Determinants of Specificity for the *Trypanosoma brucei* A to I tRNA Editing Deaminase

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by

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Dedicated to members of both my family and the Alfonzo lab. Without their guidance, support and encouragement, I would have never been able to complete this project.

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ABSTRACT

Trypanosomatids are responsible for causing illness and death among millions of humans and animals worldwide. Two main genera of these unicellular flagellated parasites are *Trypanosoma*, which cause African sleeping sickness and Chagas disease, and *Leishmania*, which cause Leishmaniasis. The World Health Organization estimates that approximately 400 million people across the globe are at risk of infections caused by these protozoa.

In addition to their pathogenic characteristics, trypanosomatids also represent some of the earliest mitochondria-containing eukaryotes. Trypanosomatids exhibit many significant and interesting biological processes, such as an extreme degree of RNA editing (as with U insertion and deletion in mRNAs) and complete tRNA import into the mitochondria, which is important for mitochondrial protein synthesis. In translation, although there are 61 amino acid codons, there is only a small subset of tRNAs to decode them. As a result, some flexibility is needed to allow for a single tRNA to decode multiple codons—as proposed by Francis Crick with the Wobble hypothesis in the 1960s (1). One nucleotide capable of increasing pairing flexibility during decoding is the nucleotide inosine, which in the third position of the tRNA anticodon, allows recognition of up to three different nucleotides in an mRNA codon: uridine, cytosine, and adenosine. The editing event that leads to inosine formation in tRNAs, is catalyzed by a heterodimeric enzyme known as ADAT2/3 (adenosine deaminases acting on tRNA). Remarkably, inosine is not encoded in DNA and is only found in RNAs as a result of an enzyme-mediated reaction, which is essential for viability.

The goal of this project was to analyze the effects various mutations at the anticodon stem loop of $tRNA^{Thr}$ have on inosine formation at the first (wobble) position of the anticodon of tRNAs in *Trypanosoma brucei*. We hypothesized that mutations at these positions of the tRNA

will decrease deamination efficiency and substrate recognition by ADAT2/3. Through *in vitro* methods, which include protein-RNA binding assays (band-shift assays) and enzyme kinetics the effect of mutations on ADAT2/3 function was evaluated. These results could shed light on how ADAT2/3 binds tRNA and catalyzes the conversion of adenosine to inosine. In addition, these observations can also lead to future design of therapeutics against parasites of such major medical importance (2).

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LIST OF ABBREVIATIONS

RNA – Ribonucleic Acid

tRNA – Transfer Ribonucleic Acid

mRNA – Messenger Ribonucleic Acid

Thr – Threonine

Val – Valine

Arg – Arginine

Tet – Tetracyline

Amp – Ampicillin

Amp^R – Ampicillin Resistance

Phleo – Phleomycin

TLC – Thin Layer Chromatography

Tb – *Trypanosoma brucei*

Ec – *Escherichia coli*

PCR – Polymerase Chain Reaction

CHAPTER 1

Overview of trypanosomatids and RNA editing

1.1 Trypanosomatids

Trypanosomatids are unicellular eukaryotes that belong to the class kinetoplastida, named after the presence of a unique kinetoplast within the mitochondrion. A kinetoplast, unlike other DNA, is comprised of a complex network of interlocked DNA rings and is associated with the flagellar basal body (Liu et al. 2005). Trypanosomatids are responsible for causing a wide range of diseases across the globe in both humans and animals. The two main genera of trypanosomatids are *Trypanosoma* and *Leishmania*. Of the genus *Trypanosoma*, there are two species that are medically important for human diseases: *Trypanosoma brucei* causes African trypanosomiasis (sleeping sickness) and *Trypanosoma cruzi* is responsible for American trypanosomiasis or Chagas disease. The three forms of leishmaniasis (cutaneous, mucocutaneous, and visceral) are the result of infection by various species of the genus *Leishmania*.

Transmitted by insect vectors, these flagellated parasites cause infection all over the world. However, they are endemic in Africa, Central and South America, Middle East, China, India, and the Mediterranean Basin. The World Health Organization estimates that there are over 30 million people infected by trypanosomatids and even more alarming is that over 500 million are at risk of infection (Table 1.1).

Disease	Causative Agent	Vector	Primary Geographic Location	Number Currently Infected	Number At Risk of Infection Per Year
African Trypanosomiasis (Sleeping Sickness)	*Trypanosoma brucei*	Tse-Tse Fly	Africa	1 million	60 million
American Trypanosomiasis (Chagas Disease)	*Trypanosoma cruzi*	Triatomine Bug (Kissing Bug)	Central and South America	16-18 million	120 million
Leishmaniasis (Cutaneous, Mucocutaneous, and Visceral)	*Leishmania* (various subspecies)	Sandfly	Middle East, Africa, South America, China, India, and Mediterranean Basin	12 million	350 million

Table 1.1: Distribution and scope of trypanosomatid infection.

1.2 Historical overview of RNA editing and modification

RNA editing and modification is a term that encompasses a plethora of molecular processes where information content is altered in an RNA molecule far and beyond what is encoded in genes. RNA editing may involve a change of one nucleotide to another. Currently, such changes have been identified in messenger RNA (mRNA), transfer RNA (tRNA) and ribosomal RNA (rRNA).

The first instance of a modified nucleotide was found in DNA by Hotchkiss (1948), which was later determined to be 5-methylcytosine (dm^5C) by Wyatt in 1950. Not even a decade later, Davis and Allen isolated an isomer of uridine, 5-riboyluracil from RNA, which they termed the "fifth ribonucleoside" (1957). Cohn later termed the nucleotide pseudouridine, abbreviated

Ψ (1960). To date, we know that pseudouridine Ψ is present in every isoacceptor tRNA and rRNAs. Since then, many other instances of RNA editing and modification has been described in organisms from all domains of life: Eukarya, Bacteria and Archaea.

Benne and colleagues in 1986 discovered a novel and interesting RNA editing process. In kinetoplastid protozoa, they demonstrated the insertion and deletion of uridines in mitochondrial mRNAs. These uridines were not genomically encoded and occur in the protein-coding sequences of the mRNA (1986). Mechanistically, this RNA editing event is protein-mediated and involves large multiprotein complexes or editosomes (Stuart 1996).

Soon after, another RNA editing event involving the conversion of cytidine to uridine (C to U) in the mRNA encoding the apolipoprotein B (apoB) in human cells (Powell et al. 1987; Chen et al. 1987). Adenosine conversion to inosine (A to I) via a deamination reaction was also demonstrated in mRNA (Bass and Weintraub 1988; Wagner et al. 1989) in RNA from viruses.

1.3 Applications to tRNA

Unlike mRNAs, transcripts of tRNA genes undergo extensive processing to become a fully functional and mature tRNA for protein translation. To generate this functionality, oftentimes it is necessary to modify or even edit nucleotides of the molecule. Editing is found in all parts of the tRNA structure: the acceptor stem, the D loop, anticodon stem loop and the TΨC loop. One of the first documented cases of tRNA editing is with *Acanthamoeba castellanii* mitochondrial tRNAs. Lonergan and Gray published data on posttranscriptional single nucleotide conversions (U to A, U to G, and A to G) in the acceptor stem of the tRNAs (1993). Some of the most heavily investigated tRNA editing events involve the deamination of cytidine

to uridine (C to U) in the tRNA backbone in plants (Fey et al. 2002). Editing of tRNAs is generally essential for cell survival.

On the other hand, the majority of tRNA modifications are not necessary for cell viability. Currently, there are over 100 posttranscriptional modifications that have been identified in tRNA from the Bacterial, Archaeal and Eukaryotic domains. Most of these modifications appear to be important for proper folding and stability of the molecule itself.

