# "Chemical Approaches to Investigate HIV-1 Rev-RRE RNA Interactions"

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A Dissertation Presented to

the Faculty of the Department of Biology and Biochemistry

University of Houston

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In Partial Fulfillment

of the Requirements for the Degree

**Doctor of Philosophy**

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By

**Nanda G. Karri**

August 2017

# Chemical Approaches to Investigate HIV-1 Rev-RRE RNA Interactions

Nanda G. Karri

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# Abstract

Two separate crystal structures of the Rev dimer have been solved. One structure features details of the A:A dimerization interface and the other features details of the B:B interface. While the binding of multiple copies of Rev is required for its RNA transport function, the details of the interactions by which Rev interacts with RRE RNA remain elusive. To investigate this, the RWZ2 fragment, which was later named sIIB was fused to a tRNA scaffold and Rev:sIIB-tRNA complexes were analyzed by various biophysical experiments. Establishing the binding strength and stoichiometry in solution has been frustrated by the need for specific chemical labeling of the Rev protein. We have successfully installed a fluorescent probe at the C-terminal end of Rev and demonstrated the binding of RevL with chimeric $tRNA^{Phe}$-sIIB RNA molecule. We sought to address whether Rev shows any preference in binding (A:A vs. B:B) and dimerization of RNA. We took the approach of making covalently crosslinked Rev dimers in a manner that is consistent with the geometry and conformation of the crystallized Rev dimers. This thesis details the solid phase semisynthetic approach, experimental procedures, and results of the studies done on Rev and the RRE.

## Table of Contents

# Chapter 1: Introduction

## 1.1 HIV-1

Human Immunodeficiency Virus (HIV), the etiological agent of Acquired Immunodeficiency Syndrome (AIDS)(Weiss et al. 1986), belongs to the genus *Lentivirus* of the family Retroviridae (Felber et al. 1989; Purcell and Martin 1993). HIV attacks T-cells, macrophages, and dendritic cells and disrupts the immune response. (McClure et al. 1987)

Upon infection, the virus gains entry into the host cell by a series of interactions. First, interaction with the cell surface primary CD4+ receptor via gp120 (glyco-protein) is followed by interactions with chemokine receptor 5 (CCR5) or another secondary receptor, CXCR4, through glycoprotein 41 (gp41)(Freed 2001; Frankel and Young 1998). Thus, fusion and the subsequent entry into cells is achieved. Once inside the cell, the viral RNA is reverse-transcribed into proviral DNA by the action of viral reverse transcriptase (RT) and integrase (IN)(Gallay et al. 1995). The proviral DNA, now called the pre-integration complex, is transported into the nucleus where it is integrated into the host's genome and may remain latent or begin replication. HIV-1 can remain latent for years and this has been a major obstacle in eradicating or finding a cure for HIV-1 infection(Frankel and Young 1998).

Once the activation of host's cellular machinery occurs, HIV-1 starts transcripting its viral DNA into messenger RNA (mRNA) and the translation of viral proteins begins. The

genome is about 9 kilobases in length and encodes fifteen proteins from nine open reading frames (ORFs). Replication of HIV is categorized by cell-entry, early and late phase replication, assembly of viral proteins and genomic RNA followed by release of virions from the cell surface (Freed 2001).

During the early phase replication, the virus takes control of the host's transcriptional machinery and begins production of accessory proteins responsible for enhanced transcription and transport of partially-spliced and fully-spliced genome(Turpin et al. 1998). The fully-spiced species encode regulatory proteins such as Rev, Tat or Nef(Maitra et al. 1991; Ahmad and Venkatesan 1988; Zhou and Sharp 1996; Frankel and Pabo 1988). The production of sufficient quantities of the regulatory proteins activates the late-phase replication. This consists of the production of singly- and doubly-spliced mRNA transcripts, which encodes env, gag, and pol proteins as well as unspliced genomic RNA(Wodrich and Kräusslich 2001; Nigg 1997; Ernst et al. 1997; Berger et al. 1991). Soon, with enough viral proteins and genome present at the cell membrane, the virus packages and releases mature, infectious virions.

## 1.2 Rev and the HIV-1 Life Cycle

Regulator of Virion Expression or Rev is a key regulatory HIV protein produced in the early-phase of HIV replication. Rev is produced from fully spliced viral mRNA(M H Malim et al. 1991); Rev helps in the transition of HIV gene expression from early to late phase. Rev was discovered in 1986 and is critical for the maturation of HIV virion(Cullen 1998; Wright et al. 1986). It enables export of unspliced and singly spliced viral RNA from the nucleus(Berger et al. 1991; Cochrane et al. 1991; Mann et al. 1994). This export is a result of Rev's ability to directly interact with the RNA and form a complex with Ran GTP and human CRM1(Henderson and Percipalle 1997; Izaurralde et al. 1997; Askjaer et al. 1998; Fischer et al. 1999). It has been reported that in the absence of Rev there is a decreased production of *gag* and *pol* encoded proteins and also the virus is unable to replicate efficiently(Malim et al. 1989; Feinberg et al. 1986).

HIV-1 Rev is a 116-residue protein, about 13 kDa with well-defined functional motifs(Pollard and Malim 1998). The structure of the 116-residue Rev is not fully known, the structure of what encompasses the N-terminal region of Rev has been elucidated by various biophysical methods(Daugherty, Liu, and Frankel 2010; DiMattia et al. 2010). Circular dichroism experiments showed that Rev's N-terminal region is α-helical(Auer et al. 1994; Daly et al. 1990).This is also proved by epitome mapping experiments using monoclonal antibody panel. A proline-rich segment was also determined to form a loop

connecting the helix regions(Jensen et al. 1997). Thus, Rev, in the N-terminal region was determined to have a helix-loop-helix motif in the N-terminal region.

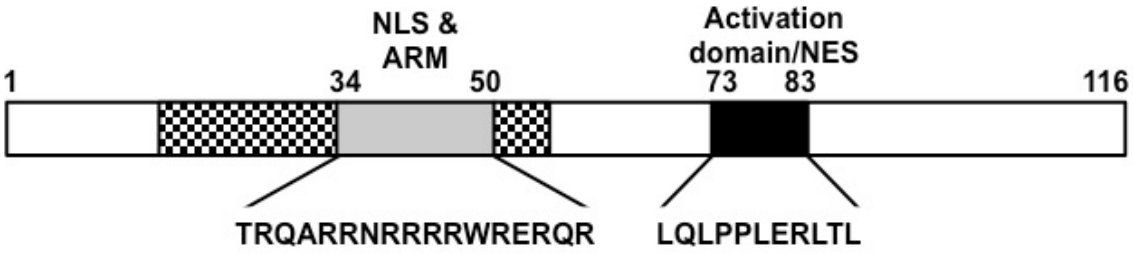


**Fig 1.1 Functional Motifs of Rev**. Rev has four distinct functional motifs. Checkered boxes represent the dimerization and oligomerization domains. The RNA binding domain, also doubles up as the Nuclear Localization Sequence comprises residues 34-50. In the C-terminal region, the Nuclear Export Sequence denoted by the black box is seen.

The N-terminal domain of Rev also houses the different domains that have distinct functions. These include the multimerization domain, the nuclear localization sequence (NLS), and the arginine rich motif (ARM), which binds to the RNA with high specificity(Berger et al. 1991; Bachelerie et al. 1997; Berthold and Maldarelli 1996). The multimerization domain contains hydrophobic residues that participate in the dimerization and higher order assembly of Rev molecules. The residues that have been implicated are Leu12, Leu13, Val16, Leu18, Ile19, Phe21 in helix-1 region and Ile52, Ile55, Ile59, Leu60 in helix-2 have been established to cause dimerization and the subsequent oligomerization of Rev(Jain and Belasco 1996; Daugherty, Liu, and Frankel 2010). Helix-2 of Rev also contains a stretch of arginines, known as Arginine Rich Motif

(ARM), which serve to specifically bind to a short stem-loop region of the rev response element (RRE) called stem-loop IIB with sub-nanomolar affinity(Bartel et al. 1991; Cullen 1998; Heaphy et al. 1990). An NMR structure of the ARM peptide in complex with the stem-loop IIB RNA has been solved, four conserved residues (Arg35, Arg39, Asn40 and Arg44) have been found to make base-specific interactions with the major groove of the RNA(Battiste et al. 1994). The ARM region also serves as nuclear localization sequence, which helps in the nuclear import of Rev after interacting with importin-β(Henderson and Percipalle 1997; Truant and Cullen 1999).

```
          10          20          30          40          50          70
MAGRSGDSDE DLLKAVRLIK FLYQSNPPPN PEGTRQARRN RRRRWRERQR QIHSISERIL

          70          80          90         100         110     116
STYLGRSAEP VPLQLPPLER LTLDCNEDCG TSGTQGVGSP QILVESPTVL ESGTKE
```

**Fig 1.2 HIV-1 Rev sequence**. Rev is 116 residues in length and has a molecular mass of ~13 kilodaltons.

The C-terminal region of Rev is intrinsically disordered and contains the nuclear export sequence (NES), the leucine-rich domain at the C-terminus region (73-84) function as the NES(Bachelerie et al. 1997). This domain interacts with human chromosome region maintenance gene-1 (CRM1) also known as exportin-1 and helps export of the Rev-RRE ribonucleoprotein complex out of the nucleus(Daelemans et al. 2002). A series of deletion and mutational studies performed on the C-terminal region have shown that such

mutants form a functionally inactive and export-deficient heteromers(Daly et al. 1993). Much of the functions of the C-terminal region of Rev still need to be explored.

### 1.3 Rev Response Element (RRE)

Rev Response Element, RRE, is a 351-nucleotide sequence found in the *env*-encoding region of the 9kb HIV-1 genome. The RRE is a *cis*-activating element, which recognizes and binds to the *trans*-activator Rev protein. The RRE when originally discovered in 1989 and was suggested to be about 240 nucleotides in length(Zapp and Green 1989; Malim, Böhnlein, et al. 1989; Malim et al. 1989). In 1994, an additional 58 nucleotides on the 5' end and 59 nucleotides on the 3' end have been included after a series of functional assays on the truncated RRE (Mann et al. 1994).The 240 base RRE is the minimally functional RRE enough interaction and subsequent export out of the nucleus. The additional nucleotides have been cited to be involved in the increased production of late-phase structural *gag* and *pol* proteins.

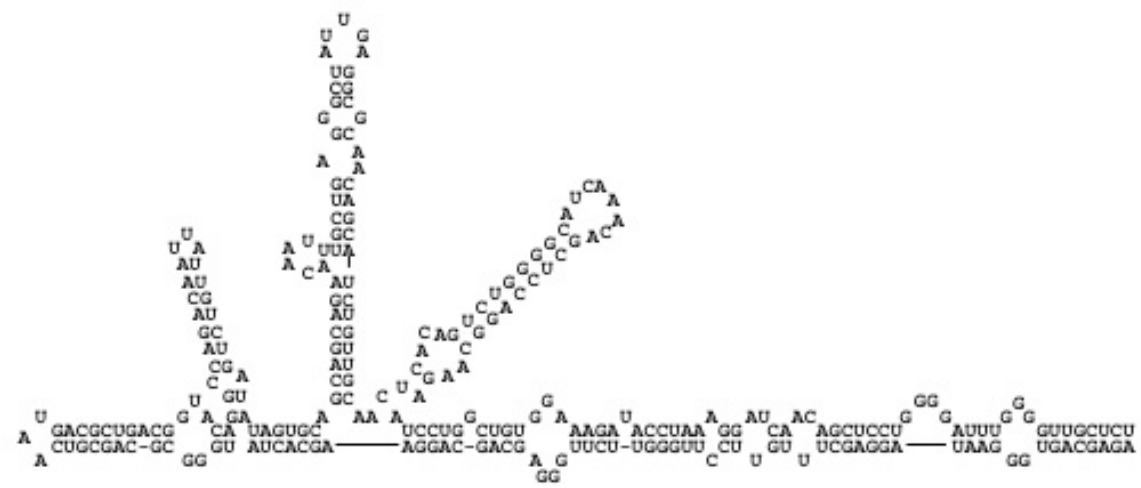

**Fig 1.3 Minimally functional RRE.** The RRE as described in 1989 consisted of 240 nucleotides

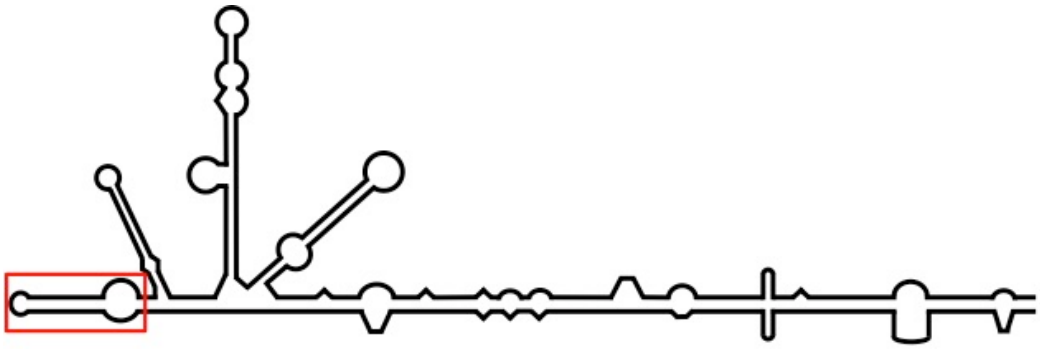

**Fig 1.4 Secondary Structure of the full length RRE.** The RRE comprises five stem loops and numerous hairpins structures. The high-affinity Rev binding site, Stem Loop-IIB, is boxed in red.

The RRE is characterized by extensive secondary structural elements such as stem loops and hairpins. Rev specifically recognizes and binds to a region of the stem-loop II of the RRE, termed as stem-loop IIB (SLIIB) with a high affinity, with a $K_d$ in sub-nanomolar range. Rev then binds across to another region of Stem I, IA site, with lower affinity. The polymerization progresses along the length of the RRE with 8-12 monomers of Rev assembling along Stem I (Zemmel et al. 1996; Lam et al. 1998; Pond et al. 2009; Vercruysse et al. 2011; Daugherty et al. 2010).

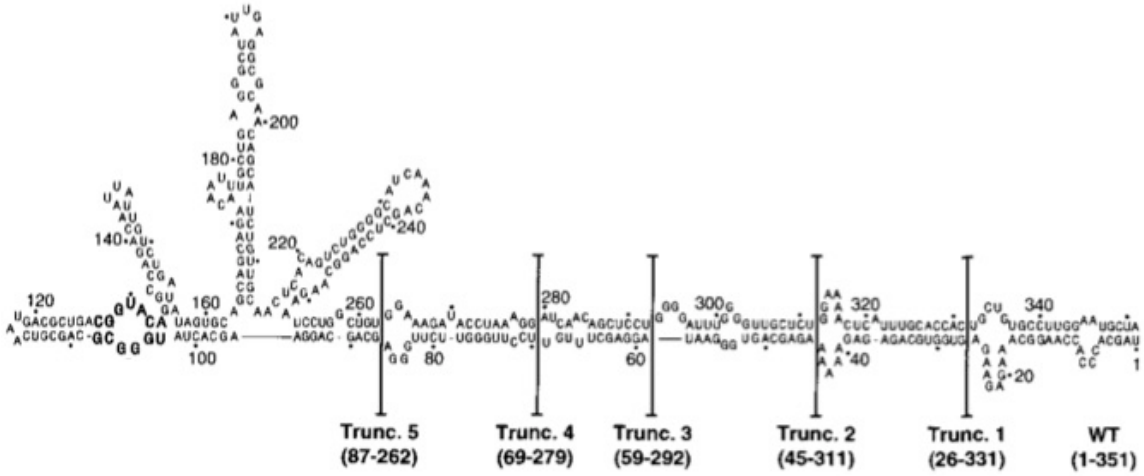


**Fig 1.5 Full length RRE.** The 351-nucleotide full length RRE and the various truncations of the RRE as studied by Karn et al. The truncations on the RRE provided information on the importance of RRE length in the context of Rev polymerization on the RRE and the subsequent export.

Rev assembly on the RRE is crucial for the export of HIV RNA from the nucleus. The assembly and the subsequent polymerization of Rev on the RRE has been studied with the aim of better understanding the interactions and mechanism of the assembly and transport. Experiments involving ribonuclease T1 digestion of the RRE in the presence and absence of Rev showed, to an extent, the specific interactions of Rev with the RRE. Regions of the RRE where Rev molecules interacted were protected from digestion by the RNase T1, while those that were accessible to the RNase were cleaved at specific nucleotides. To fully validate the length of RRE involved in Rev binding and export out of the nucleus, experiments have been performed using various truncated versions of the RRE. These experiments established the minimal length of the RRE involved in export and the decrease in Rev/RRE complexes on a gel-shift assay showed the importance of length of the RRE(Mann et al. 1994; Zemmel et al. 1996). In 2015, studies on RRE conformations using in-gel SHAPE analysis and they concluded that a variant of the HIV-1, wild-type NL4-3, exists in an near equimolar mixture of 4 stem-loop and 5 stem-loop conformations(Sherpa et al. 2015).

In 2013, Fang *et al.,* using a solution-based method- Small Angle X-Ray Scattering determined the structure of the 240 nt minimally functional RRE. The RRE constructed from the SAXS analyses adopted an "A" shaped structure with 45 – 60 Å separation between the two legs. Furthermore, mutational analysis and modeling on the RRE showed that this distance is essential for effective binding and polymerization of Rev molecules(Fang et al. 2013).

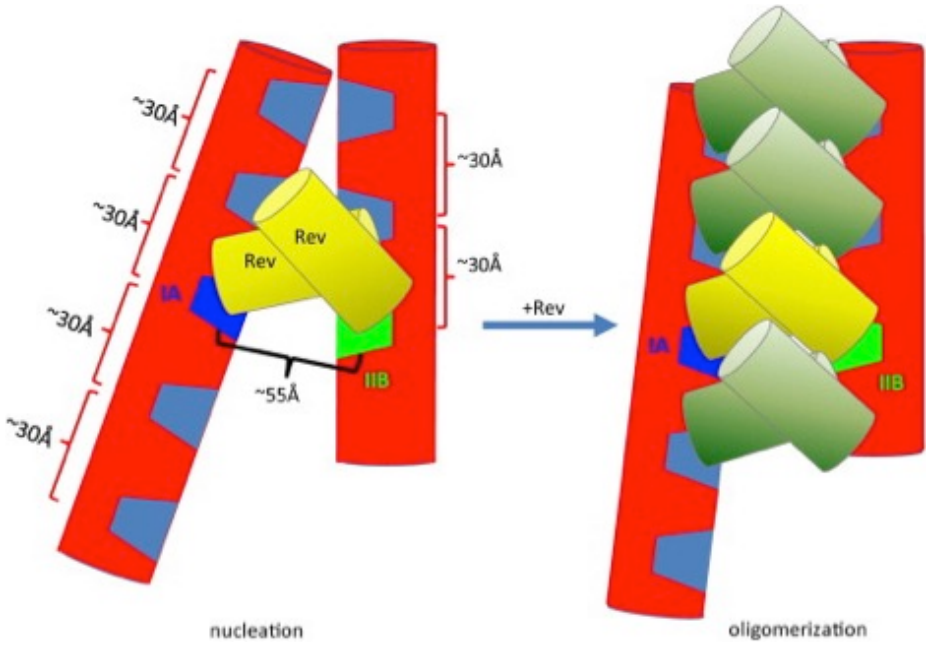


**Fig 1.6 SAXS model of the 240 nt RRE.** SAXS experiments showed that the RRE adopts an "A" shape.

More recently, SAXS and SHAPE-seq experiments on of the 351 base full-length RRE proposed a model that reiterated that the RRE adopted an A-shape. More importantly, the long Stem I of the RRE was determined to make tertiary interactions folding back towards the core of the 3-way junction of the RRE making the overall fold more compact(Bai et al. 2014).

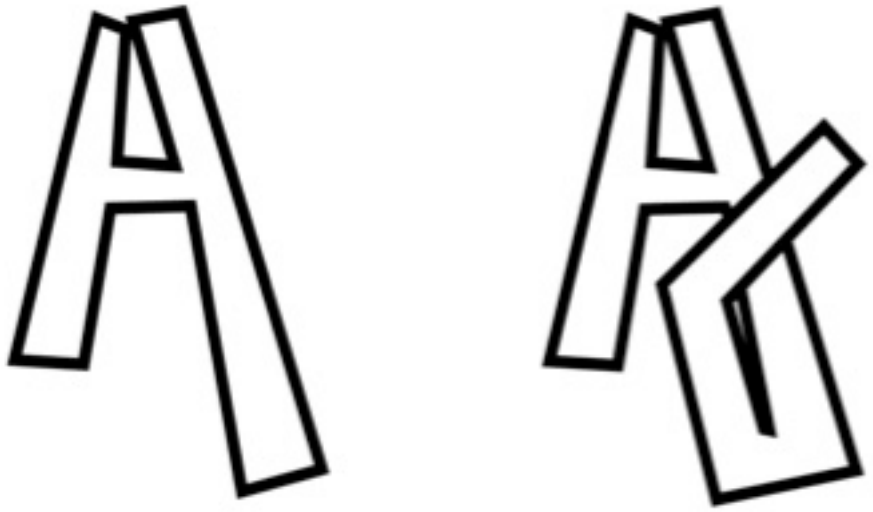

**Fig 1.7 SAXS model of the 351 nt RRE.** Stem I of the RRE was determined to fold back towards the core of the RRE. This model agrees with the previously described "A" shape for the 240 nt RRE.

## 1.4 Rev Shuttle System

During the early phase of HIV replication, HIV regulatory proteins such as Rev are translated from completely spliced RNAs. These completely spliced mRNAs are exported in a similar fashion as cellular mRNAs. The virus switches to late-phase replication characterized by the expression of viral proteins encoded by the unspliced (9 kb) or partially spliced (4 kb) RRE-containing mRNAs(Muranyi and Flügel 1991; Chang and Sharp 1989; Emerman, Vazeux, and Peden 1989). These introns are retained in the

nucleus and exported out of the nucleus once after there is a sufficient accumulation of Rev. The virus then uses Rev-dependent pathway for the export of the unspliced or singly spliced introns(Meyer and Malim 1994). The assembly of the Rev-RRE complex and followed by the recruitment of host proteins CRM1 and Ran-GTP constitute this pathway.

Since Rev accumulation occurs in the cytoplasm, it needs to be translocated to the nucleus. The arginine-rich domain of Rev has a dual role in that it not only binds to the RRE but also mediates the nuclear and nucleolar localization of Rev. Therefore, the ARM region is also the nuclear localization sequence (NLS). This overlapping RNA binding and NLS function of the ARM ensures that Rev cannot be imported while bound to the RRE; thus HIV-1 guarantees there is an efficient relocation intron-containing HIV messages. Karyophilic NLSs are those sequences that contain a cluster of positively charges residues such as lysines or arginines(Ullman, Powers, and Forbes 1997). These NLSs interacts with importin-α, which then interacts with importin-β forming a trimeric complex, a motif found in importin-β further leads to interactions with the nuclear pore.
Ran, a GTPase, plays a critical role in the nuclear import and export of proteins. Ran-GDP, a GDP form of Ran, is in abundance in the cytoplasm while Ran-GTP predominates in the nucleus. A gradient is thus created between the cytoplasmic and nuclear levels of Ran and is maintained by Ran-specific exchange factor (RCC1) in the nucleus. RCC1 also helps in the conversion of RanGDP to RanGTP(Izaurralde et al. 1997). The NLS of Rev has been shown to interact directly with importin-β

biochemically, and by forming a complex with RanGDP, Rev/importin-β/RanGDP is translocated to the nucleus.

