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# Establishment of a tRNA over-expression system in *Trypanosoma brucei* to study the role of post-transcriptional modifications on function.

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for graduation
*with research distinction* in Microbiology in the undergraduate colleges
of The Ohio State University

by

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ABSTRACT

Trypanosomatids are parasitic protozoa which infect millions of humans and animals across the globe every year. The two primary types of these unicellular flagellates are *Trypanosoma* and *Leishmania*, and the World Health Organization estimates that approximately 400 million people worldwide are at substantial risk to develop infections caused by these pathogens.

Trypanosomes also possess many significant biological and biochemical properties, and in particular the tRNAs of trypanosomatids undergo substantial *in vivo* chemical modifications. We focus on understanding how one particular set of modifications, i.e. adenosine to inosine changes in the anticodon, impact tRNA function. We are also interested on how other modifications like methylations may affect inosine formation.

The goals of this project are two-fold: first, to construct a vector capable of successfully over-expressing tRNA in *Trypanosoma brucei*, and second, to successfully introduce two non-endogenous, tagged tRNAs into *T. brucei* in order to further analyze *in vivo* modifications. To this end, we have engineered two distinct tRNA variants with mutations in the anticodon arm (Tag 22) and anticodon arm and loop (Tag 23). These mutations serve as tags to differentiate them from endogenous tRNAs naturally found in *T. brucei* cells. Prior to testing our overexpression system, the two "tagged" variants were analyzed for their ability to support known modification reactions *in vitro*. Since these variants are derived from a tRNA that undergoes adenosine to inosine changes in their anticodon *in vivo*, we tested and further showed that the mutations used to generate the

variants do not interfere with this reaction *in vitro*. In the next phase of the project, the two variants will be cloned in a plasmid vector and expressed under the control of an endogenous T7 promoter and tetracycline (TET) operator, followed by transfection into *Trypanosoma brucei* cells. Total RNA isolated from the transfected cells will thus provide a measure of overexpression of our two tRNA variants. Successful completion of this phase of the project will allow further exploration into the *in vivo* posttranscriptional modifications of tRNAs through analysis of the tagged tRNA constructs. By comparing the tagged tRNA construct to endogenous tRNAs, important modification reactions such as adenosine to inosine and cytidine to uridine editing can be further studied. As mentioned earlier, since these modifications play a vital role in the function of tRNA, their identification could be useful for future design of anti-trypanosomal treatments.

**Dedicated to my parents, Cliff and Ellyn Fill, as without their guidance and unfaltering support throughout the last 21 years, I know that I would not be the person that I am today.**

## ACKNOWLEDGEMENTS

I would first and foremost like to thank my family, as they have always encouraged me to follow my dreams and never accept anything but the best from myself. Without their love and support I never would have had the courage to attend The Ohio State University and become involved in undergraduate research.

I also could not imagine my life at Ohio State without all of the friends who have touched my life. Each person I have met here has made an impact on me and influenced my life in some way. There are, of course, certain individuals who have made a lasting impact on me and helped shape me into the person that I have become in the last four years and I will never forget them.

There is no way that I can begin to thank all of the members of the Alfonzo lab for all of their support and friendship throughout the last two years. They have truly been a second family to me, and I have found stronger relationships there than I ever thought possible.

Last but not least, I could not imagine my research experience the past two years without Juan and Mary Anne. You have both been like a second set of parents to me, and I cannot tell you how much I appreciate your wisdom, intelligence, support, career advice and friendship. There is no doubt in my mind that we will stay in touch, and I wish you both the best of luck in everything that you do.

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LIST OF ABBREVIATIONS

PCR – Polymerase Chain Reaction

RNA – Ribonucleic Acid

tRNA – Transfer Ribonucleic Acid

Thr – Threonine

Tet – Tetracycline

Amp – Ampicillin

$Amp^R$ – Ampicillin resistance

Phleo – Phleomycin

TLC – Thin Layer Chromatography

Tb – *Trypanosoma brucei*

Chloramphenicol acetyltransferase – CAT

Prehyb – Pre-hybridization

Hyb – Hybridization

ssDNA – Single stranded DNA

# CHAPTER 1

## Overview of the Trypanosomatids

### 1.1 Introduction

In the class kinetoplastida, the trypanosomatids are unicellular eukaryotes marked by the presence of a kinetoplast, a mass of DNA located within a single mitochondrion. The trypanosomatids are a group of flagellated protozoa, which cause a wide range of parasitic diseases, both in humans and in animals. The three primary human infections caused by trypanosomatids are African Sleeping Sickness, or African Trypanosomiasis (causative agent *Trypanosoma brucei*), Chagas Disease (causative agent *Trypanosoma cruzi*) and the cutaneous, mucocutaneous and visceral forms of leishmaniasis (causative agent *Leishmania* species). The primary regions of infection are Central and South America, Africa, the Mediterranean Basin, the Middle East and parts of Asia. Each parasitic infection is transmitted to a human host by an insect vector indigenous to each particular region. In recent years, The World Health Organization has estimated that over 30 million people around the world are infected with these three diseases, and that over 500 million more are at risk for infection (Table 1.1).

