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GEARING UP TO UNWIND: DESIGNING STRUCTURED MESSENGER RNA TO BIOCHEMICALLY AND STRUCTURALLY INVESTIGATE THE RIBOSOMAL mRNA HELICASE

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by

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Abstract:
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Although the ribosome is known to function as a helicase, unwinding downstream secondary structured messenger RNA while simultaneously translating upstream mRNA into a polypeptide chain, the intricacies of these unwinding interactions are not well understood. mRNA containing secondary structure can result in ribosomal pausing, as well as frameshifting and mRNA decay pathways. While previous structural studies imply delays due to issues with mRNA-tRNA translocation, the precise interactions and resulting events when an mRNA stem-loop is poised at the entry tunnel of the ribosome, remain elusive. We have designed an mRNA specifically configured to highlight these interactions. Our results illustrate where we propose the ribosome should be stalled to elicit unwinding activity and set the stage for the determination of a new cryo-EM structure which should illuminate mechanistic details of the ribosomal helicase.

Acknowledgements

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Introduction

The ribosome is a fundamental molecular machine, illustrated by its existence and convergent evolution in all living things, from Archaea, to bacteria, yeast, and humans. Utilizing a template of messenger RNA, the ribosome generates every polypeptide chain and therefore, every protein. This is the process of translation, in which the ribosome reads the messenger RNA nucleotides in triplet as codons, and matches them with their opposing transfer RNA molecule, where a bond is formed between the amino acids, ultimately resulting in a full-length polypeptide chain. This complicated process of tRNA selection according to the deciphering of the mRNA template occurs through rotation between the large and small subunits of the ribosome, alternating between classical, hybrid, and chimeric hybrid states. While our understanding of the ribosome has advanced significantly in recent decades with the use of X-ray crystallography and cryogenic electron microscopy to visualize these intricate details, with the emergence of more antibiotic resistant strains of bacteria and recently, the RNA virus SARS-CoV-2, it is evident that we have only scratched the surface in our understanding of the functionality and efficiency of the ribosome.

During formation of the polypeptide chain, the ribosome elongates by moving from the 5’ end toward the 3’ end of the mRNA. The small subunit, the 30S in bacterial ribosomes, contains a small tunnel between the head and the shoulder which allows for entry of the mRNA strand. The depth of the tunnel occupies 11 nucleotides downstream from the P-site codon at the +1 nucleotide position. However, the downstream entry tunnel is narrow, preventing any mRNA containing secondary

structure from fitting. As secondary structure is an extensive part of nearly all mRNAs, the ribosome must address and unwind the structure into a singular RNA strand for translation to occur. Therefore, the ribosome can also be classified as a helicase, moving forward on the mRNA in a processive fashion and handling three base pairs at one time, while simultaneously translating the upstream mRNA. Ribosomal processivity has been shown to be affected by the relative availability of aminoacyl-tRNA, as well as encountering mRNA with secondary structure (Jacobson and Clark, 2016, Rodnina and Wintermeyer, 2016). As mRNA containing secondary structure is unavoidable, it has been proposed that the resulting ribosomal pausing events are in fact signals to elicit populations of ribosomes to decode an alternative reading frame or delay for appropriate folding of the emerging polypeptide chain, playing a role in gene activity and the overall abundance of certain proteins (Farabaugh 1996, Giedroc and Cornish, 2009, Dinman 2012, Atkins et al., 2016, Rodnina and Wintermeyer, 2016).

Previous structural data have shown that the universally conserved ribosomal proteins uS3, uS4, and uS5 form a sort of triangle at the mRNA entry tunnel, and it was proposed that they may be involved in the ribosome's helicase activity (Yusupova et al., 2001). Specifically, mutations in uS3 were shown to cause a decrease in ribosome helicase activity (Takyar et al., 2005). However, it is not fully understood why certain sequences of secondary structure are more likely to induce ribosomal frameshifting or decay pathways. A cryo-EM structure utilizing 70S *T. thermophilus* ribosomes and an mRNA sequence similar to the E.coli *dna*X sequence,

which induces hyper-rotation and ribosomal frameshifting, shows as the mRNA stem loop is poised near the entry tunnel, the tRNA leaving the E site is sterically hindered and therefore unable to exit (Zhang et al., 2018). This implies that ribosomal frameshifting and decay pathways may be affected not by delays due to the actual unwinding process itself, but also due to the ribosome being sterically hindered from moving the tRNA to a P/E hybrid state and fully translocating. Another cryo-EM structure utilizing *E.coli* 70S ribosomes and sequences from E.coli *dna*X and the *gag-pol* transcript of the Human Immunodeficiency Virus (HIV) implicates the stem loop itself as an inhibitory element for translocation elongation by two plausible mechanisms. First, the secondary structure can evade the ribosomal helicase activity altogether and bind to the A site as a stem loop, sterically hindering incoming aminoacyl-tRNA. This mechanism has also been identified with a short, four-basepair long hairpin loop entering the A site (Yusupova et al., 2001) as well as a five-basepair long hairpin loop (Tesina et al., 2020). Secondly, the mRNA stem loop can inhibit mRNA-tRNA translocation through direct interaction with the mRNA entry tunnel of the ribosome, and specifically with ribosomal proteins uS3, uS4, and uS5, preventing tRNA from binding to the A site (Bao et al., 2020).

While each of these structures has implied that mRNA with secondary structure poses a barrier to full and seamless mRNA-tRNA translocation, the finite mechanisms and order of events when a ribosome encounters a strong stem loop at the mRNA entry tunnel remain unclear. Is translocation inhibited because of steric hinderances with the exiting tRNA of the E site, or because the stem loop itself is

docking into the A site and obstructing the incoming tRNA? If both can occur, do specific sequences or sizes of stem loops preferentially result in one translocation complication over the other? How does the mRNA stem loop at the entry tunnel interact to inhibit tRNA binding to the A site when the decoding site is physically distanced away from the entry tunnel? As a whole, how do these technicalities affect the functionality and processivity of the ribosome, such as seen through ribosomal stalling events and No-Go mRNA decay? Although previous structural and biochemical data have provided a glimpse into the unique position of the ribosome as a helicase, much remains elusive.

The goal of my project is to set the stage for future structural and biochemical studies which will show the physical interactions of a stem-loop being within the active site of the ribosomal helicase, which has not been seen before. I will accomplish this by designing an mRNA with a strong stem-loop which is specifically structured with enough clearance to account for the mRNA entry tunnel and still elucidate the physical unwinding activity of the helicase. I found that my designed mRNA meets this criteria and stalls at the desired location to depict the complete unwinding activity, which will be used for future data collection using cryo-EM.

