites are once again proliferative and, after establishing an infection in the midgut of the fly, migrate to the salivary gland where they further differentiate into the epimastigote stage. In this stage, flagellar attachment occurs which adheres the cell to the salivary gland of the fly, allowing further differentiation. Epimastigotes develop into metacyclic trypanosomes, which are cell cycle arrested while in the salivary gland, but quickly re-enter the cell cycle when injected into a mammal (Matthews 2005). Once metacyclic trypanosomes are developed within the fly, the infection is chronic, and it will continue to infect new mammalian hosts for the rest of its life (Gibson and Bailey 2003).

The trypanosome life cycle is summarized in Figure 2. Briefly, the stages are most easily distinguished by: whether they express VSG molecules or procyclins, and whether or not they are proliferative. The major morphologies of the parasites are pictured.

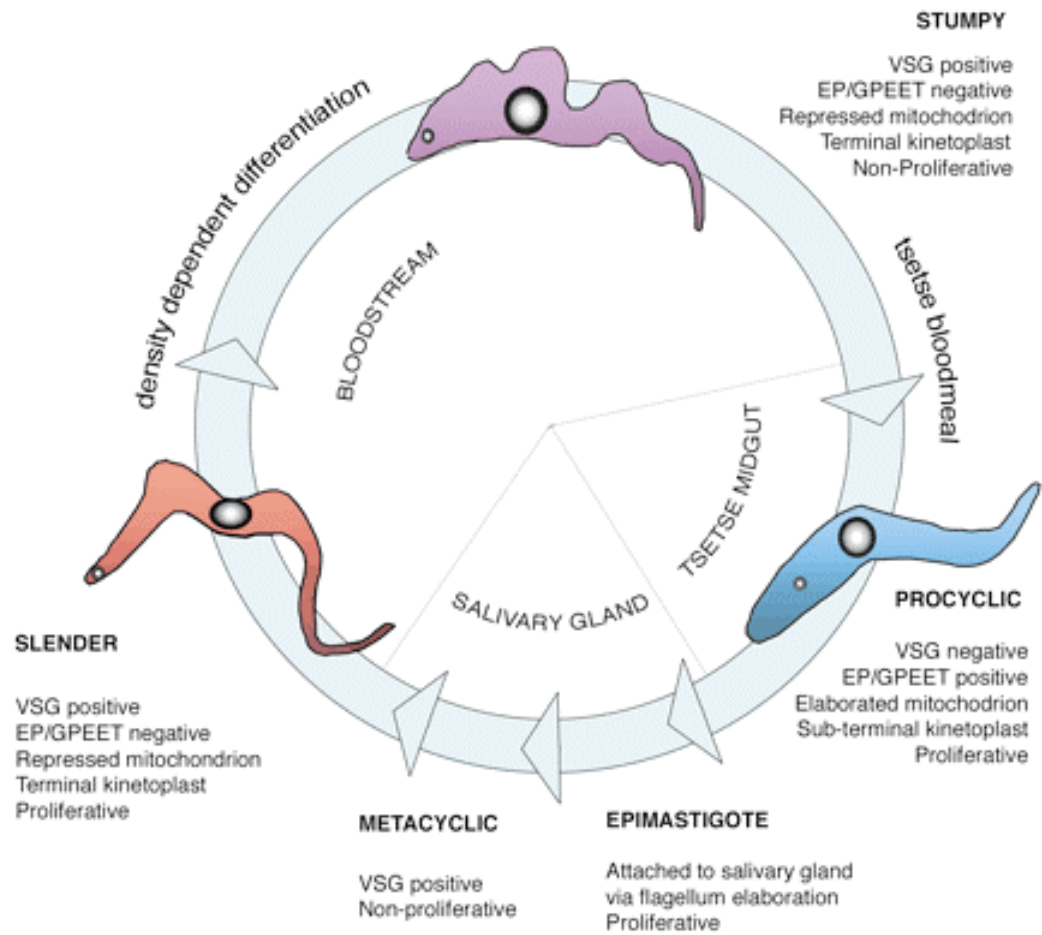**Figure 2: The Life cycle of *Trypanosoma brucei*.** This chart shows the different stages of the trypanosome life cycle. The part of the lifecycle taking place in the tsetse fly is indicated by slices dividing the stages between the midgut and the salivary gland. Differentiation is indicated by arrows and the major morphologies of bloodstream form and procyclic form parasites are pictured (Picture: Matthews 2005).

The Trypanosome Flagellum

Although the trypanosome flagellum possesses several unique features, it is becoming a popular model for studying aspects of eukaryotic cilia and flagella (Hill 2003).   Figure 3A shows a trypanosome with the components of its flagellum labeled. Figure 3B shows the trypanosome flagellum in cross section. The flagellum emerges from an invagination of the cell membrane known as the flagellar pocket (Bastin *et al.* 2000).   Within the flagellar pocket, the flagellum is attached to the cell body through four specialized, electron dense, microtubules (Kohl and Gull 1998).   The flagellar membrane is adhered down the length of the cell to the plasma membrane, except for the tip, which extends for a short distance past the end of the cell. The area where the flagellum membrane is in contact with and adhered to the cell membrane is known as the flagellar attachment zone (FAZ) (Landfear and Ignatushchenko 2001). The flagellum itself is a membrane bound axoneme of microtubules, supported by a structure known as a paraflagellar rod (PFR). The PFR lies parallel to the flagellum and although its function is unknown, one hypothesis suggests that it lends the structure the rigidity it needs to create a waveform (Santrich *et al.* 1997).
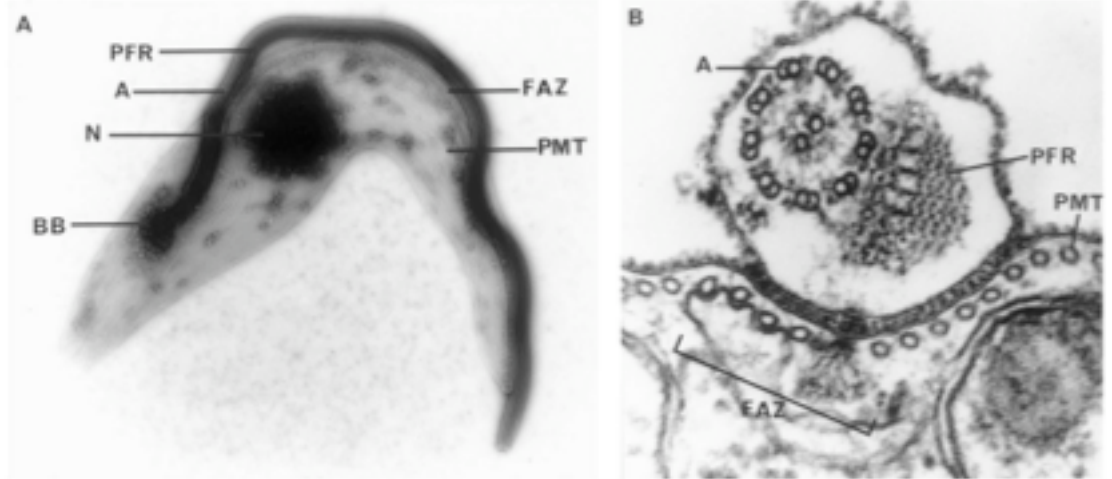
Figure 3: (A) A procyclic trypansome that has been processed with detergent to show cytoskeletal anatomy (B) A transverse section of a trypanosome flagellum and surrounding region (A: axoneme, BB: basal body, N: nucleus, PMT: pellicular microtubules, FAZ: flagellar attachment zone, PFR: paraflagellar rod)(Reproduced from Kohl and Gull 1998).

The trypanosome plasma membrane is divided into three distinct but contiguous regions (Vaughn and Gull 2003). The first covers the main body of the cell, and contains many surface molecules, primarily VSGs in the bloodstream form, and procyclins in the procyclic form (Landfear and Ignatushchenko 2001). The membrane of the flagellar pocket is responsible for all endocytosis and exocytosis, and is the site where new proteins are transferred to the cell surface (Landfear and Ignatushchenko 2001). The membrane of the flagellum itself is thought to be a sensory instrument (Landfear and Ignatushchenko 2001). It contains many proteins distinct from the rest of the plasma membrane (Bastin *et al.* 2000). The sorting of membrane proteins has been found to occur in the flagellar pocket (Bastin *et al.* 2000). While all the membranes may share

common proteins such as procyclins and VSGs, other surface proteins are sorted into the correct region of the membrane (Bastin *et al.* 2000).

The axoneme of the trypanosome flagellum is typical of eukaryotic motile flagella (Hill 2003). It is formed by nine pairs of microtubules surrounding a central pair (Hill 2003). This is commonly referred to as a '9+2' arrangement (Bastin *et al.* 2000). Dynein motors along the axoneme are activated in series by ATP, allowing them to bind to adjacent microtubules (Hill 2003). This causes microtubules to slide over one another creating a bend in the axoneme. The motion is continued along the length of the structure in order to create a waveform (Walker and Walker 1963). Directional motility is attained through tight regulation of dynein proteins via signaling from the central pair (Hutchings *et al.* 2002). This is achieved using a set of regulatory proteins known as the dynein regulatory complex (DRC) (Ralston *et al.* 2006). The DRC is not only essential for directional motility, but also for the viability of bloodstream form parasites (Ralston *et al.* 2006).