1.4 Adenosine to inosine editing at the wobble position

The standard genetic code is composed of 64 different triplets (codons), with 61 of them encoding amino acids. The last three codons signal for translation termination. As there are many more amino acid codons than amino acids themselves, most amino acids are encoded by several related codons in what is referred to as degeneracy. The only exceptions to the degeneracy are methionine and tryptophan (Agris 2004). The mechanism through which an organism can read all 61 amino acid codons to for protein translation was first hypothesized by Francis Crick in the 1960s with his Wobble Hypothesis (1966). Now it is widely known that a tRNA has the ability to decode multiple codons through flexibility in base-pairing between the third position (3' position) of the mRNA codon and the first position (5' position) of the tRNA anticodon, otherwise known as the wobble position (Table 1.2).

The nucleotide inosine, when in the wobble position of a tRNA, provides the greatest flexibility by allowing the recognition of up to three different nucleotides in an mRNA codon: cytidine, uridine, and adenosine. Not encoded by DNA, this nucleotide is found in RNAs by an enzyme-mediated deamination reaction. Hydrolytic deamination of adenosine by the removal of an amino group results in the formation of inosine (Gerber et al. 1999). In Bacteria, this editing

event at the wobble position only occurs with one tRNA—tRNA ^{Arg}. However, in the Eukaryotic domain, deamination is present with eight different tRNAs. In both domains, the conversion of adenosine to inosine in tRNA is essential for survival (Gerber et al. 1999; Wolf et al. 2002).

The family of enzymes responsible for catalyzing this reaction is called adenosine deaminases acting on tRNA (ADATs). In Bacteria, this enzyme is referred to as TadA or ADATa. Previous studies with *Escherichia coli* have shown that the TadA enzyme is a homodimer made of two identical subunits. This homodimer, in addition to acting on the full-length $tRNA^{Arg}$ substrate, can also catalyze deamination in a minimized portion (the anticodon stem loop mini-helix) of $tRNA^{Arg}$ just as efficiently. However, eukaryotic tRNAs were able to be deaminated by TadA if they contained the anticodon stem loop of $tRNA^{Arg}$ from *E. coli* (Wolf et al. 2002). In addition, crystal structure of the ADATa enzyme from *Staphylococcus aureus* has shown that the binding site is the same as the catalytic site that causes deamination of adenosine to inosine (Losey et al. 2006). Wolf and colleagues demonstrated that the *tadA* gene is essential for cell viability in *E. coli*, which suggests the importance of inosine at the wobble position in bacteria (2002).

Pairing at the third position of the codon

Base on the anticodon	Bases recognized on the codon
U	A, G
C	G
A	U
G	U, C
I	U, C, A

Table 1.2: Wobble base-pairing as described by Francis Crick (1966).

In contrast, the ADAT enzyme in eukaryotes is a heterodimer composed of two different subunits, ADAT2 and ADAT3. Previous studies in *Saccharomyces cerevisiae* have shown that the ADAT2 subunit is for catalytic activity, while the ADAT3 subunit is important for recognition of the tRNA substrate (Gerber et al. 1999). Combined together, the ADAT2/3 enzyme can act on up to eight different substrates. However, unlike the bacterial TadA, it will not deaminate a minimized tRNA substrate (Wolf et al. 2002). Gerber and Keller showed in *Saccharomyces cerevisiae* that, similar to bacteria, the *ADAT2* and *ADAT3* genes were essential for growth, again stressing the significance of this editing event (Gerber et al. 1999). Currently, no crystal structure exists for ADAT2/3 from eukaryotes, but some initial enzyme mutational studies and kinetic analyses suggest that the active site is separate from the tRNA binding site (Ragone et al. unpublished results)

1.5 *In vitro* challenges

There are undoubtedly hundreds of chemical modifications and editing events present *in vitro* that have yet to be characterized by scientists. These may play an important role in the molecular biology of the cell and could be crucial in maintaining homeostasis. In addition, though it is possible to synthetically transcribe and edit tRNAs, it is costly. As such, it is difficult to construct *in vitro* a system identical to the organism's system to accurately assess the editing and modification states of tRNA. There is optimism, however, that an editing pathway unique to *Trypanosoma brucei* could be utilized in the future as a target for anti-trypanosomal treatments once the modifications and editing events have been characterized.

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CHAPTER 2

In vitro studies for specificity requirements of the *Trypanosoma brucei* adenosine to inosine tRNA editing deaminase ADAT2/3

2.1 Introduction

One editing event in *T. brucei* that has been identified is adenosine to inosine (A to I) deamination. Like other eukaryotes, these flagellated protozoa deaminate A to I at the wobble position in up to eight different tRNAs. Figure 2.1, taken from Losey et al. (2006), illustrates the proposed mechanism of hydrolytic deamination. The active site of the ADAT enzyme contains a zinc ion (Zn^{2+}) that is tetrahedrally coordinated to a histidine and two cysteine residues (or three cysteine residues as in some cases); the fourth ligand is a zinc-activated water molecule. Zn^{2+} enables the water molecule to perform a nucleophilic attack on the carbon at position 6 of the adenine ring, forming a tetrahedral intermediate. The intermediate will then lead to the release of ammonia, producing the nucleotide inosine. A conserved glutamate residue mediates the proton transfers during the deamination process (Losey et al. 2006).

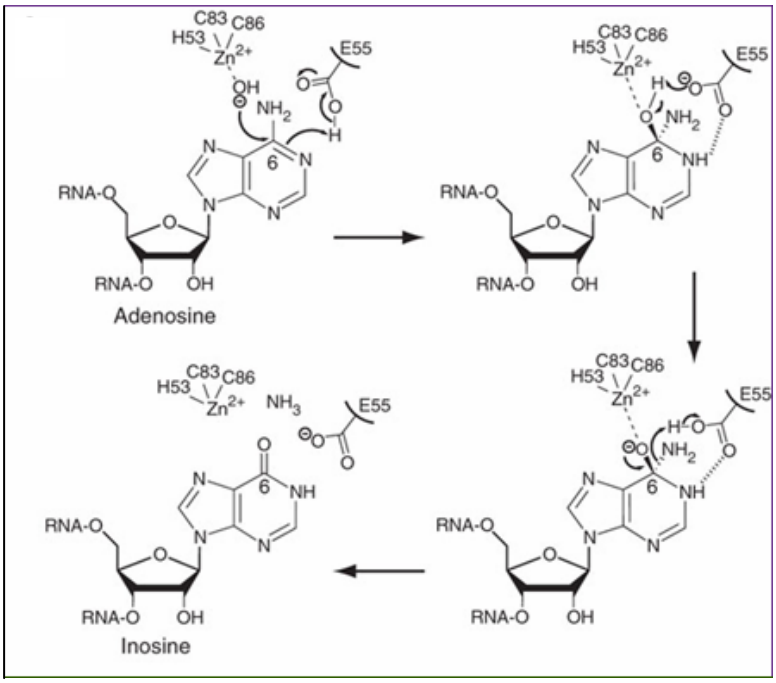

Figure 2.1: Proposed mechanism of hydrolytic deamination of adenosine to inosine. A zinc-bound water molecule attacks at position 6 of the adenine ring yields a hydrated tetrahedral intermediate that collapses to expel ammonia and produce inosine. Taken from Losey, Ruthenburg, and Verdine (2006).

Rubio and colleagues identified a homolog to the yeast ADAT2/3 in *T.brucei* (2006). When the *T. brucei* ADAT2/3 was aligned with other known deaminases, it was shown that there were a number of conserved residues known to be essential for catalysis of cytosine and adenosine deaminase families (Figure 2.2). TbADAT2 and TbADAT3 both contain the conserved histidine and two cysteine residues, however TbADAT3 does not contain the proton-shuttling glutamate. This suggests that like *Saccharomyces cerevisiae*, the ADAT3 enzyme does not play a catalytic role, but more of a structural role.

The purpose of this project was to identify the specificity requirements for TbADAT2/3 activity. This was first done by making mutations in one of the enzyme's tRNA substrates, $tRNA^{Thr}$, and observing effects on deamination activity. The specificity determinants of the

eukaryotic TbADAT2/3 were then compared to its bacterial counterpart, TadA, from *Escherichia coli*.