| Disease | Causative Agent | Primary Geographic Location | Number Currently Infected | Number At Risk Of Infection Per Year |
|---|---|---|---|---|
| **African Trypanosomiasis** | *Trypanosoma brucei* | Africa | 50,000-70,000 | 60 million |
| **Chagas Disease** | *Trypanosoma cruzi* | Central and South America | 16-18 million | 100 million |
| **Leishmaniasis** (Visceral, Cutaneous and Mucocutaneous) | *Leishmania* (variety of subspecies) | Middle East, Africa, South America, China, India, and Mediterranean Basin | 12 million | 350 million |

Table 1.1: Analysis of the distribution and scope of human diseases caused by parasitic protozoa in the order Trypanosomatida.

## 1.2 Historical Overview of RNA Editing and Modification

Beginning in the 1950's, scientists began to make important discoveries concerning modified nucleosides in nucleic acids. Hotchkiss (1948) discovered the first modified nucleoside in DNA, which was later identified by Wyatt (1950) as 5-methylcytosine ($dm^5C$). In 1956, Davis and Allen (1957) isolated 5-ribosyluracil, an isomer of uridine, later designated pseudouridine (ψ) by Cohn (1960), marking the first modified nucleosides identified in RNA. Since those discoveries in the mid-20$^{th}$ century, numerous RNA modifications have been identified, and it has been established that mRNA, tRNA, rRNA and other small RNAs all undergo modification (Table 1.2). The majority of RNA modifications occur posttranscriptionally, and many modifications have important biochemical properties that can affect the folding, function or interactions of ribonucloeosides. However, it is also important to note, that many of the known RNA

modifications have no identifiable impact on cellular metabolism, leading to many questions concerning their purpose.

| RNA | Source | | |
|---|---|---|---|
| | Archaea | Bacteria | Eukarya |
| tRNA | 36 | 42 | 47 |
| rRNA | | 1[a] | 4[a] |
| SSU | 11 | 8 | 18 |
| LSU | 8 | 14 | 12 |
| 5S | 3 | | 1 |
| 5.8S | | | 5 |
| mRNA | | | 13 |
| tmRNA | | 2 | |
| snRNA | | | 11 |
| Chromosomal RNA | | | 2 |
| Other small RNA | | | 1 |

[a] Subunit origin not shown, or is reported in a mixture of SSU and LSU rRNAs.

Table 1.2: Phylogenetic distribution of modified ribonucloeosides reported in RNA
Adapted from Rosenski, et al. 1999.

In 1986, Benne and collaborators discovered a surprising RNA modification in the mitochondrial mRNA of kinetoplastid protozoa. They showed evidence of the addition and deletion of several uridine residues in the mitochondrial mRNA that were not encoded in the organism's genome. Shortly after this discovery, the conversion of cytidine to uridine (Powell et al. 1987; Chen et al. 1987) and the conversion of adenosine to inosine (Bass and Weintraub 1988; Wagner et al. 1989) were identified in human mRNA and viral RNAs respectively. This sub-class of posttranscriptional modifications was later designated RNA editing, a term which refers to the "posttranscriptional alteration of sequence information in mRNA beyond what is encoded in the DNA genome from various

organisms" (Benne et al. 1986). Shortly after the identification of nucleotide changes in mRNA, similar alterations were noted in non-coding RNA, such as tRNA, instigating a new assessment of the definition of RNA editing to include "any alteration in the sequence of an RNA (coding or non-coding) that leads to the introduction of one of the four canonical nucleotides" (Covello and Gray 1998).

In addition, there have been repeated attempts to distinguish between RNA editing and modification, as the two have been lumped into the same category since their original discovery. A more literal definition of editing has been assumed in recent years as any sequence alteration that changes the genetic meaning of a transcript, and modification has been limited only to structural change (Grosjean and Bjork 2004).

### 1.3 Applications to tRNA

Transfer RNAs play a crucial role in the translation of the genetic code (as mRNA) into functional proteins. However, it is important to note that tRNAs undergo an extensive maturation process which allows them to function at their highest potential within the cell. Of all of the types of RNA, the most modifications have been identified with respect to tRNAs. To date, over 100 posttranscriptional modifications have been identified in Bacterial, Archaeal and Eukaryotic tRNA, and it is estimated that there are many more which are yet to be isolated. Most of the identified modifications appear to play a role in tRNA structure and appear to assist in achieving accurate folding. However, many of these modifications are not necessary for cell survival, leading to their classification as structural modulators.

Although the other major type of RNA modification, RNA editing, was first discovered in mRNA, it has since been found in the tRNAs of many species. Unlike many other modifications discussed earlier, tRNA editing is frequently essential for cell viability. To date, editing of tRNA has been found at many positions in the classical tRNA structure, including: the acceptor stem, tRNA backbone and anticodon stem and loop. This was first reported in 1993 when Gray and co-workers published data supporting the addition of nucleotides at the acceptor stem of *A. castellani* tRNAs (Lonergan and Gray 1993). However, some of the best studied tRNA editing events are the cytidine to uridine (C to U) changes that occur in the tRNA backbone in various plant species (Fey et al. 2002).