Materials & Methods

Materials

The designed sequences for SS and SK_TP constructs were cloned into plasmid pH03_fMVVV using site-directed mutagenesis (Kunkel 1985) and verified by sequencing conducted at UC Berkeley and Sequetech. SS and SK_TP mRNAs were transcribed by T7 RNA polymerase from linearized pH03_fMVVV plasmid and purified on a 7% denaturing polyacrylamide gel.

SS_mRNA

5’GGAAAGGAAAUAAAAAUGGUUGUUGUUGUUGUUGUCGUCGUCGGGC

UCGGUGCAGGCAACUGCACCGAGCCCGACGACGACUGU

SK_TP_mRNA

5’GGAAAGGAAAUAAAAAUGGUUGUUGUUGUUGUUGUCGUCGUCGGGC

UCGGUGCAGGAAAAUAAUAGAAGAAUCGGAUAAGAGAACACAGGAUCC

AGCUGGCGUAAUA**GCGAAGAGGCCCGCACC**

The TP_short cloning construct was created using two overlapping, complementary oligonucleotides synthesized by IDT, which were annealed together at 90º, then incubated with 1x RT buffer (IDT), 0.5mM dNTP’s, and M_MuLV Reverse Transcriptase (NEB). After phenol/chloroform extraction and ethanol precipitation, the construct was transcribed using T7 RNA polymerase and purified on an 8% denaturing polyacrylamide gel.

SK_TP_short mRNA

5’GGAAAGGAAAUAAAAAUGGUUGUUGUUGUUGUCGUCGUCGGGC

UCGGUGCAGGAAAAUAAUAGAAGAAUCGGAUAAGAGAAC

Tight-couple 70S ribosomes were purified as described (Lancaster et al. 2002). To prepare in vitro-transcribed $tRNA^{Val}$, the gene from *E.coli* strain MRE600 was cloned into plasmid pRZ (Walker, Avis, and Conn 2003), which is designed to produce RNA transcripts with homogenous 3’ ends using the catalytic HDV ribozyme cleavage of the transcript. To prepare *in vitro*-transcribed $tRNA^{Gly}$, the Glycine CCC sequence from tdbD00004169 *E.coli* K-12 within the NCBI Taxonomy browser (NCBI:txid83333) was cloned into the pRZ_200 construct using site-directed mutagenesis (Kunkel 1985) and confirmed by sequencing. Following transcription, cleavage from the HDV ribozyme construct and gel purification, 2’,3’-cyclic phosphate was removed from the 3’ end of tRNA via incubation with T4 polynucleotide kinase (NEB) (Schurer et al. 2002). Aminoacylation was completed by incubation of purified tRNA, 5-fold molar excess of the corresponding amino acid, 4 mM ATP and DEAE-purified S100 enzymes. Aminoacylation was shown to be greater than 90% as monitored by acid gel electrophoresis (Varshney, Lee, RajBhandary 1991). fMet-tRNA-fMet (Sigma) and NAcMet-tRNA-Met (transcribed) were prepared as described (Lancaster and Noller 2005; Moazed and Noller 1989a).

Methods

Toeprinting Assay

mRNA SK_TP (5'GGAAAGGAAAUAAAAAUGGUUGUUGUUGUUGUUGUCGUCGUCGGGC UCGGUGCAGGAAAAUAAUAGAAGAAUCGGAUAAGAGAACACAGGAUCC AGCUGGCGUAAUA**GCGAAGAGGCCCGCACC**), coding for MVVVVVVVVG, was pre-annealed to 5'[32]P-labeled DNA primer (5'GGTGCGGGCCTCTTCGC), then utilized to form P-site tRNA complexes which contained: 0.4 μM 70S ribosomes, 0.8 μM NAc-Met-tRNA, and 1.2 μM SK_TP mRNA/primer, in 25 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl, 15 mM $MgCl_2$, and 1 mM DTT, and incubated at 37ºC for 20 minutes. Ternary complex was formed using 19.2 μM EF-Tu, 12.8 μM Val-$tRNA^{Val}$, and 2 mM GTP in 25 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl, 1 mM DTT, and 1 mM GTP and incubated for 5 minutes at 37ºC. 4 pmol of Gly-$tRNA^{Gly}$ was added separately to glycine reactions prior to the addition of ternary complex and P-site complex. For translocation, 6 pmol ternary complex were added to 10 pmol EF-G followed by the addition of 2 pmol of P-site complex, with the final condition of 25 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl, 7.5 mM $MgCl_2$, 1 mM DTT, and 1 mM GTP, in a total volume of 10 μL. Following a 5 minute incubation at 37ºC, the primer extension was initiated by the addition of 1 μL of extension mix, containing 0.55 mM of each dNTP and 0.05 μL of AMV reverse transcriptase (Seikagaku) in the same buffer, and incubation continued for 5 minutes at 37ºC. After ethanol precipitation, samples were run on an 8 M urea, 7.5%

polyacrylamide gel at 60W for 1 hour and 20 minutes. The gel was dried, exposed in a phosphor screen cassette overnight, and imaged on a Typhoon laser scanner.

Filter-Binding Assays

Nitrocellulose filter binding assays for P-site mRNA dependence were performed by premixing 2 pmol of *Thermus thermophilus* 30S ribosomes and 2.4 pmol of ^{35}S-labeled fMet-tRNA^{fMet} in buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl, 10 mM mM MgCl2, and 1 mM DTT, followed by the addition of serial dilutions (0-16 pmol) of SS mRNA (5'GGAAAGGAAAUAAAAAUGGUUGUUGUUGUUGUCGUCGUCGGGC UCGGUGCAGGCAACUGCACCGAGCCCGACGACGACUGU) in a total volume of 10 µL, and incubation at 37°C for 20 min for P-site tRNA binding. After chilling on ice for 5 minutes, 10 µL of each reaction was blotted on a presoaked nitrocellulose HA filter (Millipore), which was then washed three times with a total of 15 mL wash buffer ($T_{2.5}N_{100}M_{10}$), dried, and quantified using a scintillation counter. Background filter binding in the absence of ribosomes was subtracted and maximum possible counts were measured by blotting 2.4 pmol of ^{35}S-labeled fMet-tRNA-fMet directly to a filter without any wash steps. Results were analyzed by plotting within Excel and determining mRNA-dependent P-site binding.

