5-2012

Probing the T-Box Riboswitch: A Novel, High-Throughput Transcription Reporter Assay Using a Fluorophore-Binding Aptamer

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Probing the T-box riboswitch: A novel, high-throughput transcription reporter assay using a fluorophore-binding aptamer

An honors thesis presented to the
Department of Biological Sciences,
University at Albany, State University
Of New York in partial fulfillment
Of the Honors Program requirements.

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Department in which Thesis was completed: **BIOLOGICAL SCIENCES**

Honors Thesis **Presentation Date**: **May 2, 2012**

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ABSTRACT

In most Gram-positive bacteria, including important pathogens, expression of many aminoacyl tRNA synthetase (aaRS) genes is controlled by the tRNA substrate specific to each of these enzymes. This riboswitch regulatory mechanism is unique to Gram-positive bacteria and because correct and efficient aminoacylation of tRNAs is essential to an organism's viability, it is an ideal target for the development of new antibiotics. The 5'-untranslated region (5'UTR) of the aaRS mRNA adopts a conformation that determines whether readthrough or termination of transcription occurs by interacting with unacylated or acylated tRNA, respectively. Our goal is to uncover a new class of small molecules that will disrupt the binding of tRNA to the 5'UTR and through that, inhibit transcription. We are creating a transcription based reporter assay to examine small molecules for their ability to disrupt gene expression. We cloned the entire 5'UTR of a B. subtilis aaRS gene upstream of the adenine riboswitch aptamer, with an intervening linker region. This aptamer binds 2-aminopurine and upon binding, quenches the fluorescence normally associated with this molecule. We have successfully transcribed the chimeric RNA. Preliminary fluorescence data shows the aptamer RNA is able to quench 2-aminopurine (2AP) fluorescence. Future work includes carrying out the fluorescence studies in real time during transcription and testing a library of small molecules to assess their ability to inhibit tRNA-mediated transcription.

Acknowledgements:

I would like to sincerely thank Dr. Paul Agris for allowing me to be a part of his lab.

Thank you to all the members of the Agris Laboratory, especially Dr. William Cantara and Dr. Jessica Spears, for the help, insight, and wonderful discussions.

To Dr. Richard Zitomer for all the useful advice.

Thank you to all my friends and family for your continuous support.

And ofcourse, thank you Dr. Caren Stark for your mentorship, encouragement, and friendship.

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INTRODUCTION

Maintenance of appropriate pools of aminoacylated tRNAs (aa-tRNAs) is essential for cell viability (1). This requires balanced levels of tRNAs and their cognate aminoacyl-tRNA synthetases (aaRSs) and adequate supply of matching amino acid. A variety of mechanisms for modulation of aaRS gene expression has been uncovered in bacteria. In *Escherichia coli,* regulation of aaRS gene expression is mediated by transcriptional control (AlaRS), translational control (THrRS), and ribosome-mediated transcriptional attenuation (PheRS) (2). In contrast, in *Bacillus subtilis* and in other Gram-positive bacteria, many of these genes are regulated by the T-box regulatory mechanism (3).

Direct sensing of a regulatory signal by the 5' untranslated region (5'UTR) of a nascent RNA (the "leader region"), termed riboswitch, has emerged as a common mechanism for regulation of gene expression in bacteria. In mechanisms of this type the regulatory signal modulates folding of the nascent RNA, which can determine whether the RNA folds into the helix of an intrinsic transcriptional terminator that results in premature termination of transcription, or an alternative structure which allows expression of the down-stream coding sequences (3).

The T-box system: Regulation of amino acid-related genes by uncharged tRNA

The T-box system was initially uncovered by the analysis of the *B. subtilis tyr*S gene, which encodes tyrosyl-tRNA synthetase (5). The T-box family of riboswitches commonly modulates the expression of genes involved in amino acid metabolism in Gram-positive bacteria. Subsequent bioinformatics analyses (4, 6, 7) have identified >1000 genes with features conserved in genes in this family. A G+C-rich helix followed by a run of U residues

was identified upstream of the *tyr*S-coding sequence, leading to the prediction that regulation occurs at the level of premature termination of transcription (3). For most operons in the T-box family, segments of these upstream leader RNAs can fold to form either of two alternative hairpin structures, an intrinsic transcription terminator or a competing transcription antiterminator (Figure 1). The signal molecule that determines which conformation forms is the tRNA acted on by the cognate synthetase gene. Proper pairing of an appropriate uncharged tRNA with the 5'UTR promotes the stabilization of the antiterminator structure and allows continued transcription into the downstream gene(s) of the operon (8, 9). The specificity of this interaction is depended primarily on the identity of three nucleotides, the Specifier Sequence, within the Specifier Loop domain (Figure 1A). Stabilization of this interaction is due to base pairing of the universal tRNA terminal 5'-NCCA-3' with complementary residues in a 7-nt bulge of the antiterminator helix. The covalently bound amino acid on an aminoacylated tRNA negates binding to the 5'UTR (Figure 1B) and allows formation of the terminator hairpin that result in a premature termination of transcription.

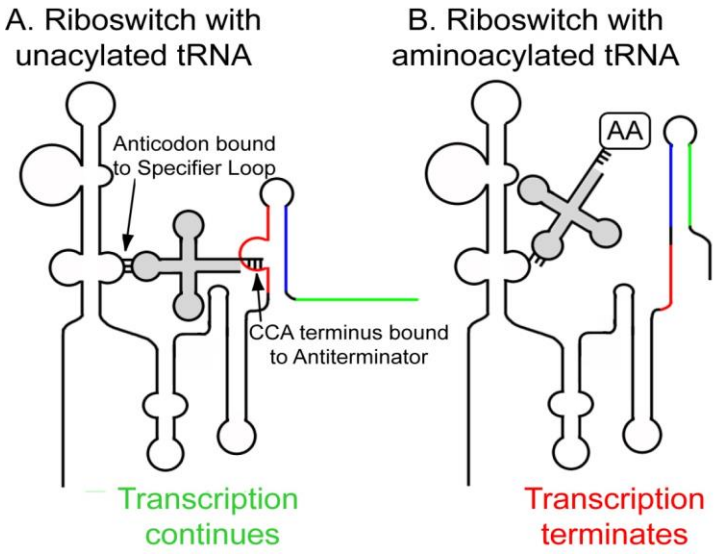


Figure 1: A. Binding of the unacylated tRNA in the 5'UTR of the nascent mRNA for the regulated aaRS gene stabilizes an antiterminator conformation allowing transcription and expression of the gene through a highly specific interaction between the tRNA anticodon and a codon-like sequence in the riboswitch Specifier Loop and a non-specific interaction of the universal 3'-terminal –CCA of the tRNA to the antiterminator. B. Binding of the covalently bound amino acid of an aminoacylated tRNA negates interaction of the tRNA's terminal –CCA with the Antiterminator allowing the formation of a Rho-independent, transcription terminator.