In the nucleus, Ran-GDP is converted to Ran-GTP, and this induces a disruption and the dissociation of importin-β(Gorlich 1997). Rev is now free to interact with the RRE and subsequently export it out of the nucleus. The ARM of Rev interacts with the RRE, specifically by binding to the high-affinity stem-loop IIB region of the RRE. Rev then polymerizes along the length of the RRE, and up to 12 monomers of Rev have been reported to assembly along stem I of the RRE. The nuclear export sequence (NES) present in the C-terminal region of Rev is a leucine-rich sequence that recognizes and binds to the CRM1 thus forming RRE/Rev/CRM1 complex(Hakata et al. 2002; Askjaer et al. 1998; Fornerod et al. 1997). The export of this complex is energy dependent and Ran-GTP, which is present in abundance in the nucleus, mediates this export out of the nuclear pores. Nucleoporins Nup214 and Nup98 have been reported to help the export of RRE/Rev/CRM1 complex(Gorlich and Mattaj 1996). This complex in the cytoplasm is dissociated by the conversion of Ran-GTP to Ran-GDP by the action of RanGAP1 and RanBP1. Once Rev leaves its cargo in the cytoplasm it returns to the nucleus for subsequent rounds of export. This shuttling of Rev between the cytoplasm and nucleus dropping off a significant amount of genomic and structural protein-encoding RNA makes it an interesting therapeutic target to block the late phase of HIV-1 replication(Daelemans et al. 2002; Dong et al. 2009).

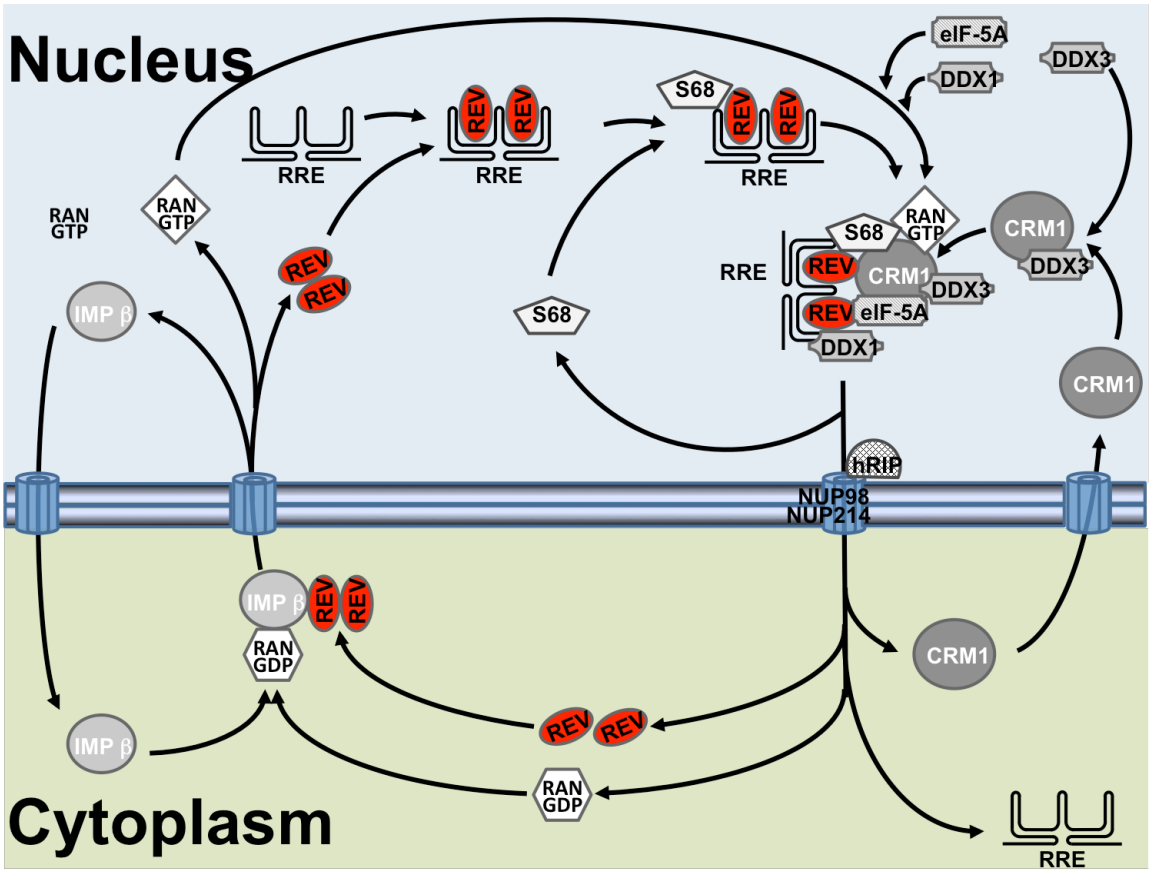


**Fig 1.8 Rev Shuttle System.** Rev interacts with many co-factors during to effectively shuttle between the nucleus and cytoplasm transporting unspliced or partially spliced RNA. [Adapted from Pollard and Malim, 1998 and Suhasini and Reddy, 2009]

### 1.5 Structural Studies on Rev

Rev is basic with an isoelectric point (pI) of about 9.06, which makes it an ideal candidate for nucleic acid binding. Phosphorylation of serine residues may be involved in the association and dissociation reactions for import and export function within infected cells. CD studies determined Rev to contain 50% α-helices and 25% β-sheet with double minima at 222 nm and 210 nm respectively. Rev was found to retain identical secondary structural elements even in complex with the RRE; the C-terminal end is intrinsically disordered. Rev forms regular, unbranched filaments of indeterminate length above a critical concentration of ~80 ng/mL (~6 μM) thus making it a complex molecule to perform any elegant biophysical studies.

Batiste *et al*., in 1996, studied ARM peptide bound to a 34-nt fragment of the RRE (SLIIB) using NMR. The structure revealed a α-helical peptide interacting with the major groove of the RRE fragment, the conserved arginines making base-specific contacts with the RNA. Jain and Belasco, in 2001, performed some elegant genetic and mutational analysis and reported the residues critical for dimerization and the subsequent assembly of Rev. These studies revealed the presence of two interfaces that Rev utilizes to dimerize and the subsequent oligomerization. The hydrophobic residues Leu18, Ile55 essential for dimerization form B:B interface; while, Leu12 and Leu60 are involved in the formation of higher-order structures and polymerization along the RRE constitutes the A:A interface interactions. This model was supported by mutant Rev:RRE complexes as studied by EMSA.

Two crystal structures of Rev were reported in 2010; these structures lead to a better understanding about the cooperative binding along the RRE. The first structure, where full-length Rev was co-crystallized with $F_{ab}$ antibodies bound to the A:A interface, showed a high-resolution structure with B:B interactions at the Rev dimer interface. Filament formation of Rev was inhibited by the interactions made with $F_{ab}$ antibodies. The Rev structure, however, lacked density in the first eight N-terminal residues and the C-terminal region including the NES. The crossing angle at the dimer interface was observed to be an obtuse 140° with the ARM regions protruded in a prong-like manner. This leads to the model where two monomers of RNA can be bound to Rev dimer in the A:A interface without any steric hindrance. Alternatively, binding to two RNA helices located in distal regions of the same RNA.

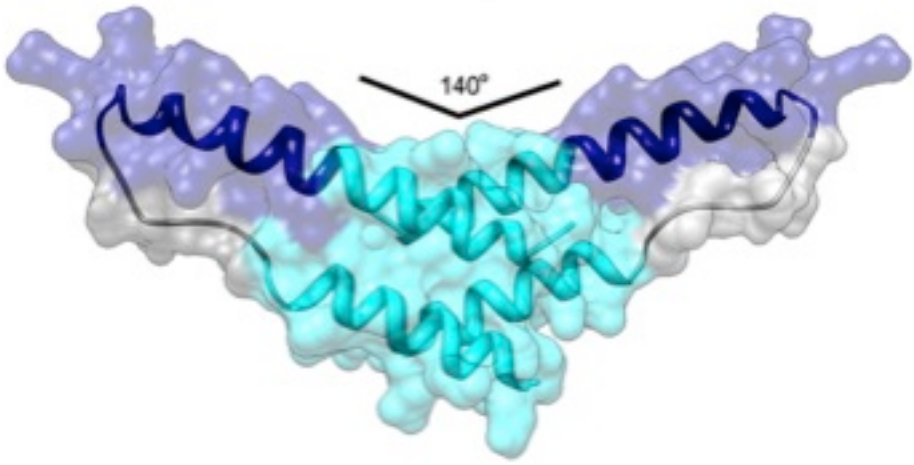


**Fig 1.9 $F_{ab}$-Rev Crystal Structure.** This structure disclosed how residues at the A:A interact, while the residues at the B:B interface were occulded by $F_{ab}$ interacting with Rev.

Details of Rev dimers with the B:B interface were elucidated in another structure by Daugherty et al. Mutant Rev species were crystallized in this set of experiments. The residues responsible for higher-order Rev assembly *viz* Leu12, Leu60 were mutated and the disordered C-terminal region truncated (RevN70) in these mutants. This made the Rev mutant amenable for crystallization. The asymmetric unit in these crystals contained four Rev molecules despite the above modifications. The packing of the hydrophobic residues on the A:A interface buried over 1500Å surface area. At the B:B interface Leu18 and Ile55 were seen to form symmetric contacts between the monomers. Phe21, Leu22, and Ile59 are also seen at the dimer interface. These two sets of residues are essential for cooperative RNA binding, export and stabilizing monomeric Rev respectively. The ARM angle in this structure is at 120° and narrower than what is observed in the B:B interface structure. A "jellyfish model" of the RNA bound to the RevN70 in B:B interface was proposed. This suggests that the ARM regions of both the dimers binding with a monomer of the short SLIIB in the major groove of the RNA helix with Rev contacts separated by one A-form helical turn. They modeled Rev assembly along the length of the RRE with the disordered C-termini projecting away from the RNA-like the tentacles of a jellyfish. In this model, the helical axis of the RNA is somewhat perpendicular to the axis of Rev oligomerization. This may be incongruous with the cooperative assembly of Rev binding to adjacent sites across the RRE.

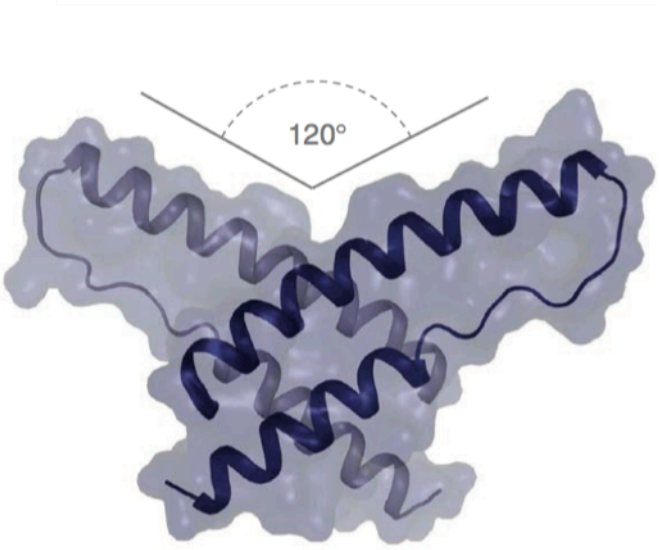


**Fig 1.10 RevN70 Crystal Structure.** RevN70, a truncated and mutated Rev revealed the interactions at the B:B interface. This mutant Rev is deficient in higher-order structure formation.

More recently, mutant RevN70 dimer (B:B interface) in complex with a modified IIB-RRE RNA was crystallized (Jayaraman *et al.*, 2014). This structure recapitulates some of the interactions seen in the other structural studies of Rev and the RRE. As expected, the tight-binding site on the RRE and the secondary Rev binding sites are occupied with Rev-Rev dimer interface interactions observed. The architecture of the RevN70 dimer in the RNA-free state displayed a V-shaped topology with a crossing angle of 120°. The conformation of the RevN70 with RNA bound altered dramatically, with the ARM regions coming closer in complex with the RNA. This complex displayed a crossing angle of 50°. Some critical residues such as Phe21 did not exhibit any interaction in this

RNA bound state. Such was not the case in RevN70 dimer structure. Also, the bound conformation buried less surface compared to the RNA-free RevN70 dimer structure (1000Å vs. 1500Å). The two RevN70 subunits are also pushed apart by 3.2Å to accommodate the RNA and showed looser hydrophobic packing. One other feature that is prominent in this structure is that Gln51 hydrogen bonds across the RevN70 dimer, which is not seen in the RNA-free state, perhaps compensating for the weaker hydrophobic interactions of the RNA-bound state.

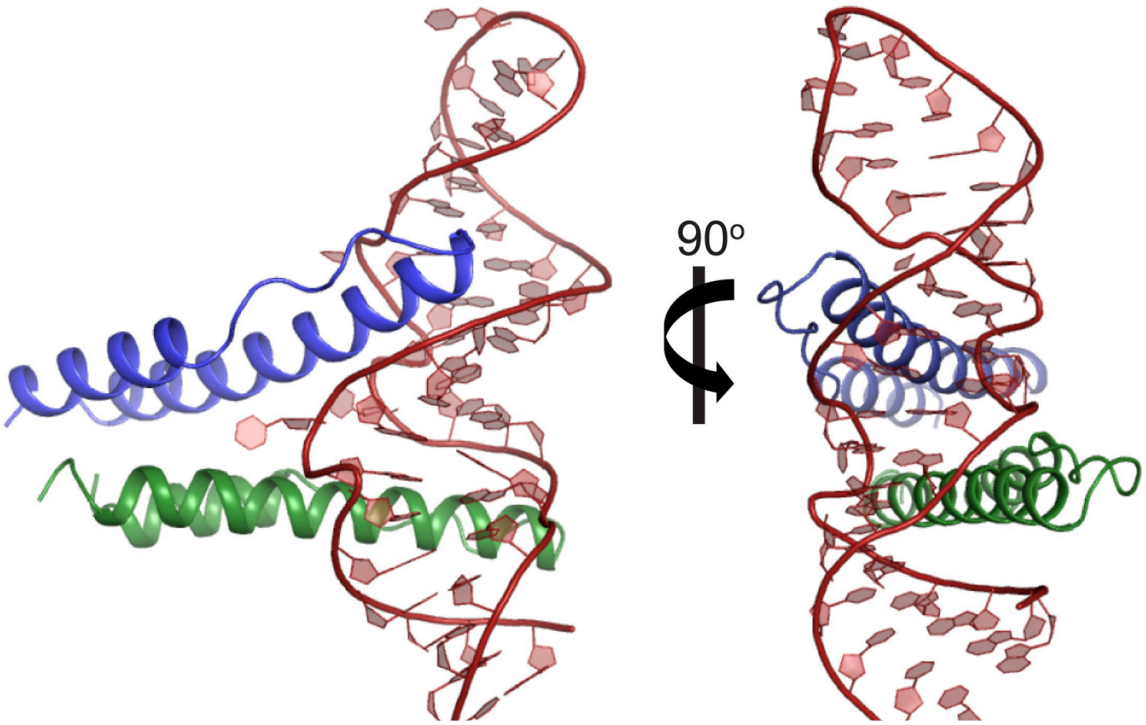


**Fig 1.10 RevN70 bound to RRE fragment.** The first crystal structure of a Rev dimer bound to an RRE RNA fragment. Many of the interactions as seen in the NMR structure of ARM peptide bound to RNA hairpin are also seen in this structure.

Though this RNA bound RevN70 dimer sheds light on the interactions between Rev and the RRE, the fact that the RNA is modified significantly and the Rev mutated to facilitate crystallization may raise questions on the actual state of the Rev-RRE conformations in the unaltered state. Also, what is the overall architecture of Rev polymerization in the context of the full-length RRE?

In a recent study, scFv-Rev (single-chain antibody variable fragment in complex with Rev) was crystallized (DiMattia et al., 2016) to explore the Rev-Rev interactions and also study the variable Rev dimer crossing angles. The study also included Rev filaments as examined by cryo-EM. The aim of the study was to investigate Rev oligomerization in the absence of RNA as well as the variable Rev dimer angles. Crossing angles from ~90° to ~140° were observed in the scFv-Rev dimers. These were measured by superposing the scFv-Rev structures over the $F_{ab}$-Rev crystal previously reported (PDB: 2X7L).

The existence of a heretofore unrecognized interface, C:C, was also reported in this study. Highly conserved residues such as P28, P29, and P31 (of the proline-rich loop) and W45 are all implicated in the C:C interface. The authors posit that the C:C interface bridges Rev dimers via the A:A oligomerization interfaces. Stacking interaction between W45 and P31 and the rigid poly-proline loop hook into each other form the interface. This interface is also stabilized by two hydrogen bonds between R46 and backbone carbonyl of the juxtaposed Rev monomer.

### 1.6 Rev Meditated RNA Dimerization

DiMattia *et al.* in their analysis of the Rev co-crystallized with Fab, superposed the NMR structure of stem loop IIB-ARM complex onto the Rev dimer and modeled two monomers of the RRE without any steric clashes with enough space to bind more rev dimers. This is supported by the geometry of the Rev monomers crossing at a 140° angle at the dimer interface. With the ARMs protruding out in a prong-like fashion, they proposed that two copies of the RRE, in an anti-parallel fashion,can interact with the two ARMs.

Electrophoretic Mobility Shift Assay (EMSA) data published by Zemmel et al in 1996 using a radio-labeled sequence of RNA with a modified stem loop-IIB (RWZ2) produced a discrete Rev:RNA species. The migration of the slower migrating Rev:RNA species according to the authors was a dimer of Rev bound to the RWZ2. This explanation, however, seemed inconsistent with the relative migration of the band on the gel. Might it be two copies of RWZ2 bound by Rev?

Dr. Jason Allison, in his graduate work asked the question, what is the stoichiometry of Rev:RRE? To address this question, Dr. Allison made a tRNA fusion of the modified RRE fragment, named sIIB, and analyzed Rev:sIIB complexes by analytical Size Exclusion Chromatography (SEC), Analytical UltraCentrifugation (AUC). The

initial experiments on SEC showed the formation of Rev:sIIB complex species which resolved as a large, discrete Rev:RRE complex. An SEC-derived Stokes radii analyses determined the discrete complex might contain two sIIB monomers bound to ARMs of Rev with additional Rev molecules participating in the complex.

In this work, Rev wt was site-specifically fluorescently labeled at the C-terminal end using Native Chemical Ligation (NCL). This allowed us to investigate further the stoichiometry of Rev:sIIB and larger RRE fragments. We found the ratio of Rev:sIIB is 4:1. The following chapters describe in detail of the experimental methods and results. As a part of this dissertation, we successfully crosslinked RevN70 using Thiol-Ene Coupling (TEC) as observed in the crystallized structures. This further allows us expand on the work, asking which Rev interface (A:A or B:B) supports the Rev mediated RNA dimerization.

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# Chapter 2: Materials and Methods

This chapter provides details on the various Rev constructs and the chimeric-RRE molecules used in the studies. This chapter also includes the experimental protocols used in the design and the purification of the molecules used in the studies.

## 2.1 Rev Constructs

### 2.1.1 Rev Wild Type (Rev WT)

Rev wild type, HIV-1 Strain HXB2, is a 116 residue protein with a mass of 13066 Daltons. Rev is a very basic protein with a pI of 9.23. Rev contains four distinct functional domains. It has an N-terminal dimerization and oligomerization domain, an Arginine-Rich Motif (ARM) region, which also serves as a nuclear localization sequence (NLS), located between 34-50 residues. A Nuclear Export Sequence (NES), which facilitates export of Rev from the nucleus to the cytoplasm upon interaction with human exportin-1 (CRM1), is found in the disordered C-terminal region of Rev.

In the Rev wt sequence from the lab, we have an extra two residues (Gly-His) at the N-terminal end that is a result of the cloning artifact. Rev wt was expressed in *E. coli* BL21(DE3) bacterial strain and grown in 2xYT media. One liter of the media yielded

about 80 mg of Rev wt. The protein was purified and stored according to the protocol described in Section 2.2

```
   1       10          20          30          40          50
GHMAGRSGDS DEDLLKAVRL IKFLYQSNPP PNPEGTRQAR RNRRRRWRER
          60          70          80          90          100
QRQIHSISER ILSTYLGRSA EPVPLQLPPL ERLTLDCNED CGTSGTQGVG
           110      116
SPQILVESPT VLESGTKE
```

Isotopically Averaged Molecular Weight = 13260.8652

Estimated pI = 9.06

**Fig 2.1 Properties of RevWT.** Number of residues, molecular weight, pI, and sequence of RevWT. The ARM and NES sequences are highlighted in red and blue, respectively. Sequence numbering starts from Methione residue. Gly-His residues upstream of Met are cloning artifacts.

### 2.1.2 RevN70

RevN70 comprises the ordered N-terminal region of HIV-1 Rev. In the C-terminal end of this construct we incorporated a Gly-Ser-Gly tripeptide. A variant of this construct was crystallized and solved in 2010 by Frankel *et al*. The disordered C-terminal region and the residues responsible for higher-order assembly *in vitro* have been terminated and mutated respectively. The structure (PDB ID: 3LPH) shows a dimer of Rev with a crossing angle of 120°. We truncated the disordered C-terminal region but did not mutate the residues responsible for higher-order assembly as seen in 3LPH. One liter of the

media yielded about 60 mg of RevN70. The protein was purified and stored according to the protocol described in Section 2.2. RevN70 plasmid was sequenced and verified.

```
  1     10          20          30         40          50
GHMAGRSGDS DEDLLKAVRL IKFLYQSNPP PNPEGTRQAR RNRRRRWRER
         60          70    75
QRQIHSISER ILSTYLGRSA EPGSG
```

Isotopically Averaged Molecular Weight = 8670.68

Estimated pI = 11.45

**Fig 2.2 Properties of RevN70.** Number of residues, molecular weight, pI, and sequence of RevN70

### 2.1.3 RevN70C

RevN70C construct represents the N-terminal helical hairpin residues that also make up the RevN70. However, we introduced a cysteine residue in the C-terminal end of this polypeptide. The purpose of this molecule is to singly label the protein on the cysteine for stoichiometry studies with RevN70 and sIIB. The protein was purified and stored according to the protocol described in Section 2.2. The RevN70C plasmid was sequenced and verified.

```
  1       10          20          30         40          50
GHMAGRSGDS DEDLLKAVRL IKFLYQSNPP PNPEGTRQAR RNRRRRWRER
          60          70    75
QRQIHSISER ILSTYLGRSA EPGCG
```

Isotopically Averaged Molecular Weight = 8686.7441

Estimated pI = 11.27

**Fig 2.3 Properties of RevN70C.** Number of residues, molecular weight, pI, and sequence of RevN70C

### 2.1.4 RevGyrA Intein Fusion Protein

RevGyrA intein fusion protein was designed to install a fluorescent label at the C-terminal region of Rev wt. Our previous findings showed that when the native cysteines in Rev were labeled with a fluorophore, there was a decrease in the binding of Rev to sIIB-RRE. So, we looked to intein fusion protein and Native Chemical Ligation (NCL) chemistry to specifically and selectively label Rev at the C-terminal end.