Nitrocellulose filter binding assays for multiple round translocation with *E.coli* 70S ribosomes were performed by premixing 2 pmol ribosomes and 4 pmol of

NAcMet-tRNA^{Met} in buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl, 15 mM mM MgCl2, and 1 mM DTT, followed by the addition of 4 pmol of either SS mRNA or MV_39 mRNA in a total volume of 10 µL, and incubation at 37°C for 20 min for P-site binding. Ternary complex was formed using 1.2 µM EF-Tu, 16 pmol ^{14}C-labeled-Val-tRNA^{Val}, and 2 mM GTP in 20 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl, 1 mM DTT, and 1 mM GTP and incubated for 5 minutes at 37ºC. For translocation, 6 pmol ternary complex was added to 10 pmol EF-G followed by the addition of 2 pmol of P-site complex, with the final condition of 20 mM Tris-HCl (pH 7.5), 100 mM NH_4Cl, 7.5 mM $MgCl_2$, 1 mM DTT, and 1 mM GTP, in a total volume of 10 µL, incubated at 37ºC for 5 minutes. After chilling on ice for 5 minutes, 10 µL of each reaction was blotted on a presoaked nitrocellulose HA filter (Millipore), which was then washed three times with a total of 15 mL wash buffer ($T_{2.5}N_{100}M_{7.5}$), dried, and quantified using a scintillation counter. Background filter binding in the absence of ribosomes was subtracted and maximum possible counts were measured by blotting 2.4 pmol of ^{14}C-labeled-Val-tRNA^{Val} directly to a dry filter without any wash steps. Results were analyzed by plotting scintillation data within Excel.

In-vitro Translation and Cleavage using RelE

mRNA TP_short

(5’GGAAAGGAAAUAAAAAUGGUUGUUGUUGUUGUCGUCGUCGGGC

UCGGUGCAGGAAAAUAAUAGAAGAAUCGGAUAAGAGAAC),

and mRNA SS
(5'GGAAAGGAAAUAAAAAUGGUUGUUGUUGUUGUCGUCGUCGGGC
UCGGUGCAGGCAACUGCACCGAGCCCGACGACGACUGU),
both coding for MVVVVVVVVG, were dephosphorylated on the 5' end using rSAP (NEB), then treated with phenol/chloroform and ethanol precipitated, for labeling with ATP [α-^{32}P] and T4 Polynucleotide kinase (NEB). Each labeled mRNA was collected from a G50 column, pre-equilibrated with Milli-Q water, and counted via liquid scintillation. Labeled mRNAs were then utilized to form P-site tRNA complexes which contained: 0.5 μM 70S ribosomes, 1 μM NAcMet-tRNA^{Met}, and 1 μM of 5'-[α-^{32}P] labeled TP_short or 5'-[α-^{32}P] labeled SS mRNA, in 20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 50 mM KCl, 10 mM NH_4Cl, and 6 mM β-Mercaptoethanol, and incubated at 37ºC for 20 minutes. Ternary complex was formed using 24 μM EF-Tu, 8 μM Val-tRNA^{Val}, and 2 mM GTP in 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 10 mM NH_4Cl, and 6 mM β-Mercaptoethanol and incubated for 5 minutes at 37ºC. 4 pmol of Gly-tRNA-Gly was added separately to corresponding reactions translating through glycine codon prior to the addition of ternary complex and P-site complex. For translocation, 4 pmol ternary complex was added to 10 pmol EF-G followed by the addition of 2 pmol of P-site complex, with the final condition of 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM KCl, 10 mM NH_4Cl, and 6 mM β-Mercaptoethanol in a total volume of 8 μL. Following a 5 minute incubation at 37ºC, 2 pmol of purified RelE enzyme (graciously provided by Scott A. Strobel) was added at room temperature, followed by a 10 minute incubation at 37ºC. Samples

were prepared for gel electrophoresis by the addition of formamide and dye, then run on an 8 M urea, 8.5% polyacrylamide gel at 60W for 1 hour and 20 minutes. The gel was dried, exposed in a phosphor screen cassette overnight and imaged on a Typhoon laser scanner.

Results

For the purpose of elucidating helicase interactions of the ribosome, an mRNA was designed with strong secondary structure but enough clearance to stall the ribosome precisely where unwinding interactions would be taking place near the entry tunnel. We focus on the designed SS mRNA, MVVVVVVVVG, which contains a strong helix with 71% G/C content. The helix structure containing complete complementarity begins immediately following the third Valine codon and allows for a proposed stall site at the Glycine codon, with 10 nucleotides before the GCAA loop structure to account for the depth of the entrance tunnel.

Toeprinting Assay

To demonstrate the ribosome's capability to undergo multiple round translocation through the corresponding Valine codons before stalling and ensure the effectiveness of the transcribed tRNAs, a toeprinting assay was utilized. Due to the limitations of extension by reverse transcriptase, the secondary structure complementarity was removed, and a DNA primer binding site was added. This resulted in a version of the SS mRNA design specifically modified for toeprinting: SK_TP mRNA. Pre-translocation complexes bound to the SK_TP mRNA, coding for

MVVVVVVVVG, were prepared by binding NAc-Met-tRNA^{Met} to the P-site. This was followed by an introduction of a two-fold excess of Val-tRNA·EF-Tu·GTP ternary complex, EF-G·GTP. The DNA primer which was annealed to the 3' end of the mRNA and labeled with 5'[32]P (GGTGCGGGCCTCTTCGC), was then extended by reverse transcriptase. The stall site of the ribosome can be determined by the length of the resulting extended DNA fragment.

The resulting DNA fragments showed the ribosome was capable of multiple round translocation during the 5 minute incubation, through the 8 Valine codons. The resulting band size and intensity was compared to the control mRNA, EL_118 coding for MVVV, chosen for its previous use in toeprinting assays and similar to the SS mRNA construct, utilization of the Val-tRNA$_2^{Val}$ as opposed to natural Val-tRNA$_1^{Val}$. The ribosome translocated through the mRNA until the next required tRNA for A-site binding was unavailable for the control EL_118 mRNA and the SK_TP mRNA, either completing through only Methionine for P-site reactions and all Valines (Figure 2). An additional doublet band is visible within the P-site and added Valine tRNA lanes for the SK_TP mRNA, which corresponds in size with the reverse transcriptase stuttering near the Glycine codon, within the sequence 'CGGGC'.