T-box structure and conservation

A T-box RNA consists of a segment of leader RNA with conserved features that allow recognition of, and pairing with, a specific uncharged tRNA (Figure 2). This in turn allows the leader RNA to form alternative secondary structures that can serve as an intrinsic transcription terminator or as an anti-Shine-Dalgarno (ASD) helix that pairs with an SD sequence. The pairing or ASD with SD sequence can block translation initiation. In addition to the segments that can form the terminator/antiterminator (ASD/anti-ASD) elements, the major structures formed within the T-box RNA are stem I, stem II, the stem IIA/stem IIB pseudoknot, and stem III (10, 11) (Figure 2).

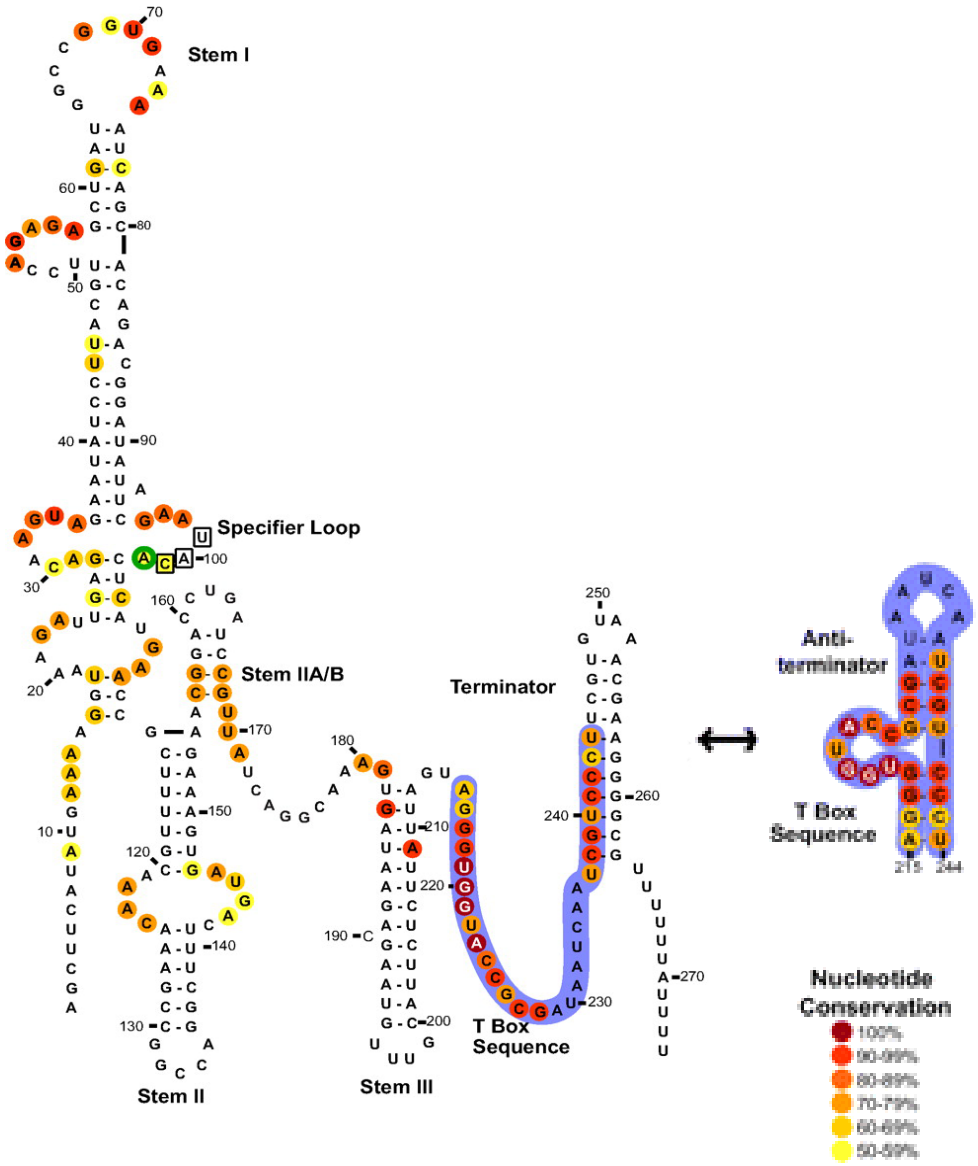


Figure 2: The T-box RNA regulatory system. Structural model of the B. subtilis tyrS T-box leader RNA. The standard T-box leader RNA arrangement consists of three major elements, stem I, stem II, and stem III plus the stem IIA/stem IIB pseudoknot, and the competing terminator and antiterminator structures. The specifier loop, an internal bulge in stem I, contains the specifier sequence (boxed UAC residues complementary to theanticodon sequence of $tRNA^{Tyr}$); the conserved purine (an adenine) following the specifier sequence is inside a green circle. The T-box sequence is unpaired in the terminator form and is paired in the antiterminator form (the antiterminator is shown to the right of the terminator). The sequence highlighted in blue shows the nucleotides involved in the antiterminator structure. The antiterminator structure has a bulge that interacts with the unpaired residues at the acceptor end of an uncharged tRNA. Nucleotide conservation in all 722 T-box sequences analyzed was evaluated using a multiple sequence alignment obtained from the Rfam database, and residues are color coded accordingly.

From Gutiérrez-Preciado, et al. MMBR (73) 2009

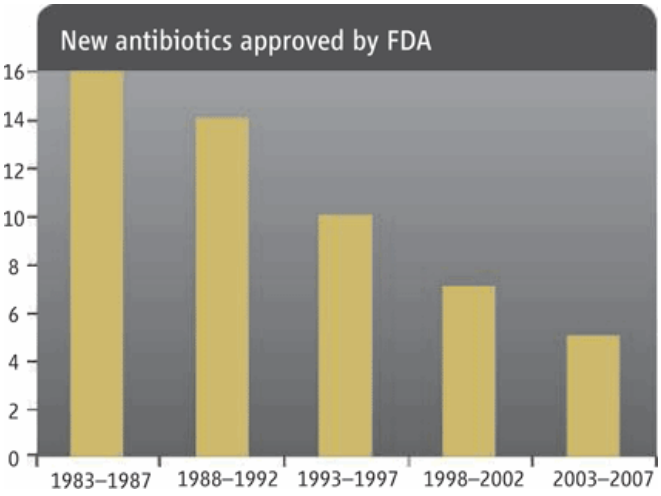
The 14 most highly conserved residues of the entire T-box RNA represent the "T-box sequence" (AGGGUGGNACCGCG). The recognition of this sequence in the leader regions of several aaRS genes led to the prediction of a conserved regulatory mechanism (12). The 3' end of the tRNA (5'-NACCA-3') with the first four residues of the antiterminator bulge (5'-UGGN-3') discriminates between uncharged and charged tRNAs. The N residue in the antiterminator bulge covaries with the corresponding position of the tRNA that plays an important role in tRNA identification for recognition by cognate aaRS. The amino acid at the 3' end of a charged tRNA prevents interaction with the antiterminator RNA. Both charged and uncharged tRNA can interact with the leader RNA at the Specifier Sequence however, only uncharged tRNA can stabilize the antiterminator sequence. Therefore, each T-box sequence monitors the ratio between the charged and uncharged forms of a specific tRNA rather than the absolute amount of the uncharged tRNA (13).