*Mycobacterium xenopi* gyraseA (Mxe GyrA) protein forms the basis of New England Biolabs IMPACT vector systems. Mxe GyrA intein was derived from NEB pTWIN1 vector. The plasmid encoding the GyrA portion was cut and expressed in pYP001 (in-house) vector. pYP001 contributes the 6x His-Tag and the Tev-protease cleavage site. Rev sequence follows Tev cleavage site and a tripeptide spacer Gly-Ala-Phe connects Rev with Mxe GyrA intein. The expression and purification of RevGyrA intein are discussed in detail in Chapter 3.

6x HiS

Tev
Cleavage Site

MGHHHHHHSS GLFKRHNDYD IPTTENLYFQ /GHMAGRSGDS
DEDLLKAVRL IKFLYQSNPP PNPEGTRQAR RNRRRRWRER
QRQIHSISER ILSTYLGRSA EPVPLQLPPL ERLTLD**C**NED
**C**GTSGTQGVG SPQILVESPT VLESGTKE**GA** **F**CITGDALVA
LPEGESVRIA DIVPGARPNS DNAIDLKVLD RHGNPVLADR
LFHSGEHPVY TVRTVEGLRV TGTANHPLLC LVDVAGVPTL
LWKLIDEIKP GDYAVIQRSA FSWKLIDEIK PGDYAVIQRS
AFSVDCAGFA RGKPEFAPTT YTVGVPGLVR FLEAHHRDPD
AQAIADELTS VTDAGVQPVY SLRVDTADHA FITNGFVSHA
TGLTGLNSGL TTNPGVSAWQ VNTAYTAGQL VTYNGKTYKC
LQPHTSLAGW EPSNVPALWQ LQ

Isotopically Averaged Molecular Weight = 46262.89

Estimated pI = 6.80

**Fig 2.4 Properties of RevGyrA.** Molecular weight, pI, and sequence of RevGyrA. Rev sequence is denoted in red, the linker in magenta, and GyrA sequence in purple. The Tev cleavage sequence is in green and underlined. 6x His-tag sequence is denoted in blue.

## 2.2. Expression, Purification and Characterization of Rev Constructs

### 2.2.1 Rev WT, N70, and N70C Purification protocol

Rev constructs were cloned in pYP001 plasmid. pYP001 is an in-house vector derived from pUC vector. pYP001 contributes the 6x His-Tag and the Tev-protease cleavage site at the N-terminal end. NdeI and NcoI restriction sites allow for the insertion of any of the Rev constructs. The plasmid was transformed into *E.coli* BL21(DE3) cells and grown in 2xYT media supplemented with 100 μg/mL of ampicillin. Cells were induced after an $OD_{600}$ reached 0.7. The culture was grown for 3 h at 37°C with shaking before harvesting. Cells were harvested by centrifugation at 4,000 rpm in a Beckman J6-HC low-speed centrifuge fitted with a JS-4.2A swing bucket rotor for 30 mins.

The cell pellet was suspended and sonicated in a denaturing sonication buffer (8M urea, 100 mM NaCl, 10 mM imidazole, 10 mM Tris, 5 mM β-ME, pH 7.2). Sonication was done for 8x30 sec cycles with the container holding the cell lysate immersed in ice. The sonicated/lysed cell lysate was centrifuged in 50 mL tubes in a Beckman J2-21 high-speed centrifuge in a F21B rotor at 10,000 rpm for an hour to pellet the cell debris. The supernatant containing the desired protein products was then loaded onto a Ni-NTA resin (Qiagen) column (15 mL bed-volume) for affinity purification. The Ni-NTA resin was pre-equilibrated with sonication buffer till the UV absorbance was stable. The cell lysate was loaded on the pre-equilibrated column and washed until the absorbance was stable. The flow-through and wash fractions were collected for analysis on SDS-PAGE. The bound protein was eluted in a buffer containing 1M imidazole (8M urea, 100 mM NaCl,

10 mM imidazole, 10 mM Tris, 5 mM β-ME, pH 7.2). The fractions were analyzed on an SDS-PAGE gel, and the elute fraction containing His-tagged protein of interest was then loaded on a SP-Sepharose 16/10 GE Life Sciences cation-exchange chromatographic column (20 mL cv) at 3 mL/min as a way of removal of bound nucleic acid.

The protein was loaded on the column equilibrated in sonication buffer. The column was extensively washed before the bound protein was bumped off the column in a buffer containing 5M GdmHCl (5M GdmHCl, 200 mM NaCl, 10 mM Tris, 5 mM β-ME pH 7.2). The eluted fractions were pooled after SDS-PAGE analysis and either diluted to a final concentration of 1M GdmHCl or dialyzed against a 1M GdmHCl buffer in preparation for 6x His-Tag-Tev removal. Rev concentration was kept around 60 μM to prevent aggregation of Rev molecules. His-tagged Tev protease was added at a ratio of 1:5 (1 mg of Tev to 5 mg of Rev) and the mixture was incubated at room temperature o/n. Tev protease cleavage reaction was quenched with the addition of Gdm HCl to make up to a final concentration of 5M Gdm HCl. This also aided in dissolving Rev precipitate that form during the cleavage reaction.

The mixture containing Tev protease was loaded on a Ni-NTA column for purifying His-cleaved Rev from His-Rev and His-tagged Tev. The Ni-NTA column (15 mL bed - volume) was pre-equilibrated with 5M GdmHCl, 200 mM NaCl, 10 mM Tris, 5 mM β-ME pH 7.2. The flow-though, which contains the His-cleaved Rev, is collected and analyzed on a SDS-PAGE for purity. As a final purification step, the flow-through from the 2nd Ni-NTA step is loaded on a C-18 RP-HPLC column after acidification with 0.1% TFA. The bound Rev was eluted over a gradient of 5%-70% acetonitrile. The eluted

protein was characterized by ESI-MS, aliquoted and lyophilized and stored at -20°C for later use.

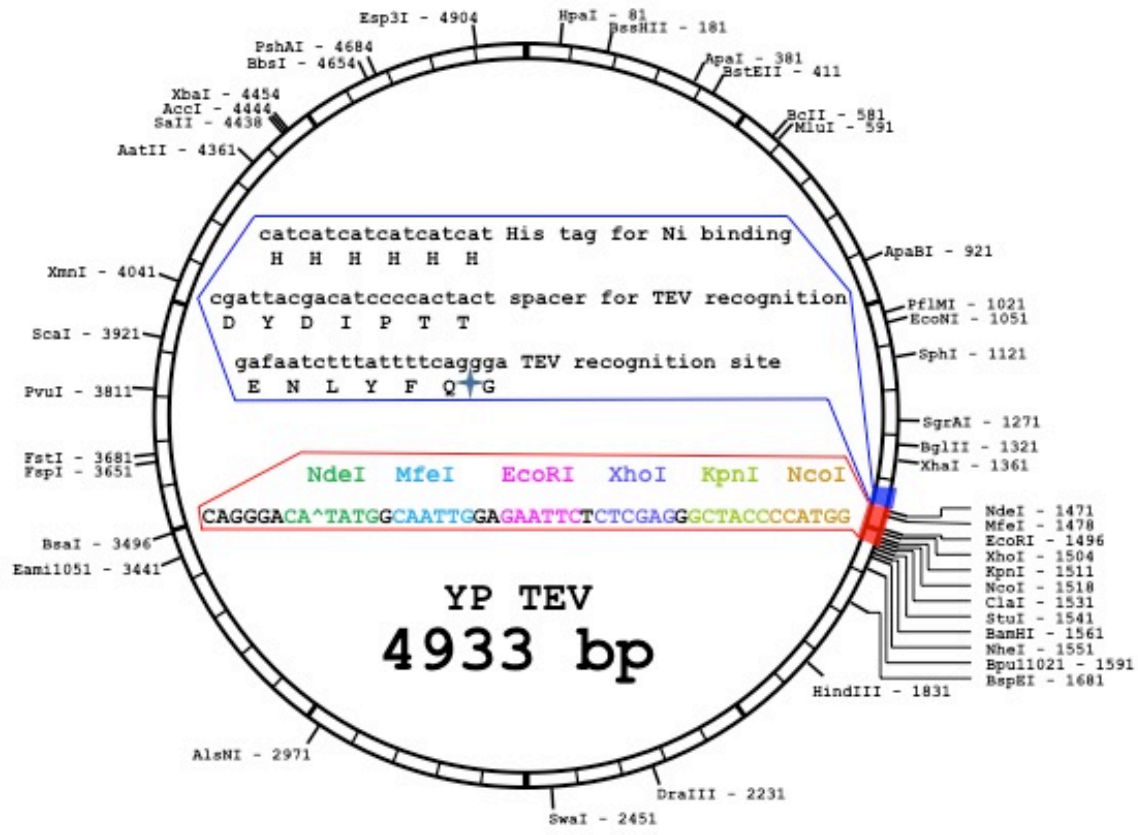


**Fig 2.5 pYP001 plasmid map.** Multiple Cloning Site and the 6X-HisTag with the Tev protease recognition site are depicted in the boxes. The cleavage site between Q and G is denoted.

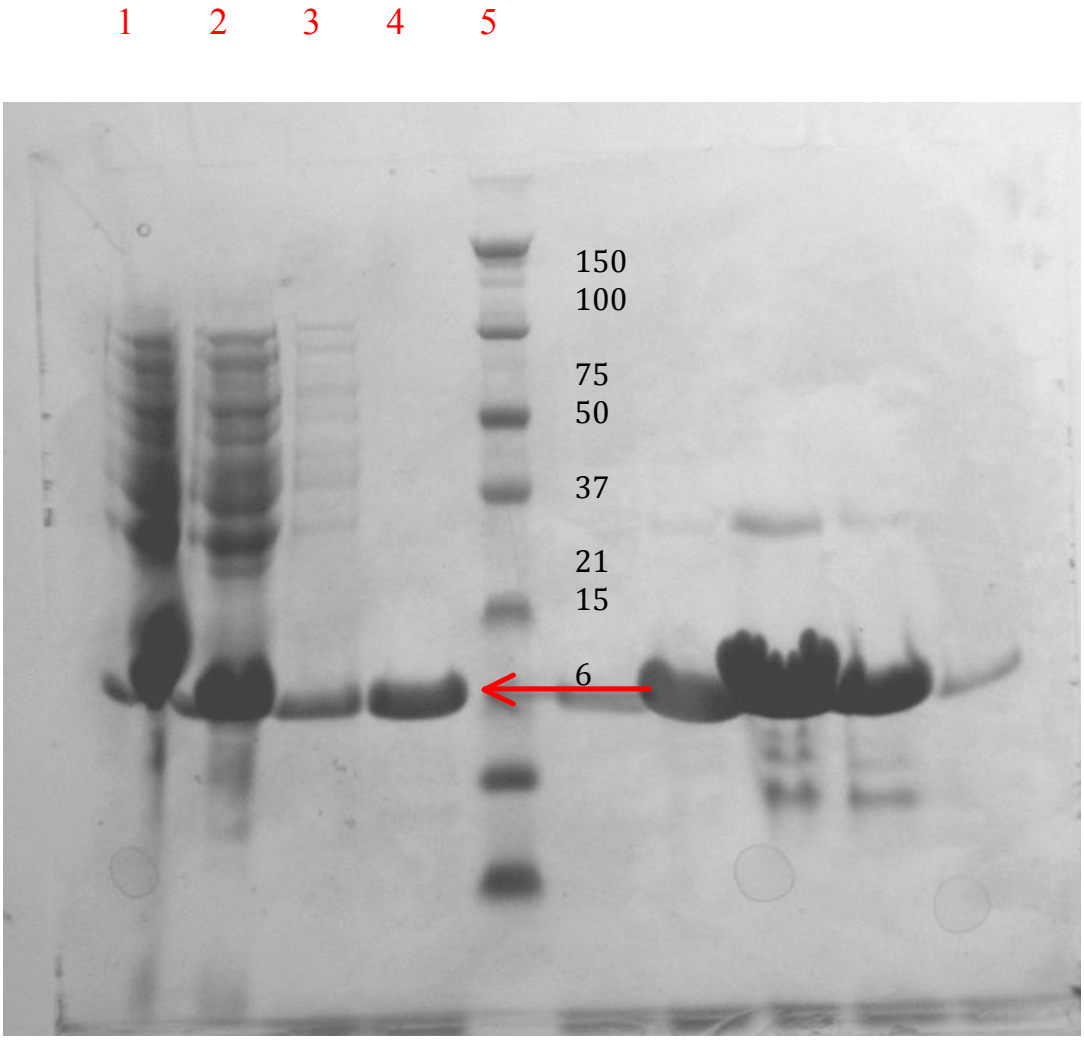


**Fig 2.6 SDS-PAGE of Ni-NTA purification of 6xHis-Rev WT.** 1: Load, 2:Flow-through, 3:Wash, 4:Elute**,** and 5:Protein Molecular wt standard. Purified 6xHis-Rev is denoted with the red arrow.

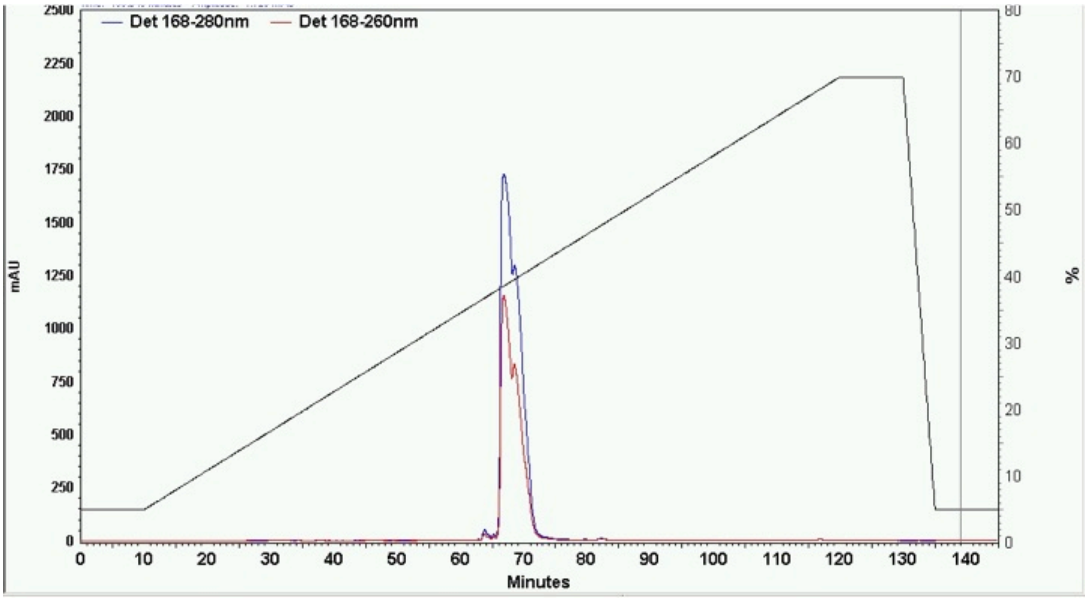


**Fig 2.7 HPLC purification trace of Rev WT using C18 RP-HPLC**. A gradient of 5% to 70% ACN was employed. Rev WT elutes at 38% to 42% ACN. The blue trace represents 280 nm, red 260 nm wavelength.

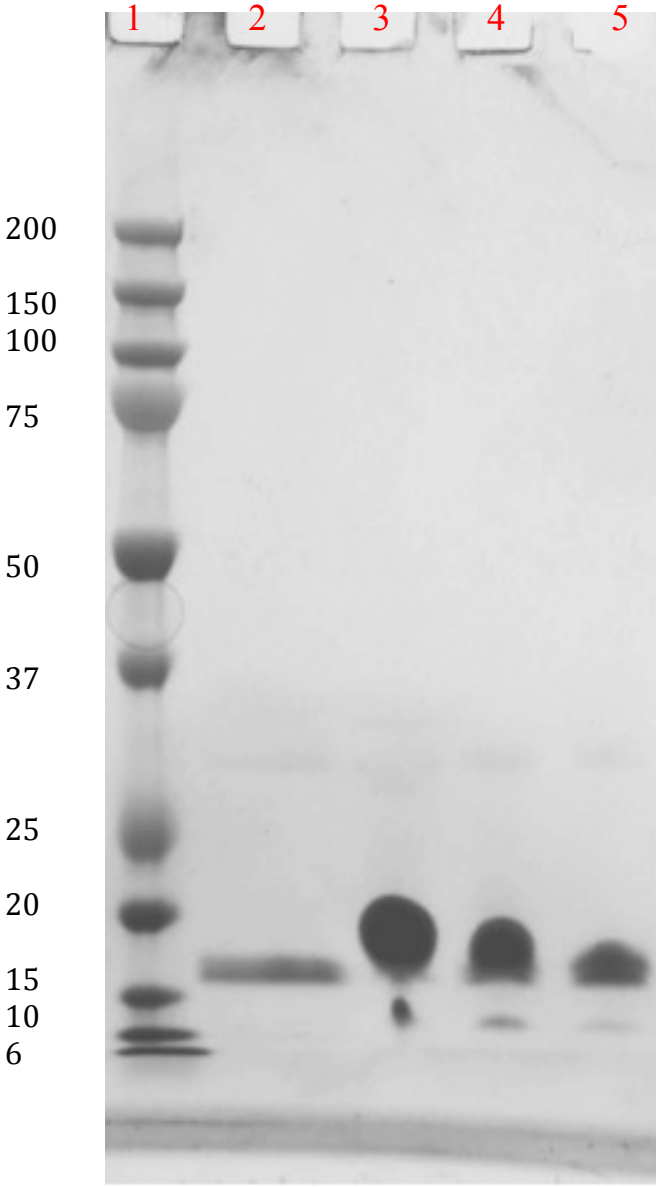


**Fig 2.8 SDS-PAGE of HPLC purified Rev WT.** 1:Protein Molecular wt standard, 2: His-cleaved Rev wt, 3,4, and 5: fractions collected from RP-HPLC purification

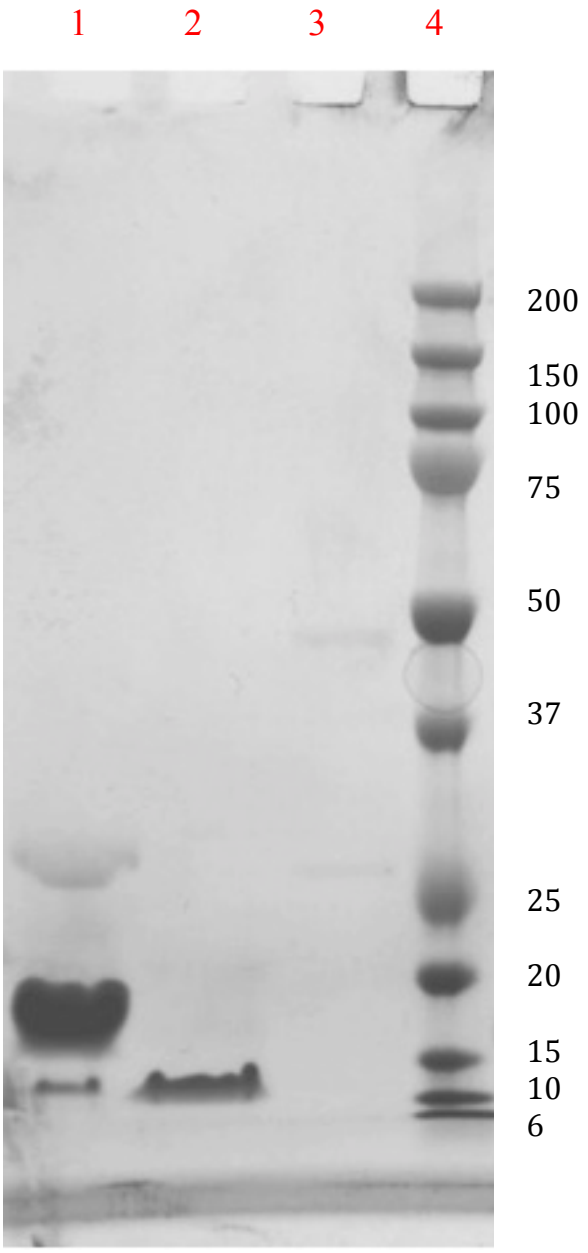


**Fig 2.9 SDS-PAGE of Rev variants.** 1: Rev WT, 2: RevN70, 3: RevGyrA, 4:Protein Molecular wt standard

### 2.2.2. RevGyrA Purification Protocol

BL21(DE3) cells transformed with the expression vector pAH-RevGyrA were plated onto LB plates supplemented with 100 μg/mL of ampicillin. The plates were incubated overnight at 37 °C temperature overnight; individual colonies were selected and used to inoculate 50 mL of LB supplemented with 100 μg/mL of ampicillin. The culture was grown overnight at 37 °C at 225 rpm in a shaker incubator. The seed was used to inoculate 2L of 2xYT medium supplemented with 100 μg/mL of ampicillin and the culture was incubated at 37 °C at 225 rpm. When the $OD_{600}$ in a 1cm path length cuvette reached 0.7-protein expression was induced with the addition of IPTG to a final concentration of 1mM. The culture was allowed to grow for 4h at 37 °C in a shaker incubator before harvesting in a Beckman J6-HC centrifuge using a JS-4.2A swing bucket rotor at 4,000 rpm. The harvested cells were resuspended in 50 mL of 40 mM sodium phosphate, 500 mM NaCl, 5 mM TCEP and pH 8. The cells were then sonicated in an ice bath and the debris spun down at 10,000 rpm in a Beckman J2-21 centrifuge using a F21B rotor at 4°C for 1h. The supernatant was loaded onto a Qiagen fast-flow Ni-NTA column (15mL bv) pre-equilibrated with the suspension buffer and washed with 40 mM sodium phosphate, 500 mM NaCl, 5mM Imidazole, 5 mM TCEP and pH 8 till the $OD_{280}$ reached base line. The bound protein was eluted with 40 mM sodium phosphate, 500 mM NaCl, 1M Imidazole, 5 mM TCEP and pH 6, care was taken to ensure that the eluted protein was not too concentrated to prevent precipitation. The eluted protein was dialyzed extensively against 40 mM sodium phosphate, 500 mM NaCl, 5 mM TCEP and

pH 6. Total yield after Ni-NTA column of the protein was about 60 mgs from 2L of 2xYT medium.