The paraflagellar rod (PFR) is a structure found in only a few groups of organisms, kinetoplasts, euglenoids and dinoflagellates (Kohl and Gull 1998). This structure runs parallel to the axoneme inside the flagellar membrane from the point where it exits the flagellar pocket to the tip (Bastin *et al.* 2000). The PFR is a lattice-like filament that has been hypothesized to lend the flagellum the rigidity required to produce the appropriate waveform (Santrich *et al.* 1997). The PFR is made up of two major proteins, PFRA and PFRC (Hill 2003). Removal of the

PRFA gene by RNA interference (RNAi) produces mutants that are paralyzed but viable (Bastin *et al.* 1998). The PFR has three distinct sections, proximal intermediate, and distal sections, based on their location relative to the axoneme. The axoneme and PFR are connected by fibers between doublets 4-7 of the axoneme and the proximal area of the PFR (Bastin *et al.* 2000).

The flagellar attachment zone (FAZ) is the region of the plasma membrane where the flagellar membrane is adhered to the cell (Kohl and Gull 1998). Although not much is known about the specific proteins and interactions in this region, the *Trypanosoma cruzi* protein GP72 was found to disrupt the attachment within the FAZ, when eliminated via double knock out (Kohl and Gull 1998).

FLA1, the *T. brucei* homolog of the *T. cruzi* FAZ protein GP72, is present in both bloodstream and procyclic forms (LaCount et al 2002). In 2002 LaCount *et al.* used RNAi to silence the expression of FLA1 in procyclic trypanosomes after several attempts at double knock outs of the gene proved unsuccessful. The resulting phenotype was the same as that of the *T. cruzi* GP72 null mutants, flagellar detachment. However, the *T. brucei* cells were also unable to initiate cytokinesis. Expression of the *T. cruzi* GP72 protein in FLA1 deficient *T. brucei* cells did not allow flagellar reattachment, but rescued the cytokinesis defect. The FLA1 mutant phenotype was identical in both procyclic form and bloodstream form parasites, However, in a northern blot showing the reduced amount of FLA1 mRNA in RNAi induced bloodstream form parasites, a second band appeared

which led to the discovery of the second FLA gene, FLA2. Both genes were targeted by this RNAi construct due to the near identity of these genes over the first 500 base pairs.

RNA Interference

RNA interference (RNAi) is a term that was coined by Fire *et al.* (1998) over the course of their research with the organism *Caenorhabditis elegans*. RNAi is defined as the process by which double stranded RNA (dsRNA) in a cell silences a gene with matching sequence, (Fire *et al.* 1998). RNAi has been characterized in many other organisms, including *Arabidopsis thaliana*, *Drosophila*, and zebrafish. It has also been shown to occur in *T. brucei* (Ngo et al. 1998). However, RNAi is notably absent in protozoans closely related to *T. brucei*, such as *T. cruzi* and *Leishmania major* (Motyka and Englund 2004)

RNAi is an extremely useful tool for studying the phenotypes of cells in the absence of a specific gene product. It is a viable alternative to difficult and time consuming gene knock outs, in which all copies of the gene of interest are eliminated by ablation (LaCount *et al.* 2000). If the gene of interest is essential to the organism, creating a knock out is actually impossible because the cells die before a phenotype can be observed. Via RNAi, it is possible to see the phenotype of cells with targeted gene products knocked down to extremely low levels. This approach to gene characterization is similar to that of a mutagenesis, where

studying the effects of losing a certain protein on a cell gives insight to the normal function of that protein.

While the complete mechanism involved with RNAi is still largely unknown, several of the proteins involved have been discovered. The best characterized of these proteins, Dicer, was first discovered in *Drosophila*, and the homolog in *C. elegans*, dcr-1 has also been extensively characterized. The Dicer family of genes contain many conserved domains, including: helicase, double stranded RNA (dsRNA)-binding and RNAse III domains (Grishok *et al.* 2001). Although the Dicer gene and its homologs have been shown to be essential for RNAi in many organisms, a homolog in *T. brucei* has only recently been identified (Shi *et al.* 2006).

When a molecule of dsRNA is introduced into a cell, it is processed by Dicer proteins into 20-25 nucleotide long single stranded fragments. These fragments, known as small interfering RNA (siRNA), guide degradative protein complexes to mRNA with complementary sequence (Grishok *et al.* 2001). LaCount *et al* (2000) tested several different forms of RNA for efficiency at producing RNAi phenotypes using the gene α-tubulin. This gene was chosen because it has a known phenotype in *T. brucei* when removed with a traditional knock out. Lack of α-tubulin causes what is known as a FAT phenotype, where the cells lose their characteristic shape and become spherical. First, they introduced dsRNA targeted to α-tubulin into *T. brucei* cells and observed that about 85% of cells showed a FAT phenotype (Ngo et al. 1998). They then

introduced only the sense strand of the same RNA construct into a different culture of cells. They observed no cells with the FAT phenotype in that culture. From this they concluded that the anti-sense strand is required for RNAi to function. Finally, they introduced only the anti-sense strand of the same RNA construct into a third culture of cells. In this culture they observed the FAT phenotype, but in only 46% of cells. They concluded that while the anti-sense strand of target RNA was required for an RNAi effect, dsRNA is much more efficient.

Although introduction of dsRNA alone can be achieved via electroporation, this technique tends to yield a phenotype that disappears after a period of time, and the amount of dsRNA introduced into each cell is variable and impossible to control (Wang *et al.* 2000). Alternatively, in order to create stable RNAi mutant lines with tight inducible regulation, recombinant plasmids have been constructed. This technique has the advantage that transfected cells can be cultured and induced repeatedly, When linearized, these plasmids integrate into specific untranscribed portions of the trypanosome genome (Morris *et al.* 2001). These plasmids express dsRNA in the cell, eliminating the need to create and purify dsRNA repeatedly to observe a particular phenotype more than once. Some of the most effective of these plasmids use dual T7 bacteriophage promoters linked to tetracycline repressor sequences (LaCount *et al.* 2000).

The promoters are placed in a head to head orientation to produce both the sense strand, which contains the same base pairs as the mRNA transcribed from

the genome, and the anti-sense strand of the target RNA, which contains base pairs complementary to the sequence transcribed from the genome, as shown in Figure 4 (La Count *et al.* 2000). Since they contain the powerful bacteriophage T7 promoter, these plasmids require special transgenic cell lines that express both the T7 RNA polymerase gene and the gene encoding the tetracycline repressor (Wirtz *et al.* 1998). These genes have been integrated into the *T. brucei* genome attached to antibiotic resistance genes, which confer resistance to G418, linked to the T7 RNA polymerase gene, and Hygromycin, linked to the tetracycline repressor gene. Antiobiotic resistance genes are used as reporter genes, so that when grown on medium containing these specific antibiotics, only cells containing these genes survive in culture (Wirtz *et al.* 1998).

The tetracycline repressor protein is expressed constantly in the cell, and the protein binds to a specific repressor sequence in front of each of the T7 promoters flanking the target gene sequence, preventing expression. In the presence of tetracycline, the repressor protein is inactivated, allowing the target sequence, which consists of approximately 400 base pairs of the gene of interest, to be transcribed (LaCount *et al.* 2000). Using this system, RNAi mutant lines can be efficiently established by integrating recombinant plasmids into the trypanosome genome, and induction of mutant phenotypes can be tightly regulated by the tetracycline repressor. A detailed diagram of p2T7, the plasmid used in this study, is shown in Appendix A.

Figure 4: This figure shows the orientation of dual T7 promoters with respect to the gene of interest. The ellipse represents the plasmid vector, and the bold area represents approximately 400 base pairs of the gene of interest. The promoters are indicated by the arrows, which also show their orientation which enables both a sense and an anti-sense strand of RNA to be transcribed (LaCount *et al.* 2000). Appendix A shows the plasmid p2T7 in greater detail.

Chemotaxis

When an organism detects a chemical gradient and in response moves along that gradient, either towards or away from the source of the chemical, it is called chemotaxis. Chemotaxis has been demonstrated in many types of organisms including: *Drosophila* larvae (Fishilevich *et al.* 2005), *Dictyostelium discoideum* (Haastert and Devreotes 2004), *Tetrahymena thermophila* (Lieck 1992), and human neutrophils (Parent 2004). Although chemotaxis has not yet been demonstrated in *T. brucei*, it is thought that the migration of procyclic trypanosomes from the midgut to the salivary gland of the tsetse fly may be controlled through chemotaxis.