Filter-Binding Assays

To determine if the designed SS mRNA is capable of binding the ribosome in a P-site dependent manner, a filter-binding assay was utilized with *T. thermophilus* 30S ribosomes versus *E.coli* 70S ribosomes to avoid external influence from magnesium concentrations while investigating P-site binding. *E.coli* 70S ribosomes

require a specific magnesium concentration, which is difficult to maintain while titrating with varying levels of mRNA. Therefore, as T. *thermophilus* 30S ribosomes provide more flexibility in regard to magnesium concentration, they were chosen as the appropriate medium to test if the SS mRNA is able to bind the ribosome in a P-site dependent manner. P-site complexes were formed (as described in Methods), with either the designed SS mRNA or MV_39 control mRNA (5'GGCAAGGAGGUAAAAAUGGUAUACAAAAAAAUCAAAAUC), coding for MVY. After blotting with P-site reactions, washing, and drying the nitrocellulose filters, the counts per minute were measured via liquid scintillation. Each P-site complex reaction and filter-binding step was completed in duplicate to avoid filter anomalies. The amount of background counts was obtained by a P-site reaction without ribosomes and was subsequently subtracted from each data point before being graphed. Therefore, the number of counts per minutes charted in Figure 3A represents the amount of radioactivity from ^{35}S-labeled fMet-tRNA bound as a P-site complex with the ribosome. Any ^{35}S-labeled fMet-tRNA^{fMet} which was not bound to a ribosome was washed through the filter during the subsequent washing steps. The percentage of ribosomes bound (Figure 3B) was calculated from the pmols of 30S ribosomes included in the reaction, relative to if all ribosomes were completely bound. This maxes out at 100% for both the SS mRNA and the MV_39 mRNA when the amount of mRNA is doubled compared to ribosomes in the P-site reaction.

As a secondary method to confirm translocation of the ribosome on the SS mRNA separate from the toeprinting assay, the filter-binding technique was also

utilized with SS mRNA and ^{14}C-labeled Val-tRNA^{Val}. The control mRNA, MVU_55, codes for MVVVV with valine codons corresponding to Val_2 tRNA^{Val} similar to the SS mRNA, rather than natural Val_1tRNA^{Val}. The nitrocellulose filters were blotted as described in the Methods section, washed, dried, and counted via the liquid scintillation counter in duplicates to avoid any filter anomalies. Although not as qualitative as the toeprinting assay to determine how many Valine codons the ribosome is translocating through before stalling, the data plotted in Figure 4 illustrate that multiple-round translocation is occurring and ^{14}C-labeled-Val is collecting on the filter as the ribosome is forming a polypeptide chain of the labeled tRNAs.

***In-vitro* Translation and Cleavage using RelE**

To investigate the ribosome's capability for multiple round translocation through the helix of the SS mRNA, the 5' end of the mRNA was labeled with ^{32}P, and P-site and ternary complexes with excess Val-tRNA^{Val} were incubated for translation with or without the addition of Gly-tRNA^{Gly}, which we would expect to allow the ribosome to complete one additional codon before stalling. After translocation, 0.6 µM of purified RelE enzyme was added. RelE is a bacterial toxin known to cleave mRNA only when bound by a ribosome. Although it does not exhibit any nucleotide specificity, RelE performs by cutting mRNA between the +2 and +3 nucleotide position of the A-site codon. Therefore, if the ribosome is completing multiple round translocation effectively, we would expect to see a single codon shift on the gel between the mRNA cleaved with the addition of the Gly-tRNA^{Gly} when compared to those only translocated with excess Val-tRNA^{Val}. Figure 5 illustrates this using a

control mRNA, TP_short, which does not exhibit secondary structure, alongside an alkaline hydrolysis digest of the mRNA. Figure 5 depicts translation reactions of the designed SS mRNA containing the helix and shows a shift equal in size and intensity to the TP_short mRNA control. Figure 6 also contains a lane corresponding to the alkaline hydrolysis digest of the SS mRNA. Several additional lanes are included for each experiment: a control with all components except ribosomes to ensure RelE is not capable of cleaving free mRNA, a control containing all P-site components but excluding RelE, and an mRNA only lane to confirm the visualized gel bands are not mRNA fragments. Figure 6 directly compares multiple round translocation through the glycine codon with TP_short mRNA and SS mRNA, as they are equivalent in size.

Discussion

Our design of a specific mRNA complex to elucidate the helicase activity of the ribosome hinged on a recently published crystal structure of the *E.coli* ribosome interacting with a single-stranded mRNA beyond the tunnel entrance, indicating the specific ribosomal protein uS3 may be responsible for stabilization and possibly unwinding of structured mRNA (Amiri, Noller 2019). The interaction between mRNA and uS3 had also been supported by earlier biochemical studies, wherein mutations in uS3 greatly reduced helicase activity of the ribosome (Takyar et al. 2005). With the emergence of cryo-EM, the ability to capture structural data and specifically, nearly real-time interactions, is now obtainable. Therefore, the purpose of this study was to set the stage for a cryo-EM structure by designing and investigating the efficacy of an mRNA designed to exemplify the ribosome's mRNA helicase activity. This entails three main components prior to future use for structural and biochemical studies: the ability to transcribe and purify the desired mRNA confirmed to have a strong helix, the ability of 70S *E.coli* and *T. thermophilus* ribosomes to translate the mRNA, and the ability to stall at the chosen site, a glycine codon, which gives enough clearance for the portion of the mRNA in the entry tunnel while the ribosome is unwinding the downstream hairpin.

Data gathered from the toeprinting assay revealed the effectiveness of both the mRNA and all transcribed tRNAs: the P-site binding is occurring and the ribosome is performing multiple round translocation through the designed eight Valine codons, into the first portion of the hairpin structure. As this was the initial investigation into

the proposed sequence and design scaffold of the secondary structure mRNA, this was encouraging. An additional doublet band is also present in the P-site and multiple round translocation lanes. We believe this can be accounted for due to limitations of reverse transcriptase, which is fundamental to the toeprinting assay. As the reverse transcriptase is transcribing DNA from the 3' end of the mRNA template, it is stalling at an additional site other than where the mRNA is occupied by ribosomes. Based upon the size of this fragment in reference to the P-site and multiple round translocation products, we believe it corresponds to the 'CGGGC' portion of the sequence. As this fragment is within the P-site lane as well, it cannot be explained by the ribosome stalling halfway through the run of eight Valine codons, but rather due to reverse transcriptase encountering a small, unintended structured region in the mRNA within the Glycine codon region, which is a limitation of the chosen assay.