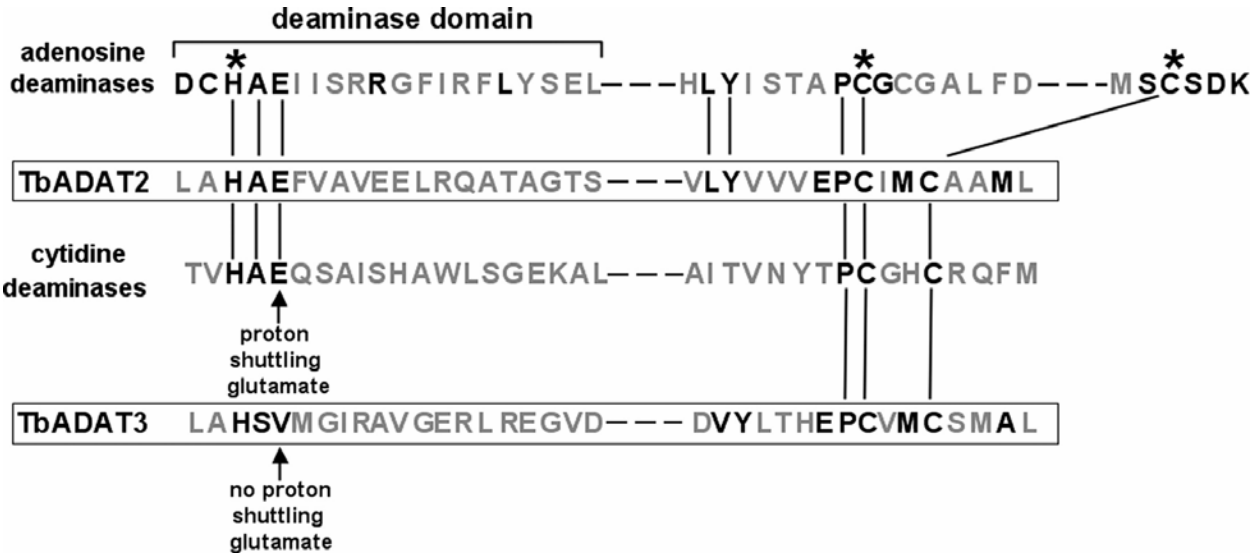

Figure 2.2: Sequence alignment of TbADAT2/3 with other known adenosine and cytidine deaminases. The boxes show the active sites of both the TbADAT2 and TbADAT3 subunits. Conserved residues are shown in boldface. Asterisks denote the conserved residues involved with Zn^{2+} coordination. Taken from Rubio et al. (2007).

2.2 Methods

2.2.1 Recombinant protein expression and isolation

The genes encoding ADAT2 and ADAT3 were cloned from *T. brucei* cDNA into an expression vector. The construct was then transformed into *Escherichia coli* cells. In a one liter culture, the cells were induced with IPTG overnight. Cells were harvested and lysed; the resulting supernatant was then applied to a TALON Superflow affinity resin. ADAT2/3 was eluted, concentrated and purified (Rubio et al 2007).

2.2.2 *In Vitro* editing assays

The tRNAs were transcribed *in vitro* with internally incorporated [α-^{32}P]-ATP. For the editing assays, the tRNAs were incubated with either *Trypanosoma brucei* ADAT2/3 (at 27˚C) or *Escherichia coli* TadA (at 37˚C). After incubation, the reactions were purified with phenol and digested with Nuclease P1 to generate 5'-monophosphates. Then, the 5'-monophosphates were separated by one-dimensional thin layer chromatography (TLC). The TLC was visualized by PhosphoImager and the radioactive adenosine (pA) and inosine (pA) monophosphates were quantified. The precise pI and pA spots were identified by running an unlabeled IMP and AMP along side the reactions as a marker. To determine the percentage of adenosine to inosine deamination, the relative fraction of adenosine to inosine was established by dividing the amount of radioactivity in the pI spot by the sum of the radioactivity in the pA and pI spots. The specific percent conversion at a single site (the wobble position) was then determined by normalizing the amount of radioactivity at position 34 to the total number of labeled adenosines in the tRNA (n=13). Specific yield of pI was then calculated by dividing the percent total by the relative percentage at one site.

2.3 Results and Discussion

2.3.1 Different tRNAs are deaminated with comparable efficiency

TbADAT2/3 can recognize and deaminate eight different tRNA substrates. The first aim of this project was to compare how different tRNA substrates are deaminated. Two different tRNAs, $tRNA^{Thr}$(AGU) and $tRNA^{Val}$(AAC), were used for *in vitro* studies (Figure 2.3). Uniformly labeled with [α-^{32}P]-ATP, these tRNAs were incubated at 27˚C for one hour with increasing concentrations of the TbADAT2/3 recombinant protein (0.05, 0.25, 0.5, 1.25 and 2.5 μg). Analysis showed that there was no significant difference in inosine formation at the wobble position between the two different tRNAs, suggesting that TbADAT2/3 can deaminate at least two different tRNAs with comparable efficiency (Figure 2.4).

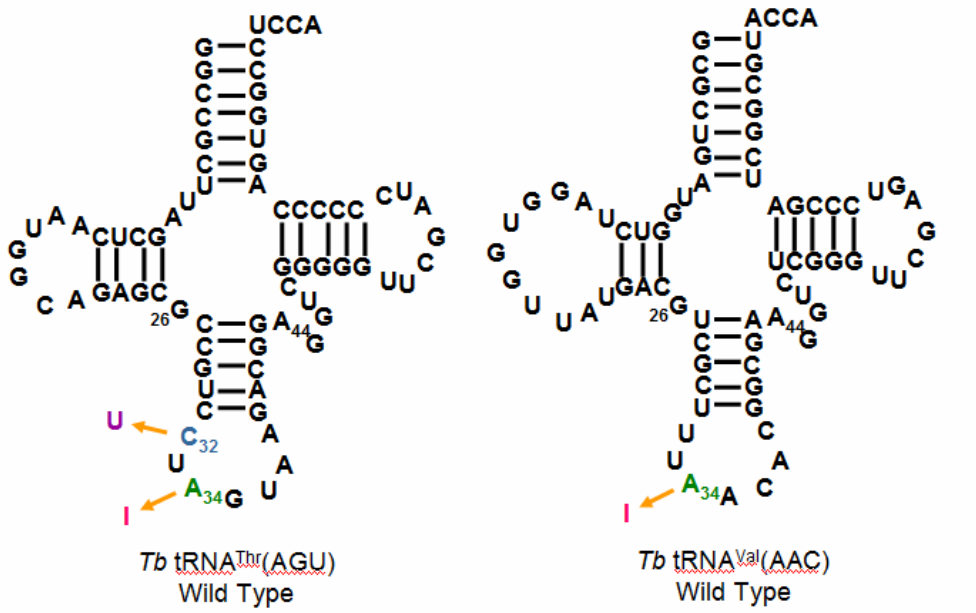

Figure 2.3: Secondary structure of the wild type $tRNA^{Thr}$(AGU) and $tRNA^{Val}$(AAC) from *Trypanosoma brucei*. Both tRNAs deaminate at position 34 to form inosine, as shown by the arrows. In addition, Rubio and colleagues discovered in *Tb*$tRNA^{Thr}$(AGU) C to U deamination at position 32.