## 1.4 Applications to trypanosomatids

Although RNA editing was first discovered in the kinetoplastids (Benne et al. 1986), the majority of work done on RNA editing has been in plants, animals, bacteria and yeast. However, the trypanosomatids have been a source of many important scientific discoveries including: RNA editing, mRNA trans-splicing, Eukaryotic poly-cistronic transcription, and a mechanism for large-scale mitochondrial tRNA import. The relatively recent discovery of C to U editing *in vivo* of a non-coding RNA, $tRNA^{Trp}$, began a period of intense investigation into RNA editing and modification events in trypanosomatids. Since then, numerous editing events have been isolated in trypanosomal tRNA, including cytidine to uridine (C to U) and adenosine to inosine (A to I) base changes in $tRNA^{Thr}$ by hydrolytic deamination (Figure 1.1). In 2006, Rubio and colleagues showed that C to U editing at position 32 of the anticodon loop stimulated A to I editing at position 34 in *T. brucei* $tRNA^{Thr}$ (Figure 1.2). The mechanism of inosine formation at position 34 has been

relatively well defined in eukaryotic systems, and it is known that the enzymes ADAT2 and ADAT3 perform the hydrolytic deamination of adenosine. This editing event is fairly common as editing at position 32 allows "wobbling" of the tRNA anticodon in order to recognize certain mRNA codons for which there is no complementary tRNA. For example, in *T. brucei*, there are four codons which code for threonine: ACC, ACU, ACG and ACA. However, there are only three $tRNA^{Thr}$, with anticodons: AGU, CGU and UGU. When $tRNA^{Thr}$ (AGU) undergoes A to I editing at position 32, the inosine can effectively wobble to bind with the ACU mRNA codon. Therefore this editing event is critical for effective translation of the ACU threonine codon in *T. brucei*.

Although *in vitro* assays show that ADAT2/ADAT3 enzymes can successfully deaminate cytidine, and a deamination motif similar to that in adenosine has been found in cytidine; the exact deamination mechanism of C to U has not been completely defined.

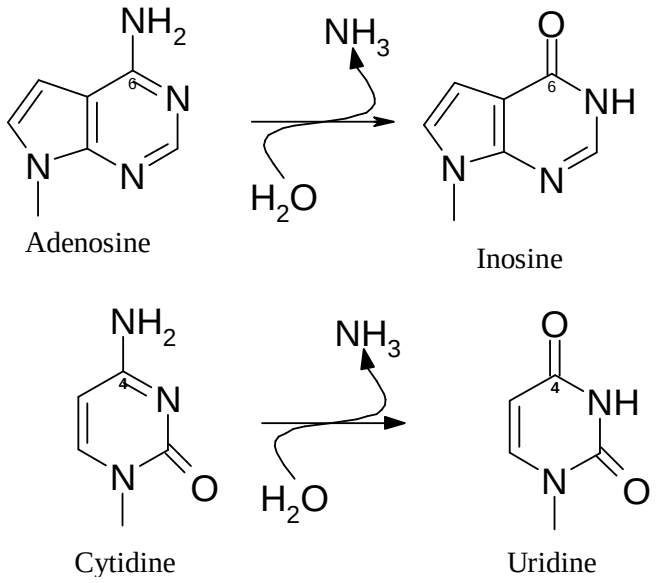


Figure 1.1: Mechanism of hydrolytic deamination of adenosine to inosine, and proposed mechanism of hydrolytic deamination of cytidine to form uridine. The deamination

enzymes have an active site which contains a proton shuttling glutamate capable of forming a complex between water and zinc to create a nucleophile. This nucleophile attacks at the C6 carbon in adenosine and removes an ammonia group creating inosine.

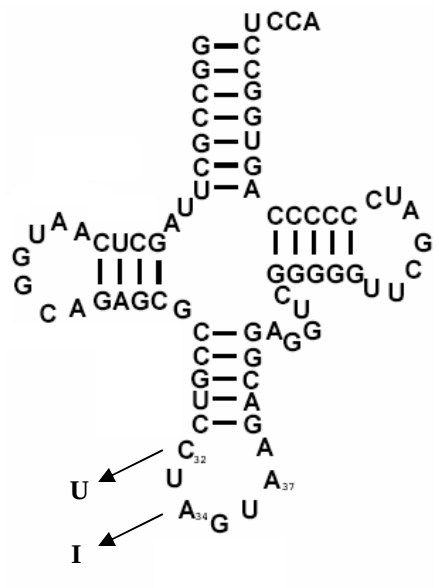


Figure 1.2: Editing events in $tRNA^{Thr}$ (AGU): cytidine to uridine (C to U) and adenosine to inosine (A to I) editing.