After confirming the performance of the transcribed tRNAs and the desired stalling portion of the sequence, we were able to begin investigation of the mRNA containing the helix. Initially, we employed filter-binding assays as we were able to utilize 70S *E.coli* and 30S *T. thermophilus* ribosomes to test both P-site binding dependence on the levels of mRNA using ^{35}S-fMet-tRNA^{fMet}, in addition to multiple round translocation through the accumulation of radioactivity on the nitrocellulose filters due to ^{14}C-Val-tRNA^{Val}. Filter-binding data was collected via liquid scintillation counting and plotted within Excel for interpretation of results.

As shown in Figures 3A and 3B, P-site binding of 30S *T. thermophilus* ribosomes is dependent upon the amount of mRNA, maxing out when the

concentration of mRNA is doubled over ribosomes. This was a vital conclusion to demonstrate both the efficacy of the helix mRNA, which compares similarly to a control mRNA (MV_39) used in past studies, as well as understanding how much mRNA is needed for complete P-site binding for *in-vitro* translocation assays. Although filter-binding is not as useful for multiple round translocation as toeprinting assays, we hypothesized that using ^{14}C-labeled Val-tRNA^{Val}, the level of radioactivity should increase as the ribosome translates through the stretch of Valine codons in the helix mRNA and labeled Valine amino acids accumulate in the P-site on the forming polypeptide chain. This is demonstrated in Figure 4. However, according to the numerical values of the accumulated filter counts when compared to a dry filter blotted with the maximum level of possible counts for the ^{14}C-labeled-Val-tRNA^{Val}, it seems the ribosome could be only performing multiple round translocation through five Valine codons. There are certainly alternative interpretations for this, such as the accumulation of deacylated over aminoacylated tRNA after five rounds of translocation, as well as issues comparing levels of a dry, unwashed filter for the maximum ^{14}C-labeled Val-tRNA^{Val} to the presoaked, washed reaction filters.

The strongest evidence for appropriate design and stalling of the helix mRNA is within the *in-vitro* translation assays. Depicted in Figures 5, 6, and 7, these gel images show strong bands at the location of P-site binding and multiple round translocation through the run of Valine codons, and ultimately, the desired stall site for structural studies in the Glycine codon position of the mRNA. The appearance of additional light background bands is due to an RNase contamination in the

preparation of 70S *E.coli* ribosomes, as is evident when ribosomes are excluded in the control lane for RelE. The sequence of the helix mRNA can be traced through the alkaline hydrolysis digest in Figure 5, which shows the same repetitive spacing of each ‘GUU’ codon and blurring of the ladder where the strong helix structure begins. When Gly-tRNA^{Gly} is added to the P-site and ternary complex reaction, the ribosome is able to perform one additional round of translocation before the mRNA is cleaved with RelE, which is shown with the same size and intensity between the control TP_short, and the SS helix mRNA in Figure 6.

Conclusion

The mechanism and precise interactions of the mRNA ribosomal helicase as well as the resulting delays in translocation due to mRNA containing secondary structure are not fully understood. The effects of mutations on ribosomal protein uS3 imply that it plays a specific role in mRNA unwinding, along with uS4 and uS5. (Takyar et al. 2005). Previous structural work has identified a variety of possible steric hinderances within mRNA-tRNA translocation resulting from strong secondary structure and stem loops known to elicit frameshifting. While further investigation into these specific sequences and interactions have increased the scope of understanding, they altogether lack a whole and conclusive mechanistic explanation. Our design and results, combined with the findings from previous studies, set the stage for the utilization of cryo-EM to grasp a more holistic view.

Corresponding mRNA Name:	Sequence:
SK_TP	GGAAAGGAAATAAAAATGGTTGTTGTTGTTGTTGT CGTCGTCGGGCTCGGTGCAGGAAAATAATAGAAG AATCGGATAAGAGAACACAGGATCCAGCTGGCGT AATAGCGAAGAGGCCCGCACCGGTAC
SS	GGAAAGGAAATAAAAATGGTTGTTGTTGTTGTTGT CGTCGTCGGGCTCGGTGCAGGCAACTGCACCGAG CCCGACGACGACTGTT
Glycine tRNA CCC	TAATACGACTCACTATAGCGGGCGTAGTTCAATGG TAGAACGAGAGCTTCCCAAGCTCTATACGAGGGTT CGATTCCCTTCGCCCGCTCCAGCCGGCCATGGTCC CAGCCTCCTCGCTGGCGGCCGGTGGGCAACATTCC GAGGGGA