Drug resistance in Gram-positive pathogens

Despite the development of new therapeutic options, antibiotic resistance is an ongoing problem. Gram-positive pathogens are of particular concern, as resistance is increasing in organisms that have been susceptible to most available antibiotics until the past decade. Vancomycin, a drug of "last resort" is currently used to treat infections caused by Gram-positive bacteria. However, with increasing frequency of multidrug-resistant bacteria, including vancomycin-resistant enterococci (VRE) over time (Figure 3A) it has necessitated the development of other agents. With increasing resistance to current antibiotics and decreasing approval of new antibiotics by the FDA (Figure 3B), it has become necessary to find a new class of antibiotics to treat infections caused by Gram-

positive bacteria. We are developing a robust and responsive transcription reporter assay to study the functional ramification of interfering with the Specifer Loop:ASL binding. Small molecules that terminate transcription are candidates for drug development against MRSA or other Gram-positive bacteria.

A.



B.

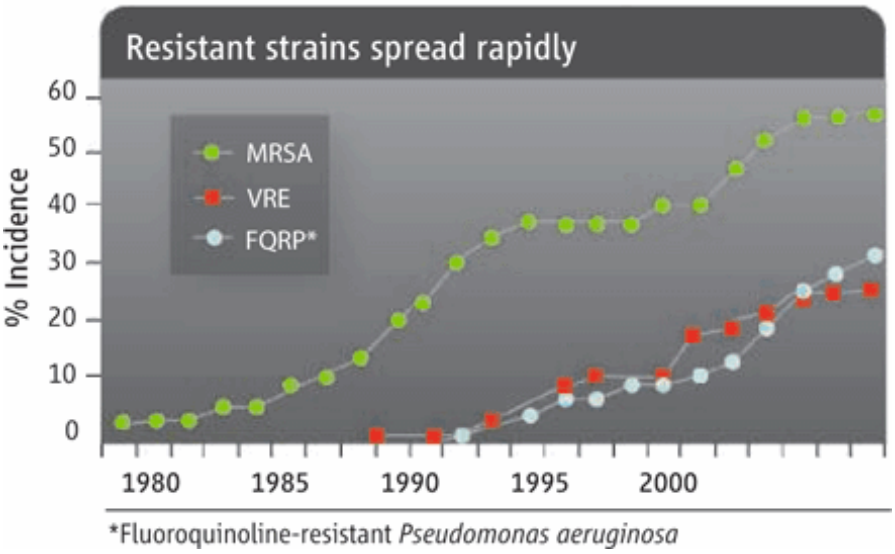


Figure. 3: A. Increasing frequency of MRSA, VRE, FORP resistance over time. B. Decreasing approval of new antibiotics by the FDA.

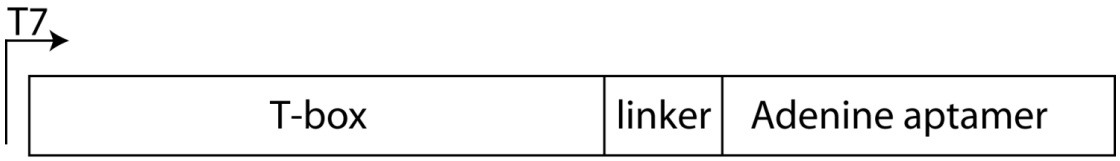
Specifier Loop - a novel drug target

The Specifier Loop domain, located in the Stem I of the 5'UTR that contains nucleotides that are complementary to and pair with the tRNA anticodon, has two major common RNA structural motifs (Loop E and K-turn motifs). These RNA structural motifs are essential for the proper function of the bacteria. The loop E motif in the Specifier Loop provides a stable platform that appears to help position the Specifier nucleotides to accept the anticodon of the cognate tRNA. This motif is found in several prokaryotic and eukaryotic rRNAs and the hairpin ribozyme (16, 17) These motifs create an intricate folding pocket in the Specifier Loop and can be used as a novel drug target against pathogenic, Gram-positive bacteria such as MRSA (methicillin-resistant *Staphylococcus aureus*) and *Bacillus anthracis*. In fact, a recently completed study of *in silico* docking simulations of 25,000 drug-like compounds on the Stem I structure (Cantara and Agris, unpublished data) indicated that 20 compounds bind to the Specifier Loop with specificity and selectivity. We are interested in targeting the Specifier Loop structure with small molecules to distort its structure and, as a consequence, look to see if they disrupt transcription function.

A transcription reporter assay:

We are developing a novel, high throughput assay to quantify transcription termination in the context of the full T-box riboswitch. An adenine-binding aptamer, which has high affinity for 2-aminopurine, has been inserted downstream of the riboswitch to monitor transcription termination.

A.



B.

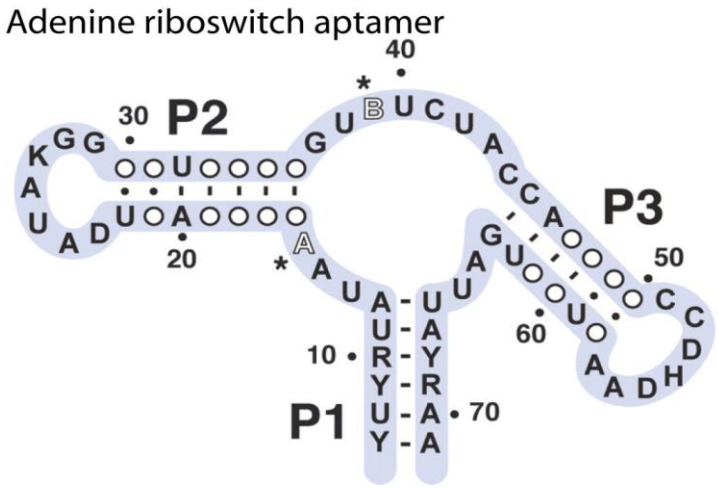


Figure 4: A. Sequence in which we designed our cassette: T-box riboswitch, linker region, and the adenine aptamer. B. Structure of the adenine riboswitch aptamer.