## 1.5 *In vitro* challenges

Although it is possible to synthetically modify and edit tRNAs, it is costly. In addition, it is only possible to synthesize RNA with modifications which are available as a phosphoramidite. There are undoubtedly hundreds of more chemical modifications and editing events which are currently unknown to scientists, yet, which play a substantial role in the molecular biology of the cell. It is much more reasonable to create a system in which the cell's machinery can perform all of the editing and modifications itself. There is optimism that an editing pathway unique to *Trypanosoma brucei* could be utilized in the future as a target for effective anti-trypanosomal treatments.

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# CHAPTER TWO

## The Creation of a tRNA Over-Expression System in *Trypanosoma brucei*

### 2.1 Introduction

The global effect of *Trypanosoma brucei* infections has been established in Chapter One of this thesis. However, it is now important to assess potential targets to anti-trypanosomal treatments. There are many editing and modification processes present *in vivo* in *T. brucei*, and there is optimism that a unique editing pathway could be an excellent drug target. In order to achieve that goal, it is crucial to continue to identify editing and modification events, to understand their mechanisms and to isolate a particular event specific only to *Trypanosoma brucei*.

The goals of this project were two-fold. The first step was to create two unique sequence-tagged variants of *T. brucei* $tRNA^{Thr}$. These constructs would then be tested for *in* vitro A to I editing. The second step was to construct an over-expression plasmid capable of creating a high concentration of these tagged tRNAs which would be edited and modified by *T. brucei* cellular machinery (Figure 2.1).

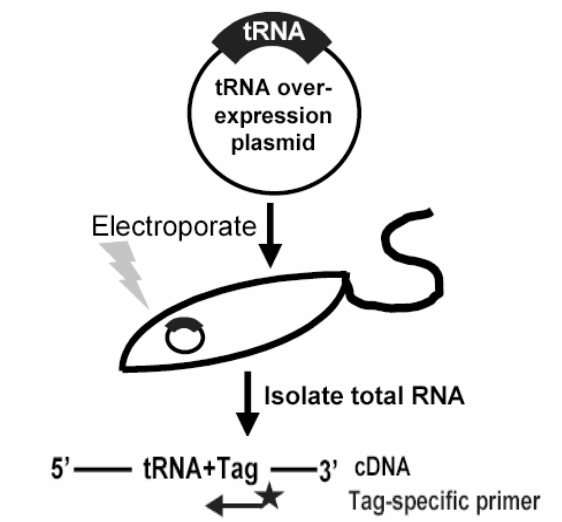


Figure 2.1: Schematic representing project design.

## 2.2 Methods

### 2.2.1 Creation and Testing of Tagged tRNAs

In order to identify and isolate modified tRNAs, two distinct $tRNA^{Thr}$ mutant constructs were created with alterations made to the anticodon arm (Tag 22) and anticodon arm and loop (Tag 23) of tRNA threonine (AGU) (Figure 2.2). However, it was critical that the sequence tags be in such a location in the tRNA as to not affect potential editing and modification by *T. brucei* machinery.

In order to confirm that that the sequence tags did not interfere with editing events, an assay was performed to measure the capability of the tagged tRNAs to undergo inosine formation at position 34 (Figure 2.3). [α-32P]-ATP uniformly labeled tRNAs were incubated with Tb ADAT2/3 enzymes for 1 hour at 27°C. Subsequently, the tRNAs were digested with Nuclease P1 to generate 5'-monophosphates, and were then separated by one-dimensional thin layer chromatography (TLC) and visualized by PhosphoImager

analysis. TLC analysis showed that inosine formation at position 34 of the tagged $tRNA^{Thr}$ constructs proceeded in a normal fashion, indicating that the sequence tags did not interfere with the mechanism of inosine formation.

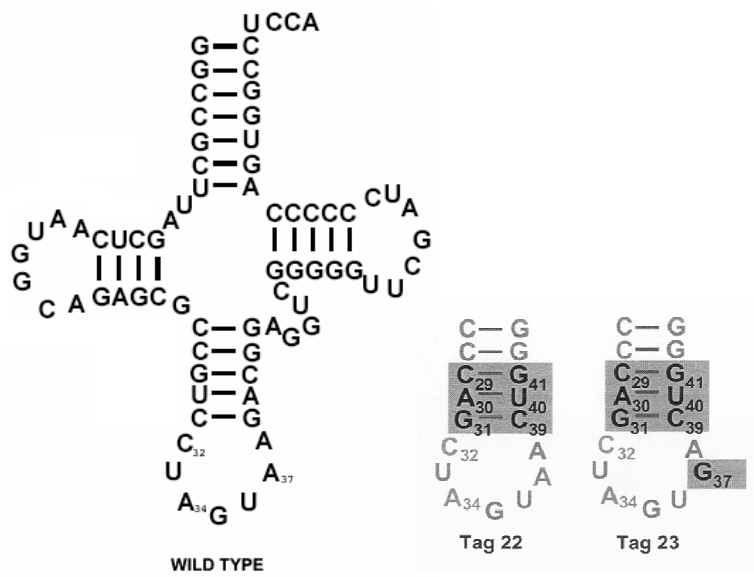


Figure 2.2: Schematic of distinct $tRNA^{Thr}$ (AGU) constructs with base changes highlighted in gray.