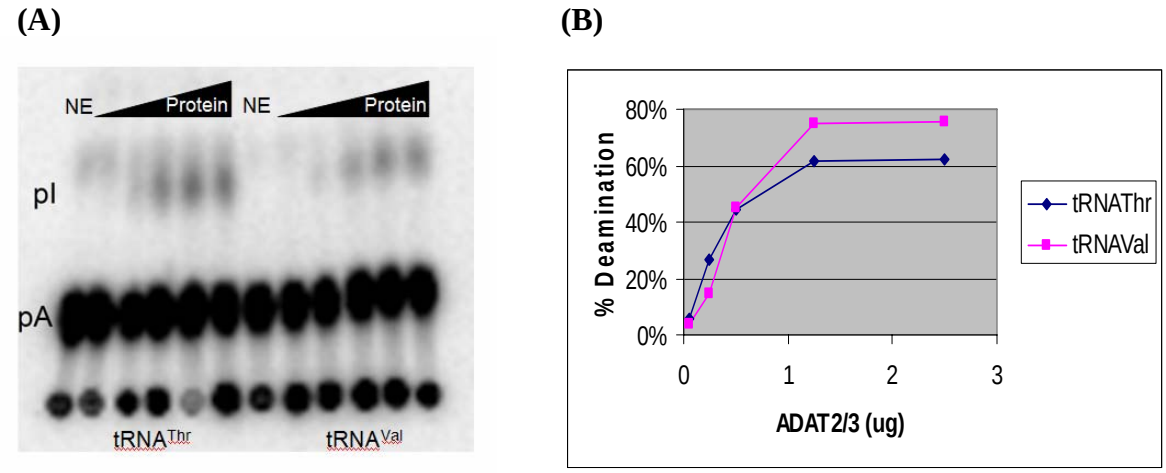

Figure 2.4: *In vitro* testing of deamination efficiency of wild type $tRNA^{Thr}$(AGU) and $tRNA^{Val}$(AAC) from *Trypanosoma brucei*. (A) TLC analysis of the two tRNAs incubated with increasing amounts of TbADAT2/3. As negative controls, lanes 1 and 7 did not contain TbADAT2/3. (B) A plot of the percentage of conversion of A to I at position 34 versus amount of protein.

2.3.2 *Trypanosoma brucei* $tRNA^{Thr}$(AGU) helices are not substrates for TbADAT2/3

The tRNA secondary structure is composed of primarily four parts: the acceptor stem, the D arm, anticodon stem loop, and the TΨC arm (Figure 2.5). To assess if any particular region of the tRNA may be influential in the ability TbADAT2/3 to catalyze hydrolytic deamination of adenosine to inosine at the wobble position, various substrate mutants were created using different regions of the tRNA (Figure 2.6). Four different mutants were tested. Incubation of the protein with Mutant A (composed of the acceptor stem and the TΨC arm) showed no enzymatic activity. No inosine formation was observed when Mutant B (TΨC arm and the anticodon stem loop), Mutant C (D arm and acceptor stem) and Mutant D (D arm, anticodon stem loop and TΨC arm) were incubated with TbADAT2/3. Individual components or combinations of different

parts of the tRNA structure were not enough to convey specificity for TbADAT2/3. Even with the majority of the tRNA present in Mutant D (missing the acceptor stem), the tRNA adenosine deaminase was unable to recognize and perform the reaction on the substrate. The data suggests that the TbADAT2/3 cannot use a minimized tRNA of any sort as substrates and that a full-length tRNA is necessary for deamination activity.

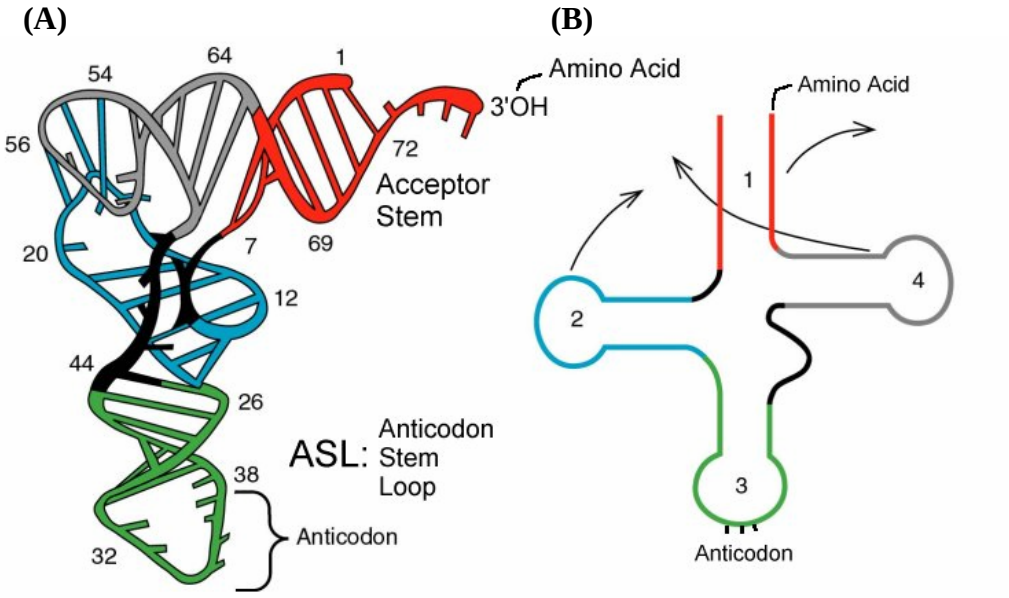

Figure 2.5: Two representations of tRNA structure. (A) Schematic of the tertiary structure of a tRNA. (B) The corresponding secondary tRNA structure with the same color-coding as with the secondary structure. Taken from Weaver (2002).

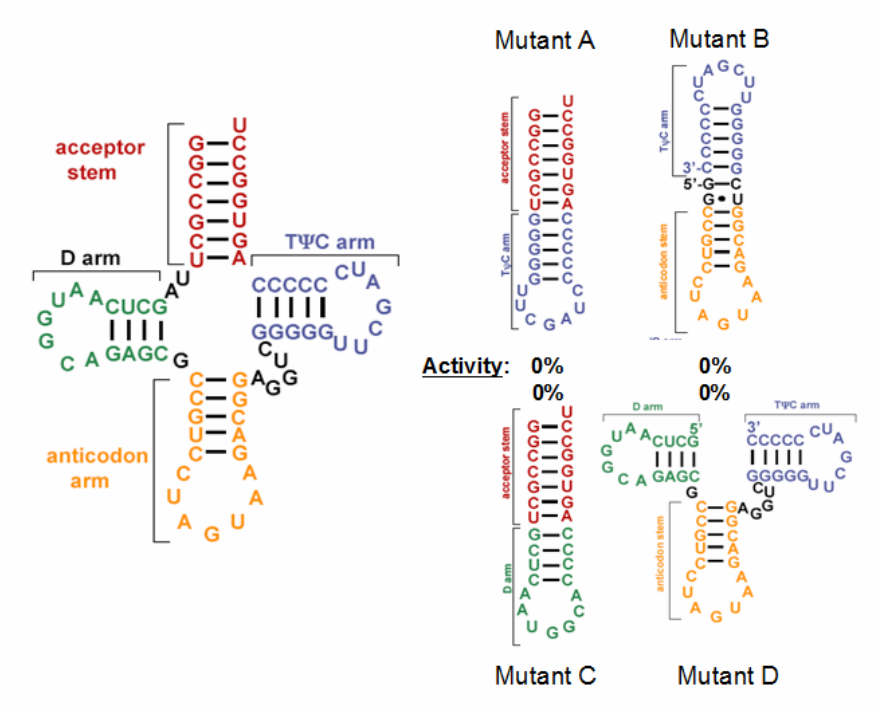

Figure 2.6: Schematic of the secondary structure of tRNAThr(AGU) and the substrate mutants. The acceptor stem was shown in red; green represents the D arm; the anticodon stem loop is illustrated in yellow; blue corresponds to the TΨC arm. Mutant A was composed of the acceptor stem and the TΨC arm. The TΨC arm and the anticodon stem loop made up Mutant B. Mutant C was derived by combining the D arm and the acceptor stem. Mutant D was constructed with the D arm, the anticodon stem loop and the TΨC arm (missing the acceptor stem).