Previously, trypanosomes have been observed to form aggregate colonies on agarose plates. These colonies exhibited complex behaviors, such as the recruitment of lone cells, directed motion of the colony towards other colonies, and the merging of colonies (McLelland and Hill, personal communication). These behaviors suggest that there is intercellular signaling taking place to affect directional motility. Further characterization of this phenomenon and the molecules involved could provide insight into the way trypanosomes respond to other factors that affect directional motility, such as chemical gradients.

The migration of trypanosome cells toward one another to form colonies is not unlike the behavior of *D. discoideum* when under stress the individual cells migrate together and form a multi-cellular organism (Parent 2004). In *D. discoideum* as well as other cells, such as neutrophils, chemotaxis is initiated by the binding of a chemoattractant to transmembrane receptors that are homogenously distributed throughout the plasma membrane (Parent 2004). This initiates a cAMP signaling cascade, which triggers several responses, including the rearrangement of cellular components within the cytoplasm (Parent 2004).

No transmembrane receptors similar to other eukaryotic chemotactic receptors have so far been identified in *T. brucei*. However, components of the cAMP signaling pathway have been identified, and localized to the flagellum (Oberholzer *et al.* 2006). This supports the idea that the flagellum functions as a sensory instrument (Landfear and Ignatushchenko 2001).

MATERIALS AND METHODS

FLA1 and FLA2

Plasmid Preparation

RNAi plasmids were produced first by amplifying an approximately 400 base pair sequence of each FLA1 and FLA2 via a polymerase chain reaction (PCR). The sequences were chosen by aligning the two genes using bl2seq tool (Tatusova and Madden 1999) online, to find areas with low identity. A *Bam*HI and a *Hin*dIII restriction site was added to each primer for later insert confirmation. The primers chosen and construct size of each fragment are shown in Table 1. PCR products were confirmed by gel electrophoresis.

The vector plasmid used was p2T7 (LaCount *et al.* 2000), a generous gift from the Hill lab. The p2T7 plasmid used was modified by Invitrogen (www.invitrogen.com), to have topoisomerase recognition sites, with topoisomerase covalently bound to them. The PCR products were ligated into the vector by combining them with the topoisomerase plasmid in a salt solution at room temperature, the optimal conditions for the topoisomerase to ligate. The ligated plasmids were then transformed into One Shot® Top 10 competent *E. coli*

from Invitrogen. The cells were then plated on LB with ampicillin and incubated at 37˚C overnight.

Several colonies from each transformation were selected and bacterial cells were grown in a liquid suspension overnight. Plasmids were isolated from these cells using a rapid boiling preparation. Briefly, cells were centrifuged to the bottom of a tube, the LB was removed and cell lysis solution (8% Sucrose, 5% Triton X-100, 50mM Na2 EDTA, 50mM Tris-HCL, Lysozyme) added. The cells were then boiled briefly and centrifuged again. From this centrifugation, the supernatant was transferred to a clean tube, and the DNA was precipitated from the supernatant using isopropanol. The DNA pellet was then washed with 70% ethanol and resuspended in elution buffer (EB) from Qiagen (http://www.qiagen.com). Several microliters of the resulting DNA were digested with *Hin*dIII and *Bam*HI and analyzed by gel electrophoresis to confirm the insertion of FLA1 and FLA2 PCR products.

To obtain the large amounts of DNA required for transfection, 50ml cultures of *E. coli,* containing FLA1 or FLA2 plasmids, were grown up overnight at 37˚C. Plasmid was then extracted from these cells using the Wizard® *Plus* Midipreps DNA Purification System from Promega (http://www.promega.com). Briefly, the cells were centrifuged, the LB removed, and resuspended in Lysis Solution. The tubes of lysed cells were then centrifuged again and the supernatant saved. DNA purification resin was then added, and the solution transferred to a DNA binding column attached to a vacuum manifold. The solution was drawn

through the column completely, and then the column was washed with column wash solution and dried by vacuum for 5 minutes. The column was then removed from the vacuum and centrifuged to remove any remaining liquid. Warmed elution buffer was then added to the column, which was allowed to incubate at room temperature for one minute before being centrifuged to elute the DNA. The resulting DNA solution was analyzed using a Nanodrop (http://www.nanodrop.com) ND-100 spectrophotometer.

Transfection of FLA1 and FLA2 p2T7 into *T. brucei*

To transfect plasmids stably into the *T. brucei* genome both the DNA and the *T. brucei* host must be thoroughly prepared ahead of time. Fifteen micrograms of each plasmid to be transfected was linearized by digestion with *Not*I overnight at 37˚C, two days before transfection. One day before transfection, the digested DNA was precipitated using 100% ethanol and resuspended in sterile cytomix (25mM HEPES, 120mM KCl, 0.15mM $CaCl_2$, 10mM K_2HPO_4/KH_2PO_4, 2mM EDTA, 5mM $MgCl_2$, 1% Glucose, 0.1% BSA, pH 7.6). A small volume of the resulting DNA solution was analyzed by gel electrophoresis to confirm linearization.

Procyclic 29-13 cells, a gift from the Klingbeil Lab, were cultured in SDM-79 medium with G418 (15μg/ml) and Hygromycin (50μg/ml). The cells are grown to log phase, approximately $1-2x10^7$ cells per milliliter, for two to three days before transfection. It is critical that the strain 29-13 was used, because this

strain expresses transgenic T7 RNA polymerase as well as the tetracycline

repressor protein, both of which are required for the proper function of the p2T7

plasmid.

To transfect the linearized plasmids into the cells, the cells were first

counted, then 1×10^8 cells per transfection were centrifuged at 2400xg for 12

minutes. The medium was then removed, and the cells were resuspended in 5ml

cytomix. The cells were then centrifuged again at 2400xg for 12 minutes and the

cytomix removed. The parasites were resuspended in 500µl of DNA suspended in

cytomix, or for the no DNA control, just cytomix. The cell suspensions were then

transferred into 4mM gap cuvettes and electroporated at 1.6kV, 25 ohms. After

electroporation cells were immediately transferred into 9.5ml room temperature

SDM-79 with G418 and Hygromycin, and incubated for 24 hours at 27˚. After 24

hours phleomycin (2.5µg/ml) was added in order to select for cells in which the

plasmid had successfully integrated into the genome.

The transfected cultures were divided in the first two to three days in order

to add nutrients to the depleted medium. They are then monitored for between two

and three weeks, until normal growth is resumed.

Establishment of Frozen Stabilites

After the cultures begin growing normally frozen stabilites are made.

When the cell titer is between 5 and 7×10^6 cells per milliliter, 800µl of cell

suspension was combined with 200µl sterile 50% glycerol per cryo-vial. These

tubes were then closed and inverted to mix, and stored overnight at 80˚C. After 24 hours, the cryo-vials were moved to liquid nitrogen.

Tetracyline Induction of Transfected Cell Lines

After the cells have recovered from electroporation, each culture was split to form two new cultures, at a titer of approximately $5\text{x}10^5$ cells per milliliter. To one of these cultures, tetracycline was added at a concentration of 3µg /ml to activate the expression of the target dsRNA. Both cultures were then counted once every 24 hours and monitored for mutant phenotypes for six days. After 48 hours RNA was isolated from both cultures using the Versagene™ RNA purification system from Gentra Systems (http://www.gentra.com).

Procyclic Trypanosome Culture on Agar Plates

Procyclic trypanosomes were cultured on agar plates using an adapted protocol from the Hill lab. Wild type trypanosomes, strain 427 obtained from the Klingbeil lab, were used in all directional motility plating observations. Spent medium was used in the agarose solution to assist Trypanosome growth by providing any molecules that the parasites secrete. Spent medium is medium collected from cultures between $5\text{x}10^5$ and $1\text{x}10^6$ cells/ml and filtered through a 0.22µm filtration system. Trypanosomes were plated on 0.8% agarose mixed with a combination of 50% fresh SDM-79 medium and 50% conditioned SDM-79

medium (1 volume of spent SDM-79, filtered with a 0.22µM filter; 1 volume

fresh SDM-79 medium and 0.4 volumes FBS).

The number of trypanosomes plated ranged from 5×10^5 to 1×10^6. Plates

were monitored for between thirty minutes and two hours. The plates were

viewed on a Nikon inverted microscope. Time-lapse pictures were taken every 10

seconds using bright field microscopy at 100x magnification using Metavue™

software (http://www.moleculardevices.com).

RESULTS

FLA1 and FLA2

Plasmid Construction

Table 1 shows the primers used for PCR amplification of FLA1 and FLA2 fragments, as well as the fragment size. It was noted that the second FLA2 primer contains a *Bam*HI site, so no restriction site was added. The PCR product sizes were confirmed by gel electrophoresis, as shown in Figure 5. Both constructs were shown to be between 400 and 500 base pairs, which was the expected product size.

Table 1: FLA Gene Primers and Their Product Sizes.