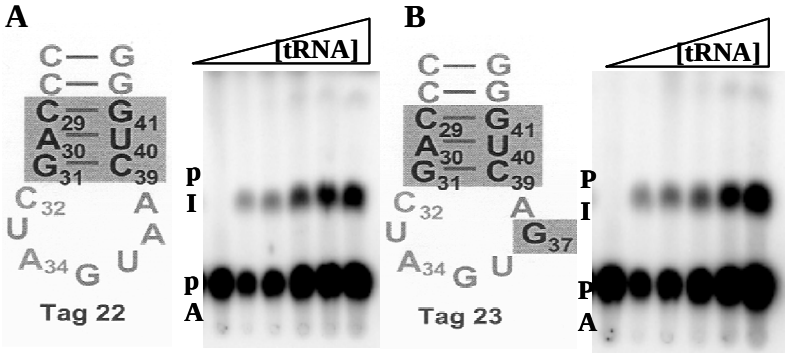


Figure 2.3: *Trypanosoma brucei* $tRNA^{Thr}$(AGU) A) Tag22 and B) Tag 23 constructs. Lane 1 is a no enzyme control with cold marker, and lanes 2-6 are in the presence of

TbADAT2/3 enzymes and increasing concentrations of tRNA (5, 10, 20, 40, 80 picomoles, respectively).

2.2.2 Vector Selection and Evaluation

Although numerous vectors were evaluated for their potential use as an over-expression system, ultimately the pLEW82 plasmid was chosen for over-expression of the tagged $tRNA^{Thr}$ constructs (Figure 2.4). The original pLEW82 plasmid contains a large luciferase gene region under the control of an endogenous 10% T7 promoter and tetracycline (TET) operator, as part of a TET "On" system. Two T7 terminators are present at the end of the coding region to ensure effective termination (Figure 2.5). Other advantages of the pLEW82 plasmid include multiple recognition sites for numerous restriction enzymes and resistance markers for ampicillin ($Amp^R$), for selection purposes in *Escherichia coli* and phleomycin (Phleo), for selection purposes in *Trypanosoma brucei*. In addition, it was hypothesized that the regulatory mechanisms present in pLEW82 should be able to effectively control gene expression within the selected *Trypanosoma brucei* cell line.

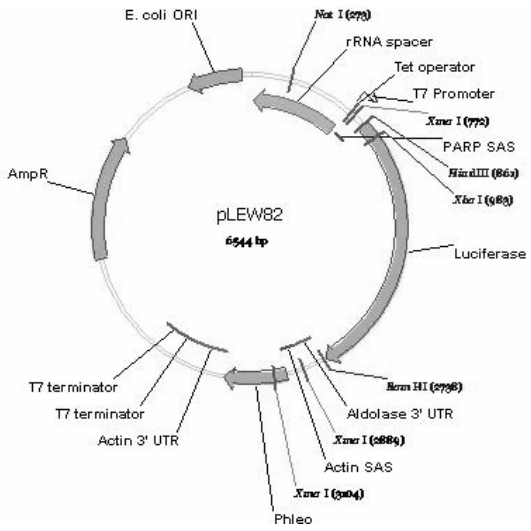


Figure 2.4: Schematic of the pLEW82 plasmid
Adapted from Wirtz and Cross, 1999.

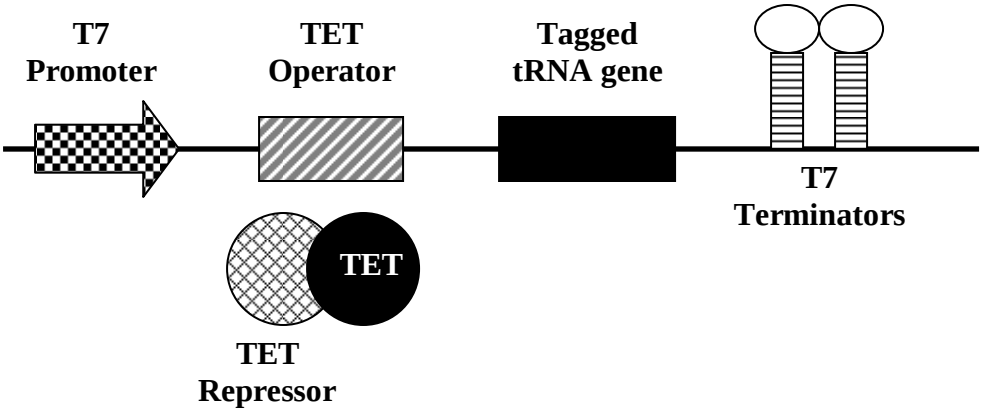


Figure 2.5: Schematic of the transcriptional control elements present in the pLEW82 plasmid.

Before proceeding with cloning the tagged tRNA constructs into the selected plasmid, a separate assay was performed to test the effectiveness of the transcriptional control elements present in pLEW82. There was some concern that T7 RNA polymerase would not be fully functional or achieve optimum control in a Eukaryotic system such as *Trypanosoma brucei*. To test this, a chloramphenicol acetyltransferase (CAT) reporter assay was performed by cloning the CAT gene into pLEW82 and measuring chemiluminescence levels at varying time points throughout an acetylation assay (Figure 2.6). The CAT assay showed that T7 RNA polymerase was fully functional in *T. brucei* and that transcriptional control could be achieved in a tetracycline-dependent manner.