Table 1. DNA Sequencing Data for Transcribed mRNAs

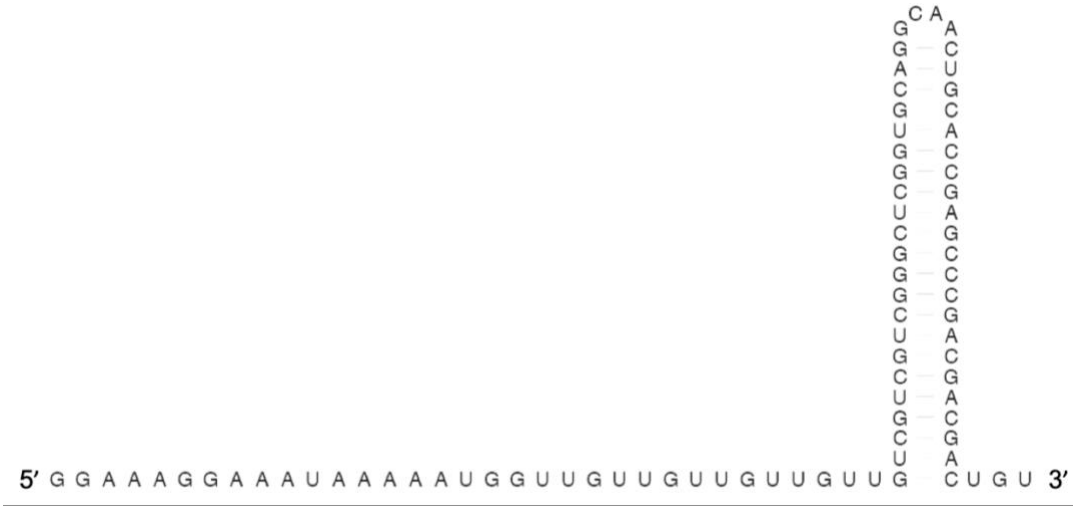

Figure 1. Design of Secondary Structure Helix mRNA

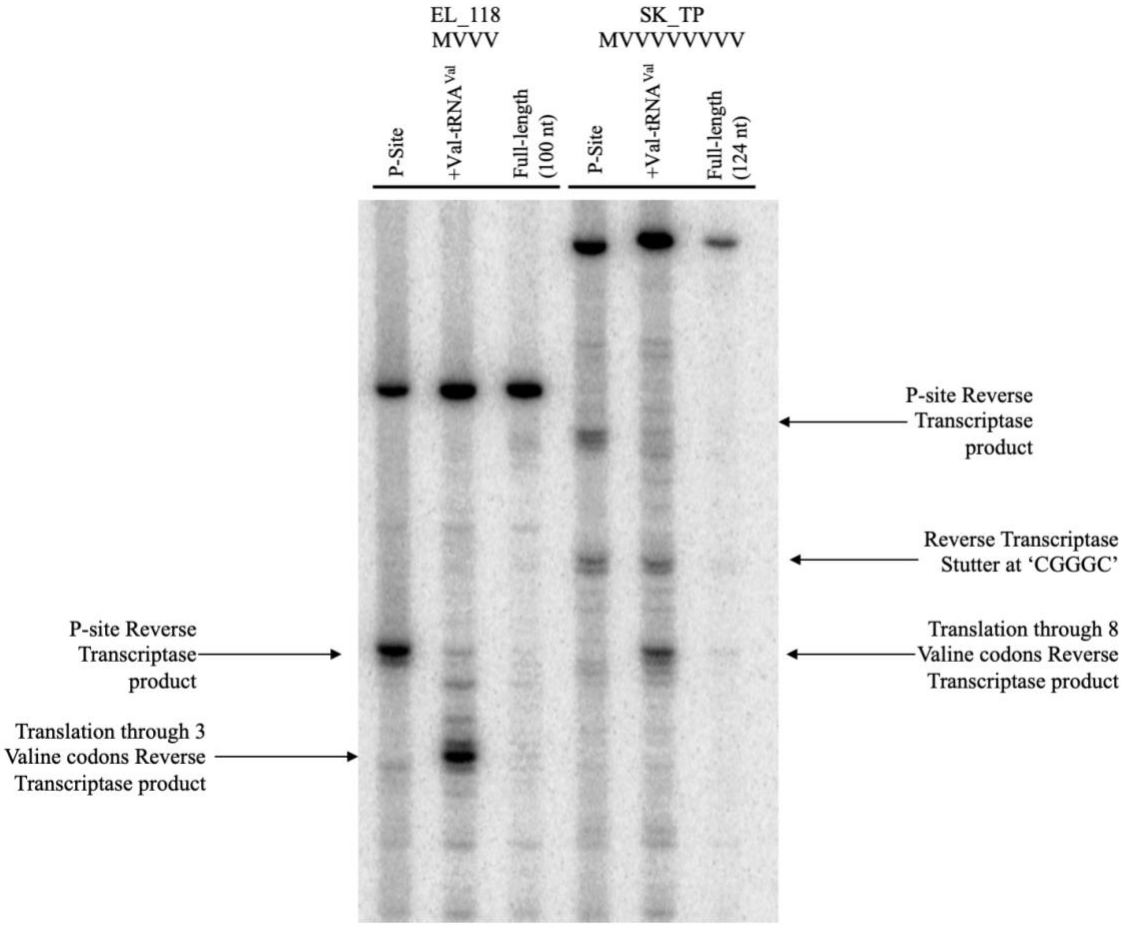

Figure 2. EL_118/SK_TP Toeprint Gel

To test the integrity of the translation system with a nonstructured mRNA (SK_TP), and especially the efficacy of the transcribed tRNAs Valine and Glycine, a toeprinting assay was performed. Multiple round translocation is analyzed to determine the stalling location of the ribosome on the mRNA by utilizing a ^{32}P labeled primer which anneals to the 3' end of the mRNA. Reverse transcriptase transcribes toward the 5' end of the mRNA and is able to continue until it encounters a ribosome, which results in a shorter product and illustrates where the ribosome is stalled.