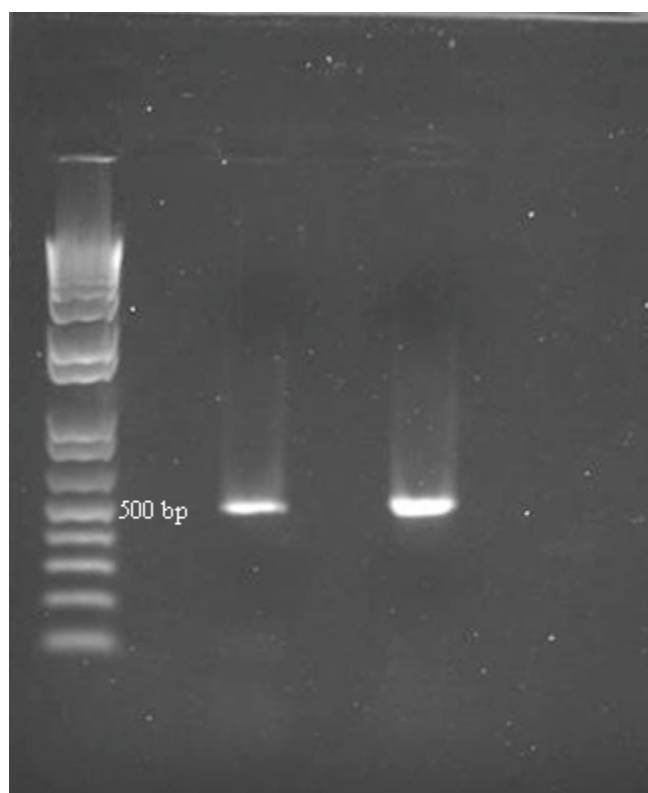|	Primers	Product Size (bp)
FLA1	CAC GGATCC GCGATTCGGATGGTAACAAG	457
	CAC AAGCTT CGAAAGGCTACCTCCACATC	
FLA2	CAC AAGCTT TTGAGCAAAACTGGGGAGTC	478
	CAC GGATCCGGATTGGGAGTAAT	

Figure 5: Gel Electrophoresis of FLA PCR Products.
Lane 1: Ladder; Lane 2: Blank; Lane 3: FLA 1 PCR product; Lane 4: Blank; Lane 5: FLA 2 PCR product. Both FLA 1 and FLA 2 PCR products ran between 4 and 500bp compared to the ladder. This corresponds with the expected product size listed in Table 1.

The PCR products were then ligated into p2T7 plasmids, and confirmed

by restriction enzyme digestion with *Bam*HI and *Hin*dIII. Figure 6 shows the gel

electrophoresis of the digestion reaction. Since the digestion cut the inserted

construct out of the plasmid, the gel was expected to show a band between 400

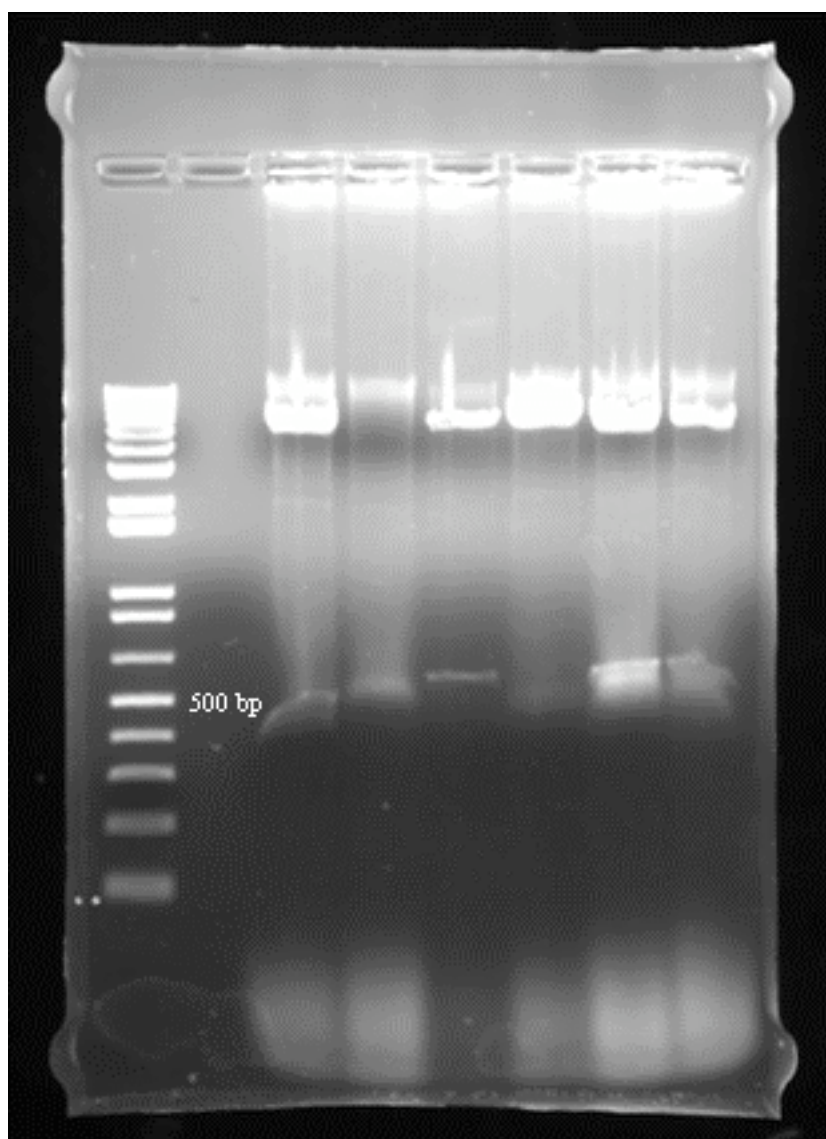and 500bp and a band in the range of 5000bp.

Figure 6 Confirmation of PCR Product Ligation into p2T7

Plasmids isolated from *E. coli* were digested with BamHI and HindIII. Lane 1: Ladder, Lane 2: Blank, Lanes 3-5: FLA1, Lanes 6-8: FLA 2. Bands visible between 4 and 500bp represent the successful insertion of the PCR product into the plasmid from that particular colony of *E. coli*. Bands running in the range of 5000bp are linearized p2T7 DNA.

FLA1 Tetracycline Induction of RNAi

A little more than 24 hours after tetracycline induction, cells with a mutant phenotype were observed. The number of cells with a mutant phenotype increased steadily until about 120 hours after induction, where the number seemed to plateau. Figure 7 shows a graph of the percentage of cells observed with a mutant phenotype over time.

Figures 8 through 11 show phase contrast photos of mutant phenotypes, taken ninety-six hours after induction. Figure 8 shows a wild type trypanosome from the uninduced culture of the same transfectant line. Figure 9 shows the initial FLA 1 phenotype, a detached flagellum. Figure 10 shows a trypanosome with two detached flagella, the result of a cytokinesis defect. Figure 11 shows a multiflagellated amorphous trypanosome, which exemplifies the cytokinesis defect created by FLA 1 silencing,
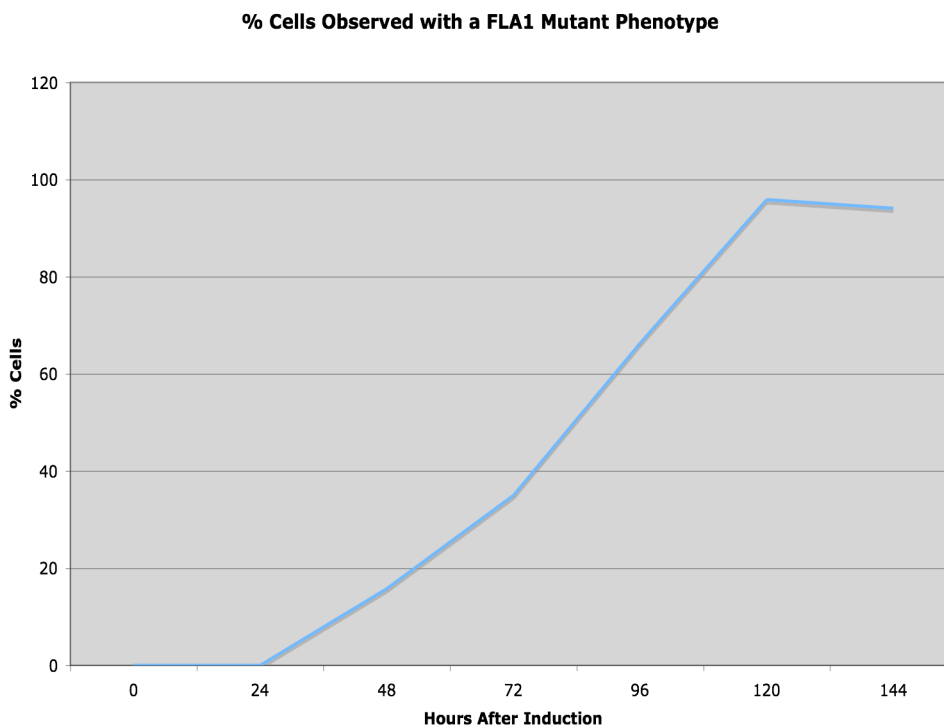
% Cells Observed with a FLA1 Mutant Phenotype