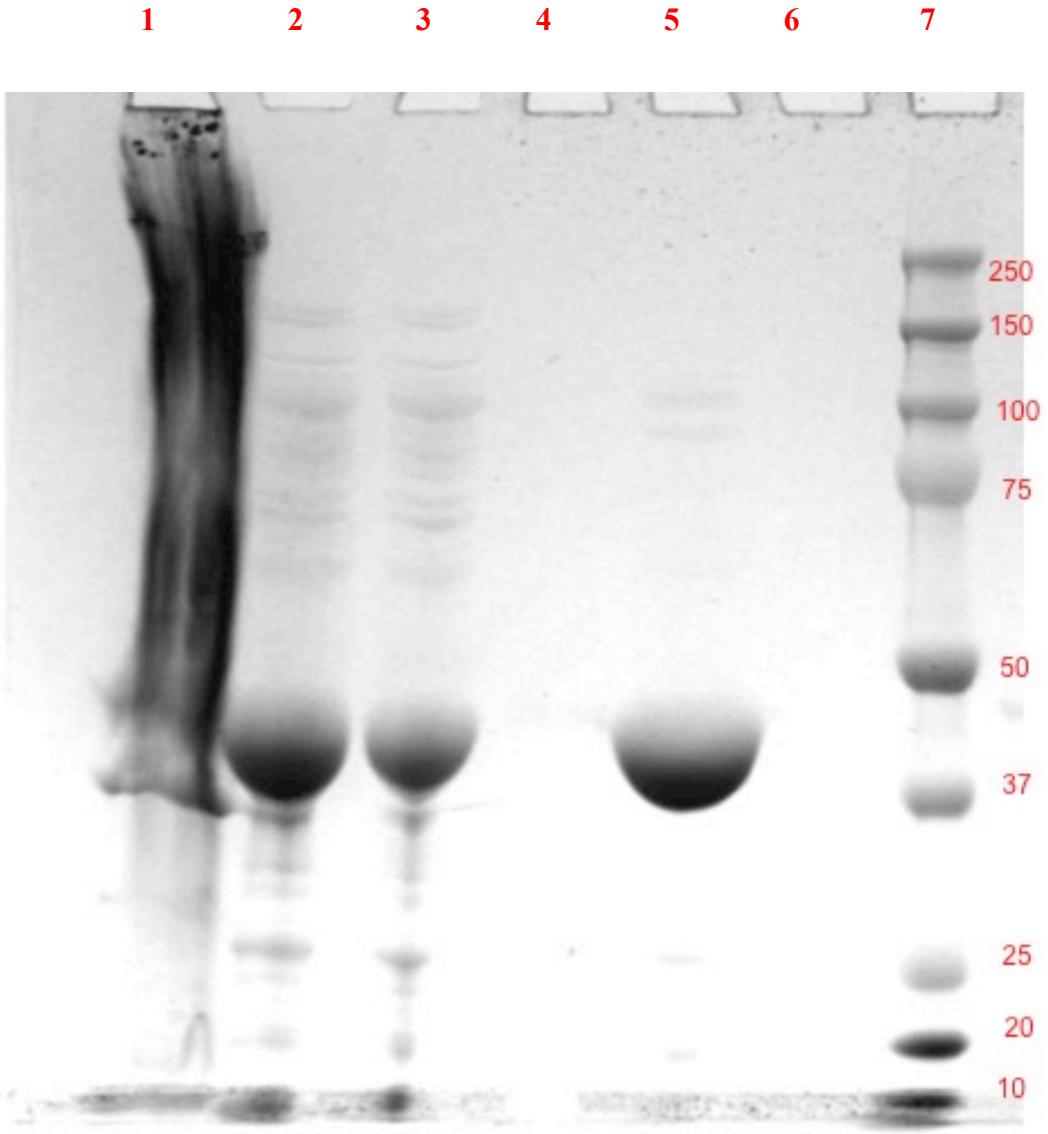


**Fig 2.10 SDS-PAGE of Ni-NTA purification of RevGyrA.** 1: Total cell extract, 2:Load, 3:Flow-through, 4:Wash, 5:Elute, 6: Blank, 7:Protein Molecular wt standard. RevGyrA, molecular wt. of 44.9 KDa.

## 2.3. Chimeric tRNA-RRE Constructs

The ability to produce and procure highly purified and copious amounts of RNA is a major limiting factor in performing structural studies. RNA can be synthetically produced using T7 polymerase. However, this technique creates heterogenous transcripts, which affects the final yield of the desired product. It is worth noting that T7 runoff transcription isn't cost-effective when one wishes to have RNA in milligram quantities.

Ponchon and Dardel( 2007) came up with an ingenious method to circumvent the production of RNA in milligram quantities. They envisioned creating chimeric tRNA molecules that contained RNA of interest and use *E. coli* cells to produce abundant quantities of chimeric RNA molecules. The target RNA sequences were cloned into the anticodon loop of tRNA. The acceptor stem, T⋃C, and D-loop of the tRNA were left untouched. The gene using an lpp promoter was subcloned into a commercially available expression vector, pBSK+. The plasmid was transformed into *E. coli* for overexpression. The lpp promoter codes for bacterial lipoprotein-most abundantly produced bacterial protein; this constitutive promoter sees that the chimeric RNA is also abundantly expressed. The expressed chimeric RNA was purified from other endogenously expressed RNAs via ion-exchange chromatography. The limitation of this technique is that the chimeric tRNA scaffold can effectively produce the RNA sequences of only up to 370 nts. Also, the yield of the chimeric RNAs goes down as the length of the insert

sequence increases. The group also showed that the chimeric RNAs were resistant to degradation by cellular ribonucleases. This technology seemed a viable and cost-effective approach to obtain RNA for structural studies.

### 2.3.1. $tRNA^{Phe}$-sIIB RRE Design

The design of the chimeric tRNA-RRE molecules was based on the size of the RRE fragment and the interactions of Rev with the RRE. We started with a small RRE fragment which encompasses the tight binding site of the Stem-Loop IIB region of the RRE as a model chimeric RNA. Dr. Jason Allison, my predecessor, on this project, first conceptualized and designed chimeric tRNA-RRE molecules with the aim of studying Rev-RRE interactions in solution, crystallizing Rev-RRE complexes, and small angle X-Ray scattering experiments to study the Rev-RRE interactions.

Initially, Dr. Allison's goal was to find a fragment of RRE, which would form a discrete complex with Rev and also should be short enough to show decent levels of expression. Studies on the Rev-RRE interactions performed by Mann *et al.* in 1994 demonstrated that a modified sequence resembling stem-loop IIB (RWZ1) bound a monomer of Rev and may not support ternary interactions of rev assembly. However, this sequence supported the formation of discrete Rev-RRE complexes making it an ideal candidate for solution biophysics. Another modified sequence, RWZ2, which contained a UC-bulge flanking the unpaired base region showed increased cooperativity of Rev binding to RWZ1. Dr. Allison based all his experimental studies involving Rev-RRE on this modified RWZ2

sequence. RWZ2 was inserted in the anticodon loop of baker's yeast phenylalanyl-tRNA, named sIIB in the studies.

In the original work with the chimeric tRNA-RNA molecules, the authors employed human lysyl-tRNA and were successful in achieving high yields of mutant RNA expression and the crystallizability of human lysyl-tRNA also was a factor in using the scaffold. We have designed and expressed chimeric RRE RNA in various tRNA scaffolds such as human lysyl-tRNA, baker's yeast phenylalanyl-tRNA, initiator methionine tRNA from *E.coli*. Dr. Allison in his work found that baker's yeast tRNAPhe was the best suited for expressing RRE fragments; this scaffold had not previously been used as a scaffold for expressing RNA sequences. It was also chosen due to its crystallizability, that these crystal contacts might help stabilizing Rev when in complex with RRE RNA in possible crystals.

All the chimeric $tRNA^{Phe}$-RRE sequences were adapted from a previously published DNA template. From 5' to 3', the DNA sequence includes the XholI restriction site, Lpp promoter, EcoRI restriction site, tRNA scaffold, Eag restriction site, sIIB sequence, Eag restriction site, tRNA scaffold, PstI restriction site, rrnC terminator, and HindIII restriction site, respectively.

**5’ *ctcgag* gtcgccccatcaaaaaaatattctcaacataaaaaactttgtgtaatacttgtaacgct *gaattc***

***XhoI1*** ***lpp promoter*** ***EcoRI***

**gcggatttagctcagttgggagagcgc ccggaattcttgggcgcagcgucattgac**

Phenylalanyl tRNA Scaffold sIIB Insert

**gctgcggtacatcggaattccggtcggcc gaggtcctgtgttcgatccacagaattcgcacca**

sIIB Insert **Phenylalanyl tRNA Scaffold**

***ctgcag* atccttagcgaaagctaaggatttttttt *aagctt* 3’**

*PstI* *rrnC terminator* *Hind*III

**Fig 2.11** Annotated gene sequence for $tRNA^{Phe}$-sIIB

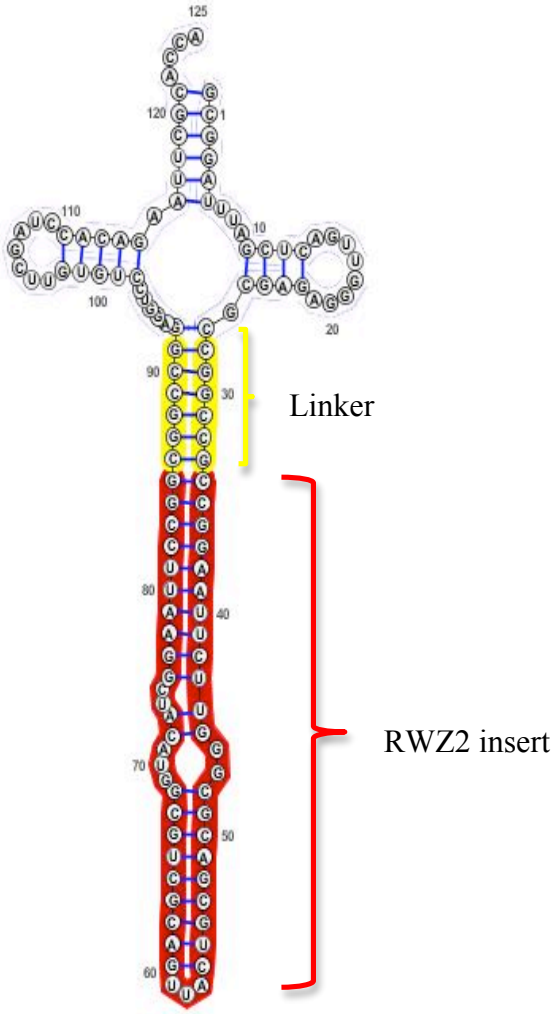


**Fig 2.12 Energy minimized (MFold) 2-dimensional representation of sIIB-tRNAPhe** (Using Varna online software). The structure is color coded and labeled as scaffold, linker, and sIIB insert

### 2.3.2. tRNA$^{Phe}$-T2 RRE Design

Previous studies on the effect of the length of the RRE in complex with Rev have shed some light on its subsequent export out of the nucleus. As mentioned earlier, Rev binds specifically and tightly to stem-loop IIB of the RRE and has been proposed to polymerize along the bubbled stem I of a monomeric RRE. A decrease in the transport of RNA has been observed in truncations of the RRE distal to the IIB binding site. Karn *et al.* identified a truncated sequence of the RRE, truncation 2 (T2), of the 351-base RRE by gel-shift analyses. This 273 base sequence is similar to the full-length RRE in the way it binds and interacts with Rev. We chose this T2 fragment of the RRE as a model to study the shortest fully functional unit of the RRE. The T2-tRNA$^{Phe}$ RNA contains a 273 base insert consisting of bases 45-311 of the RRE. This RRE truncation contains the natural hairpin regions of the RRE and the IIB Rev tight binding site.

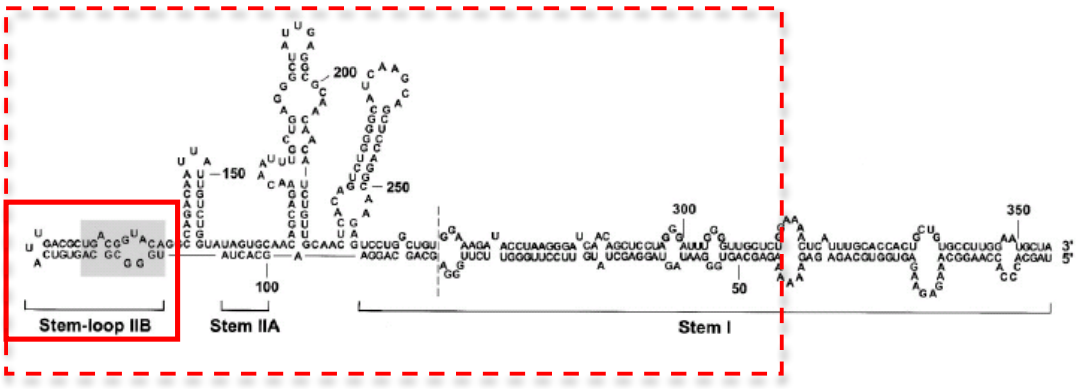


**Fig 2.13 T2 in context of the RRE.** The dotted boxed sequence corresponds to T2-RRE

**Gene sequence for tRNA$^{Phe}$- T2**

All the chimeric tRNA$^{Phe}$-RRE sequences were adapted from a previously published DNA template. From 5' to 3', the DNA sequence includes the XholI restriction site, Lpp promoter, EcoRI restriction site, tRNA scaffold, Eag restriction site, T2 sequence, Eag restriction site, tRNA scaffold, PstI restriction site, rrnC terminator, and HindIII restriction site, respectively.

5' *ctcgag* gtcgccccatcaaaaaaatattctcaacataaaaaactttgtgtaatacttgtaacgct
*XhoI* *lpp* promoter

*gaattc* gcggatttagctcagttgggagagcgc ccgg
tRNA$^{Phe}$ Scaffold Linker

AGAGCAGTGGGAATAGGAGCTTTGTTCCTTGGGTTCTTGGGAGCAGCAG
GAAGCACTATGGGCGCAGCGTCAATGACGCTGACGGTACAGGCCAGACA
ATTATTGTCTGATATAGTGCAGCAGCAGAACAATTTGCTGAGGGCTATTG
AGGCGCAACAGCATCTGTTGCAACTCACAGTCTGGGGCATCAAACAGCT
CCAGGCAAGAATCCTGGCTGTGGAAAGATACCTAAAGGATCAACAGCTC
CTGGGGATTTGGGGTTGCTCT
T2-RRE

ccgg gaggtcctgtgttcgatccacagaattcgcacca
Linker tRNA$^{Phe}$ Scaffold

*ctgcag* atccttagcgaaagctaaggatttttttt *aagctt* 3'
*PstI* Terminator *HindIII*

**Fig 2.14 Annotated gene sequence for tRNA$^{Phe}$-T2**

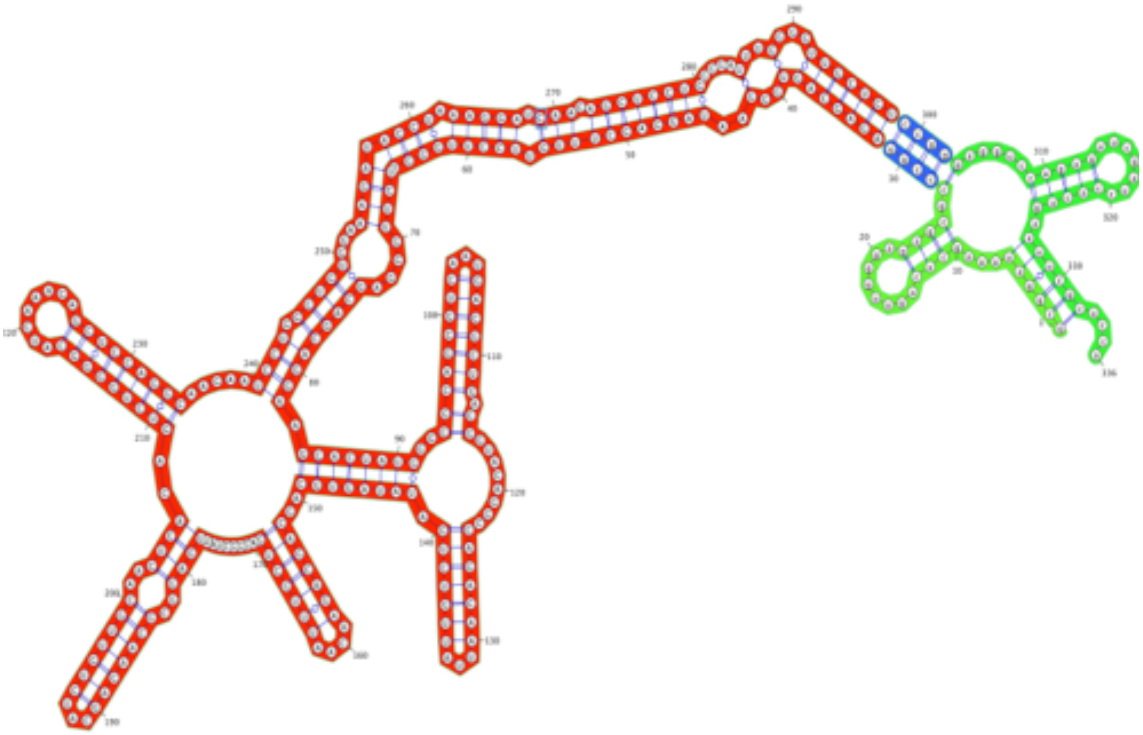

**Fig 2.15 Energy minimized (MFold) 2-dimensional representation of T2-tRNA$^{Phe}$** (Using Varna online software). The structure is color coded with tRNA$^{Phe}$ scaffold in green, linker in blue and the T2-RRE in red.

# CHAPTER 3: SOLID PHASE SEMI-SYNTHESIS OF C-TERMINALLY LABELED HIV-1 REV

### 3.1 Introduction:

The Regulator of Virion Expression or Rev is a regulatory, shuttle protein produced during the early phase of HIV development. Rev is essential for the transport of unspliced and partially spliced viral RNA from the nucleus in complex with RanGTP and CRM-1(Felber, Drysdale and Pavlakis, 1990; Purcell and Martin, 1993). Without Rev; there is a decreased production of *gag* and *pol* proteins and genomic-length RNA in HIV-1.(McClure *et al.*, 1987; Lowenstine *et al.*, 1986) HIV-1 Rev is 116 residues in length and 13 kDa in mass with well-defined functional motifs.(Felber, Drysdale and Pavlakis, 1990; Hirsch *et al.*, 1993).

The N-terminal residues 34-50 function as the nuclear localization sequence (NLS) and Rev Response Element (RNA) binding region. This region is flanked on both sides by oligomerization domain(s) (Fig 3.1) The C-terminal residues 73-83 function as the nuclear export sequence (NES) and an activation domain. It is classified as a trans-activating, and shuttle protein which interacts with a specific RNA sequence known as the Rev Response Element (RRE) (cis-target) contained within a ~351 nucleotide sequence within genomic HIV RNA and shuttles between the nucleus and cytoplasm of an infected cell.(Henderson and Percipalle, 1997; Izaurralde *et al.*, 1997; Askjaer *et al.*, 1998; Fischer *et al.*, 1999; Daelemans *et al.*, 2002)

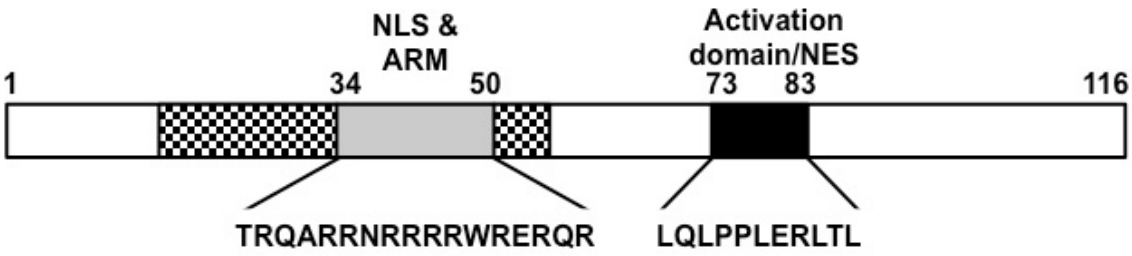


**Fig 3.1 Functional Motifs of Rev.** Rev is 116 residues in length. The N-terminal residues 34-50 function as the nuclear localization sequence (NLS) and Rev Response Element (RNA) binding region. This region is flanked on both sides by oligomerization domain(s) (checkered). The C-terminal residues 73-83 function as the nuclear export sequence (NES) and activation domain.

The RRE is a ~351 nucleotide sequence contained within the *env* encoding region HIV-1 genomic RNA (Malim *et al.*, 1989; Mann *et al.*, 1994). This region contains many secondary structural elements, which Rev recognizes. Once there is a sufficient accumulation of Rev in the nucleus of the infected cell, Rev recognizes Stem-Loop IIB within the RRE with high affinity ($K_D$ = 1 to 5nM) and subsequently assembles along Stem Loop-I, followed by the interaction with exportin-1 and Ran-GTP for the transport of singly-spliced and genomic-length RNA(Bartel *et al.*, 1991; Heaphy *et al.*, 1990).

Rev alone was determined to contain 50% alpha helices and 25% beta- sheet content with double minima at 222nm and 210nm, respectively(Auer *et al.*, 1994; Daly *et al.*, 1990). The disordered C-terminus was critical for assembly onto the RRE. Gel shift analysis demonstrated that removal of C-terminal residues 92-116 was sufficient to disrupt Rev's ability to assemble the RRE(Daly et al. 1993).

A helix-loop-helix model was proposed for Rev, which suggested that residues essential for dimerization were located at the A interface and residues at the B interface were required for oligomerization or Rev assembly(Jain and Belasco 2001). In 2010, two crystal structures of Rev were reported. In one of the crystal structures, the disordered C-terminal region was truncated and the residues necessary for higher order structure formation mutated(Daugherty, Liu, and Frankel 2010). Although the other structure is the full-length Rev co-crystallized with $F_{ab}$ antibodies, it, however, had no density in the intrinsically disordered region(DiMattia et al. 2010).

Several studies have been done to investigate the assembly of Rev on the RRE. Given, the propensity of Rev to oligomerize and aggregate at concentrations as low as 8 μg/mL has hindered any elegant structural or biophysical studies (Wingfield *et al.*, 1991; Cole *et al.*, 1993).Several groups have reported varying numbers of Rev monomers (6-12) assembling on the RRE using various biochemical and biophysical techniques.(Daly *et al.*, 1989; Cook *et al.*, 1991; Mann *et al.*, 1994; Pallesen *et al.*, 2009; Daugherty, Liu and Frankel, 2010).

In the recent years, Native chemical ligation (NCL) has come to the forefront of utilizing chemical, and biological principles in modifying biological macromolecules.(Dawson and Kent 2000) NCL involves the regioselective and chemoselective coupling of two protein or peptide moieties. It involves the generation of the N-terminal component with a C-terminal thioester and the C-terminal component with an N-terminal cysteine residue. The components combine to give a native peptide bond at the point of ligation(Dawson et

al. 1994). We have utilized this approach and created a RevGyrA fusion protein which when treated with MesNa releases an active thioester at the C-terminal end of Rev. The thioester can then be attacked by a cysteine-containing peptide which contains a fluorophore at the C-terminal end of the peptide.

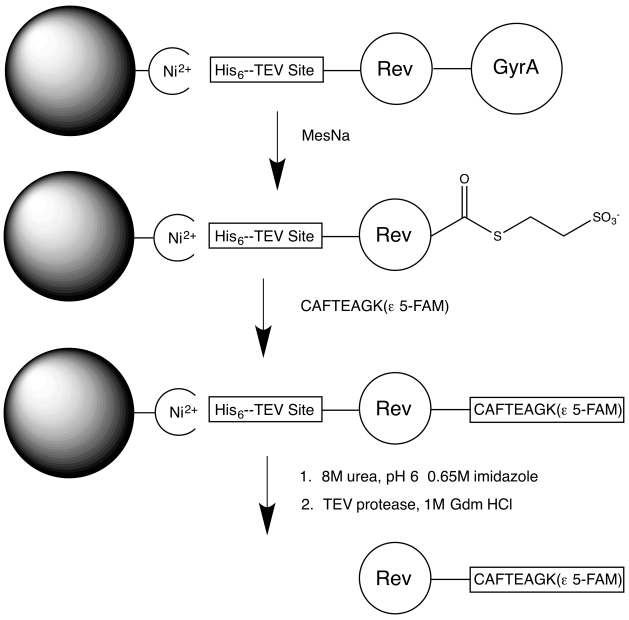


**Fig 3.2: General route of the preparation of RevL.** RevGyrA fusion protein was immobilized on the N-terminal end via the 6xHis-Tag. Treatment with MesNa released the GyrA portion and an active thioester generated at the C-terminal end of Rev. Upon treatment with a cysteine containing peptide, the cysteine attacks the thioester and there is an S-N arrangement with the formation of an amide bond. The ligated Rev was bumped off the column with an imidazole-containing buffer.