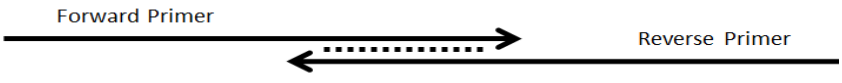
The fluorescence associated with this molecule is quenched once bound by the aptamer, therefore fluorescence will serve as a reporter for transcription termination. Unacylated tRNA is titrated in the presence and absence of small molecules shown *in silico* to bind the 5'UTR near the Specifier Loop to assess their ability to inhibit tRNA-mediated transcription. With the success of this system *in vitro*, future work would involve demonstrating its efficacy in cells.

MATERIALS AND METHODS

To create the T-box-aptamer (adenine binding aptamer) clone, an intervening linker sequence is required to ensure proper folding of the neighboring RNAs. 15-mer sequences were screened using M-fold to identify linkers that would not interfere with T-box or aptamer folding and AAAAAUAAAAAUAA was initially found to fit our criteria (this sequence was later changed later on to the naturally occurring sequence found in *B. subtilis*).

Initially, the T-box sequence was amplified by PCR from *B. subtilis* genomic DNA and cloned into pGEM-3Zf via PstI and HindIII restriction sites and resulting clones were sequenced. Using two overlapping primers, the cassette to clone the T-box-aptamer chimeric DNA was created. These primers were hybridized and filled in with Klenow to create a double stranded DNA (Figure 5) containing the linker and adenine aptamer with PstI and HindIII ends. The DNA was digested using PstI and HindIII and the gene fragment cloned into a vector and sequenced. We were not able to recover any wild type clones using this method, therefore ordered custom gene synthesis (Figure 6) from Integrated DNA Technologies (IDT) containing T-box-linker-aptamer sequences (Table 1).

A.



B.

Seq Name	Seq 5' to 3'	OD
Linker-VV1PstI	CCGCTGCAGAAAAATAAAAATAAATCA AGGCTTCATATAATCCTAATGATATGG TTTGGGAGTTT	7.9
Linker-VV1HindIII	AAGCTTACAGACTTCATAATCAAGAGT TTAAGGCTCTTGGTAGAAACTCCCAAAC CATATCATTA	8.7

Figure 4: A. Construction of Cassette, used to clone the T-box-aptamer chimeric DNA, using two overlapping primers. B. Oligonucleotide with regional 20 base pair overlap with 43 nt unpaired.

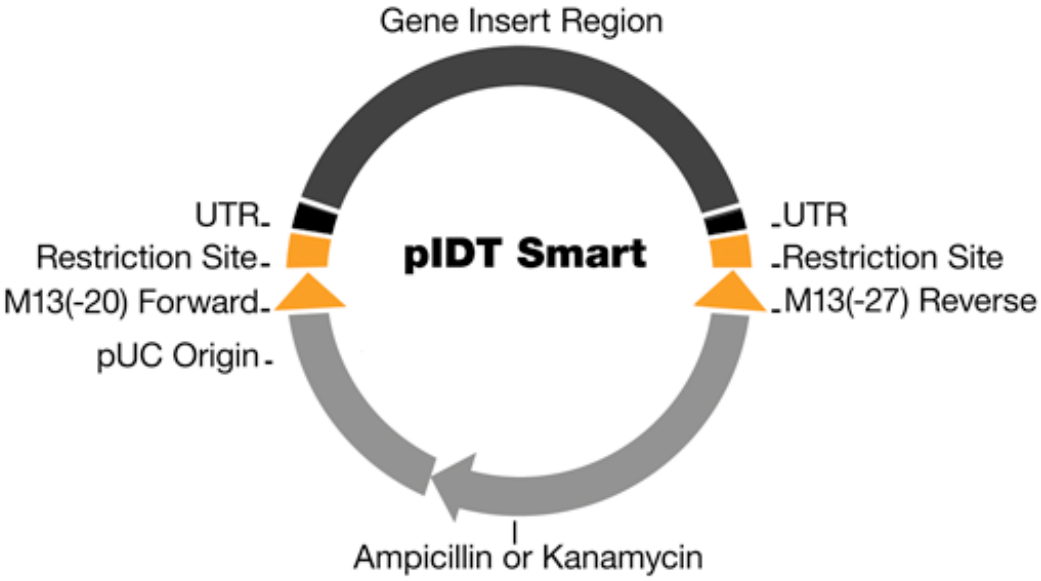


Figure 6: IDT's proprietary cloning vector, pIDTSmart. This vector has been specifically engineered to remove most common restriction endonuclease cleaving sites and does not contain a promoter within the cloning region. We used the kanamycin resistance cassette.

Tbox-Linker-adenine binding aptamer cassette	5'GAATTCactataaaatatgttgcagtgagagaaagaagtact tgcgtttacctcatgaaagcgaccttagggcggtgtaagctaag gatgagcacgcaacgaaaggcattcttgagcaattttaaaaaag aggctgggattttgttctcagcaactagggtggaaccgcgggag aactctcgtccctatgtttgcggctggcaagcatagagacggga gttttttggttAAAAATAAAAATAAAtcaaggcttcatataatc ctaatgatatggtttgggagtttctaccaagagccttaaactct tgattatgaagtctgtCTGCAG3'
Adenine binding aptamer	5'GAATTCtcaaggcttcatataatcctaatgatatggtttggg agtttctaccaagagccttaaactcttgattatgaagtctgtCT GCAG3'
Tbox-Linker-malachite green (MG) binding aptamer cassette	5'GAATTCactataaaatatgttgcagtgagagaaagaagtact tgcgtttacctcatgaaagcgaccttagggcggtgtaagctaag gatgagcacgcaacgaaaggcattcttgagcaattttaaaaaag aggctgggattttgttctcagcaactagggtggaaccgcgggag aactctcgtccctatgtttgcggctggcaagcatagagacggga gttttttggttAAAAATAAAAATAAAggatcccgactggcgaga gccaggtaacgaatggatccCTGCAG 3'

Table 1: T-box-linker-aptamer sequence. The yellow highlights are the restriction sites.

Cloning cassettes into pGEM-3Zf(+) for transcription

Restriction analysis and gel purification:

The custom plasmids from IDT were digested using EcoRI and PstI and cleaned using Qiagen PCR purification kit. The digested product was run on a 2% low melt agarose gel and bands corresponding to each released insert were excised and the DNA purified (Qiagen gel extraction kit). Purified DNA was checked on 1% agarose gel (Figure 7; result section).

Ligation and Transformation:

The DNA fragments, T-box-MG aptamer, T-box-2AP aptamer, 2AP aptamer, were ligated to EcoRI and PstI cut pGEM-3ZF plasmid using 200 U (in a 10 µL reaction) of T4 DNA ligase. Using the heat shock protocol, 2 µL of each ligation mix was used to transform 50 µL of XL-10 competent *E. coli* cells. The cells were plated on LB+ampicillin (100µg/ml) plates and grown overnight at 37 °C.