Figure 7: Percentage of Cells observed with a Mutant Phenotype

This graph shows the percentage of trypanosome cells exhibiting a detached flagellum or cytokinesis defect. Cytokinesis defective cells were identified by multiple flagella, or large deformed cell shape.
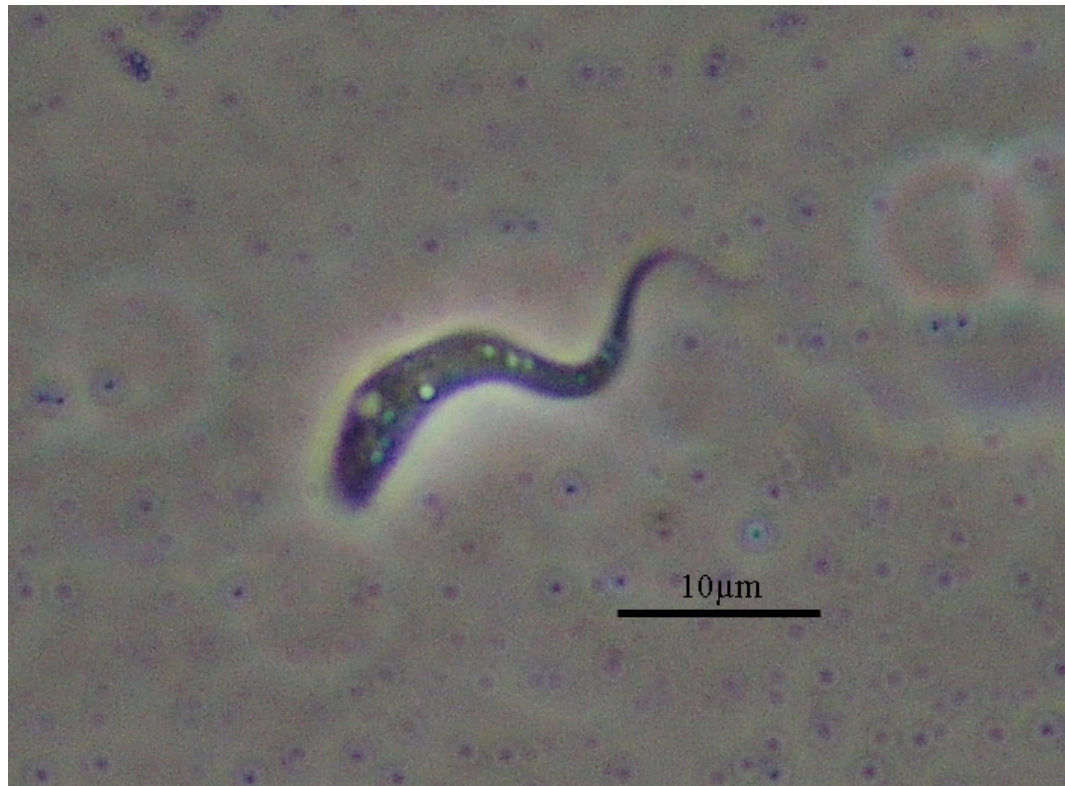
10μm

Figure 8 Uninduced FLA1 Trypanosome

This cell, viewed with a 100x objective, has been transfected with the FLA1 RNAi plasmid. There is no tetracycline in the cell suspension to induce the expression of dsRNA, and the cell exhibits a wild type phenotype. The wild type flagellum originates in the flagellar pocket and is attached down the length of the cell.

Figure 9 FLA1 Trypanosome exhibiting a detached flagellum

This picture shows a trypanosome transfected with the FLA1 RNAi plasmid, which has been activated by the presence of tetracycline. This cell exhibits one of the FLA1 phenotypes, a detached flagellum. Unlike the wild type cells, the flagellum of this cell is detached along the length of the cell, remaining connected only by the point of origination in the flagellar pocket. This image was obtained with a 100x objective.
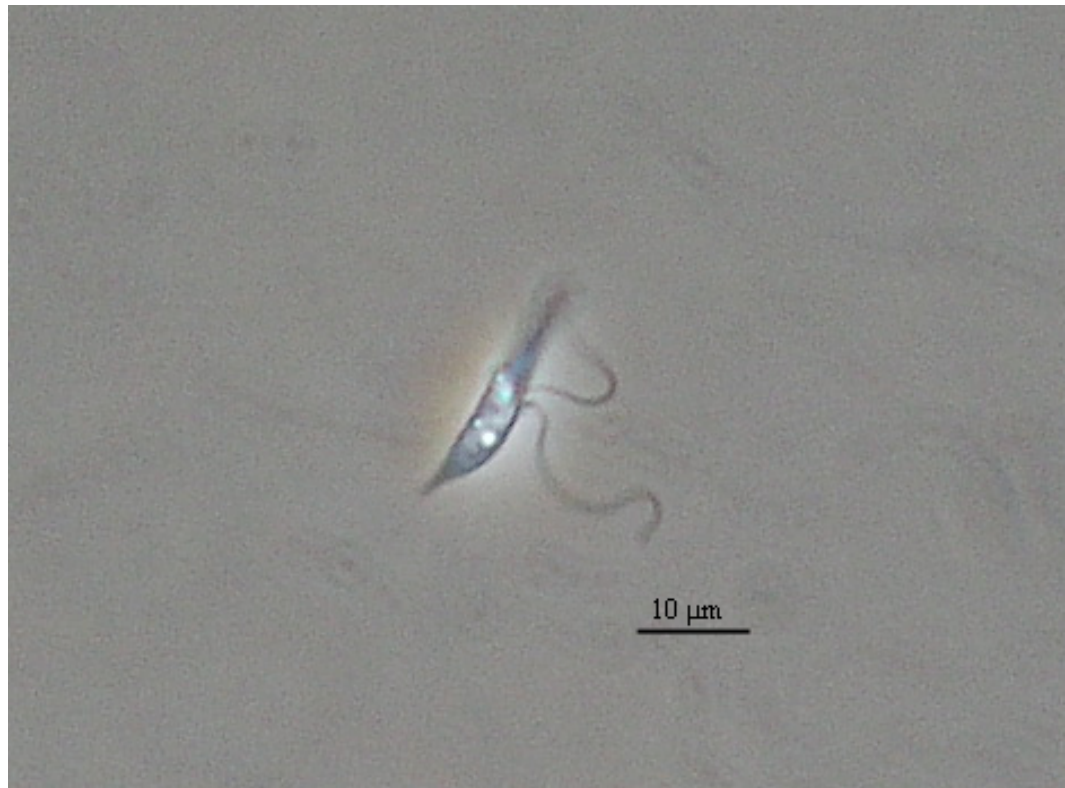
Figure 10 FLA1 Multi-flagellated trypanosome

This image, taken with a 40x objective, shows a later consequence of FLA1 protein loss. This cell has undergone mitosis and replicated its flagellum, but failed cytokinesis. The cell still appears relatively normal, aside from the two detached flagella.
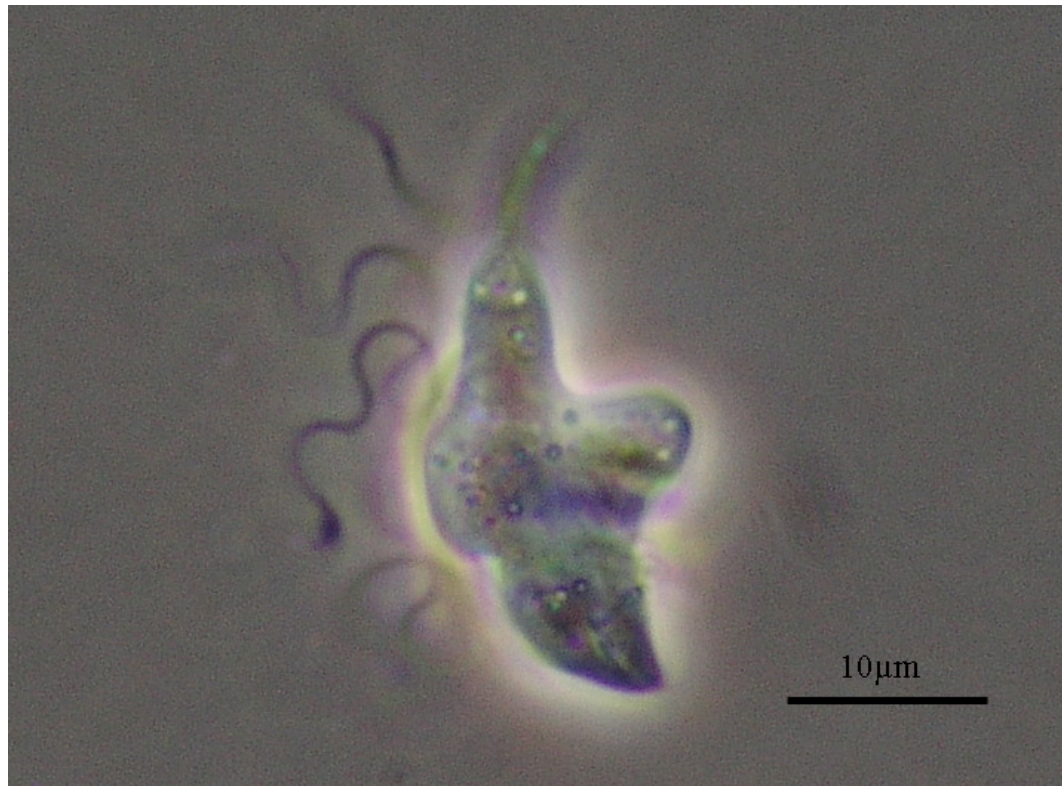
Figure 12 FLA1 Trypanosome exhibiting cytokinesis defect

This picture, taken with a 100x objective, shows a FLA1 RNAi induced trypanosome unable to undergo cytokinesis. The cell is multi-flagellated, and amorphous.