## 3.2 RESULTS AND DISCUSSION:

Previously, we labeled the cysteines in the C-terminal region; however, a loss in function of Rev when the cysteines were labeled was observed (data not shown). Other labeling methods such as labeling the N-terminal lysines have been shown to be deleterious to Rev's functionality. We, therefore, decided to pursue the NCL route to singly label the C-terminal region of Rev. The labeling reaction was done with His-tagged RevGyrA fusion protein immobilized on Roche cOmplete His-Tag purification Resin™ (gift from Roche GmBH). The resin was tested for its ability to withstand high concentrations of MesNa used in the NCL reactions. The best conditions for the cleavage, labeling reaction were determined as 37°C, 50 mM MesNa, pH 6 in native phosphate buffer conditions.

After successful analytical reactions, the reactions were scaled up to preparative scale. We have immobilized 1.5 micromoles of RevGyrA fusion protein and used a 2-fold excess of the fluorescent peptide: Fl8RP. Fl8RP is a 7-residue peptide, CAFTEAGK (ε5-FAM), purchased from Biomatik Corporation, Canada. To the fusion protein immobilized on the resin, a mixture of Fl8RP, 50 mM MesNa in phosphate buffer at pH 6 was added. Although the reaction was allowed to go for 3 days, we have observed the reaction to go to completion around 48 h. Elute from the labeling reaction contains labeled Rev (RevL), unlabeled Rev and unreacted RevGyrA. In order to purify RevL away from unlabeled Rev and RevGyrA, traditional methods of purification such as cation-exchange chromatography and RP-HPLC were unsuccessful. However, we

serendipitously found that when the mixture was loaded on a MonoQ column, RevL bound to the column while the unlabeled Rev and RevGyrA fusion protein eluted in the flow-through. A quick literature survey showed that MonoQ columns have been previously employed to selectively purify labeled protein molecules away from the unlabeled molecules(Jäger, Nir, and Weiss 2006; Sims and Cohen 2009). RevL was bumped off the MonoQ in 5M Gdm HCl buffer and treated with Tev protease to cleave the 6X His-Tag. After Tev protease treatment, Ni-NTA column was employed to separate the His-tag removed RevL from His-tagged RevL(Fig 3.3). RevL was purified on a Vydac C-18 column; this is to ensure there is no nucleic acid bound to RevL.

Mass spectrometry was used to further confirm the identity of the molecule. ESI-MS did not yield data with good resolution; hence we resorted to a tryptic digest of RevL. Trypsin digestion of RevL protein and LC MS/MS analysis confirmed 92.25% of the RevL sequence (Table 3.1) with 15 unique and 86 total peptides. The list of peptides is presented in the supplementary data. The peptide "EGAFCAFTEAGK(ε5-FAM)" confirms the ligation of the peptide, Fl8RP, to the C-terminal end of Rev.

We, then, asked whether the installation of a peptide is in any way hindering Rev's interactions with the RRE by gel-retardation assay. To test RevL's binding to the RRE, we prepared a 120 base chimeric $tRNA^{Phe}$-RRE fragment. The chimeric RNA contained the tight binding site of the RRE for which Rev has nanomolar affinities. The chimeric RRE fragment (0.5 μM) was incubated with increasing molar concentrations of both RevL and Rev wild-type. The complexes were incubated on ice for 30 min before loading

on to a continuously running 7% native PAGE gel. A constant voltage of 150 V was applied and the protein:RNA complexes were analyzed under UV and after ethidium bromide staining(Fig 3.4). No change in the migration or the appearance of the complexes was observed. Both RevL and Rev wild type were similar in their apparent interaction with the RRE.

We have successfully employed NCL to install a fluorescent peptide at the c-terminal end of Rev. Unlike traditional methods, NCL allowed to specifically and selectively install only one label per Rev molecule without inhibiting and hindering other residues which are critical for Rev's intrinsic functions with respect to the RRE. With the labeled Rev, RevL, one can address the stoichiometry question and perhaps can expect a more precise answer to the number of Rev molecules per RNA.

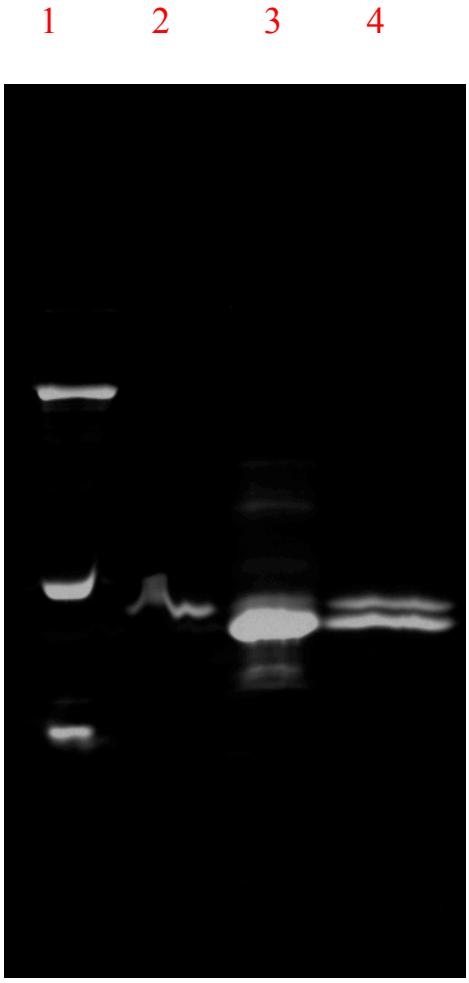


**Fig 3.3. RevL Tev protease cleavage.** Lane 1: Protein Mol. Wt standard, 2: 6x-His-RevL, 3:His-cleaved RevL, 4: 6xHis cleavage in 2M GdmHCl conditions shows uncleaved upper migrating RevL

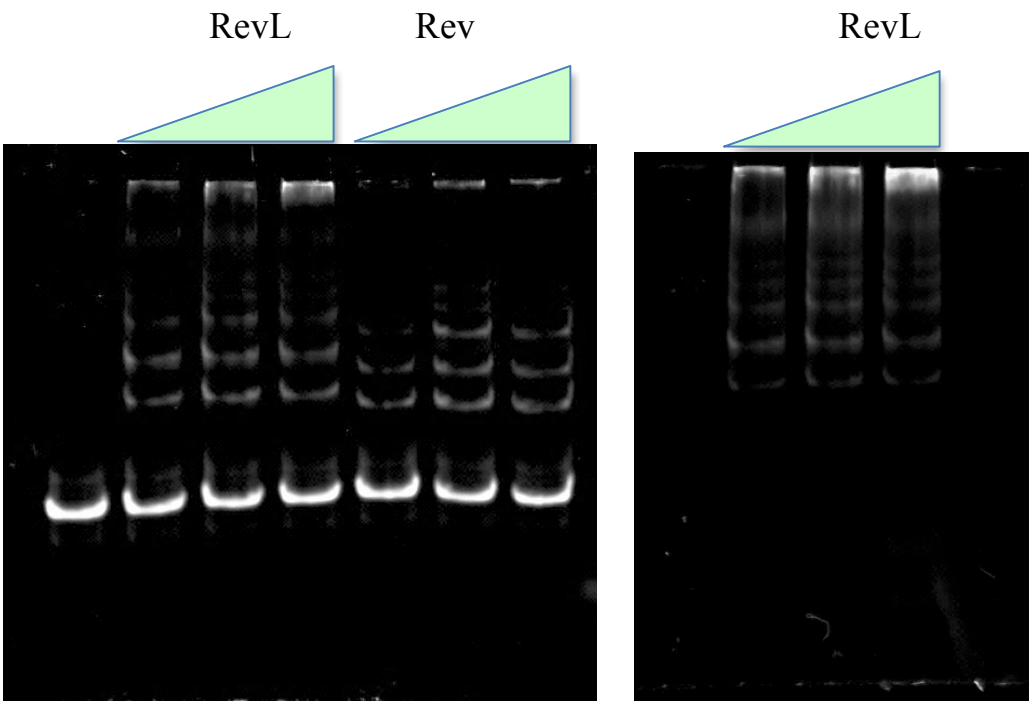


**Fig 3.4. Comparison of RevL and Rev wt binding to chimeric tRNA$^{Phe}$-sIIB RRE.** 7% Native PAGE shows that the binding of RevL to tRNA$^{Phe}$-sIIB RRE is similar to Rev wt binding. The picture in the right panel shows RevL migration under UV illumination prior to EtBr staining. [sIIB]= 0.5 μM.

### 3.3 Materials and Methods:

#### 3.3.1 Construction of the RevGyrA Fusion Expression Vector

The fusion gene for RevGyrA was constructed by overlapping PCR. The Rev gene from vector RevTG was amplified using the primers

Rev-GyrA-R:

5`-CTGGAAAGCGGTACCAAAGAGGGCTCTTCCTGCATCACGGGAGAT

Rev-GyrA-F:

5`- CCATGGTTATCATTGAAGCTGCCACAAGGCAGGAAC

The Mxe-GyrA gene from pTWIn1 vector (NEB biolabs) was amplified using the primers

Antiparallel Rev-GyrA

5`-CTGGAAAGCGGTACCAAAGAGGGCTCTTCCTGCATCACGGGAGAT

Rev-GyrA-R

5`-ATCTCCCGTG ATGCAGGAAGAGCCCTCTTT GGTACCGCTTTCCAG

The PCR products from individual amplifications were run on a 1.5% agarose gel and the bands corresponding to the product of interest excised out and gel purified. The purified DNA were mixed in a 1:1 stoichiometric ratio and amplified by PCR using the flanking primers. The PCR product was ligated to a pCR 2.0 TA cloning shuttle vector

(Invitrogen) and transformed into Turbo cells (NEB). Colonies containing the gene of interest was identified by colony PCR and plasmid DNA isolated from them. The genes were sequenced using M13 forward and reverse primers to confirm the sequence. The confirmed gene was excised out of the shuttle vector using the restriction enzymes Nde1 and Nco1. The insert was then ligated into the *E. coli* expression vector pYP001 previously linearized by the same enzymes. The ligated vector was transformed into Turbo and the colonies containing the gene of interest identified. Purified expression vector from the identified colonies were sequenced using the T7 promoter and T7 terminator primers. The sequence confirmed pAH-RevGyrA plasmid was then transformed into BL21 (DE3) cells for protein expression.

### 3.3.2 Protein Expression and Purification

BL21 DE3 cells transformed with the expression vector pAH-RevGyrA were plated onto LB plates supplemented with 100 μg/mL of ampicillin. The plates were incubated overnight at 37°C temperature overnight; individual colonies were selected and used to inoculate 50 mL of LB supplemented with 100 μg/mL of ampicillin. The culture was grown overnight at 37°C at 225 rpm in a shaker incubator. The seed was used to inoculate 2L of 2YT medium supplemented with 100 μg/mL of ampicillin and the culture was incubated at 37°C at 225 rpm. When the $OD_{600}$ in a 1cm path length cuvette reached 0.7-protein expression was induced with the addition of IPTG to a final concentration of 1mM. The culture was allowed to grow for 4h at 37°C in a shaker incubator before harvesting in a Beckman J6-HC centrifuge using a JS-4.2A swing bucket rotor at 4,000

rpm. The harvested cells were resuspended in 50 mL of 40 mM sodium phosphate, 500 mM NaCl, 5 mM TCEP and pH 8. The cells were then sonicated in an ice bath and the debris spun down at 10,000 rpm in a Beckman J2-21 centrifuge using a F21B rotor at 4°C for 1h. The supernatant was loaded onto a Qiagen fast-flow Ni-NTA column preequilibrated with the suspension buffer and washed with 40 mM sodium phosphate, 500 mM NaCl, 5mM Imidazole, 5 mM TCEP and pH 8 till the $OD_{280}$ stabilized. The bound protein was eluted with 40 mM sodium phosphate, 500 mM NaCl, 1M Imidazole, 5 mM TCEP and pH 6, care was taken to ensure that the eluted protein was not too concentrated to prevent precipitation. The eluted protein was dialyzed extensively against 40 mM sodium phosphate, 500 mM NaCl, 5 mM TCEP and pH 6. Total yield after Ni-NTA column of the protein was about 60 mgs from 2L of 2YT medium.

MGHHHHHHSS GLFKRHNDYD IPTTENLYFQ GHMAGRSGDS DEDLLKAVRL
IKFLYQSNPP PNPEGTRQAR RNRRRRWRER QRQIHSISER ILSTYLGRSA
EPVPLQLPPL ERLTLDCNED CGTSGTQGVG SPQILVESPT VLESGTKEGA
FCITGDALVA LPEGESVRIA DIVPGARPNS DNAIDLKVLD RHGNPVLADR
LFHSGEHPVY TVRTVEGLRV TGTANHPLLC LVDVAGVPTL LWKLIDEIKP
GDYAVIQRSA FSWKLIDEIK PGDYAVIQRS AFSVDCAGFA RGKPEFAPTT
YTVGVPGLVR FLEAHHRDPD AQAIADELTS VTDAGVQPVY SLRVDTADHA
FITNGFVSHA TGLTGLNSGL TTNPGVSAWQ VNTAYTAGQL VTYNGKTYKC
LQPHTSLAGW EPSNVPALWQ LQ

**Fig 3.5 Sequence of RevGyrA fusion protein** indicating 6x Histag (red) and Tev Cleavage site (underlined)

### 3.3.3 Labeling of Rev by Native Chemical Ligation

Dialyzed RevGyrA fusion protein (55 mg) was immobilized on a preequilibrated 5 mL Roche cOmplete™ His-tag purification resin-containing column. The fusion protein was incubated with the resin for 1h at room temperature on an orbital shaker. The flow-through after incubation was collected and the resin was then washed with 5 bed volumes (bv) of dialysis buffer-Buffer A. A 2-fold mole excess of the peptide, Fl8RP, CAFTEAGK(ε5-FAM), was carefully weighed and dissolved in buffer A containing 50 mM MesNa. This was then added to the resin bed containing the immobilized fusion protein and incubated at 37°C for 72 hours.

At the end of the incubation period, collecting the flow-through, which contains excess peptide and the released GyrA moiety, stopped the reaction. The resin bed was further washed with 5 bv of Buffer A followed by an o/n incubation of the labeled protein in Buffer A containing 5M Gdm HCl at room temperature on an orbital shaker. The purpose of this incubation is to break any Rev aggregates that may have formed during the labeling procedure. The resin bed was washed with 5 bv of Buffer A + 5M Gdm HCl and 5 bv of Buffer A+8M Urea and 5M Gdm HCl. This was followed by an extensive wash with Buffer A+ 8M Urea; 4 bv of Buffer B, which contains 40 mM Tris, 100 mM NaCl, 1M imidazole 5 mM β-ME and 1 M imidazole at pH 8 was added and the resin incubated at room temperature for an hour and the bound protein eluted.

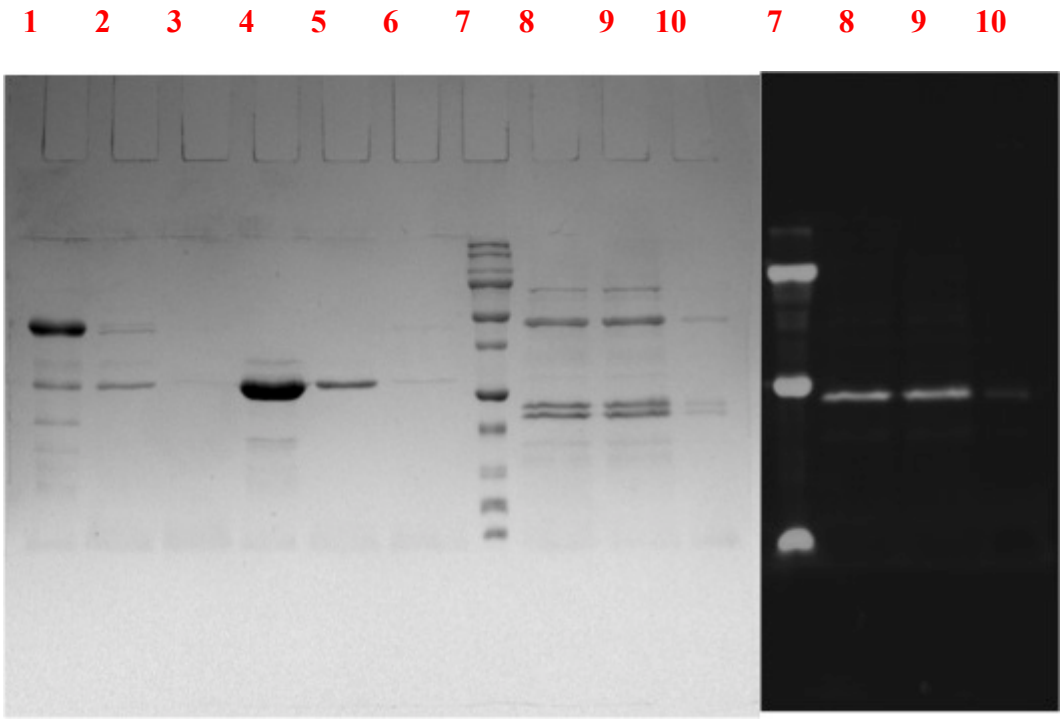


**Fig 3.6 Labeling of Rev immobilized on cOmplete resin.** 1: RevGyrA load, 2:Flow-through, 3:Wash, 4:Flow-through after reaction with Fl8RP, 5:Wash with 5M Gdm HCl buffer, 6:Wash with 5m Gdm HCl and 8M urea buffer, 7: Protein Molecular wt standard, 8,9, and 10: Elute samples containing RevL. The panel on the right is the gel illuminated under UV-light. Lanes 8,9, and 10, which contain the fluorescent label, fluoresce under UV.

### 3.3.4 Purification of RevL on a MonoQ column

The eluted fraction from the labeling reaction contains fusion protein, RevGyrA, hydrolyzed Rev and labeled species, RevL. This mixture was diluted 5-fold with Buffer C containing 40 mM Tris, 8M urea, 5 mM β-ME, 100 μM EDTA at pH 8 and incubated for an hour on ice. MonoQ 16/10 column (20 mL column volume) was equilibrated with Buffer C at pH 7.4 with the buffers sitting on ice. The mixture was loaded on to the column and wash with 5 column volumes of Buffer C. The flow-through contains the unbound fraction, which include the fusion protein, RevGyrA and unlabeled Rev. The column was then washed with 1 column volume of Buffer D, 40 mM Tris, 5 mM β-ME, 100 μM EDTA at pH 7.4. The bound RevL was bumped off the column with 2M Gdm HCl, 40 mM Tris, 200 mM NaCl, 5 mM β-ME at pH 7.2.

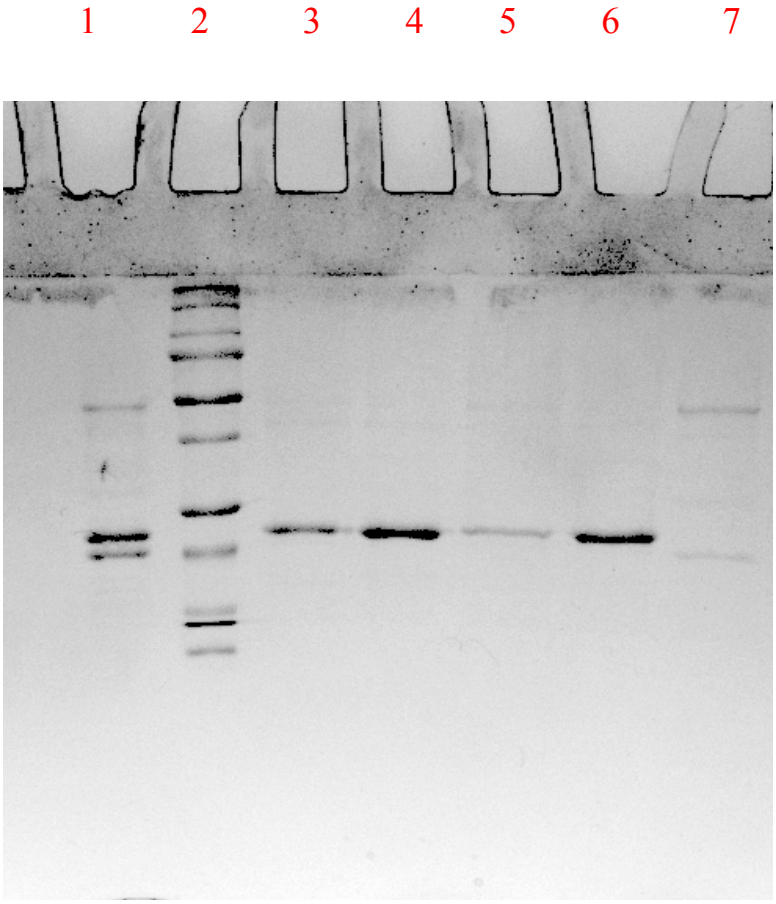


**Fig 3.7 SDS-PAGE of MonoQ separation of RevL.** 1: Load containing mixture of RevGyrA, RevL, and unreacted Rev 2: Protein Molecular wt standard, 3,4,5 and 6: RevL fractions from MonoQ, 7: Flow-through containing RevGyrA and unreacted Rev

### 3.3.5 Tev Proteolysis and separation of Tev cleaved RevL

The fraction bumped off the MonoQ column contains RevL species only. This was diluted with 40 mM Tris at pH 7.2 to keep the RevL concentration below 60 μM; Tev protease (1:5 RevL) was added and the mixture was incubated at room temperature under dark conditions o/n.

Tev cleavage was quenched by making the solution up to 5M Gdm HCl with the addition of solid Gdm HCl. Ni-NTA column was equilibrated with Buffer G which contains 5M Gdm HCl, 40 mM Tris, 200 mM NaCl, 5 mM β-ME at pH 7.2. The protein solution was loaded on the column and the flow-through containing His-tag cleaved RevL was collected. The bound species, Tev protease and uncleaved RevL were eluted with Buffer G containing 1 M imidazole.

### 3.3.6 RP-HPLC of RevL

RevL was acidified with 0.1% TFA (Sigma), 5% acetonitrile was added to the acidified RevL and loaded on a Vydac TP-54 C18 column. Running solution A used is water in 0.045% TFA and solution B is acetonitrile in 0.05% TFA. A gradient of 5% to 80% B at 1.5% per min was employed. RevL eluted at 40% B. RevL was aliquoted and lyophilized.