DNA purification and restriction analysis to look for presence of proper insert:

Picked 4 colonies from each plate and inoculated 3 mL LB+ampicillin. Grew the cells overnight at 250 rpm at 37 °C. Purified the plasmid DNA using Qiagen spin miniprep kit followed by restriction analysis with EcoRI and PstI. Checked products on a 1% agarose gel (Figure 8; result section).

Sent the plasmid with insert for sequencing and all were wild type.

Correct mutant $tRNA^{gly}$/pUC18 plasmid

It was found that all the sequenced plasmid had the CCA-AAG mutation. When looking at the sequence, it was noticed that two mutations were present in the "wild type" sequence. The first mutation had a missing A, in the 6^{th} position from the 5' end, in the

acceptor stem of the tRNA. The second mutation was a G missing downstream of the tRNA sequence in the pUC18 plasmid in the M13-pUC reverse primer binding site. While the second mutation has no effect on the tRNA being produced, the first acceptor stem mutation could have a major effect on the structure in that portion of the tRNA. Used the following primers to correct mutant $tRNA^{gly}$/pUC18 plasmid.

Sequence name	Seq 5′ to 3′	OD
$tRNA^{gly}$+A6Forward	CTCACTATAGGCGGAAGTAGTTCAGTGGTAG	24.2
$tRNA^{gly}$+A6Reverse	CTACCACTAACTACTTCCGCCTATAGTGAG	23.9

Table 2: Primers used to quickchange pUC18 plasmids with $tRNA^{gly}$ and $tRNA^{gly}$AAG to add A6 back o the sequence

Did 18 cycles of PCR, annealing temperature of 55 °C, to quickchange pUC18 plasmids with $tRNA^{gly}$ and $tRNA^{gly}$AAG to add A6 back to the sequence. In a total reaction mixture of 50 µL, 125 ng of reverse and forward primer, 10 MM of dNTPs, 1 U of Phusion protein (Finnzymes), and 20 ng of "wild type" tRNA (reaction 1) and 20 ng of tRNA AAG (reaction 2) were added. Used XL-10 cells and heat shock method for transformation. Picked up colonies and inoculated it in 3 mL LB+ampicillin overnight culture at 250 rpm. Did miniprep to purify the DNA using the Quigen kit.

Transcription

Linearized the template with T-box-2AP and 2AP using HindIII restriction enzyme. Did phenol chloroform extraction and ethanol precipitation to purify the DNA. Used 0.43 pmol of DNA, 100 µM NTPs, 10x reaction buffer, 150 mM of $MgCl_2$, T7 polymerase and varying amounts of tRNA (1 fold molar excess and 10 fold molar excess) for a 20 µL

transcription reactions. Incubated the mixture for 2 hrs at 37 °C. Ran the transcription product on a 6% denaturing gel (Figure 9; result section).

Redid the transcription (2nd time) with 1:1000 dilution of NTP and equimolar, 10 fold molar excess and 100 fold molar excess of tRNA. Did a 1:10 dilution of the transcription product and loaded 2 µL on a 6% denaturing gel. No bands were observed on the gel.

We later redesigned the T-box-linker-apatmer cassette by using the naturally occurring sequence found downstream of the T-box in *B. subtilis* as the linker (highlighted in red).

Tbox-Linker-adenine binding aptamer cassette	5′GAATTCactataaaatatgttgcagtgagagaaagaagtacttgcgt ttacctcatgaaagcgaccttagggcggtgtaagctaaggatgagcacg caacgaaaggcattcttgagcaatttaaaaaagaggctgggattttgt tctcagcaactagggtggaaccgcgggagaactctcgtccctatgtttg cggctggcaagcatagagacgggagtttttggttGCTGCCGCAGTCAA CTTATGAtcaaggcttcatataatcctaatgatatggtttgggagtttc taccaagagccttaaactcttgattatgaagtctgtCTGCAG3′
Adenine binding aptamer	GAATTCtcaaggcttcatataatcctaatgatatggtttgggagtttct accaagagccttaaactcttgattatgaagtctgtCTGCAG
Tbox-Linker-malachite green binding aptamer cassette	5′GAATTCactataaaatatgttgcagtgagagaaagaagtacttgcgt ttacctcatgaaagcgaccttagggcggtgtaagctaaggatgagcacg caacgaaaggcattcttgagcaatttaaaaaagaggctgggattttgt tctcagcaactagggtggaaccgcgggagaactctcgtccctatgtttg cggctggcaagcatagagacgggagtttttggttGCTGCCGCAGTCAA CTTATGAggatcccgactggcgagagccaggtaacgaatggatccCTGC AG 3′

Table 3: T-box-linker-aptamer sequence with revised linkers – T-box naturally occurring downstream sequence (21 nt; highlighted in red). The yellow highlights are the restriction sites.

For transcription using the revised linker sequences, we used 45.36 pmol DNA for Tbox-lnk-2AP template and 51.5 pmol DNA for Tbox-lnk-MG template, 100 µM NTPs, 10x reaction buffer, 150 mM of $MgCl_2$, T7 polymerase and 10 fold molar excess of tRNA for a 30

μL transcription reaction. Incubated the mixture for 2 hrs at 37 °C. Ran the transcription product on a 6% denaturing gel (Figure 12).

This transcription reaction, with the naturally occurring sequence, was repeated with different NTP concentration and $MgCl_2$ concentration (data not shown).

Fluorescence Studies

We carried out the fluorescence studies using PerkinElmer's Envision® Multilabel Plate Reader. Inorder to find the optimum amount of 2AP needed to do the fluorescence studies we did several dilution series (100 mM to 1 pM). Excitation for 2AP fluorescence was done at 309 nm to obtain a good separation between the Raman peak and the 2AP-fluorescence signal. The emission wavelength was set 375 nm with the measured height of 8.2 nm. From the data we concluded that 100 nM of 2AP is the optimum concentration to carry out the fluorescence studies (Figure 13). To do the transcription studies using fluorescence 100 nM of 2AP was used. RNA was transcribed from a double-stranded DNA template (1 μg) using 10x reaction buffer, $MgCl_2$ (150 mM), NTPs (100 μM), and T7 RNA polymerase (30 μL reaction). The RNA was purified with ethanol precipitation.

Prior to 2AP fluorescence measurements, the RNA was heated for 1 min to 95°C in water and then preincubated at 23°C for 10 min in the reaction buffer (10 mM $MgCl_2$, 50 mM Tris–HCl, pH 8.3, and 100 mM KCl) to ensure homogeneous folding of RNA species. All data were collected at 25°C at a fixed 2AP concentration (100 nM) with decreasing amount of RNA (1μM to 12.8 pM). Spectra were corrected for background, and intensities were determined by integrating the data collected over the range 330–450 nm.