The silencing of FLA1 in the induced culture turned out to be a lethal defect, indicating that the gene is essential in procyclic form *T. brucei*. Figure 12 shows a growth curve, graphed for both the induced and uninduced cultures. The induced cultures grow normally until about 72 hours after induction, at which point the cell density declines. RNA was harvested from each culture 48 hours after induction.
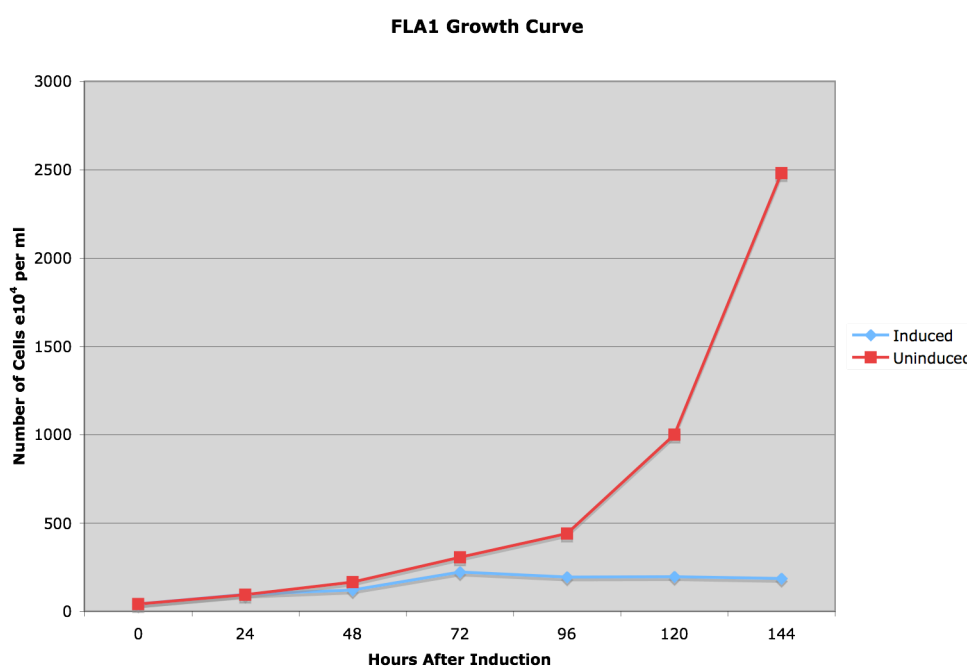
FLA1 Growth Curve

Figure 12 Growth Curve of FLA1 Transfectants

This linear graph shows the number of cells per milliliter in both the induced and uninduced cultures for 144 hours after induction. The uninduced culture grows exponentially, but the induced culture eventually peaks and begins to decrease.

FLA 2 Tetracycline Induction of RNAi

The FLA2 plasmid was successfully transfected into *T. brucei*, and induced. There was no observable growth or motility phenotype observed after tetracycline induction. RNA was harvested from both the uninduced and induced FLA2 cultures after 48 hours.

Directed Motility

Trypanosome colony formation was viewed on agarose plates using bright field microscopy and a 10x objective. Time-lapse photography was employed to observe the plates over a period of between half an hour and 2 hours. Colony formation

Figure 13 shows a time-lapse sequence of a trypanosome colony aggregating. The sequence was observed approximately 70 minutes after plating. Figure 14 shows a single cell joining the newly formed colony. Figure 15 shows two small colonies merging to form a larger colony. Figure 15 was observed about 75 minutes after the plating, and approximately 5 minutes after the aggregation of the first colony.
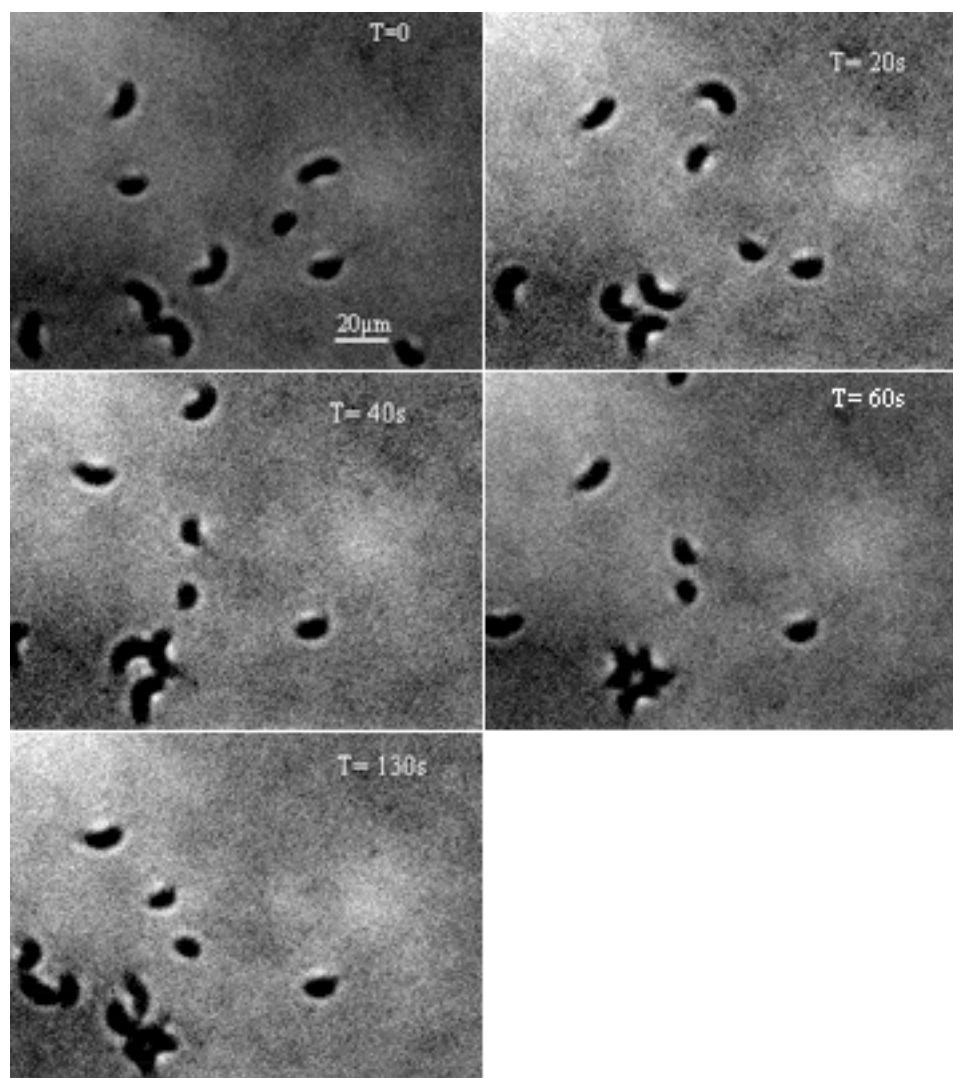
Figure 13 Trypanosome Colony Formation