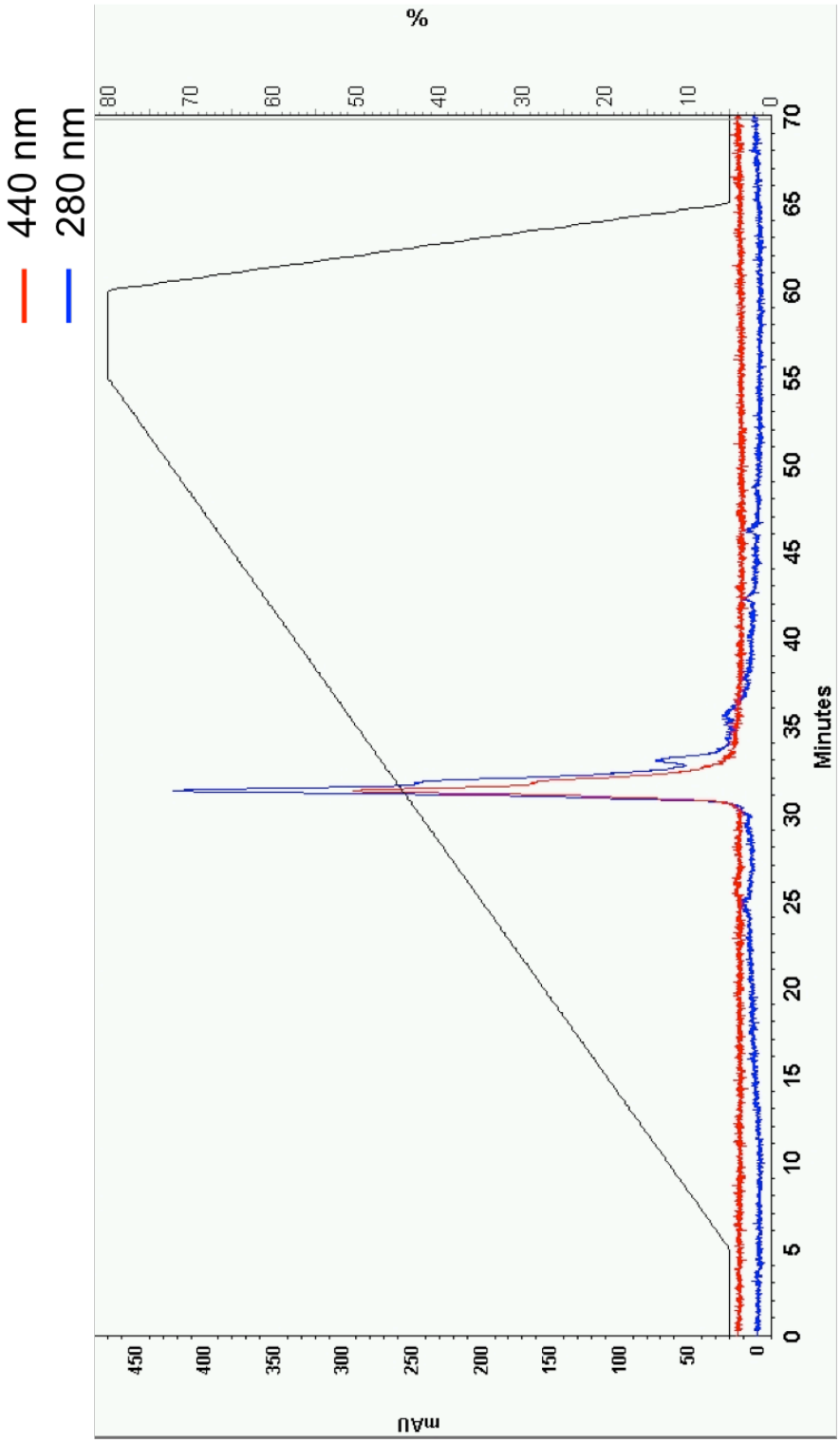


**Fig 3.8 RP-HPLC purification of RevL.** RevL purified on a C18 RP-HPLC column elutes at 45% ACN.

### 3.3.7 RevL trypsin digestion and Mass Analysis

Lyophilized protein sample was dissolved in 6 M urea and protein concentration was measured. Protein disulfide bonds were reduced by adding 25 mM tris 2-carboxyethyl phosphine (TCEP) in 300 mM ammonium bicarbonate buffer pH 8 and incubating at 37°C for 30 minutes. The free cysteine residues were alkylated by the addition of 20 mM iodoacetamide and incubating in the dark at room temperature for 30 minutes. 5 µg of reduced and alkylated sample was diluted with 100 mM ammonium bicarbonate buffer to bring the urea concentration below 0.5 M. Then, 100 ng of trypsin was added (1:50 ratio) and incubated overnight at 37°C. The reaction was stopped by the addition of formic acid (final concentration of 1%). Sample was centrifuged at 14,000 rpm for 5 minutes and 1 µl of sample (15 pmoles) was injected for each mass spectrometry analysis.

Liquid chromatography tandem mass spectrometry (LC MS/MS) was performed on a ThermoFinnigan LTQ equipped with an Agilent 1290 Infinity UPLC system using Solvent A (water + 0.1% formic acid) and Solvent B (methanol + 0.1% formic acid). The sample was run for 60 minutes with the solvent gradient of 20% Solvent B to 70% over 33 minutes. A home-packed 500 µm x 6 cm C18 reversed phase column was used with a flow rate of 30 µl/min. Electrospray voltage was set to 3.78 kV with sweep, auxiliary, and sheath gas set to 0 on a standard IonMax ESI Source. The capillary temperature was set to 250°C and the mass spectrometer was set for Dynamic Exclusion Data Dependent MS/MS with the three highest intensity masses observed in the MS scan targeted for MS/MS fragmentation. The RAW data files were converted to MGF format using the MSConvert utility program from the ProteoWizard program suite

(http://proteowizard.sourceforge.net/tools.shtml). Spectra from the three technical replicates LC-MS/MS run were output into a single MGF file for analysis in OMSSA against RevL protein database. The OMSSA database search was performed with the following setting: tryptic cleavage, 1 missed cleavage allowed, precursor mass tolerance 2 Da, product mass tolerance of 0.8 Da, carbamidomethyl cysteine (+57.02) as fixed modification, 5FAM (+358.31104) at lysine as variable modification and E value threshold was set to 1.000e-001.

Trypsin digestion of RevL protein and LC MS/MS analysis confirmed 92.25% of the RevL sequence with 15 unique and 86 total peptides. The peptide "EGAFCAFTEAGK(ε5-FAM)" confirms the addition of sub-peptide with 5-FAM to the RevL protein.

Peptide Hit Detail View @Peptide E-Value Cut-off = 1.000E-001 for OMSSA file: 20160627_RevL_Merged.oms

| E-Value | Peptide | Modification | Mass | M-Diff | Charge (z) | Position |
|---|---|---|---|---|---|---|
| 1.51E-07 | GHMAGRSGDSDEDLLK | | 1687.105 | 0.343 | 2 | 1~16 |
| 1.25E-06 | SGDSDEDLLK | | 1077.785 | 0.302 | 2 | 7~16 |
| 5.98E-07 | SGDSDEDLLKAVR | | 1404.185 | 0.496 | 2 | 7~19 |
| 2.34E-13 | LIKFLYQSNPPPNPEGTR | | 2069.708 | -0.383 | 3 | 20~37 |
| 2.00E-09 | FLYQSNPPPNPEGTR | | 1716.265 | 0.437 | 2 | 23~37 |
| 1.18E-04 | FLYQSNPPPNPEGTRQAR | | 2072.528 | 1.503 | 3 | 23~40 |
| 1.65E-03 | QRQIHSISER | | 1253.125 | 0.46 | 2 | 51~60 |
| 2.06E-03 | QIHSISER | | 968.745 | 0.24 | 2 | 53~60 |
| 8.05E-07 | ILSTYLGR | | 921.945 | 0.417 | 2 | 61~68 |
| 2.77E-08 | ILSTYLGRSAEPVPLQLPPLER | | 2449.448 | 1.073 | 3 | 61~82 |
| 2.61E-12 | SAEPVPLQLPPLER | | 1545.345 | 0.487 | 2 | 69~82 |
| 4.32E-04 | SAEPVPLQLPPLERLTLDCNEDCGTSGTQGVGSPQILVESPTVLESGTK | | 5176.898 | 1.34 | 3 | 69~117 |
| 7.59E-08 | LTLDCNEDCGTSGTQGVGSPQILVESPTVLESGTK | | 3654.248 | 5.537 | 3 | 83~117 |
| 2.53E-12 | LTLDCNEDCGTSGTQGVGSPQILVESPTVLESGTKEGAFCAFTEAGk | K 5FAM:47 | 5280.758 | 5.189 | 3 | 83~129 |
| 6.46E-05 | EGAFCAFTEAGk | K 5FAM:12 | 1644.785 | -0.084 | 2 | 118~129 |

**Table 3.1 MS/MS Analysis of RevL identified the peptide containing the 5-FAM group.**

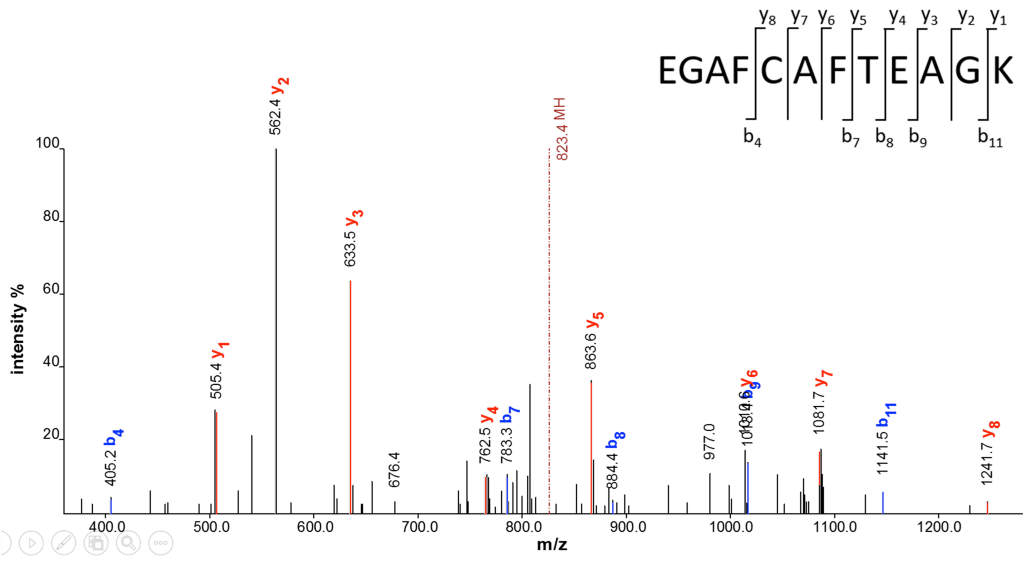


**Fig 3.9 MS/MS of RevL**. The peptide with the fluorescent label is identified.

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# CHAPTER 4: HIV Rev mediates the dimerization of HIV RRE RNA

This chapter discusses the experimental details and results of sIIB-tRNA$^{Phe}$ in complex with Rev and RevL. Most of the experiments have been done with Dr. Jason Allison and some of the experimental data has been a part of Dr. Allison's dissertation work.

## 4.1 INTRODUCTION

The HIV-1 Regulator of Virion Expression (Rev) a regulatory protein which is critical for the late-phase development of the Human Immunodeficiency Virus-1 (HIV-1) (Bartel et al. 1991; Benko et al. 1990;Berger et al. 1991;Calnan et al. 1991;Cullen 1991, Fankhauser et al. 1991). During early-phase development of HIV-1, Rev accumulates in the cytoplasm and is transported into the nucleus through interactions with Importin-β via its Nuclear Localization Sequence (NLS) (Love, Sweitzer and Hanover 1998;Panaro et al. 2008;Pollard and Malim 1998;Richard, Iacampo and Cochrane 1994;Wolff et al. 2006). Following sufficient accumulation in the nucleus, Rev recognizes and assembles on the Rev Response Element (RRE), a 351nt region contained within singly and unspliced HIV RNAs (Anazodo et al. 1995;Tan et al. 1993;Venkatesan et al. 1992; Venkatesh, Mohammed and Chinnadurai 1990;Williams and Leppard 1996;Williamson et al. 1995;Wodrich and Kräusslich 2001;Xu et al. 1996;Zapp and Green 1989). This ribonucleoprotein (RNP) complex allows for the export of these RNA transcripts encoding viral structural proteins and genomic RNA to the cytoplasm without further splicing. Of particular interest is the interaction of Rev with the Rev Response Element (RRE) RNA. It is known that Rev initially binds to a structured Stem Loop-IIB on the RRE and subsequently assembles along flanking sequences of the RRE; however, details of this interaction are not completely understood. RRE-containing tRNAs were designed for biophysical studies of Rev:RRE complexes. The tRNA chimeras employ natural tRNA from human lysyl tRNA, bacterial initiator methionyl tRNA, and baker's yeast (*S. cerevisiae*) phenylalanyl tRNA for use as scaffolds to express RRE sequences. Using

size-exclusion chromatography (SEC), analytical ultracentrifugation (AUC), and small angle X-ray scattering (SAXS), we demonstrate the formation of a discrete Rev:RRE RNA complex. Data analysis suggests that Rev is mediating a dimer of RRE RNA fragment used in these studies.

### 4.2 Implications for Rev-mediated dimerization of RRE RNA

It is known that a mature HIV-1 virion carries a diploid copy of genomic RNA in the capsid. The dimerization of the RNA is mediated by cis-acting signals contained within secondary structural elements of HIV RNA synergistically with transactivating viral proteins. The cis-acting signals for encapsidation and RNA dimerization are contained within the 5' untranslated region (UTR) and include the "kissing" loop (Ψ), dimer linkage structure (DLS), primer binding site (PBS), and the dimerization initiation site (DIS) (Laughrea and Jetté 1996;Paillart et al. 1996;Russell, Liang and Wainberg 2004). The transactivating proteins promoting HIV RNA dimerization are nucleocapsid (NC) and the group associated antigen (gag) proteins. Recently, it was shown using a Murine Leukemia virus (MLV)-derived viral vector that the Rev/RRE system is required with the 5' UTR cis elements to augment encapsidation of heterologous RNA into a HIV-1 viral particle (Cockrell et al. 2011). For these experiments, the Rev/RRE system as well as 5' UTR *cis* packaging elements were reconstructed in a retroviral vector RNA to investigate the additive, and independent, impact on the gain of encapsidation function into HIV-1 derived viral particles. The Rev/RRE system increased the packaging efficiency of a diploid copy of the heterologous RNA ~100 fold. Although the physical basis by which

the Rev/RRE system increases the efficiency of packaging a diploid copy of genomic RNA is unknown.

Experiments performed on Rev/RRE complexes since the discovery of the RRE in 1989 have all been interpreted as that Rev interacting with one copy of RRE RNA. The position of the RNA binding regions of the Rev dimer(s) as revealed by the two crystal structures solved suggest that an oligomer of Rev could bridge a dimer or bind a single copy of RRE RNA (Daugherty et al. 2010;DiMattia et al. 2010). Both crystal structures reveal that Rev monomers interact at an obtuse angle to each other with the RNA binding residues (residues 34-50) which protrude in a prong-like manner and could bridge two copies of RRE RNA in an antiparallel fashion. DiMattia *et al.* (2010) suggests that Rev binds to one copy of the RRE in a manner in the first monomer binds to stem loop-IIB followed by stem loop-I of the RRE "wrapping" around and interacting with the oligomer of Rev via the available RNA binding regions. Gel shift experiments performed by Daugherty and colleagues suggest that Rev binds to one stem loop-IIB (34 nucleotides) and an extended version of stem loop-IIB (42 nucleotides) as a dimer.

Gel retardation data published in 1996 demonstrated that a radio-labeled sequence of RNA containing a modified stem loop-IIB sequence (named RWZ2) with the introduction of a UC bulge flanking the unpaired residues produced a soluble, discrete Rev:RNA species at a Rev/RNA molar ratio of 16/1 (Zemmel et al. 1996;Heaphy et al. 1990). The disparity of the migration position of the unbound RWZ2 RNA and the Rev:RWZ2 complex was curious. The authors claim that the upper band was a dimer of Rev bound to RWZ2. However, from the position of the complex band on the gel, this

did not seem consistent. It seemed plausible that Rev may have been binding two copies of the RWZ2 RNA hairpin. To investigate this, the RWZ2 fragment, which was later named sIIB was inserted into a tRNA scaffolds and Rev:sIIB-tRNA complexes were analyzed by SEC, AUC, and the monomeric sIIB-$tRNA^{Phe}$ was examined by SAXS.

## 4.3 RESULTS AND DISCUSSION

### Rev does not bind to scaffold RNA at low concentrations

Following calibration of the Shodex KW803 column, Rev was mixed with the phenylalanyl tRNA (Yeast) at different Rev:RNA molar ratios and RNA concentrations as a control to determine if Rev interacts with the tRNA scaffold at these concentrations. The running buffer composition was 200 mM NaCl, 100 μM MgCl, 50 mM Tris, 2 mM beta-mercaptoethanol, pH 7.2. At 0.5 μM $tRNA^{Phe}$, Rev did not bind the tRNA at any tested molar ratios (Fig 4.1): the elution of the $tRNA^{Phe}$ monomer at 9.1 mL is unperturbed, and no RNA elutes at an earlier position. Curiously, at increasing Rev:$tRNA^{Phe}$ molar ratios, the $A_{260}$ decreased monotonically. This may be due to the RNA interacting with Rev, which is nonspecifically bound to the column matrix or precipates on sample preparation. At [$tRNA^{Phe}$] = 5 μM, Rev formed multiple higher-order species at all Rev:$tRNA^{Phe}$ ratios, indicative of nonspecific binding to the scaffold (Fig 4.1). At a 1:1 Rev:RNA, peaks at 6.5 and 8.0 mL appeared and became more apparent as the Rev:RNA molar ratio increased. The elution peak at 8.0 mL may represent increasing numbers of Rev bound to a single RNA. The trailing 6.5 mL peak

may represent Rev:RNA aggregates containing more than one RNA. Conversely, at a [sIIB-tRNA$^{Phe}$] = 0.5 μM the Rev:sIIB-tRNA$^{Phe}$ species eluted as a discrete species at 6.9 mL (Fig 4.1). The 6.9 mL species represented a large complex that elutes as a 600 kDa spherical protein. The remainder of this section describes the behavior of Rev:sIIB-tRNA$^{Phe}$ complexes on the Shodex KW803 column.

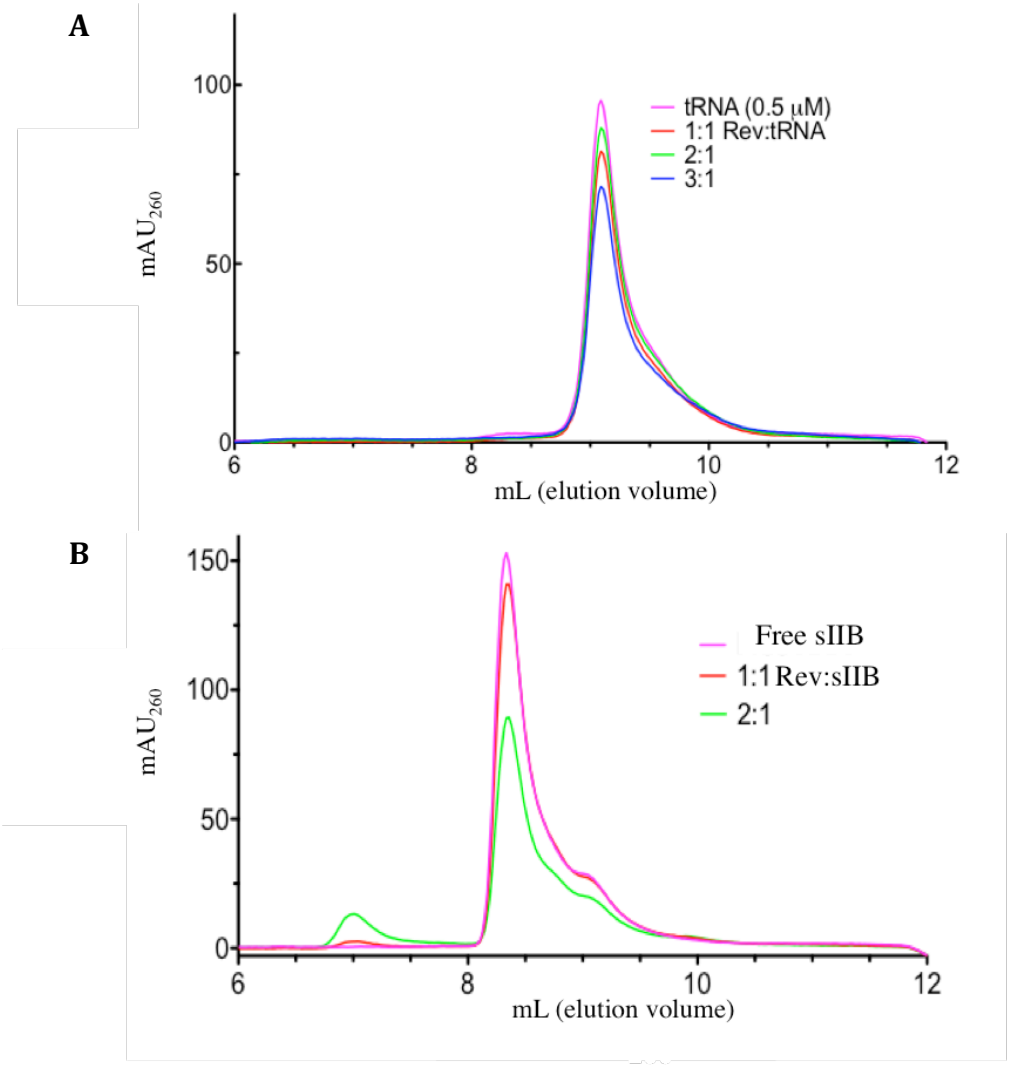


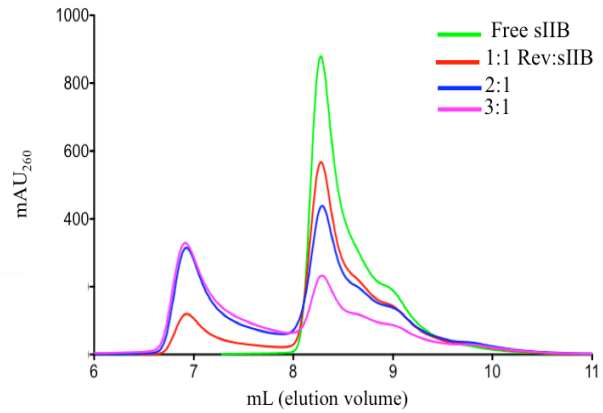

**Fig 4.1.** (A) SEC elution profiles of Rev:tRNA$^{Phe}$. At [tRNA$^{Phe}$] = 0.5 μM, Rev did not bind to the tRNA$^{Phe}$ at Rev:RNA ratios of 1:1, 1:2, 1:3, or 1:4. (B) SEC elution profiles of Rev:sIIB-tRNA$^{Phe}$. At [sIIB-tRNAPhe] = 0.5 μM, Rev formed a discrete species with sIIB-tRNA$^{Phe}$ which eluted at 6.9 mL. The sIIB-tRNA$^{Phe}$ monomer eluted at 8.23 mL on a Shodex KW-803 column at a flow-rate of 0.350 mL/min.