2.3.3 Anticodon stem loop determinants for deamination activity

The tRNA anticodon stem loop contains the adenosine that undergoes inosine formation. Because the anticodon stem loop is positioned in the catalytic site, nucleotides in this portion of the tRNA may contact the protein in the catalytic pocket or may be important for binding recognition. Three mutant threonyl tRNA constructs were created with mutations in the anticodon stem (Tag 22), at the anticodon loop at position 37 (Tag 23) and at position 38 (Tag 24) of the *Trypanosoma brucei* threonyl tRNA(AGU) (Figure 2.7). These "tagged" tRNAs were

then used to assess whether or not particular residues in the anticodon stem loop were important for deaminase activity.

In the same assay used to test the different *T. brucei* tRNAs, the wild type $tRNA^{Thr}$, Tag 22, Tag 23 and Tag 24 tRNAs were uniformly labeled with [α-^{32}P]-ATP. They were then incubated with TbADAT2/3 enzymes for various times: 5, 15, 30, 45 and 60 minutes. The percent deamination of adenosine to inosine at the wobble position was determined in the method described above (Figure 2.8). The results showed that changes in the anticodon stem (Tag 22) do not affect the enzyme's ability to deaminate the substrate, as the activity levels did not differ from wild type threonyl tRNA(AGU). In addition, a change to guanosine at position 37 does not appear to affect activity (Tag 23) but the change was from a purine to another purine. However, the nucleotide at position 38 (Tag 24) decreased activity drastically as compared to rest of the constructs. While the wild type $tRNA^{Thr}$, Tag 22 and Tag 23 maximally deaminates around 60%, Tag 24 yields about 20% deamination activity. This suggests that the mutation at position 38 (A to C) altered the structure of the anticodon stem-loop and impaired the ability of TbADAT2/3 to catalyze the deamination reaction. The change from a purine nucleotide (A) to a pyrmidine (C) in this Tag mutant may have destabilized $tRNA^{Thr}$(AGU). However, another possibility for the decrease in deamination activity may be the result of a combination of effects of both mutations at positions 37 and 38. The two changes together may have affected the overall stability of the secondary and tertiary structure of the threonyl tRNA.

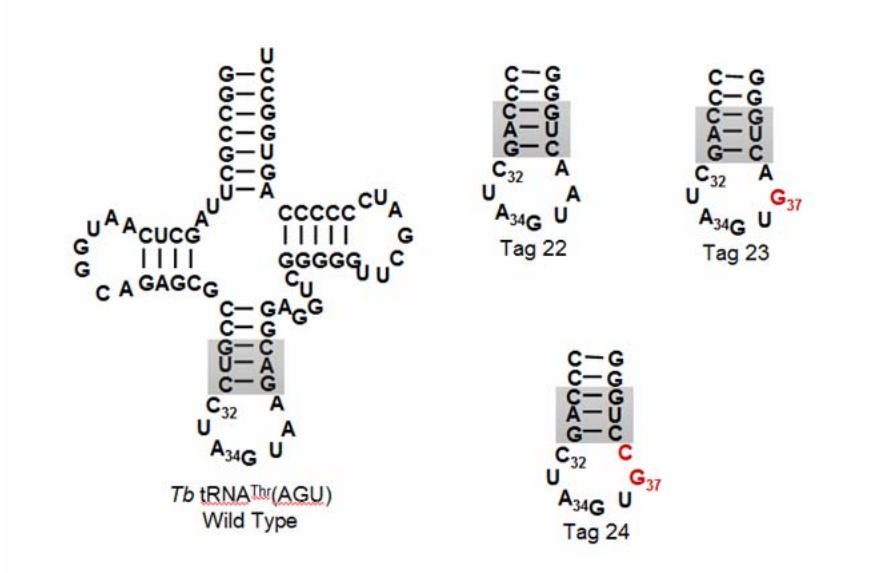

Figure 2.7: Secondary structure of *Trypanosoma brucei* tRNA^{Thr}(AGU) anticodon mutant constructs. Changes in the anticodon stem are highlighted in gray. Anticodon loop changes are shown in red.

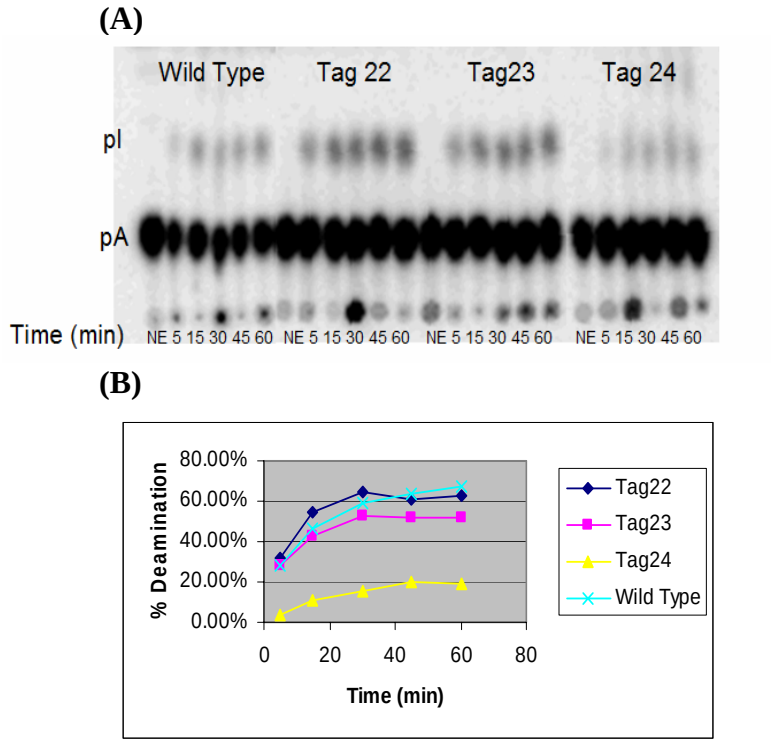

Figure 2.8: Effect of anticodon stem loop mutations on *Tb*tRNA^{Thr}(AGU) deamination. Reactions were incubated with no enzyme (NE) as a negative control and for increasing amounts of time: 5, 15, 30, 45 and 60 minutes.

2.3.4 Comparison of the sequence determinants for TbADAT2/3 versus the bacterial EcTadA

The next step of the project was to compare the eukaryotic and bacterial tRNA adenosine deaminase systems from *T. brucei* and *E. coli* respectively. The *T. brucei* $tRNA^{Thr}$(AGU) and the *E. coli* $tRNA^{Arg}$(ACG) were used for these studies (Figure 2.9). Various mutations were made in the two tRNAs in different regions. The mutants were then assessed to see how the changes affect activity in both TbADAT2/3 and EcTadA. These experiments were dependent on the fact that the EcTadA cannot deaminate $tRNA^{Thr}$(AGU) and TbADAT2/3 cannot deaminate the $tRNA^{Arg}$(ACG). The objective was to determine which specific nucleotide positions are important for each enzyme's ability for substrate recognition and subsequent deamination.

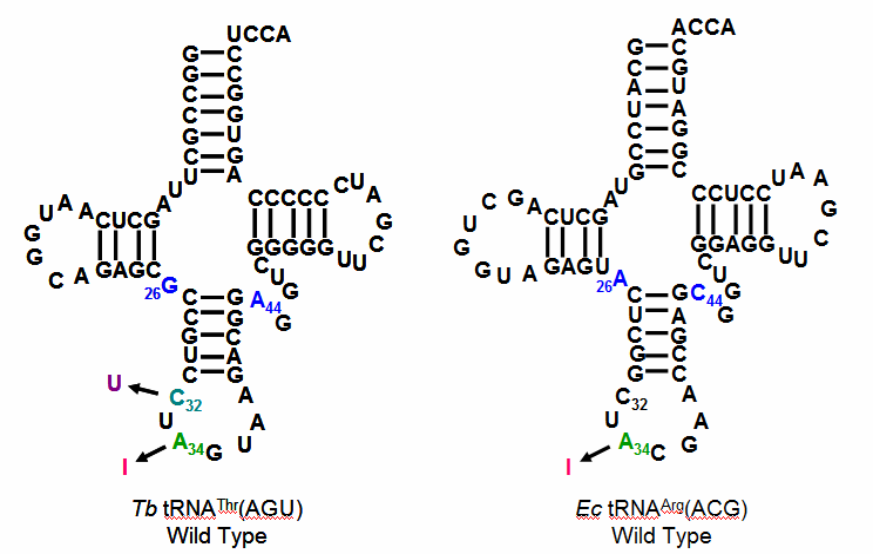

Figure 2.9: Secondary structures of *Trypanosoma brucei* $tRNA^{Thr}$(AGU) and *Escherichia coli* $tRNA^{Arg}$(ACG). The alignment of the tRNAs shows that the only major difference between the two nucleotides that may be important for contact with the ADAT enzymes are the nucleotides at position 26 and 44 (shown in blue).