Results

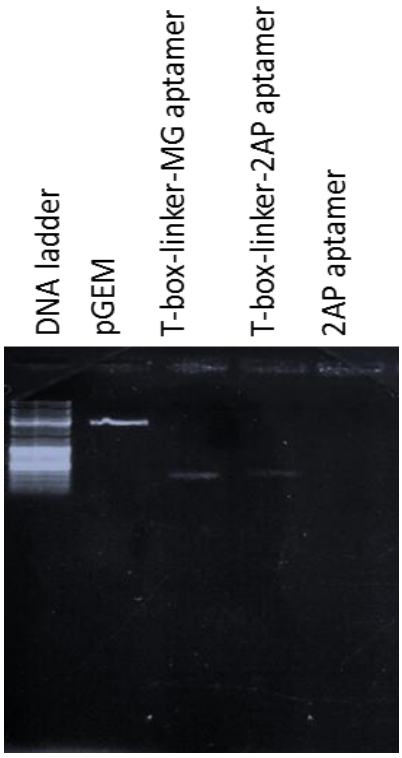


Figure 7: From left to right: DNA ladder, pGEM, T-box-linker-MG aptamer, T-box-linker-2AP aptamer, 2AP aptamer. 1% agarose gel.

Due to the small size (90 bp) of the DNA fragment the 2AP aptamer was not seen on the gel (Figure 7). The size of the DNA fragment might be too small for the gel purification column. We excised the area where the DNA fragment should be and did gel purification with it. Ligation studies showed that 2AP aptamer was present in the gel during purification.

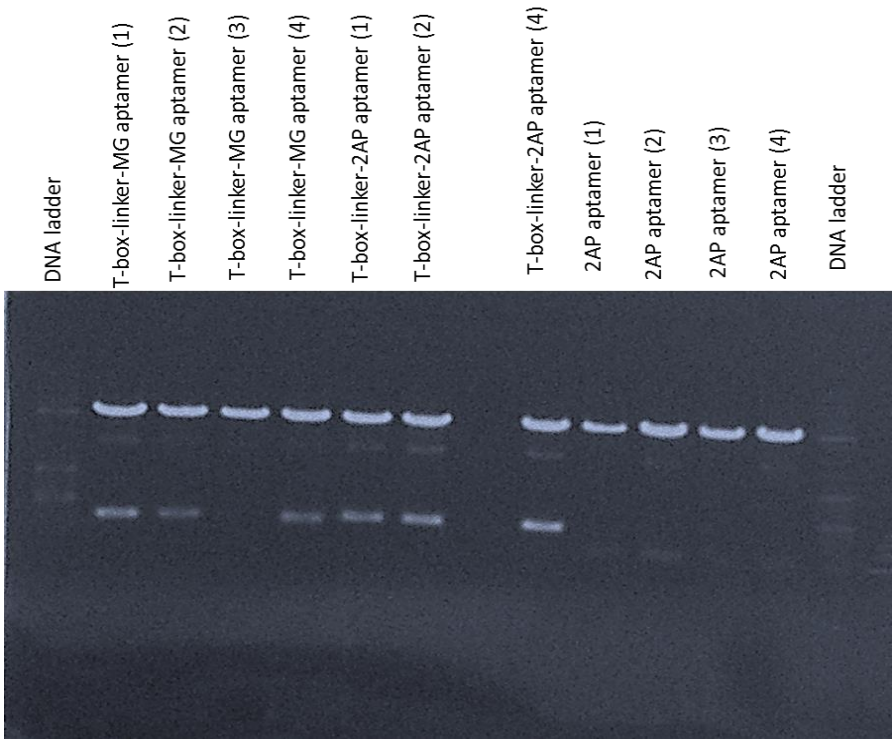


Figure 8: Miniprepred cultures with 4 different colonies with each insert and ran the samples on 1% agarose gel. From left to right: DNA ladder, T-box-linker-MG aptamer sample 1, 2, 3, 4; T-box-linker-2AP aptamer sample 1, 2, 3, 4; 2-AP aptamer sample 1, 2, 3, 4; DNA ladder

With increasing concentration of tRNA there should be a shift towards the readthrough product. However, with increasing concentration of tRNA a shift in the formation of the termination product was noticed. With 10 fold molar excess tRNA a 3rd band was seen (Figure 9). Results (Figure 10, 11) suggested that we might have created a second termination site in the linker region into the aptamer region. One of the reasons for the formation of this termination site might be the presence of so many adenine residues.

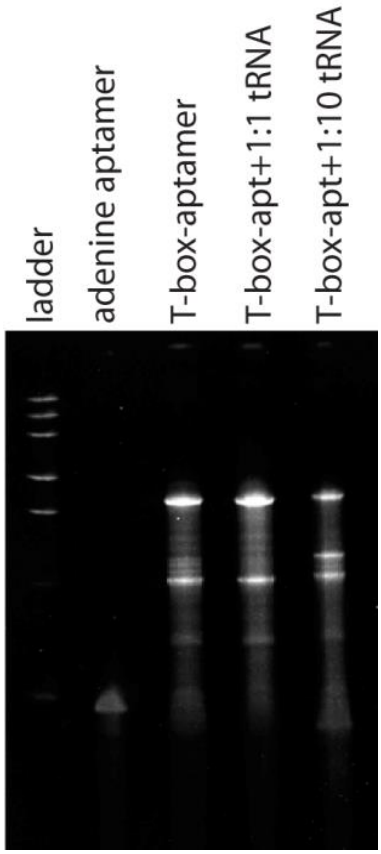


Figure 9: A 6% denaturing gel with 2 µL of transcription products (1:10 dilution). From left to right: RNA ladder, 2AP template, T-box-linker-2AP template, T-box-linker-2AP+tRNA (1 fold molar excess), T-box-linker-2AP+tRNA (10 fold molar excess).

Henkin lab showed that as the NTP concentration was increased, the level of readthrough increased in the absence of tRNA and at the highest concentration of NTP, all of the observed readthrough was tRNA independent (16). This indicated that both termination and tRNA-dependent antitermination are sensitive to NTP concentration. Knowing this, we redid the transcription with varying amount of NTPs and tRNA. However, our results (Figure 10) showed no transcription activity when the NTP concentration was lowered. And when 100 fold molar excess of tRNA was used, a complete shift towards the termination product was noticed (Figure 11).