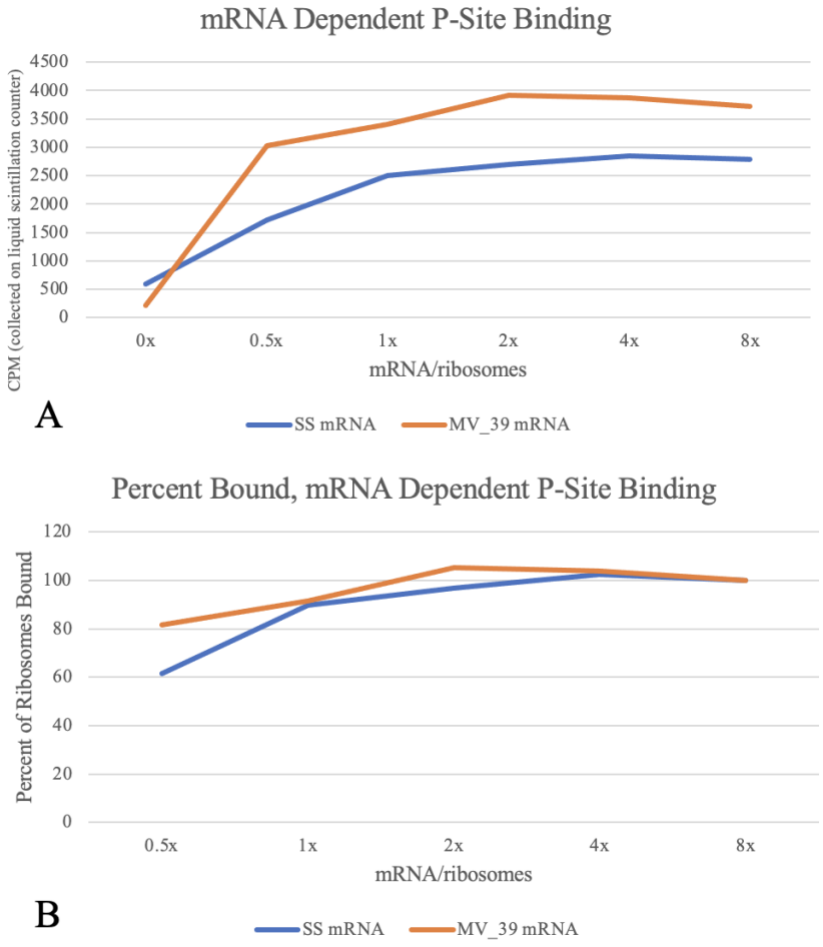

Figure 3. Filter-Binding with ^{35}S-fMet tRNA^{Met}

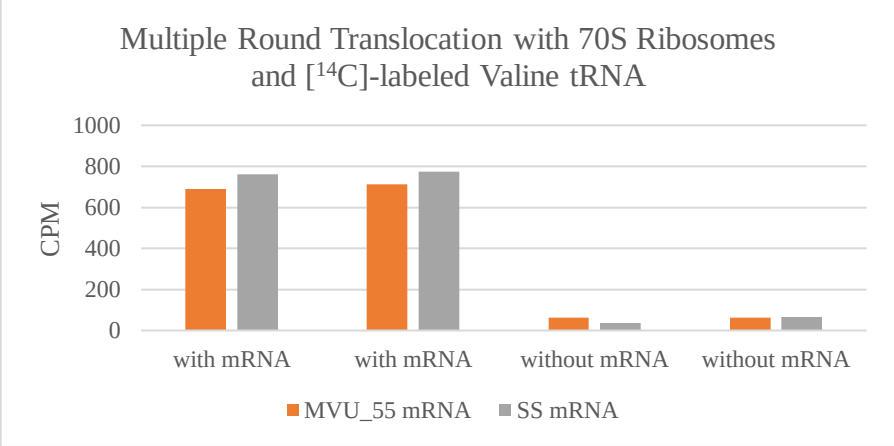

Figure 4. Filter-Binding with ^{14}C-Val-tRNA^{Val}

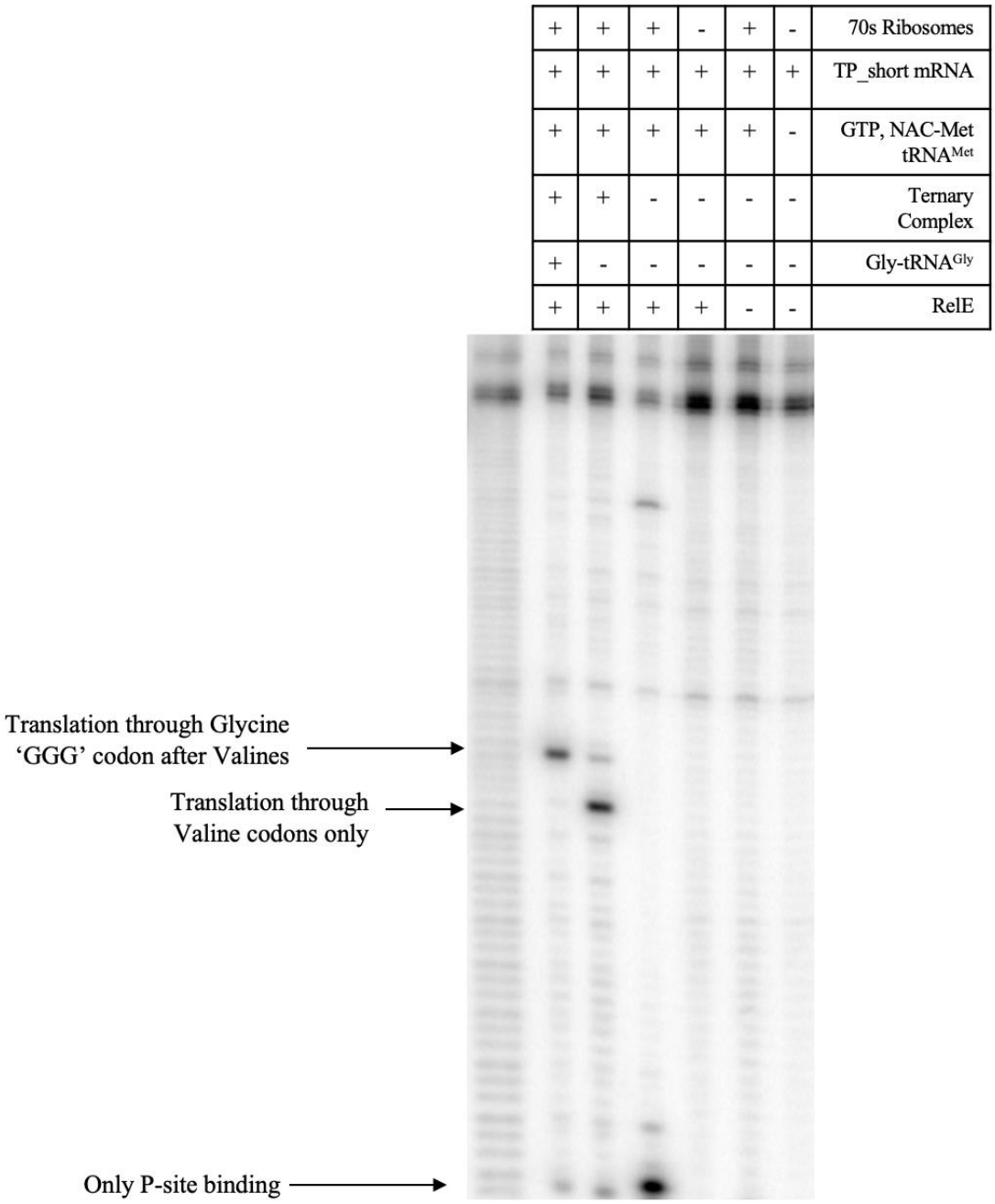

Figure 5. In-vitro Translation with TP_short mRNA & RelE Cleavage

After multiple round translocation, the 5' ^{32}P-labeled TP_short mRNA (lacking secondary structure) was cleaved with RelE, which results in a gel band corresponding to the location of the stalled ribosome on the mRNA. When Valine tRNA is added, the ribosome translates through 8 Valine codons before being cleaved by RelE in the vacant A-site. When Glycine tRNA is added to the translation system, the ribosome is able to complete one additional round of translocation, resulting in a cleaved mRNA product one codon longer. An Alkaline Hydrolysis digest of the unstructured mRNA is depicted in the leftmost lane.