The first set of studies involved making mutations at different positions in the *Tb*$tRNA^{Thr}$(AGU) to the corresponding nucleotides of *Ec*$tRNA^{Arg}$(ACG). The nucleotide

changes were made based on a comparison of the two tRNAs (Figure 2.9). The major difference between the two nucleotides were those at positions 26 and 44—just outside of the anticodon stem loop. Other differences were present in the base-pairing nucleotides from various arms, but these were not investigated as specific residues in these regions most likely would not contact the protein directly to affect deaminase activity. Indeed, as shown with Tag 22 in Figure 2.8, the base-paired nucleotides in the anticodon stem did not seem to be important for specificity with TbADAT2/3. Figure 2.10 illustrates the results of mutational studies with *Tb*tRNA^{Thr}. When positions 26 and 44 (Mutant 1) were changed to A and C respectively, deamination activity by ADAT2/3 was completely abolished as compared to deamination of the wild type tRNA^{Thr}. However, these changes did not rescue activity with TadA. This implies that these nucleotides just outside of the anticodon stem loop may be important for contact with the TbADAT2/3 protein. Mutant 2 additionally changed the threonyl tRNA anticodon AGU to the tRNA^{Arg} anticodon ACG. As expected, TbADAT2/3 was unable to deaminate this mutant, but inosine formation was possible with EcTadA to some degree. The entire anticodon stem loop from *Tb*tRNA^{Thr} was replaced by the *Ec*tRNA^{Arg} anticodon (Mutant 3), which again yielded no production of inosine with TbADAT2/3. However, EcTadA was able to deaminate to an even greater degree than with Mutant 2. When replacing the AGU anticodon with the *E. coli* ACG anticodon (Mutant 4), as expected, TbADAT2/3 was unable to produce inosine at position 34. Incubation with EcTadA resulted in a comparable deamination efficiency to the previous two mutants. These results suggest that TbADAT2/3 requires a greater degree of specificity for its substrate than EcTadA, which seems to only require the anticodon nucleotides. The next two mutants focused on one nucleotide change each in the anticodon positions 35 and 36. Mutant 5, U36G, was unable to be deaminated by either TbADAT2/3 or EcTadA. The change to C from G

at position 35 in Mutant 6 demonstrated the same results. The last mutant to be tested was just a minimized threonyl tRNA substrate of just the anticodon stem loop. Neither the *T. brucei* nor the *E. coli* enzyme could catalyze deamination on this substrate. In summary, for TbADAT2/3 activity, the nucleotides immediately 5' and 3' to the anticodon stem loop appear to be essential for deamination. EcTadA however requires only needs the anticodon sequence ACG in a full-length tRNA to be form inosine at the wobble position.

Trypanosoma brucei $tRNA^{Thr}$

tRNA	Mutations
Mutant 1	G26A, A44C
Mutant 2	G26A, A44C, G35C, U36G
Mutant 3	C28U, G29C, U30G, C31G, G35C, U36G, G39C, A40C, C41G, G42A (EctRNAArg Anticodon Stem Loop)
Mutant 4	G35C, U36G
Mutant 5	U36G
Mutant 6	G35C
Mutant 7	Anticodon Stem Loop only

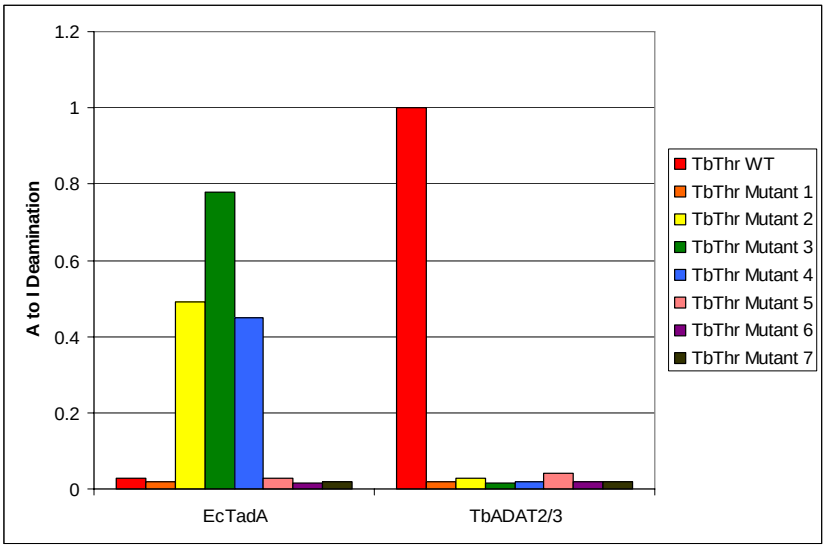

Figure 2.10: Comparison of TbADAT2/3 and EcTadA specificity determinants with *Tb*$tRNA^{Thr}$(AGU). TbADAT2/3 requires a greater deal of specificity than EcTadA. The substrates were incubated for one hour. EcTadA reactions were incubated at 37˚C and TbADAT2/3 reactions were tested at 27˚C, the optimal temperature for each enzyme.

To determine is the sequence determinants were similar in the bacterial system, changes were then made to the arginyl tRNA(AGU) from *E. coli* using the same strategy as above (Figure 2.11). Mutant 1 (A26G, C44A) was not enough to recover deamination activity with TbADAT2/3, but did not abolish activity when incubated with EcTadA (unlike its threonyl counterpart). However, EcTadA activity with Mutant 1 decreased sixty percent. Again, this suggests that the nucleotides at these positions are important for deaminase activity, though less so than with its *T. brucei* equivalent. Mutant 2 involved the change of the anticodon from ACG to AGU. These mutations completely abolished activity with EcTadA. Not surprisingly, ADAT2/3 was unable to deaminate this substrate. No inosine was formed in Mutant 3 (which contained the mutations at positions 26 and 44 and the anticodon) with either system's deaminase enzyme. Mutant 4, which was the anticodon stem loop only of *Ec*tRNA^{Arg}, was deaminated by EcTadA to some degree; incubation of this mutant with ADAT2/3 showed no deamination activity. The results from with the arginyl tRNA mutants suggest that even though the nucleotides just outside of the anticodon stem loop are important for EcTadA activity, they are less of a determinant for deamination than with TbADAT2/3. The mutations made were not enough to rescue TbADAT2/3 activity.