## Hydrodynamic data indicates that Rev dimerizes RRE RNA

### Size Exclusion Chromatography Experimental Data

The Rev:sIIB-tRNA$^{Phe}$ complex elutes from the Shodex KW803 column as discrete species at 6.9 mL (± 0.023 mL) at all Rev:sIIB-tRNA$^{Phe}$ ratios at both [RNA] = 0.5 μM and 5.0 μM. The position of the Rev:sIIB-tRNA$^{Phe}$ complex at 6.9 mL is equivalent to the elution position of a spherical protein of 400 kDa or a spherical RNA with a mass of 600 kDa. As the molar ratio of Rev:sIIB-tRNA$^{Phe}$ was increased there was more observable complex with less unbound, monomeric sIIB- tRNA$^{Phe}$ (Fig 4.2). At a Rev:RNA ratio of 4:1, all of the sIIB-tRNA$^{Phe}$ was complexed with Rev forming a Rev:RNA species (Fig 4.2).

**A**




**B**

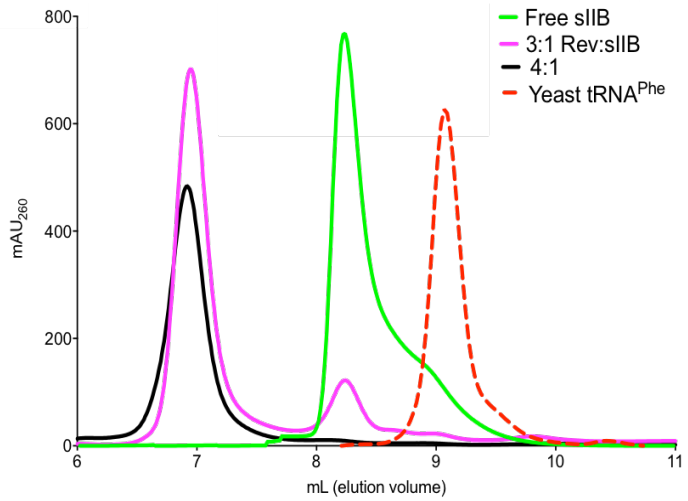



**Fig 4.2.** (A) Shodex KW803 elution profiles of Rev:sIIB-tRNA$^{Phe}$, [RNA] = 5 µM. Increasing the molar equivalents of Rev results in more of the Rev:sIIB-tRNA$^{Phe}$ complex as observed at the elution position of 6.9 mL. (B) SEC Elution profiles complexed sIIB-tRNA$^{Phe}$, [RNA] = 5 µM. At a Rev:sIIB-tRNA$^{Phe}$ of 4:1 all of the RNA is complexed with Rev elution at 6.9 mL (black). Elution position of a 3:1, Rev:RNA complex (magenta). Elution position (8.23 mL) of sIIB-tRNA$^{Phe}$ monomer (green). Elution position (9.1 mL) of yeast phenylalanyl tRNA (dotted red).

**Analytical UltraCentrifugation Experimental Data**

Rev:sIIB-tRNA$^{Phe}$ mixtures were analyzed by AUC. Complexes were prepared at 5 μM RNA at Rev:RNA molar ratios of 1:1, 2:1, and 3:1. The mixtures were dialyzed overnight into 200 mM NaCl, 100 μM $MgCl_2$, 50 mM Tris, 2 mM β-mercaptoethanol, pH 7.2. Two species were detected: the 4.8S species, which likely corresponds to the sIIB-tRNA$^{Phe}$ monomer, and a second species that sediments more rapidly at 9.8S, which we infer corresponds to a Rev:RNA complex (Fig 4.3). Both of these species were observed at 1:1, 2:1, and 3:1 Rev:RNA ratios. To determine the compositions consistent with the 9.8S species, we calculated frictional ratios ($f/f_0$) for complexes containing different numbers of RNA and Rev monomers. Complexes containing a single sIIB-tRNA$^{Phe}$ give $f/f_0$ values (and corresponding axial ratios) that are not physically realistic unless bound to more than a dozen equivalents of Rev. Since more than half the RNA sediments as complex at a 3:1 Rev:RNA ratio, we conclude that the complex must contain more than one copy of sIIB-tRNA$^{Phe}$. Complexes containing two RNAs and from zero to ten equivalents of Rev produce results that are consistent with 9.8S and are physically possible. The top portion of (Table 1) gives molecular weights for two RNAs with increasing equivalents of Rev (2, 4, 6, and 8 Revs). The bottom part of the table gives calculations for $\bar{\upsilon}$, hydrodynamic radius, hydration radius, density, frictional ratios, and axial ratios. From these calculations, it is reasonable to conclude that the 9.8S species is a Rev-mediated sIIB-tRNA$^{Phe}$ dimer, however, the Rev stoichiometry is not clear.

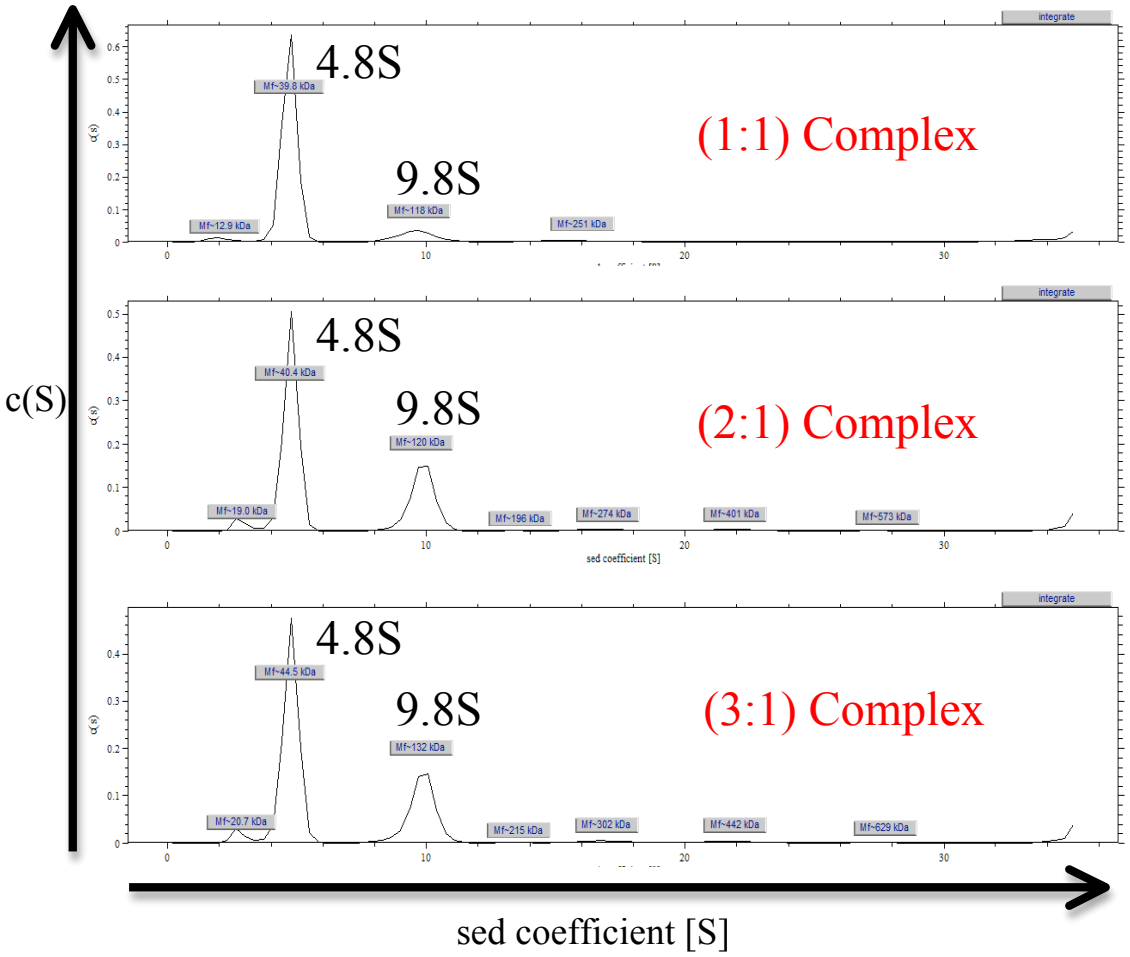



**Fig 4.3.** S-values extracted from SV-AUC data for Rev/sIIB-tRNA$^{Phe}$ mixtures. Monomeric or unbound sIIB-tRNA$^{Phe}$ sedimented at a value of 4.8S. The Rev: sIIB-tRNA$^{Phe}$ sedimented with a value of 9.8S.

**C-terminal truncation of Rev abrogates RRE RNA dimerization**

C-terminal residues of Rev have are important for RRE binding. Rev:RRE gel shift experiments demonstrated that the removal of C-terminal residues perturb binding to the RRE which may explain the aberrant binding behavior of RevN70 (Daly et al. 1993). The C-terminal truncation of Rev complexed with RRE RNA migrated faster than full-length Rev:RRE complexes indicating a smaller Rev:RNA complex. This property of a C-terminal truncation of Rev was confirmed in the SEC data collected on RevN70:sIIB-$tRNA^{Phe}$ complexes. The RevN70 did bind to sIIB-$tRNA^{Phe}$, however, the complexes eluted very differently than Rev:sIIB-$tRNA^{Phe}$ complexes. The data show that the Rev:sIIB-$tRNA^{Phe}$ complexes eluted at 6.9 mL, much earlier than the RevN70:sIIB-$tRNA^{Phe}$ which eluted at 8.1 mL (Fig 4.4). The error of ±0.017 shows that the elution position of the RevN70:sIIB- $tRNA^{Phe}$ is significantly different from that of the sIIB-$tRNA^{Phe}$ monomer which elutes at 8.28 mL. This may be due to RevN70 being unable to self-assemble or the protein is unable to mediate an oligomer of RNA as is proposed in this work. The role of the C-terminus in RRE binding and assembly is not well understood.

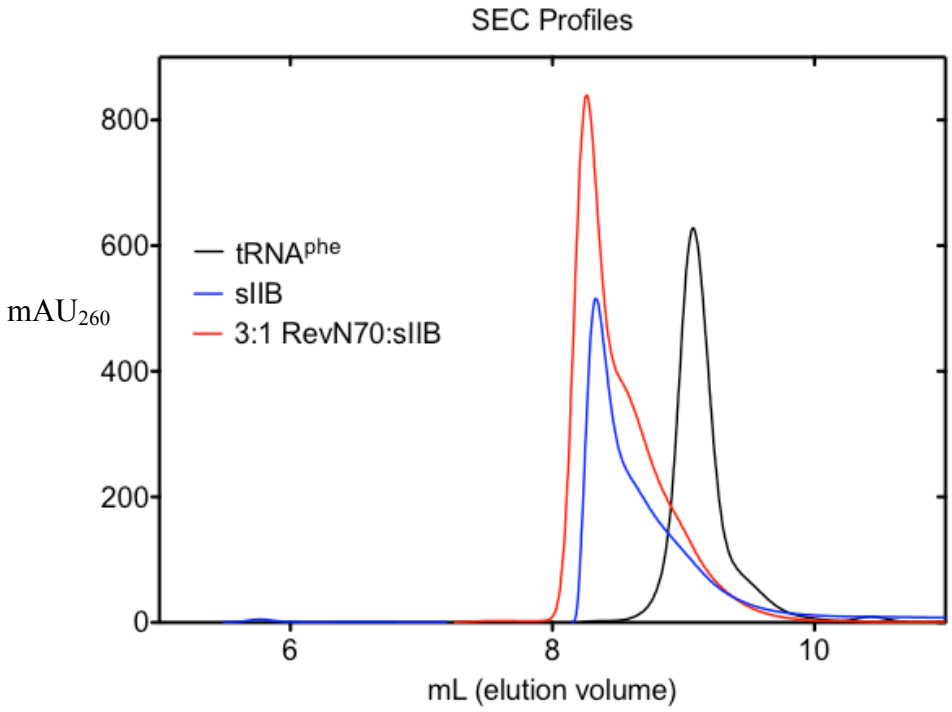



**Fig 4.4.** SEC Elution RevN70 complexed sIIB-tRNA$^{Phe}$, [RNA] = 5 µM. Removal of C-terminal residues (71-116) disrupts the assembly of Rev onto RRE RNA as evidenced by the elution of the RevN70:sIIB-tRNA$^{Phe}$ complex (red) at 8.13 mL. The sIIB-tRNA$^{Phe}$ monomer elutes at 8.23 mL (blue) with tRNAPhe eluting at 9.1 mL (black).

**Rev is complexed with RRE RNA**

In order to confirm the stoichiometry of the Rev:sIIB-tRNA$^{Phe}$ complex contained Rev the protein was labeled with a 5-carboxyfluorescein containing peptide using Native Chemical Ligation, RevL (See Chapter 3). Mass spectrometry was performed on RevL. It was determined that the protein was singly labeled at the C-terminal end. RevL-sIIB-tRNA$^{Phe}$ complexes were mixed at 1:1, 2:1, and 4:1 molar ratios and resolved on the Shodex column with UV detection at 260 nm, 280 nm, and 495 nm. In Fig 4.5, only the $A_{260}$ is shown. The elution peak at 6.9 mL was the only peak which contained absorbance at 495 nm indicating the presence of Rev. The $OD_{495}$ nm/$OD_{260}$ nm ratios across the 6.9 mL peak at Rev:RNA ratios of 2:1 and 3:1 indicates a stoichiometry of ~4:1 Rev:RNA (Fig 4.5). The molar ratios were calculated using the formula given below:

5-FAM : $\varepsilon_{260}$ 20,900 $M^{-1}cm^{-1}$ $\varepsilon_{495}$ 75,000 $M^{-1}$ $cm^{-1}$

Calculated Extinction Coeff of all aromatics in RevL at $A_{260}$: 5,418 $M^{-1}$ $cm^{-1}$
Extinction Coeff from 5-FAM and aromatics = 26,500 M-1 cm-1

Formula:

$C_L = A_{495}/\varepsilon_{495}$ $\varepsilon L_{495}$=75,000 $M^{-1}cm^{-1}$

$C_{RNA} = [(A_{260}-\varepsilon L_{260}*C_L)/\varepsilon_{260}RNA$ $\varepsilon_{260}$ RevL= 26,500 $M^{-1}$ $cm^{-1}$
$\varepsilon_{260}$ RNA= 840,000 $M^{-1}$ $cm^{-1}$

$$C_L/C_{RNA} = \frac{A_{495}/\varepsilon_{495}}{[(A_{260}-\varepsilon_{260}*CL)/\varepsilon_{260RNA}}$$

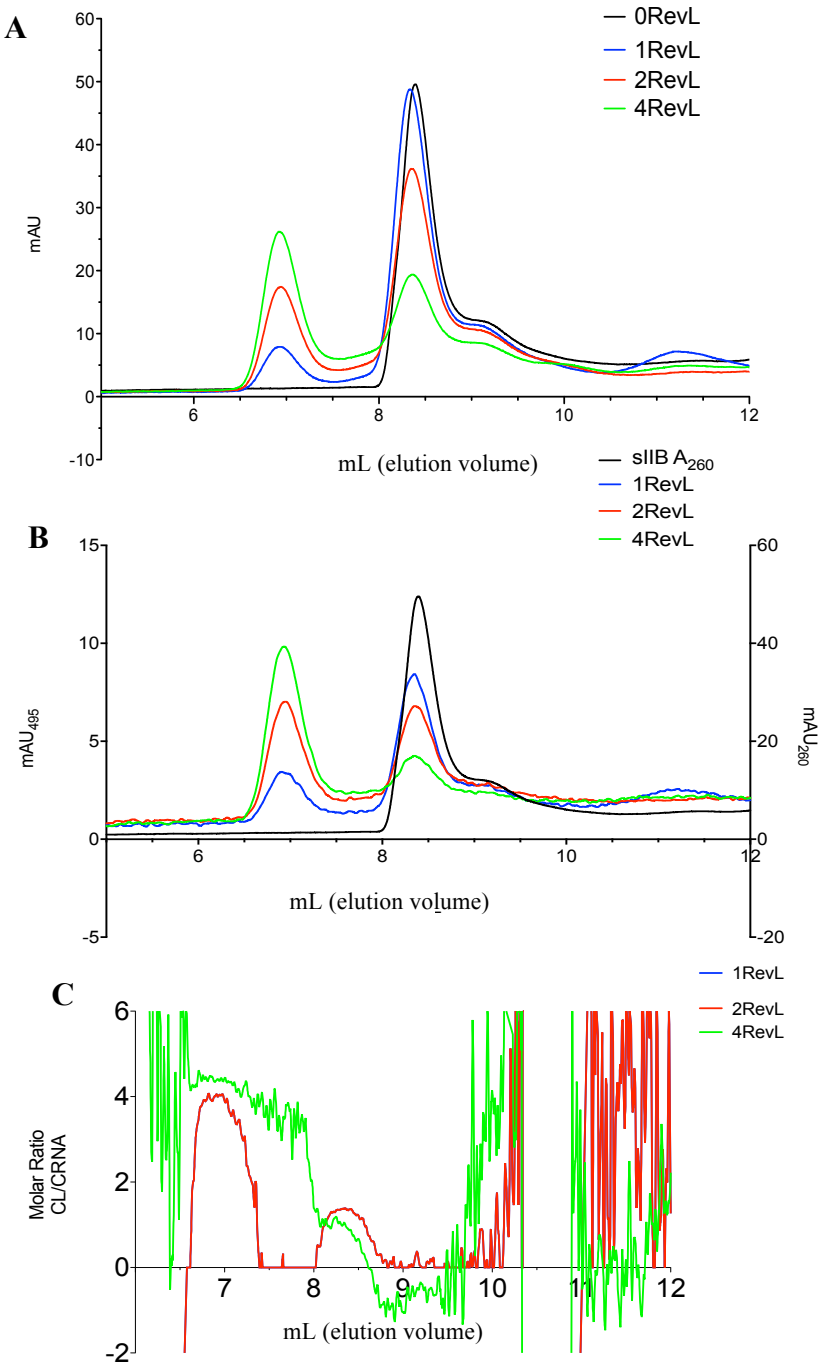



**Fig 4.5. (A)** SEC Elution profiles of RevL:sIIB-tRNA$^{Phe}$ showing 260 nm absorbance of complexes, [RNA] = 0.5 µM. **(B)** As evidenced by UV absorbance at 495 nm, all of the fluorescein-labeled Rev is contained in the Rev:sIIB-tRNA$^{Phe}$ complex observed at 6.9 mL. The sIIB-tRNA$^{Phe}$ monomer elutes at 8.23 mL (black), the right axis gives $A_{260}$. **(C)** Calculated molar ratio shows a stoichiometry of 4:1 in the 6.9 mL complex peak, while the monomer peak shows a 1:1 ratio

**Atomic model of Rev:RRE RNA dimer matches experimental hydrodynamic data**

The sedimentation properties and behavior of macromolecular assemblies on size exclusion chromatography can be particularly informative about shape when the particles deviate strongly from spherical geometry. Particles with $f/f_0 = 1.2$ deviate only modestly from spherical geometry, and so they sediment or elute from SEC columns much like a sphere of the same density that has been hydrated with 0.3 g of water per g of macromolecule. However, particles that deviate considerably from spherical geometry give large measured (and calculated) frictional ratios and elute from SEC columns much earlier than a sphere of the same volume. Given that the sIIB-tRNA$^{Phe}$ monomer is itself highly oblong, we reasoned that its complex with Rev might also be quite non-spherical, and that this could explain the SEC elution volume for the Rev:sIIB-tRNA$^{Phe}$ complex (6.98 ml, equivalent to an RNA sphere of 640 kDa). No experimental structure for the Rev:RRE is available, so we developed models for Rev:sIIB-tRNA$^{Phe}$ by superposing our model for the sIIB-tRNA$^{Phe}$ monomer onto crystal structures of Rev dimers.

We developed two models for the Rev-mediated dimer of sIIB-tRNA$^{Phe}$ (Tables 4.2 and 4.3). One model used the crystal structure of the Rev dimer (PDB ID 2X7L) with each monomer interacting via the A:A (Rev:Rev) interface (DiMattia et al. 2010) (Fig 4.6 and 4.7). The second model utilized the Rev dimer structure (PDB ID 3LPH) where the monomers interact via the B:B (Rev:Rev) interface (Fig 4.8 and 4.9). The model for sIIB-tRNA$^{Phe}$ was built using the NMR structure of an RRE fragment bound to the ARM peptide from Rev (PDB 1ETF), so we superposed one copy of sIIB-tRNA$^{Phe}$ onto each Rev monomer in a dimer by superimposing the ARM peptide Cα atoms. The spacing between the RNA monomers in the two models is very different due to the relative

positions of the ARM regions in the A:A and B:B dimers. As calculated by Hydropro, the elution volume of the sIIB-tRNA$^{Phe}$ dimer model (Rev$_2$:sIIB-tRNA$^{Phe}{}_2$, Table 4.1) using the Rev dimer from DiMattia *et al.* (PDB ID 2X7L) is 7.09 ml. This value is in close agreement with the experimentally measured elution volume of the Rev:sIIB-tRNA complex at 6.98 mL (Table 4.2). The sIIB-tRNA$^{Phe}$ dimer model constructed using the Rev dimer associated by the B:B interface elutes at 7.30 mL as calculated by Hydropro (Fig 4.7 and 4.8) (Table 4.1). This is in contrast to the experimentally determined position of 6.9 ml. The disparity between the two models indicates that it is likely that the initial dimer binding to stem loop-IIB on RRE RNA is dictated by the residues interacting by the A:A interface, and the subsequent monomer binds via the B:B interface.

The calculated sedimentation coefficient for the Rev$_2$:sIIB-tRNA$^{Phe}{}_2$ (A:A) model was 7.09S, and the calculated S-value of the Rev$_2$-sIIB$_2$ (B:B) was 7.68S (Table 4.2). The experimentally measured value for the complex was 9.8S (Table 4.3). This disparity in sedimentation rates between calculated data from the models and experimental data could be explained by the fact that the stoichiometry of the Rev:sIIB-tRNA$^{Phe}$ remains unknown and the partial specific volume ($\bar{\upsilon}$) would be dependent on the number of Rev's mediating the RNA dimer. Small changes in the partial specific volume of the Rev:RNA complex would represent large changes on the S-value.