70S Ribosomes	+	+	+	-	+	-
SS mRNA	+	+	+	+	+	+
GTP, NAc-Met $tRNA^{Met}$	+	+	+	+	+	-
Ternary Complex	+	+	-	-	-	-
Gly-$tRNA^{Gly}$	+	-	-	-	-	-
RelE	+	+	+	+	-	-

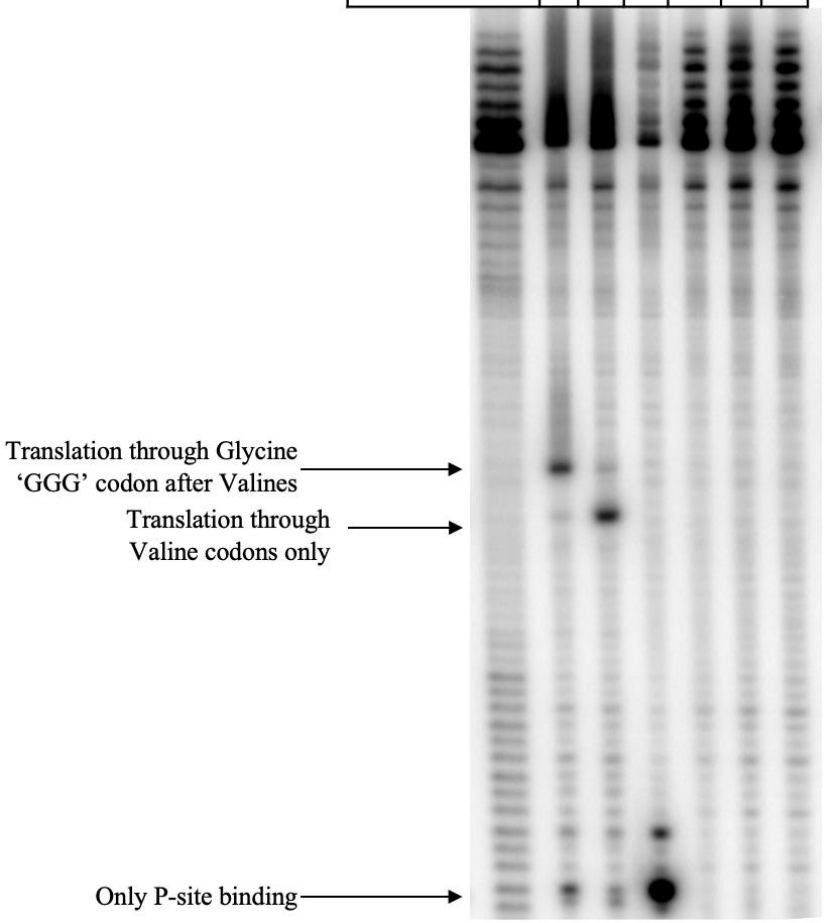

Figure 6. In-vitro Translation with SS mRNA & RelE Cleavage

After multiple round translocation, the 5' ^{32}P-labeled Helix mRNA (SS mRNA) was cleaved with RelE, which results in a gel band corresponding to the location of the stalled ribosome on the mRNA. When Valine tRNA is added, the ribosome translates through 8 Valine codons before being cleaved by RelE in the vacant A-site. This occurs ~11 nucleotides into the helix secondary structure. When Glycine tRNA is added to the translation system, the ribosome is able to complete one additional round of translocation, resulting in a cleaved mRNA product one codon longer. An Alkaline Hydrolysis digest of the Helix mRNA is depicted in the leftmost lane.

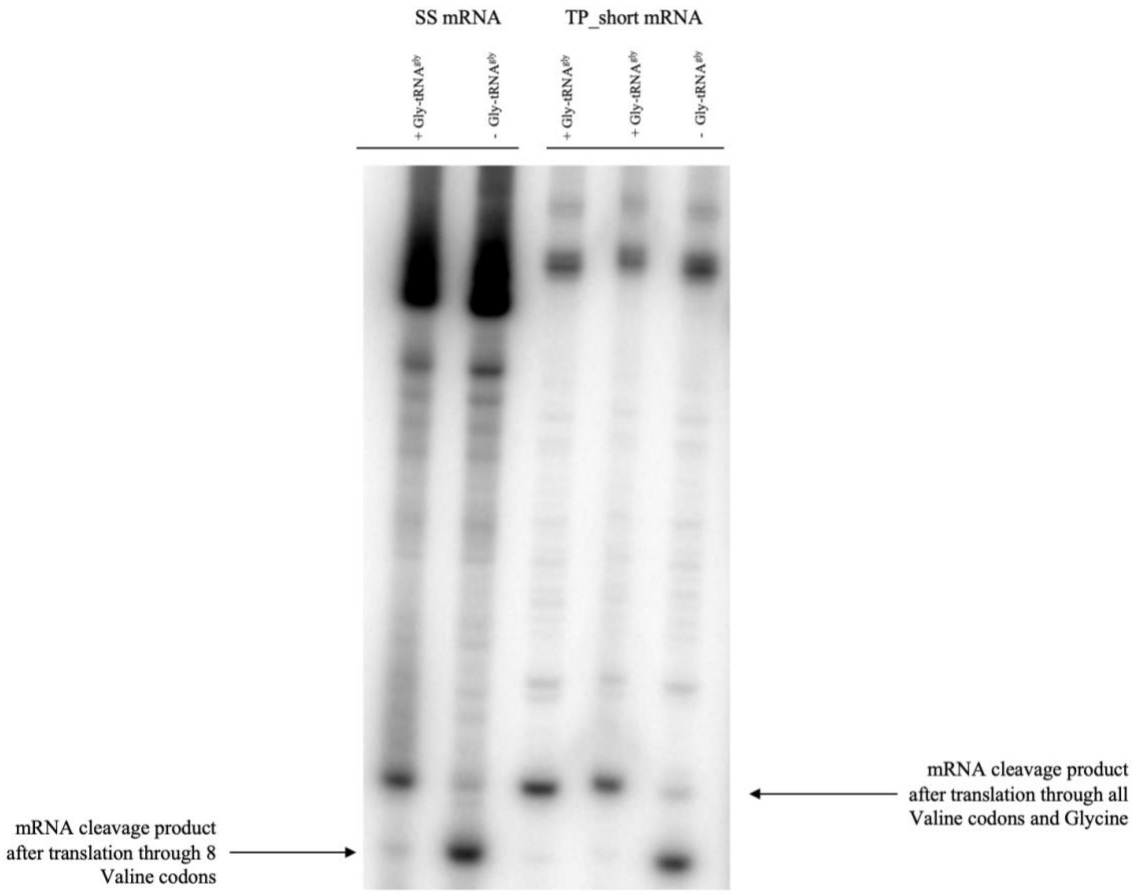

Figure 7. In-vitro Translation of Helix (SS) and TP_short mRNAs +/- Gly-tRNA^{gly} with RelE Cleavage

The multiple round translocation and RelE experiment was completed again to confirm the results of previous gels. These products were loaded on the same gel to compare the relative sizes of each cleaved mRNA product when only Valine tRNA, or Valine and Glycine tRNA were added to the system and the mRNA was subsequently cleaved with RelE.

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