Escherichia coli $tRNA^{Arg}$

tRNA	Mutations
Mutant 1	A26G, C44A
Mutant 2	C35G, G36U
Mutant 3	A26G, C44A, C35G, G36U
Mutant 4	Anticodon Stem Loop only

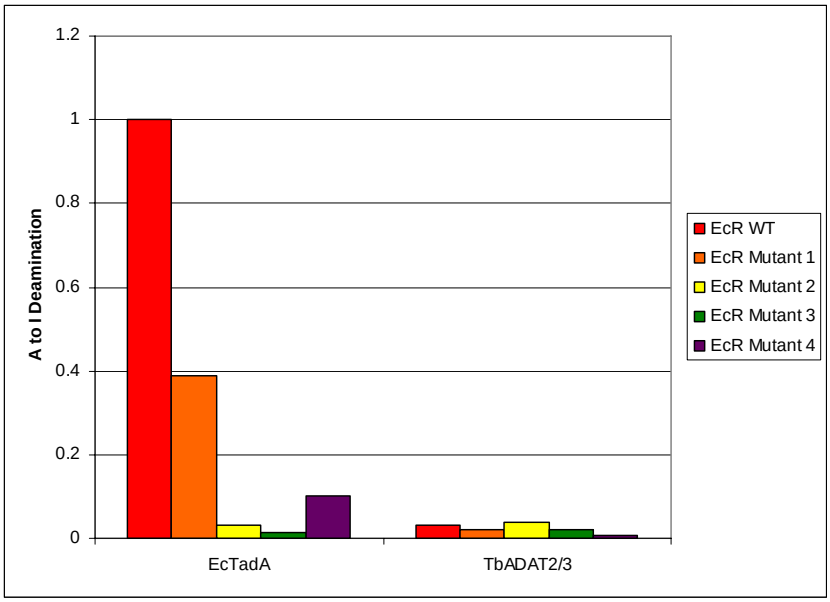

Figure 2.11: Comparison of TbADAT2/3 and EcTadA specificity determinants with *Ec*$tRNA^{Arg}$(ACG). TbADAT2/3 requires a greater deal of specificity than EcTadA. The substrates were incubated for one hour. EcTadA reactions were incubated at 37˚C and TbADAT2/3 reactions were tested at 27˚C.

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CHAPTER 3

Creation of a tRNA over-expression system to assess editing states of tagged threonyl tRNAs *in vivo*

3.1 Introduction

The previous studies tested the effects of tRNA mutations on ADAT2/3 using *in vitro* methods. To assess the editing state of mutant tRNAs *in vivo*, the tagged tRNAs will be introduced into *T. brucei*. Tag 22, Tag 23 and Tag 24 (Figure 2.7) will be produced at high levels in *T. brucei* using the expression vector pLEW100, which can then be edited and modified by the parasite's cellular machinery, particularly by endogenous ADAT2/3. Once introduced into *T. brucei*, total RNA from the cells will be isolated and then analyzed to evaluate the ability of ADAT2/3 to deaminate these mutants *in vivo*. Figure 3.1 illustrates the process for creating the over-expression system. The tagged tRNAs will be ligated into pLEW100 and electroporated into *T. brucei*. Once the cells recover, total RNA will be isolated from the cells and the goal is to determine the ability of endogenous ADAT2/3 to catalyze the formation of inosine at the wobble position.

3.2 Methods

3.2.1 Vector selection

Numerous vectors are available for over-expression in *T. brucei*. However, two of the more common over-expression plasmids used in *T. brucei* studies are pLEW82 and pLEW100 (Figure 3.2). Both vectors utilize a tetracycline (Tet) to induce expression (Figure 3.3). The pLEW82 plasmid is set up so that the gene of interest is under the control of an endogenous T7

promoter and Tet operator. Two T7 terminators are present at the end of the coding region to ensure efficient termination. Using this system, pLEW82 has demonstrated the highest expression levels of known expression vectors for *T. brucei* (Wirtz et al. 1999). The presence of multiple resistance markers for ampicillin (Amp^R) for *Escherichia coli* selection and phleomycin (Phleo) for selection in *T. brucei* allow for effective selection during cloning and transfection for the cells that contain the plasmid construct. The plasmid pLEW100 also contains resistance markers for ampicillin and phleomycin. However, expression of this vector is under the control of a dual-promoter system. The selectable marker is under the control of a constitutive T7 promoter. A Tet-regulated PARP promoter drives expression of the gene of interest.

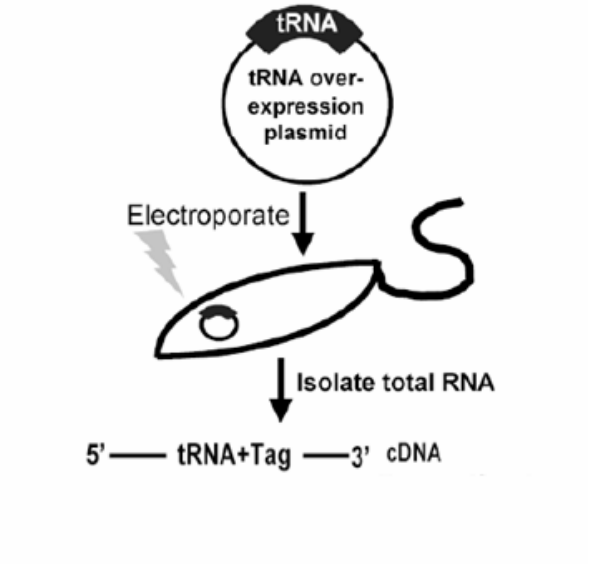
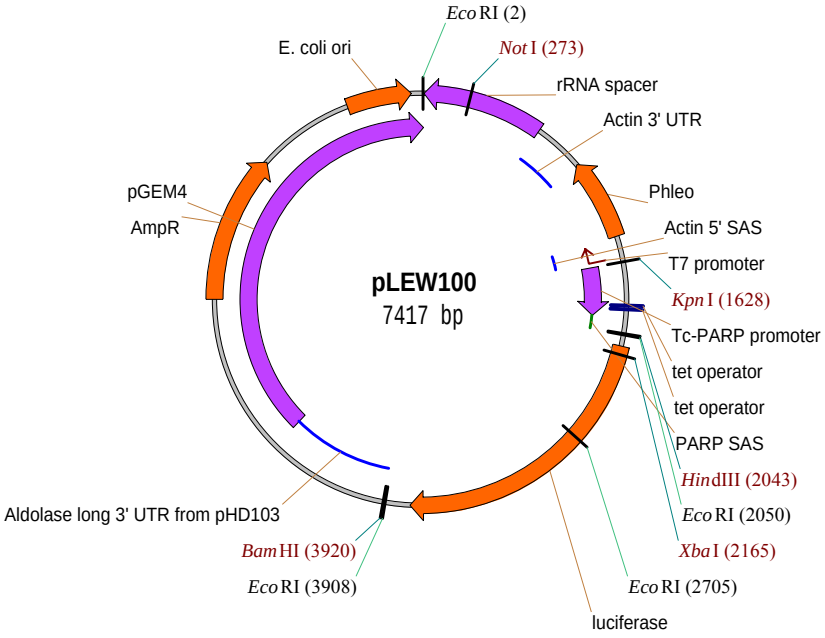


Figure 3.1: Schematic of the tRNA over-expression plan. Once the plasmid is electroporated into *T. brucei* cells, total RNA will be isolated. cDNA copies of the tRNAs are then sequenced to assess the editing state of the RNA.

A first-generation inducible expression vector, pLEW82 utilizes a single Tet-responsive T7 promoter to drive expression of the experimental gene and at the same time expressing the drug-resistance marker gene. The use of a dual-promoter however is more strongly regulated;

plasmid expression or “leakiness” without induction by tetracycline addition is much less with pLEW100 than with pLEW82.