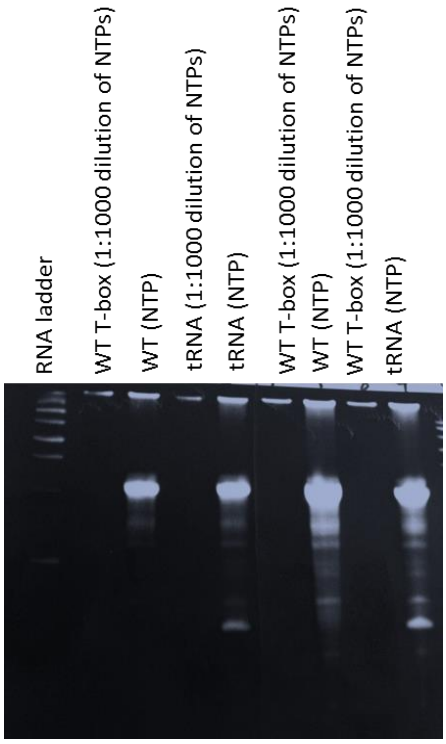


Figure 10: A 6% denaturing gel with varying amount of transcription product, NTPs, and tRNA. From left to right: 2 µL of RNA ladder, WT T-box (1:1000 dilution of NTPs), WT (NTP), tRNA (1:1000 dilution of NTPs); tRNA (NTP); 5 µL of WT T-box (1:1000 dilution of NTPs), WT (NTP), tRNA (1:1000 dilution of NTPs); tRNA (NTP), RNA ladder.

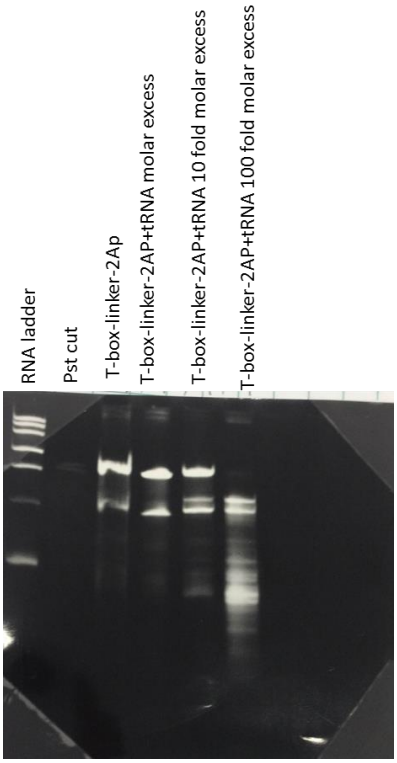


Figure 11: A 6% denaturing gel with varying amount tRNA. From left to right: 2 µL of RNA ladder, plasmid cut with Pst; T-box-linker-2AP (HindIII cut), T-box-linker-2AP+tRNA (one fold molar excess; HindIII cut), T-box-linker-2AP+tRNA (10 fold molar excess; HindIII cut), T-box-linker-2AP+tRNA (100 fold molar excess; HindIII cut)

Due to the formation of a potential 2nd termination site in the linker region, we changed the linker region to the naturally occurring sequence found in *B. Subtilis*. The studies do not show a shift to formation of readthrough product in the presence of uncharged tRNA, even at 10-fold molar excess (Figure 12). Terminated products were produced even in the absence of tRNA. However, no 3rd band was noticed with 10 fold molar excess tRNA.

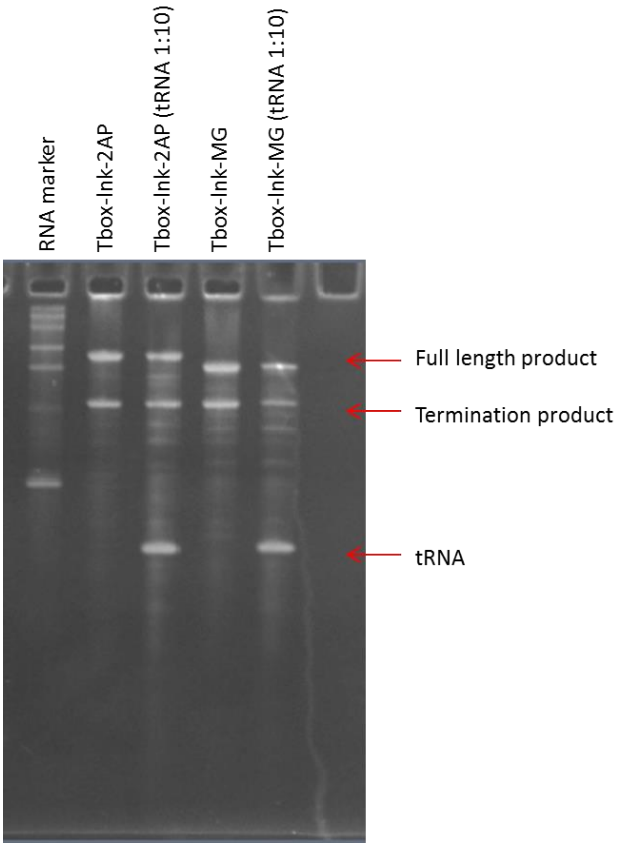


Figure 12: A 6% denaturing gel with varying amount of RNA (with naturally occurring linker region). From left to right: RNA marker, Tbox-lnk-2AP, Tbox-lnk-2AP + tRNA (10 fold molar excess), Tbox-lnk-MG, Tbox-lnk-MG + tRNA (10 fold molar excess).

Fluorescence data:

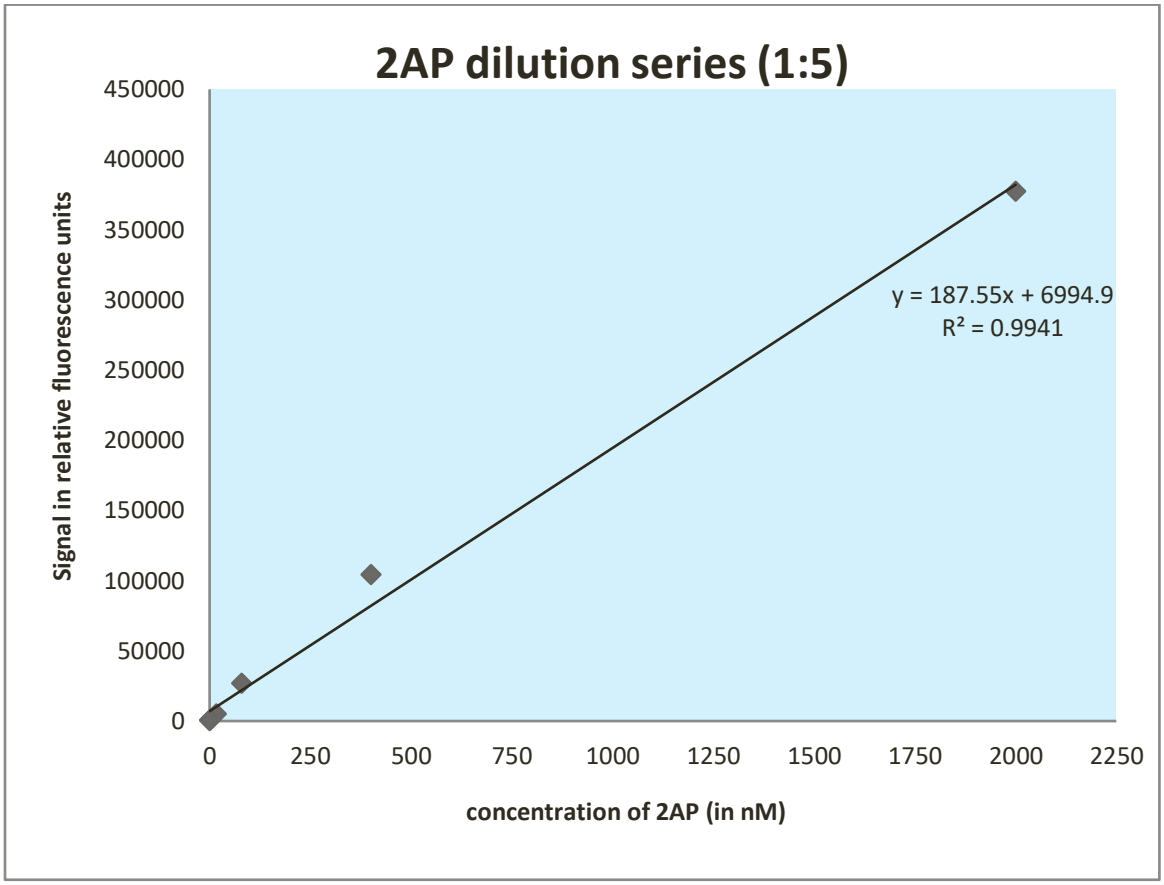


Figure 13: Dilution series (100 mM to 1 pM) of 2AP. Excitation wavelength and emission wavelength for 2AP fluorescence was done at 309 nm and 375 nm respectively.

After doing serial dilution it was concluded that 100 nM of 2AP is the optimum concentration to carry out the fluorescence studies. Preliminary studies have been done using 100 nM of 2AP with increasing concentration of RNA (1 μM to 12.8 pM) and the data showed that the aptamer RNA is able to quench 2AP fluorescence.

DISCUSSION

With the addition of uncharged tRNA, there should be a shift towards formation of the readthrough product. However, the pilot studies do not show shift to formation of readthrough product in the presence of uncharged tRNA, even at 10-fold molar excess. Instead, the results show that addition of unacylated tRNA is resulting into termination (Figure 10, 11) and formation of a 3rd band, that were are unable to explain to explain (Fig. 9). The results suggest that we might have created a second termination site in the linker region into the aptamer region. One of the reasons for the formation of this termination site might be the presence of so many adenine residues. We therefore redesigned the T-box-linker-apatmer cassette by using naturally occurring sequence found downstream of the T-box in *B. subtilis* as the linker. The studies with the naturally occurring linker region did not show a shift to the formation of readthrough product in the presence of uncharged tRNA, even at 10-fold molar excess. Terminated products were produced even in the absence of tRNA. However, no 3rd band was noticed with 10 fold molar excess tRNA (Figure 12).

Choice of the promoter and cognate RNA polymerase is important for transcription antitermination assay. While we have transcribed both cassettes using T7 promoter for determining the optimal conditions for the fluorescence assay, we are currently cloning the MG aptamer downstream of the T7 promoter for similar control experiments. While T7 RNA polymerase does respond to rho-independent terminators (17, 18, 19), the terminator sequence plays a role in responsiveness to these sites (19). T7 RNA polymerase is often used for its robust activity as compared to bacterial polymerases. The transcription rate of T7 and bacterial polymerases varies, with bacterial polymerases synthesizing RNA at 10-35 nt sec^{-1} *in vitro* (20, 21) and T7 polymerase incorporating nucleotides at 200-400 nt sec^{-1} *in*

vitro (22). This might be part of the reason for the inefficient termination with T7 polymerase.

We therefore explored the use of alternative bacterial RNA polymerases and corresponding promoter such as the commercially available *E. coli* RNA polymerase holoenzyme and *E.coli* KAB-TTTG promoter. The KAB-TTG promoter is a derivative of the KAB-TG promoter, a factor-independent derivative of the *gal*P1 promoter with a unique *Sph*I site between the -35 and -10 hexamer elements (Cite this). The results showed that the formation of the readthrough product was independent of the uncharged tRNA.

Studies have been done to show that there is RNA-RNA interaction between the *Bacillus subtilis* 5'-UTR Tbox riboswitch and its cognate tRNA. Dr. Kun Lu, at Dr. Paul Agris's lab, used isothermal titration calorimetry (ITC) to study this interaction. No interaction was seen at physiological salt conditions of ~1 mM Mg^{2+}; however, at 10 mM, the RNA:RNA interaction was observed. An increase in binding affinity was observed with the increase of Mg^{2+} up to 40 mM. The thermodynamic parameters obtained for this interaction by ITC showed significantly tighter binding compared to those previously determined by filter binding studies (40 nM vs 5 uM). Electrophoretic mobility shift assays confirmed the Mg^{2+} dependence for the Tbox riboswitch-tRNA interaction. Therefore, we tried varying amount of Mg^{+2} to do the transcription reaction however, there was still no shift to towards the readthrough product with the addition of unacylated tRNA. The studies by Dr. Lu show that there is interaction between the Tbox riboswitch and its cognate tRNA however, we are not certain if the addition of the adenine aptamer at the end of our cassette is interrupting this interaction.

With the naturally occurring linker we did not see any second terminated product (as seen in Figure 12) however, we still were not seeing any tRNA dependent readthrough. This might be because of the promoter and the polymerase we are using. The T7 promoter and polymerase that we are using are not naturally occurring sequences. Previous transcription studies done by the Henkin lab used a naturally occuring *B. subtilis* promoter and polymerase. The naturally occurring *B. subtilis* polymerase is not commercially available and we currently do not have the resources to purify it. However, it has been shown that *E. coli* RNA polymerase exhibited $tRNA^{Gly}$-dependent read-through similar to that observed with *B. subtilis* RNA polymerase (cite it) This indicated that the antitermination event is dependent on features of the transcript, but not on the enzyme that generates this transcript. Next, we will be exploring the use of the naturally found *B. subtilis* promoter and the *E. coli* polymerase to do the transcription studies and eventually test small molecules that disrupt gene expression or use S. aureus RNA polymerase and sigma factor. Then carry out the fluorescence studies in real time during transcription and determine the effects of these small molecules on transcription readthrough.

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