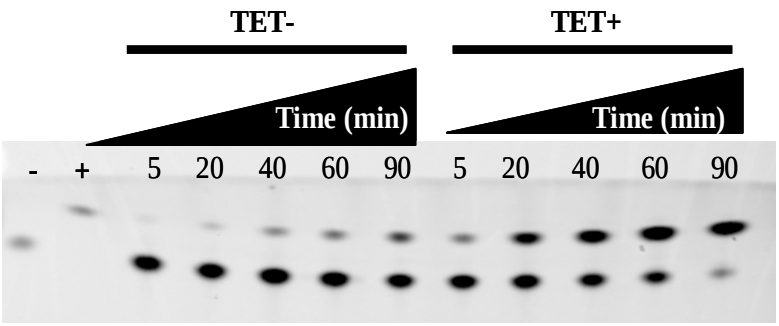


Figure 2.6: *Trypanosoma brucei* cells are capable of expressing active T7 RNA Polymerase in a tetracycline-dependent manner. This is demonstrated by using a chloramphenicol acetyltransferase (CAT) reporter assay.

2.2.3 Cloning

In order to effectively clone the tagged tRNA constructs into a *Trypanosoma brucei* cell line, the following cloning scheme was used. The tagged tRNAs were first ligated into TOPO®, then transformed into *Escherichia coli* DH5α competent cells, and digested with *EcoRI* restriction endonuclease to confirm the correct fragment size (Figure 2.7). Following completion of that phase of cloning, each tagged tRNA was ligated into pLEW82, transformed into *Escherichia coli* DH5α competent cells and digested with *HindIII* and *XbaI* restriction endonucleases. After screening colonies and confirming the desired product size, 250 milliliters of two selected clones were midi-prepped and run through a QIAGEN column to extract purified plasmid DNA.

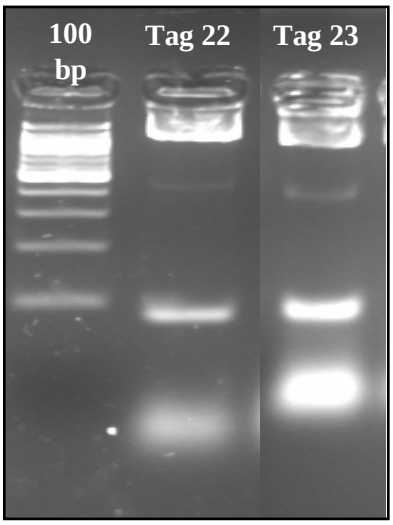


Figure 2.7: 1% Agarose gel confirming sizes of Tag 22 and Tag 23 tRNA constructs in TOPO®.

2.2.4 Transfection

Following isolation of purified plasmid DNA, *Trypanosoma brucei* cells were prepped for electroporation.

After transfection, the *T. brucei* cells were recovered for a period of two to three weeks, allowing them sufficient time to repair from the electroporation and incorporate and translate the plasmid.

2.2.5 Preparations for Northern Blotting

Following the recovery period, stable transfectents were chosen to extract total RNA from. The *T. brucei* cells were spun down, and total RNA was isolated by guanidine extraction techniques. Next, the RNA was run on a denaturing acrylamide gel and the area where tRNAs are known to migrate was cut out and transferred to a membrane (Figure 2.8).

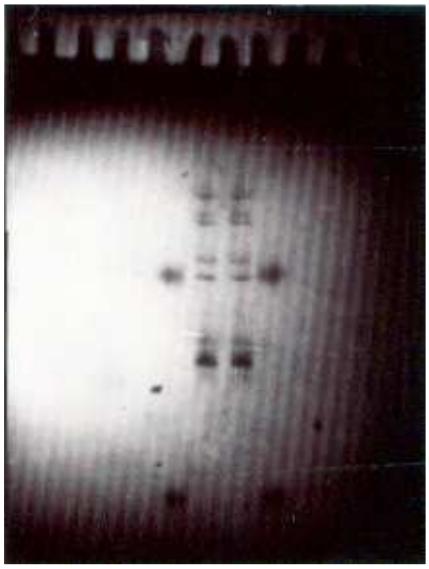

Figure 2.8: 6% Acrylamide RNA gel. Markers are loaded on either side of total RNA samples.

In order to perform Northern blots, a probe was constructed which would ideally hybridize only to its complementary sequence of either the tagged $tRNA^{Thr}$ sequences of Tag 22 and Tag 23 or wild-type $tRNA^{Thr}$. Initially two probes were created, WT 833R and TAG 834R; however a third probe was also constructed, TAG 677R (Figure 2.9). The WT 833R probe was designed to hybridize to wild type $tRNA^{Thr}$ on the D loop and anticodon stem, whereas the TAG 834R probe was constructed to hybridize to the same region of the tagged $tRNA^{Thr}$ construct. The third probe, TAG 677R, was designed to hybridize to the TΨC loop, variable loop and anticodon stem of the tagged $tRNA^{Thr}$ construct. Each probe was end-labeled with Gamma $^{32}$P-ATP, and evaluated with the scintillation counter to achieve satisfactory radioactivity levels (normally 3.00 – 4.00 x $10^5$ counts/ml).