(A)



(B)

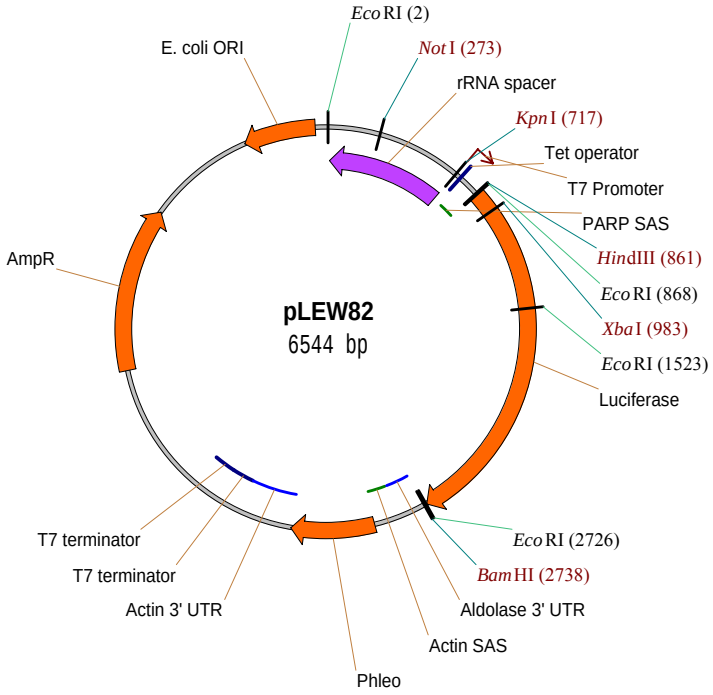


Figure 3.2: Schematic of the over-expression vectors. (A) The pLEW100 vector is under the control of a dual-promoter (T7 and PARP promoters. (B) The pLEW82 vector is controlled by a single T7 promoter. In both expression vectors, the luciferase gene is replaced by the gene of interest.

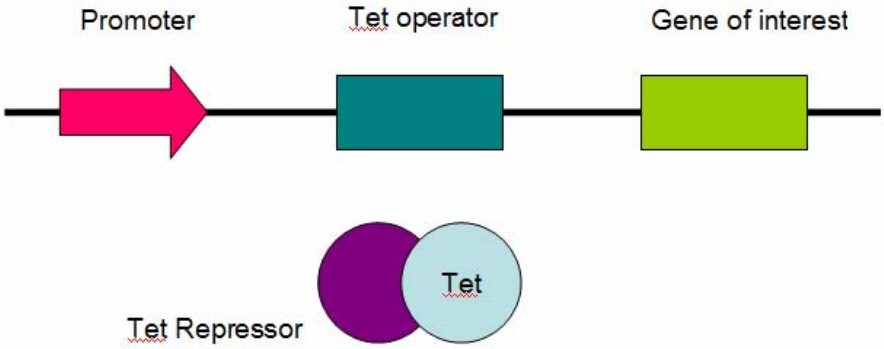


Figure 3.3: Schematic of the Tet-regulator system. Under normal conditions, the Tet Repressor binds to the Tet operator and prevents expression of the gene of interest. However tetracycline, when added, will bind to the Tet Repressor and prevent it from blocking gene transcription.

3.2.2 Cloning

In order to clone the tagged tRNA (Tag 22, Tag 23, and Tag 24) into a *Trypanosoma brucei* cell line, the following cloning plan was used. pLEW100 was digested with *BamHI* and *HindIII* to release the luciferase gene from the original plasmid. After digesting the tagged constructs with the same enzymes, they were then ligated into the expression vector so that its expression will be under the control of the PARP promoter. The constructs were then transformed into *Escherichia coli* DH5α competent cells. To confirm that the constructs were taken up the *E. coli* and that they contained the correct tagged tRNAs, the plasmids were isolated from the *E. coli* cells and were sequenced. Once verified that the tagged tRNAs were successfully ligated in, 200 milliliters of the correct clones were grown, midi-prepped and run through a QIAGEN column to extract purified plasmid DNA.

3.2.3 Transfection

Following isolation of purified plasmid DNA, *Trypanosoma brucei* cells were prepared for electroporation. Electroporation is a process where a current passes through the cells' membrane to allow for uptake of the plasmid. The vector carries an antibiotic gene (in this case Phleo), which allows for growth of positively transformed cells. 100 μg of each plasmid DNA construct were used for the transfection. After the electroporation, the *T. brucei* cells were recovered over a period of a few weeks to allow incorporation and expression of the plasmid and recovery from the electroporation process.

3.3 Results and Discussion

To observe the effects of mutant tRNAs *in vivo*, the tagged $tRNA^{Thr}$ mutants (Tag 22, Tag 23, Tag 24) were over-expressed in *Trypanosoma brucei* to assess the editing and modification states of the mutant tRNAs *in vivo* by *T. brucei* cellular machinery, especially by ADAT2/3. Previously, it was shown that changes in the anticodon loop did not affect deamination activity (Figure 2.8). As such, these mutations effectively created a "tag" to differentiate the endogenous tRNAs from the tRNAs introduced by over-expression and can be used as a sequence tag for reverse-transcription analysis.

Earlier research with pLEW82 has demonstrated high levels of over-expression. However, Wirtz and colleagues demonstrated that regulation of the dual-promoter constructs (pLEW100) was better than the original single-promoter system (pLEW82) with respect to background expression in the absence of tetracycline (Wirtz et al. 1999). As such, pLEW100 was selected for over-expression of the tagged tRNAs.

The plasmids have been electroporated twice and are in the recovery phase of the second electroporation. Before the cells are grown in the antibiotic to select for positively transfected cells, they are first grown in plain media until the cell density is optimal for growth in the selective media with Phleo. The entire process takes weeks to complete. Once the cells recover, total RNA will be isolated from the *T. brucei* cells. From the total RNA, a cDNA copy of the $tRNA^{Thr}$ mutants will be made from using reverse-transcriptase, and then will be amplified by PCR. These PCR products will then be purified, cloned and sequenced to assess the editing state of the tRNAs from the endogenous ADAT2/3.

3.4 References

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CHAPTER 4

Conclusions and Future Work

As *Trypanosoma brucei* and other trypanosomatids have a profound effect on both human health and global economy, it is important to look for possible targets for therapeutics against these parasites. Because of the editing and modification events that exist *in vivo* in *T. brucei*, there is potential for discovering a unique biological process that can serve as an ideal drug target. Identification and characterization of these editing and modification events can lead to the development of a therapeutic that can specifically target and kill the parasite without having any adverse effects on the human or animal host.

The *T. brucei* ADAT2/3 enzyme can act on up to eight different tRNA substrates. It was found that it could deaminate at least two of the tRNAs with comparable efficiency *in vitro* (Figure 2.4). With *Tb*tRNA^{Thr}(AGU) in particular, several conclusions can be made. The first conclusion that can be drawn is that the full-length tRNA is necessary for *in vitro* hydrolytic deamination of adenosine to inosine at the wobble position (Figure 2.6). Second, changes to the anticodon stem and to position 37 do not appear to affect enzymatic activity. Mutation of the tRNA nucleotide at position 38 affected deamination most likely by changing the anticodon loop structure of the tRNA (Figure 2.8).

The eukaryotic and bacterial ADAT specificity determinants were then compared using *T. brucei* and *Escherichia coli* as representatives for each domain, respectively. The observations suggest that the TbADAT2/3 enzyme requires a greater deal of specificity than EcTadA. EcTadA, when acting on a full-length substrate, only requires the correct anticodon sequence for deamination. In addition, the nucleotides just 5' and 3' to the anticodon stem loop

appear to be important for activity, though more so with TbDAT2/3 than EcTadA (Figure 2.9 and 2.10).

The aim of this study was to elucidate what nucleotides and/or regions of the tRNA that are important for recognition and deamination by TbADAT2/3 to produce inosine at position 34. Although this data gives a good indication of the requirements of tRNA for activity, further studies must be performed to determine if the mutations made during this study affect catalysis or impacts substrate recognition and binding (and thus impairing the deamination reaction). In addition, *in vivo* studies should be made with the tRNA mutants to find out if other components of *T. brucei* cellular machinery may contribute to the editing event.

With the establishment of an expression vector, once electroporation is completed, analysis of tRNA must be made to assess the editing states of the tagged tRNAs in comparison with the wild type $tRNA^{Thr}$(AGU). The findings may yield insight on how the tRNAs are edited *in vivo* and also may identify additional unique modification and editing events in hopes of finding a novel editing mechanism that is unique to *Trypanosoma brucei*. These distinctive events can then be further investigated as the target for anti-trypanosomal therapeutics.

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