Approximately 1.5 hours after plating, small colonies of trypanosomes began to form. In just over two minutes, a colony coalesces, and cells in the surrounding area begin to congregate around it.
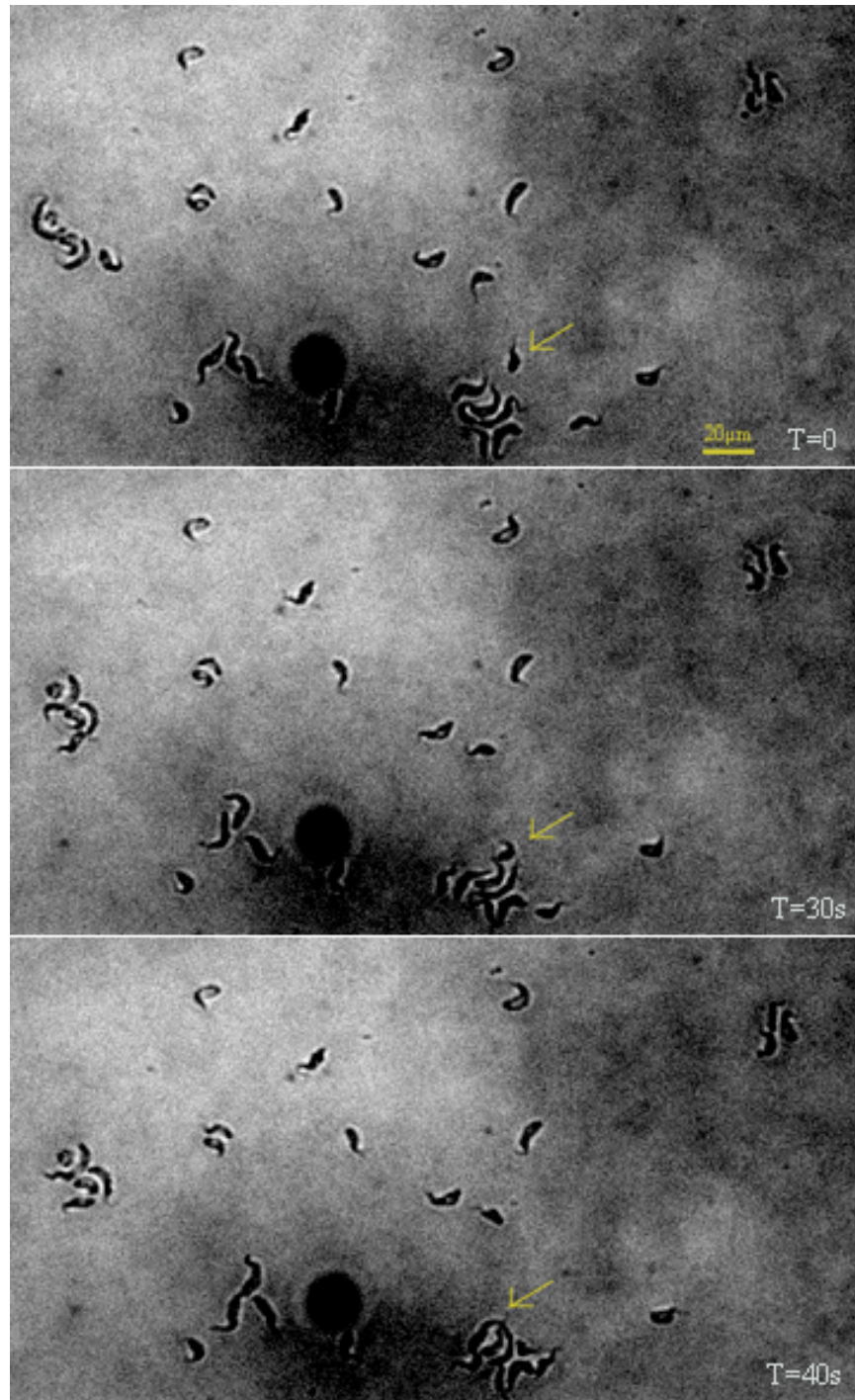
Figure 14 A Single Cell Joins a Colony

This figure shows the course of forty seconds, during which a single cell, shown by an arrow, makes contact with a colony, tumbles in place, and moves to join the colony.

Figure 15 Two Colonies of Trypanosomes Merging

During this two-minute time segment, a small colony of two to three cells, indicated by a yellow arrow, merges with a newly formed colony of five to seven cells, indicated by a green arrow. As the two colonies detect one another, they lengthen and meet in the middle.

Discussion

FLA1 and FLA2

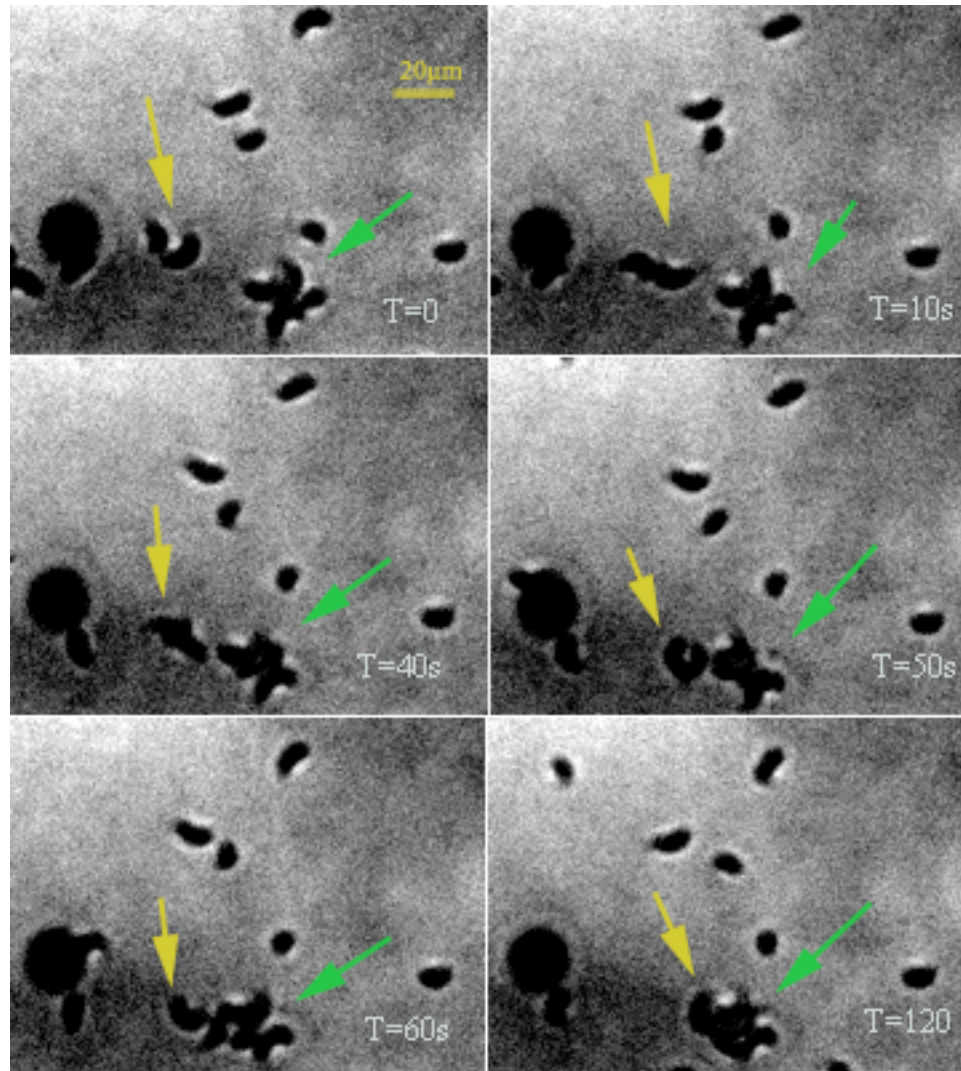The phenotype resulting from RNAi induction of the FLA1 construct was consistent with the phenotype observed by LaCount *et al (*2002). Because the depletion of FLA1 protein caused eventual cell death, FLA1 is considered essential to procyclic *T. brucei*. The reduction in mRNA could not be confirmed by Northern Blot at this time due to time constraints; however, RNA isolation was performed in preparation for confirmation.

The FLA1 cytokinesis defect is particularly interesting, because it is unclear how the protein plays a role in cytokinesis or if motility is disrupted enough to stop the cells from dividing. Although it is unlikely that FLA1 plays a direct role in cytokinesis, the separation of the flagellum from the cell membrane may disrupt intracellular signaling, or another process critical to the initiation of cytokinesis.

The multi-flagellated "monster" phenotype shown in Figure 12 is somewhat similar to the phenotype observed in bloodstream form trypanin knockdown lines (Ralston and Hill 2006). Trypanin is an integral part of the dynein regulatory complex, which regulates the flagellar beat (Ralston and Hill

2006). Silencing trypanin caused a different effect in bloodstream and procyclic form trypanosomes. In the bloodstream form, the cells were unable to move directionally, and unable to divide, forming large multiflagellated, amorphous cells. This was due to the accumulation of duplicate organelles resulting from multiple rounds of mitosis.

When this gene was silenced in the procyclic form, the cells were unable to move directionally, but exhibited only a minor cytokinesis defect. In contrast to the bloodstream form parasites, which failed cytokinesis at the beginning of the process, the procyclic cells failed cytokinesis at the end. The trypanin deficient procyclic cells remained attached at the tips, but were able to be separated by mechanical agitation (Ralston and Hill 2006). The phenotype of procyclic cells lacking FLA1 protein is more similar to that of the bloodstream trypanin knockdown. This suggests that something more than directional motility is lost when FLA1 is silenced that additionally disrupts cytokinesis.

The flagellar detachment component of the FLA1 phenotype is exciting because it supports the previous immunoflourescence evidence (Nozaki *et al.* 1996) suggesting that FLA1 is part of the flagellar attachment zone. Further studies could employ immunoflourescence to probe specifically for FLA2 to identify where it localizes relative to the FAZ and FLA1.