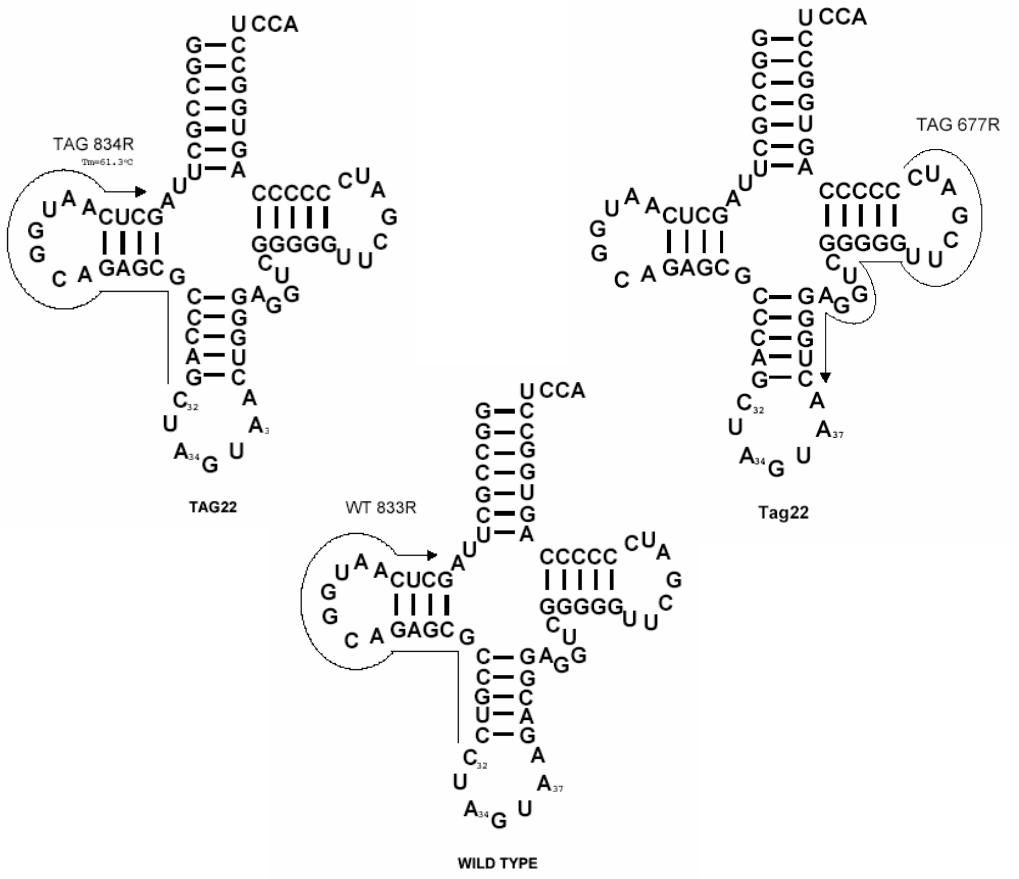


Figure 2.9: Maps of distinct oligonucleotide probes created for hybridization to wild type or tagged constructs in Northern blotting.

2.2.6 Northern Blotting

The membrane was pre-hybridized for at least 30 minutes with pre-hybridization (prehyb) solution (150µl per $cm^2$ of membrane) and ssDNA (10µl per ml prehyb) at 45ºC. The membrane was then hybridized with prehyb solution (150µl per $cm^2$ of membrane), ssDNA (10µl per ml prehyb/hyb) and end-labeled probe ($10^6$ cpm/ml of hyb) overnight at 45ºC. Following overnight hybridization, the hyb was saved and the membrane was washed with solutions 1, 1.5, 2.0 and stripping solution to achieve optimum hybridization of the probe to the desired tRNA (350µl wash required per $cm^2$ of membrane).

Visualization of hybridization was achieved by using the Phospho-Imager and ImageQuant analysis.

## 2.3 Discussion

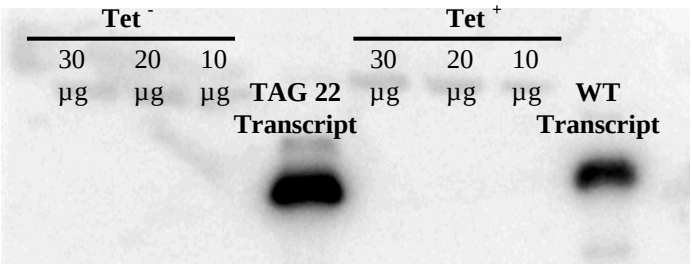


Figure 2.10: Northern blot using TAG 834R and WT 833R probes. Lanes 1-3, Different concentrations of RNA isolated from $Tet^-$ *T. brucei* cells. Lane 4, Tag 22 transcript. Lanes 5-7, Different concentrations of RNA isolated from $Tet^+$ *T. brucei* cells. Lane 8, Wild Type transcript.

There is strong hybridization to the Tag 22 transcript with the TAG 834R probe and substantial hybridization to the wild type transcript with the WT 833R probe. However, there is little to no significant hybridization to the $Tet^+$ and $Tet^-$ *T. brucei* cell RNA extracts.

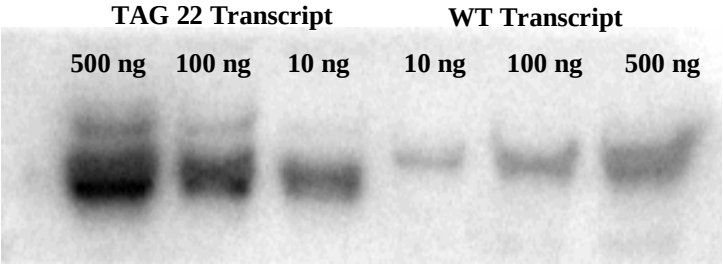


Figure 2.11: Northern blot using TAG 677R probe. Lanes 1-3, Different concentrations of Tag 22 transcript. Lanes 4-6, Different concentrations of Wild Type transcript.

Using a different oligo designed to hybridize to the TΨC loop, variable loop and anticodon stem of the sequence tagged $tRNA^{Thr}$; Tag 22 transcript and WT transcript were probed. Significantly different levels of hybridization were achieved between the sequence tagged and wild type transcripts.

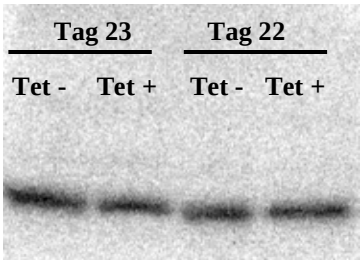


Figure 2.12: Northern blot using TAG 677R probe. Lane 1, $Tet^-$, Tag 23 cells isolated from *T. brucei*. Lane 2, $Tet^+$, Tag 23 cells isolated from *T. brucei*. Lane 3, $Tet^-$, Tag 22 cells isolated from *T. brucei*. Lane 4, $Tet^+$, Tag 22 cells isolated from *T. brucei*.

RNA was isolated from four different *T. brucei* cell cultures: Tag 23, $Tet^-$; Tag 23, $Tet^+$; Tag 22, $Tet^-$; and Tag 22, $Tet^+$; and probed with the TAG 677R probe. Significant levels of hybridization were achieved with all cell cultures, although no significant difference was observed between $Tet^+$ and $Tet^-$ cultures.

Thus far, the work that has been completed on the over-expression plasmid has yielded several conclusions. First, we can conclude that T7 RNA polymerase is fully functional in the *Trypanosoma brucei* cell line, and that the transcriptional control elements present in the pLEW82 plasmid effectively control transcription and in a tetracycline dependent manner. Second, we can conclude that both sequence tags on the $tRNA^{Thr}$ (AGU) constructs do not affect inosine formation *in vitro*. We have also established through Northern blot analysis that the probe to the sequence tag of $tRNA^{Thr}$ (AGU) is specific for the tagged tRNA constructs.

Although the $tRNA^{Thr}$ (AGU) over-expression vector is fully functional, further optimization of the current version of this vector is needed to achieve maximal over-expression.

## 2.4 References


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# CHAPTER 3

## Conclusions and Future Work

Although many discoveries have been made in the study of RNA editing and modification, there is still much work to be done. By creating large amounts of tagged tRNA which is edited and modified by the *Trypanosoma brucei* cellular machinery, there is optimism that more can be discovered about these editing and modification events.

Several substantial steps have been made in the creation of an over-expression vector. It has been shown that T7 RNA polymerase is fully functional in *T. brucei*, and that transcriptional expression can be controlled in a tetracycline-dependent manner. It has also been shown that the sequence tagged $tRNA^{Thr}$ (AGU) constructs undergo normal adenosine to inosine formation and that the probe created for Northern blot analysis is specific for the tagged constructs, Tag 22 and Tag 23.

Although these are substantial steps toward creating the ideal over-expression system, there are still more optimizations which can be made to the plasmid. We plan to insert an integration site into the plasmid so that the contents of the plasmid will undergo homologous recombination and be inserted into the *T. brucei* chromosome. Other research has been done concerning potential replacement of the mutant 10% T7 promoter endogenous to pLEW82 with either a *T. brucei Parp* promoter or WT 100% T7 promoter to achieve maximum over-expression.

Once optimum over-expression levels have been achieved, there are many potential directions to be undertaken. Larger quantities of *in vivo* modified tRNAs can be isolated for further analysis of their chemical modifications. These modified tRNAs may then be used to study editing pathways to determine specific components of the editing machinery. Although many of these pathways remain unknown, the underlying goal is to find novel editing mechanisms unique to *Trypanosoma brucei* as a stepping stone towards the design of ideal drug targets to combat disease.

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