Due to difficulties caused by contamination, the FLA2 plasmid transfection was delayed. FLA2 was eventually transfected successfully but again due to time constraints the data is at this time unavailable.

In the future, it may be worthwhile to pursue the biochemical differences between FLA1 and FLA2 by elucidating their protein structural domains. FLA2 has been predicted to be approximately 73% similar to FLA1 at the amino acid level, with the most notable difference being a 44 amino acid long insertion in the middle of the protein, which is rich in proline residues (LaCount *et al.* 2002). Another related experiment could be to clone the entire FLA2 gene and express it in the FLA1 knockdown line to see whether FLA2 can rescue the FLA1 phenotype.

Directional Motility

Although trypanosomes have previously been observed to begin forming aggregate colonies within minutes of being plated (McLelland, personal communication), in this study colonies could only be observed after a period of hours. After colony formation, the motility of both colonies and single trypanosomes seemed greatly reduced, and approximately ten minutes after the end of the time-lapse sequence, the cells appeared to be lysed. This could have occurred for several reasons. The temperature of the room where the microscope was located was not optimal for procyclic trypanosomes, due to the temperature restrictions of the equipment and the danger of overheating. Consequently, the room temperature was approximately 21˚C, about 6˚C lower than the optimal growing temperature for procyclic trypanosomes. In the future finding a way to raise the temperature of the stage area of the microscope would be beneficial. Another factor that may not have been optimal for the cells was the microscope

light, which remained on for the duration of the time-lapse sequence, and may have caused the agarose gel to dry out, lysing the cells.

Once formed, those colonies behaved as previously observed (Hill, McLelland, personal communication). When the cells began to form colonies, they formed relatively quickly, over a period of two minutes. Figure 13 shows a group of random cells coalescing over a period of two minutes to form colonies. Although the motility of all of the cells was greatly diminished by the time colonies were able to form, I was able to document several of the behaviors previously observed, including a single cell joining a colony, in Figure 14, and two colonies merging, in Figure 15.

It is probable that there is a signaling pathway involved in colony formation, because of the concerted movement of cells to aggregate into a colony, and their directional behavior once the colony is formed. It is possible that the temperature, light or nutrient conditions interfered with this pathway. If this is true, it might explain why the formation of colonies was only observed after a long period of time. It could be that it took longer for critical signaling molecules to accumulate in the environment, or that when the cells are under high levels of stress, or are dying off that they secrete more of these factors. This could explain why the cells formed colonies only shortly before they appeared to be lysed.

As previously mentioned, the cyclic AMP signal transduction pathway is a candidate for mediating a chemotaxis response. cAMP is an important secondary messenger in many eukaryotic cells. It is produced by the enzyme adenylate

cyclase from ATP and can be degraded by phosphodiesterases (PDEs). In humans, cAMP is responsible for cellular responses to hormones like glucagons, insulin, and adrenaline (Robinson *et al.* 1968)

In *D. discoideum* chemotaxis is mediated by cAMP waves (Robinson *et al.* 1968). It is thought to be a mediator for chemotaxis in trypanosomes for several reasons. Many of the molecules associated with the cAMP signal transduction pathway have been found in the trypanosome genome. This is also true for closely related organisms such as *T. cruzi* and *L. major* (D'Angelo *et al.* 2004)

In the *T. brucei* genome, five genes coding for PDE proteins have been identified. One of these, called TbrPDEB1 has been shown to localize almost exclusively to the flagellum, specifically the paraflagellar rod (Oberholzer *et al.* 2006). Due to the special sorting of molecules in the three main divisions of the trypanosome cell membrane, and the previously stated hypothesis that the flagellum functions as a sensory organ, the presence of a key component of the cAMP signaling cascade exclusively in the flagellum is encouraging evidence that such a model may in fact be true. Although the cAMP signal transduction pathway has not yet been definitively shown to play a role mediating directed motility in *T. brucei* its location in the flagellum makes it a strong theoretical candidate.

Conclusion

African trypanosomiasis is a problem of epidemic proportions in parts of Sub-Saharan Africa. This study has shown that the FLA1 mutant phenotype in *T. brucei* is identical to the FLA1/FLA2 mutant phenotype that was previously observed. Additionally, a form of directed motility response to other trypanosomes was documented that could indicate that trypanosomes possess other forms of directional motility, such as chemotaxis. These findings could one day lead to the discovery of a new drug target against trypanosomes.

Appendix A: p2T7
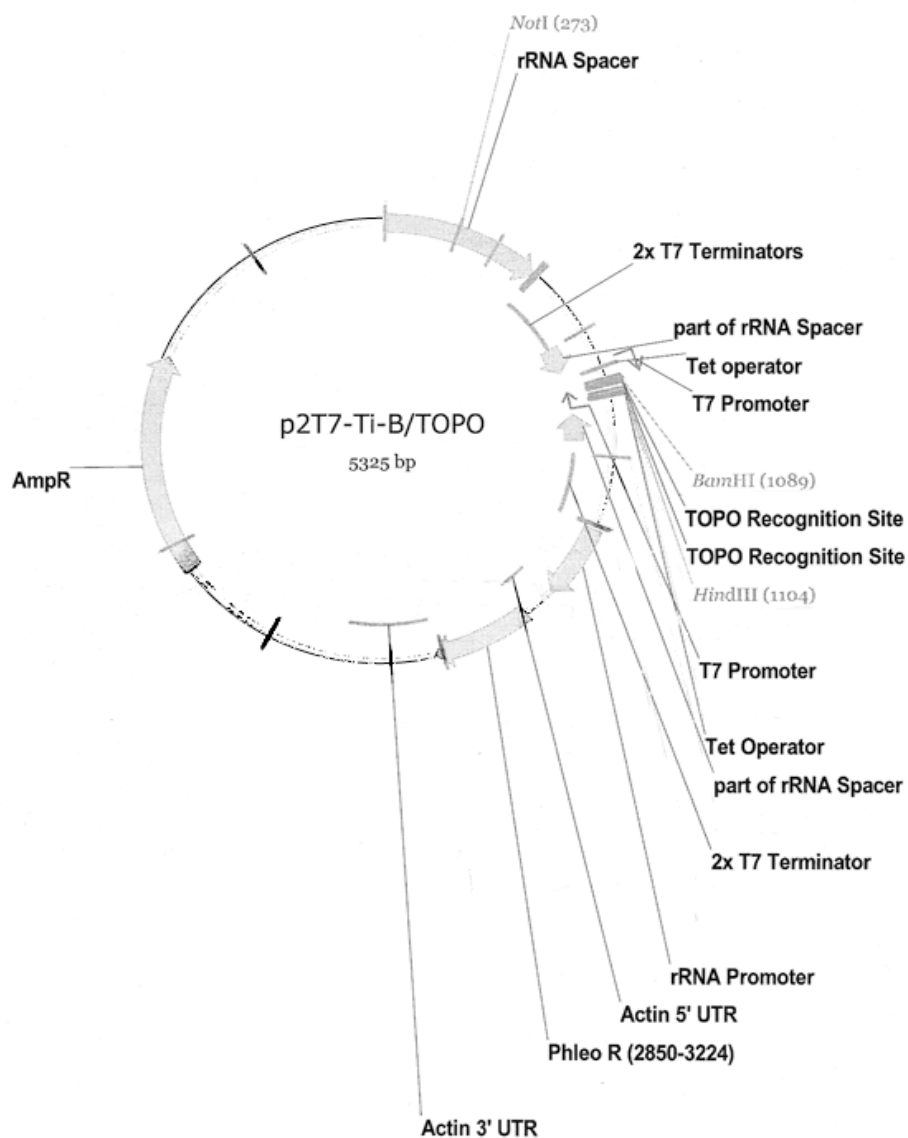
A detailed diagram of p2T7

This diagram shows relevant sites on the p2T7 plasmid including the restriction enzyme sites for *Not*I, *Bam*HI, and *Hin*dIII. The T7 promoter transcription directions are indicated with thin arrows.

Appendix B: Trypanosome Plating Protocol

Agarose Preparation

To make 10ml of the agarose plating solution, first combine 8mg of agarose with 0.92ml distilled water and adjust by weight to 1g. To prepare the medium, combine 3.75ml fresh medium, 3.75ml spent medium, and 1.5ml FBS. Heat the now conditioned medium to 55˚C. Melt the agarose solution and combine with the conditioned medium. Keep this solution at 55˚C for approximately 10 minutes, swirling occasionally.

Plate Preparation

Pour approximately 4ml of agarose solution into each 60x15mm plate. Let the plates cool, covered, for 10-15 minutes or until set. Remove the covers in a laminar flow hood, or a similar area where moisture can evaporate from the plates without dust falling into them. The plates should dry for 5-10 minutes so that the optimal 0.8% concentration of agarose is achieved.

Cell Preparation

Count the cells, and adjust the concentration accordingly, avoiding centrifuging the cells. Plate between 25 and 50μl of the resulting cell suspension. Spread using a sterile glass spreader if desired.

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