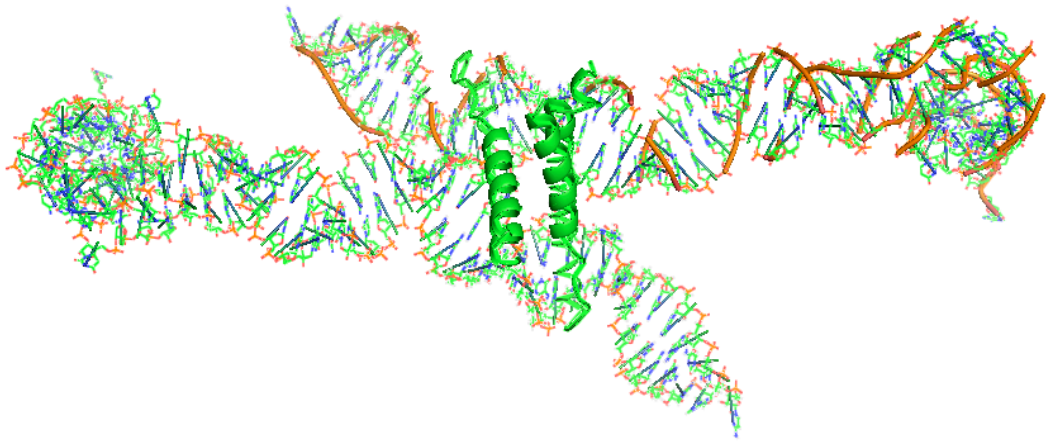


**Fig 4.6 Model of a $Rev_2$: sIIB-$tRNA^{Phe}_2$.** The Rev-mediated dimer model of sIIB-$tRNA^{Phe}$ using crystal structure of the A:A Rev dimer interface (PDB ID 2X7L)

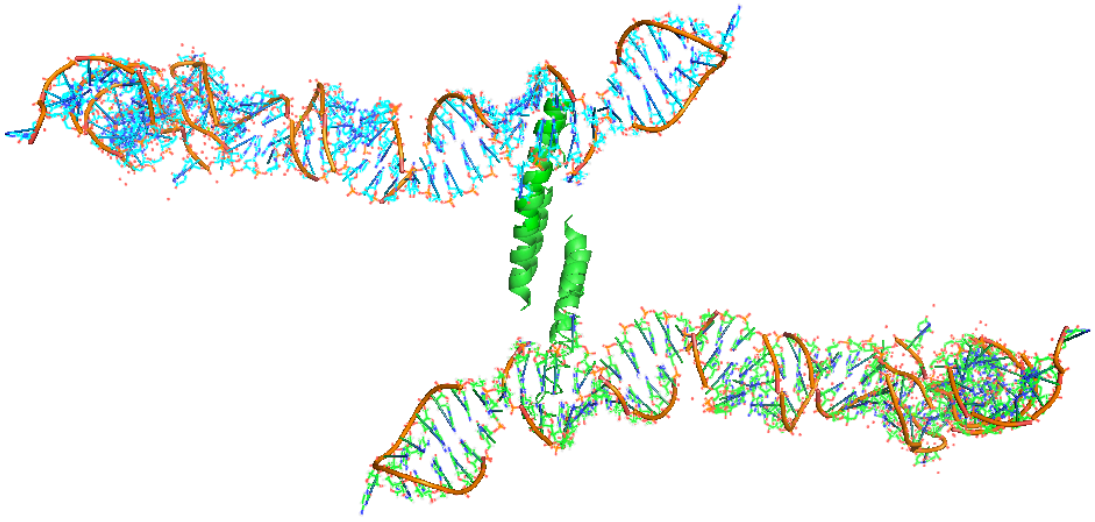


**Fig 4.7. Model of a $Rev_2$: sIIB-$tRNA^{Phe}_2$.** The Rev-mediated dimer model of sIIB-$tRNA^{Phe}$ using crystal structure of the A:A Rev dimer interface (PDB ID 2X7L) rotated to show the spacing between the sIIB-$tRNA^{Phe}$ monomers.

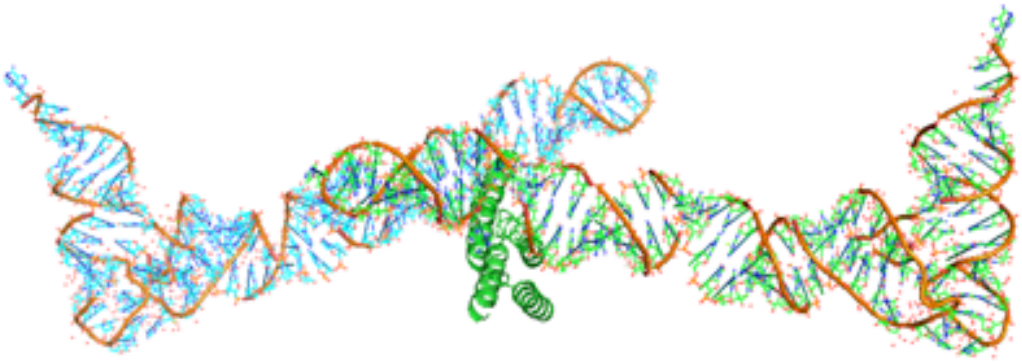


**Fig 4.8. Model of a $Rev_2$: sIIB-$tRNA^{Phe}_2$.** The Rev-mediated dimer model of sIIB-$tRNA^{Phe}$ using crystal structure of the B:B Rev dimer interface (PDB ID 3LPH) rotated to show the clashes between the sIIB-$tRNA^{Phe}$ Monomers.

| sIIB-$tRNA^{Phe}$ | Elution (ml) | $MW_{app}$ (kDa) | sed (S) | $R_g$ (Å) |
|---|---|---|---|---|
| **Model** | **8.36** | 174 | **4.77** | 43 ±1 |
| **Experiment** | **8.28** | 161 | **4.8** | 42.2±0.90 |

**TABLE 4.1** Comparison of experimental and calculated (model) hydrodynamic

| **Model** | **#** | **MW (kDa)** | **Elution (ml)** | **$MW_{app}$ (kDa)** | **sed (S)** | **$R_g$ (Å)** |
|---|---|---|---|---|---|---|
| $tRNA^{Phe}$ | | 25 | 9.3 | 64 | 3.90 | 23.9 |
| sIIB-$tRNA^{Phe}$ | 1 | 40 | 8.28 | 174 | 4.77 | 42.2 |
| $Rev_1$-$sIIB_1$ | 2 | 53 | 8.12 | 204 | 5.04 | 42.5 |
| $Rev_2$-$sIIB_1$ | 3 | 66 | 8.06 | 217 | 5.70 | 42.0 |
| **$Rev_2$-$sIIB_2$ (A:A)** | **4** | **107** | **7.05** | **600** | **7.09** | **62.1** |
| $Rev_2$-$sIIB_2$ (B:B) | 5 | 107 | 7.30 | 463 | 7.68 | 53.7 |
| $Rev_2$-$sIIB_4$ | 6 | 137 | 7.04 | 600 | 8.21 | 59.4 |

**Table 4.2** Measured hydrodynamic properties of tRNA, sIIB-$tRNA^{Phe}$, and its complexes with Rev and RevN70

| **Species** | **MW (kDa)** | **Elution (ml)** | **$MW_{App}$ (kDa)** | **sed (S)** | **$R_g$ (Å)** |
|---|---|---|---|---|---|
| $tRNA^{Phe}$ | 25 | 9.09 | 78 | n.d. | 24.3 |
| sIIB-$tRNA^{Phe}$ | 40 | 8.36 | 161 | 4.8 | 43±1 |
| sIIB + Rev | ?,a | 6.98 | 640 | 9.8 | _ |
| | ?,a | 8.23 | 183 | n.d. | n.d. |
| sIIB + RevN70 | ?,b | 8.23 | 183 | n.d. | n.d. |
| ? unknown but (a) Rev = 13kDa, (b) RevN70 = 8.5 kDa | | | | | |

**TABLE 4.3.** Calculated hydrodynamic properties of models for sIIB-$tRNA^{Phe}$ with Rev

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# Chapter 5: Chemical Crosslinking of Rev

### 5.1 RevN70 cysteine mutant selection

Rev's propensity to aggregate and form filaments in vitro, and its dynamic conformational nature (Wingfield et al. 1991) have been well established. The crystal structures of Rev (DiMattia et al., 2010; Daugherty et al., 2010a) have provided information on how Rev dimerizes through hydrophobic surfaces in the N-terminal domain. A crystal structure of the RevN70 bound to an RRE fragment was determined (Jayaraman et al. 2014); this structure showed that the quaternary structure of the RevN70 with RNA bound altered dramatically, with the ARM regions coming closer in complex with a single RNA helix. There is a disparity in the conformations observed in these structures, especially the variable crossing angles at the dimer interface.

The variability of the crossing angles at the dyad axis in all the crystallographically observed dimers and taking into consideration that two monomers of a short RNA were successfully superposed on to the two ARMs of the $F_{ab}$-Rev dimer, we hypothesized, is there a preference of the Rev interface (A:A vs B:B) for binding two monomers of the RNA? To address this question, we decided to install a covalent crosslinker that would make "obligate" Rev dimers while being consistent with the geometry and conformation as observed in the crystallized dimers.

### 5.1.1 Rationale and Design

The goal was to find site(s) in the Rev dimers that would be amenable to mutation to a cysteine for crosslinking. Finding such a site was dependent on several factors:

a) The need to preserve the geometry and conformation of the dimers; b) The distance between the residues close enough to be crosslinked by a short crosslinker; c) Importantly, the chosen site(s) should preferably make only one kind of Rev homodimer while being inconsistent with the other dimer forms i.e. crossing angle of 140° (A:A) vs. 120°(B:B) vs. 50°(B:B) as observed in $F_{ab}$-Rev, RevN70 mutant, and RevN70 mutant bound to RNA respectively.

Also, we didn't want to mutate any of the residues that are required for the various functions of Rev such as dimerization, oligomerization, and RNA binding. We were left with only few residues in the Rev NTD to accomplish our aim to crosslink after addressing all the above concerns. We looked at residues in all the Rev dimers and calculated the distances between the Cα of monomer 1 to Cα of monomer 2 such that it is 8Å across.

### 5.1.2 Residue Selection for Cysteine Mutation

**A:A/140° Rev homodimer**

The crossing angle at the dyad axis observed in the B:B structure is 140°. In this structure, the oligomerization domain and the CTD of Rev were occluded by the interactions with $F_{ab}$ (DiMattia et al. 2010). Two monomers of RNA can be superposed on the two ARMs of this Rev dimer without any steric hindrance. When we looked at the residues that satisfy our set conditions with a relative distance of ~8Å, only Glu57 in the $F_{ab}$-Rev dimer with a $C\alpha_{57}$-$C\alpha_{57}$ of 8.8Å was found to be the right candidate for mutation. Glu57 in mutant RevN70 (B:B) dimer and Rev-RNA (B:B) structure were 21.3Å and 21.2Å apart respectively. So, it is possible upon crosslinking at this site, the conformation and geometry observed in the $F_{ab}$-Rev will be preserved. Thus, Glu57 was mutated to a cysteine by site-directed mutagenesis and expressed in *E.coli* BL21 (DE3) cells.

**B:B/120°, RevN70 homodimer**

RevN70, B:B, dimer is the crystal structure with a truncated CTD and mutation of the higher order residues *viz* Leu12 and Leu60 (Matthew D Daugherty, Liu, and Frankel 2010) . It presented a 120° crossing angle at the dyad axis. To achieve the geometry and conformation of the RevN70 dimer, we identified 4 residues that fulfill our conditions. These are Gln51, Lys14, Ser54, and Arg17. Out of these four residues, Arg17 is a promising candidate with 8.5Å distance between the two monomers. Arg17 in $F_{ab}$-Rev is 14.3Å and 18.4Å in RevN70-RNA structure. Arg17 was mutated to cysteine by site-directed mutagenesis.

**B:B/50°/ RevN70 homodimer bound to RNA**

RevN70-RNA (B:B) dimer is Rev as seen in the mutant RevN70 dimer structure along with a Glu57 to Ala57 mutation to reduce surface entropy. Complexes of a short RRE fragment and RevN70 were crystallized(Jayaraman et al. 2014). The resulting structure showed a marked difference in the geometry when bound to the RNA. There was a drastic shift in the crossing angle at the dyad axis to 50° and interestingly Gln51 in the dimer shifted much closer to 4.8Å vs. 8.2Å in the RevN70 dimer vs. 27.4Å in the $F_{ab}$-Rev structure. The Cα-Cα distances between RevN70 dimer and RevN70 bound to RRE fragment at Gln51 are quite close and we anticipate conformational heterogeneity after crosslinking. However, this is the only site that fit our needs and therefore we mutated Gln51 to Cys and expressed the mutant in *E.coli* BL21 (DE3).

| Xtal Structure | Glu57 Å | Gln51 Å | Lys14 Å | Ser54 Å | Arg17 Å | Angle at the dyad axis | A:A or B:B | PDB ID | WT/Mutant | Residues in Xtal Structure |
|---|---|---|---|---|---|---|---|---|---|---|
| RevN70 | 21.3 | 4.8 | 9.9 | 10.7 | 8.5 | Obtuse, 120° | A:A | 3LPH | Mutant*, RevN70 | 9-70 |
| Rev-RRE | 21.2 | 8.2 | 12.2 | 15.8 | 18.4 | Acute, 50° | A:A | 4PMI | Mutant*, RevN70 | 9-70 |
| Fab-Rev | 8.8 | 27.4 | 16.2 | 17 | 14.3 | Obtuse, 140° | B:B | 2X7L | WT | 9-65 ordered |

**Table 5.1 Rev Homodimer selection.** The relative Cα-Cα distances as seen in the crystallzied Rev dimers. * denotes mutations L12S, L60A residues responsible for the formation of higher ordered structures.

## 5.2 Chemistry for Crosslinking

### 5.2.1 Crosslinking using Thiol-ene Chemistry

We looked at alternative approaches to crosslinking and found thiol-ene chemistry to be a good route to crosslink two monomers, being specific to cysteine residues chemically. Thiol-ene chemistry has been applied successfully in polymer chemistry (Hoyle, Lee, and Roper 2004), dendrimer formation (Killops, Campos, and Hawker 2008), hydrogel (Cushing and Anseth 2007), and thin films but had not been used in the production of protein homodimers. In biological macromolecules, this chemistry has been applied to protein glycoconjugation (Dondoni et al. 2009), peptide and protein glycosylation (Lin, Chalker, and Davis 2009), and protein tagging (Floyd et al. 2009). Thiol-ene coupling (TEC) is mediated by UV-light in the presence of a photoinitiator that can activate a sulfhydryl group to a thiol radical and has proven to be regioselective with high yield. This method is compatible with oxygen and water conditions and orthogonal to a variety of functional groups.

A typical TEC involves a free sulfhydryl group which can be activated in the presence of a radical initiator e.g. AIBN or photointitiators e.g. Vazo-44, DPAP and UV. Some of the reactions are also induced thermally. The coupling occurs by a radical mechanism in anti-Markovnikov regioselective fashion.

Dondoni et al.,(2009) in their efforts to glycosylate BSA using TEC identified and optimized conditions, which led to a maximum yield of glycoconjugated BSA. The conditions are as follows: 80 mol% DPAP, hυ 365 nm, 20mM phosphate buffer pH 7.4 irradiating for 5 min under argon. The allyl galactoside used to conjugate to BSA was 30-fold mole excess to the BSA.

Floyd et al., (2009) followed a similar approach where they used Vazo-44, a water-soluble radical initiator, under a variety of pH conditions for site-specific ligation of glycosyl thiols to proteins. They also incorporated a non-natural amino acid (L-homoallylglycine) into a protein, which also functioned as a tag. They found that 0.2 eq of Vazo-44 under acidic conditions (pH 4-6) and UV gave the most efficient ligation.

### 5.2.2 Crosslinking using Diiodoacetamide Crosslinkers

Cysteine residues selectively react with electrophiles such as Michael acceptors e.g. maleimides and α-halocarbonyls e.g. iodoacetamide. Alkylation of cysteine residues is a common method used biochemically to cap the thiol groups before digestion for protein sequencing; iodoacetamide (IAM) is routinely used for this purpose. IAM typically forms a covalent adduct with sulfhydryl group by nucleophilic substitution ($SN_2$). The reaction of IAM to thiols is more specific than maleimides, which at a basic pH or when used in large excess can modify other residues such as lysine and histidine(Hill et al. 2009). Iodoacetamide is more electrophilic than alkyl iodides due to the presence of the carbonyl

group. The reaction proceeds with the nucleophilic attack of iodine by the sulfur atom of the sulfhydryl group forming a stable thioether linkage. This reaction is irreversible and selective and thus makes an ideal candidate to consider as a crosslinker.

After careful consideration, taking into account the Cα distances between the residues at the dimer interface and the flexibility of the linker candidates we zeroed on two diiodoacetamide linkers:

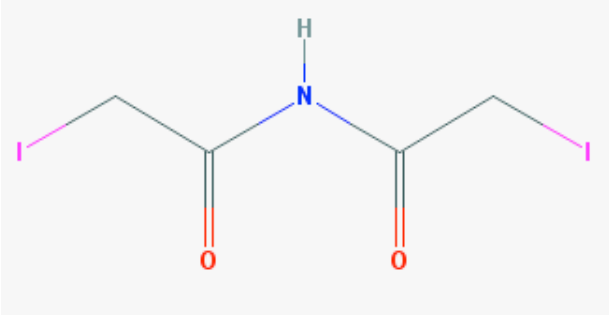



**Fig 5.1** N,N'-Bis(iodoacetamide)

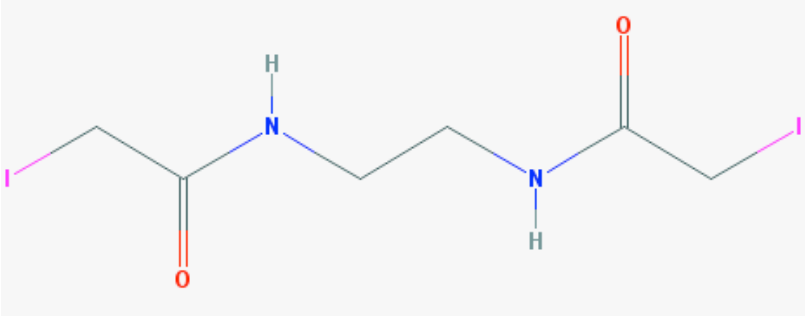



**Fig 5.2** N,N'-Ethylenebis(iodoacetamide)

N,N’-bis(iodoacetamide) is an ideal crosslinking because of the length (8.23 Å) and it is conformationally constrained. There, however, were a few drawbacks with these compounds. These are expensive to purchase, 100 mg of N,N’-bis(iodoacetamide) costs $ 1200 and the published synthesis involves using nitrogen triiodide, which is explosive at the slightest touch(Fellman et al. 1956). Also, these compounds are similar to mustard gas (Fig.5.3), which may make it sufficiently reactive toward amine, amino groups. N,N’-Ethylenebis(iodoacetamide) is available commercially at $300 for 100 mg (Santa Cruz Biotechnology). This compound measures 12.13 Å, a little over our targeted 8Å distance.

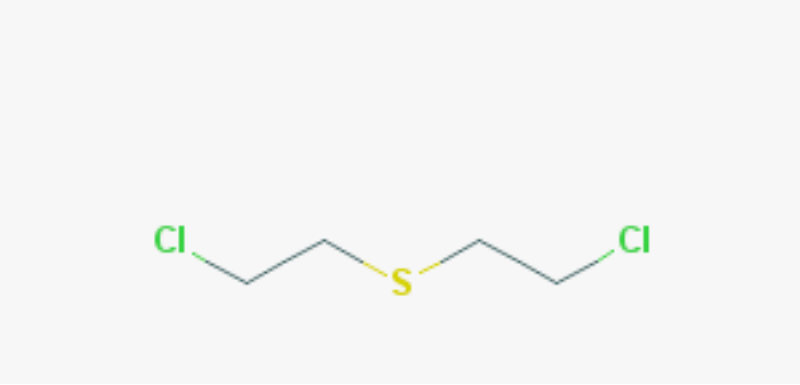



**Fig.5.3 Mustard Gas**

**5.3 General route for crosslinking using Thiol-ene chemistry:**

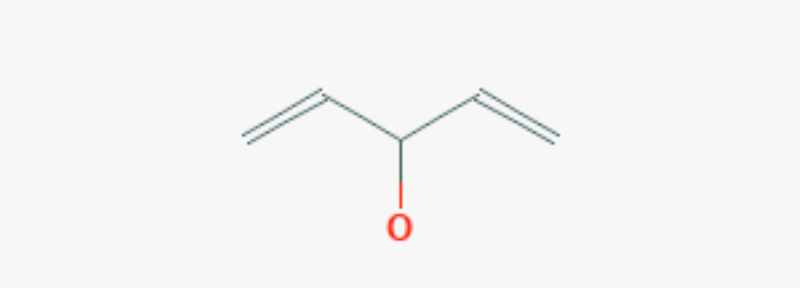
We took a solid phase synthetic approach to prepare the RevN70 dimers to avoid protein aggregation problems presented by Rev. The immobilization and the subsequent labeling of Rev wt on cOmplete™ resin yielded much success (See Chapter 3). This approach seemed to be the best route to pursue the chemical crosslinking of RevN70 for many reasons. The aggregation problem of RevN70 is curbed to an extent by immobilizing Rev. We desired 6xHis-RevN70 and 6xHis-cleaved crosslinked dimers. Therefore, we decided on the immobilization of RevN70 and treated the immobilized RevN70 in the presence of an excess of the crosslinker followed by the treatment with His-cleaved RevN70 to achieve RevN70 crosslinked dimers.

Vazo-44 (2,2'-Azobis2-(2-imidazolin-2-yl) propanedihydrochloride) is a water-soluble, non-nitrile initiator and has been used to radicalize thiol groups in polymer chemistry. For our purposes, we needed an initiator, which is relatively miscible in buffers containing GdmHCl or urea, and Vazo-44 was chosen over DPAP (2,2-Dimethoxy-2-phenylacetophenone). The solubility of DPAP is fairly low at around 200 mg/L in water.

### 5.3.1 Selection of crosslinker candidates

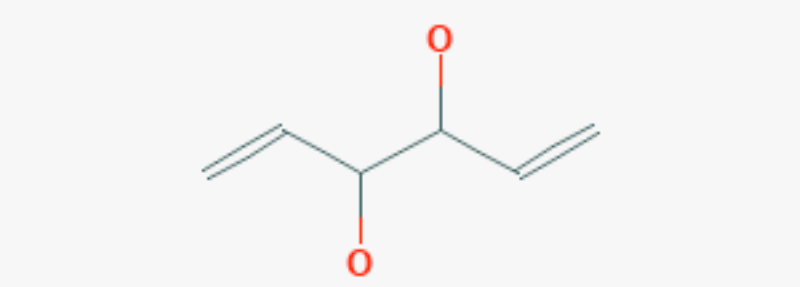
We looked for compounds that are applicable in our goal to achieve crosslinking across two monomers. Divinyl compounds were well suited for this purpose. Solubility of such compounds is an issue that we had to address. In selecting the ideal compounds, we had to make sure that the compound is fairly soluble in aqueous medium. As our protocol involves using the crosslinkers in a huge mole-excess in order to achieve ample modification at the radicalized thiol, the cost of such crosslinkers is also a factor. Since, the sites for cysteine mutation we have selected are between 4.8Å to 8.8Å, we decided on the following compounds for crosslinking via thiol-ene chemistry.

A) 1,4-pentadiene-3-ol



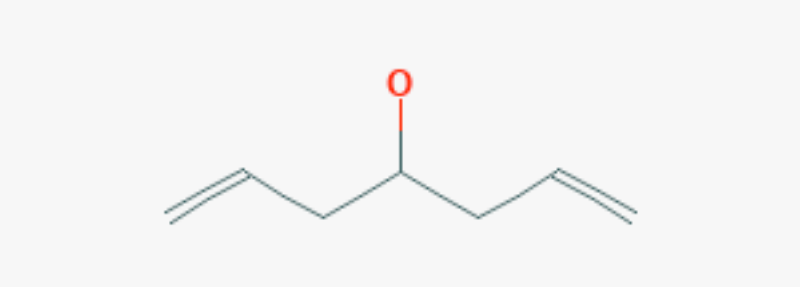

Solubility in water: 60 g/L

B) 1,5-hexadiene-3,4,-diol




Solubility in water: 50 g/L

C) 1,6-heptadiene-4-ol




Solubility in water: 7 g/L

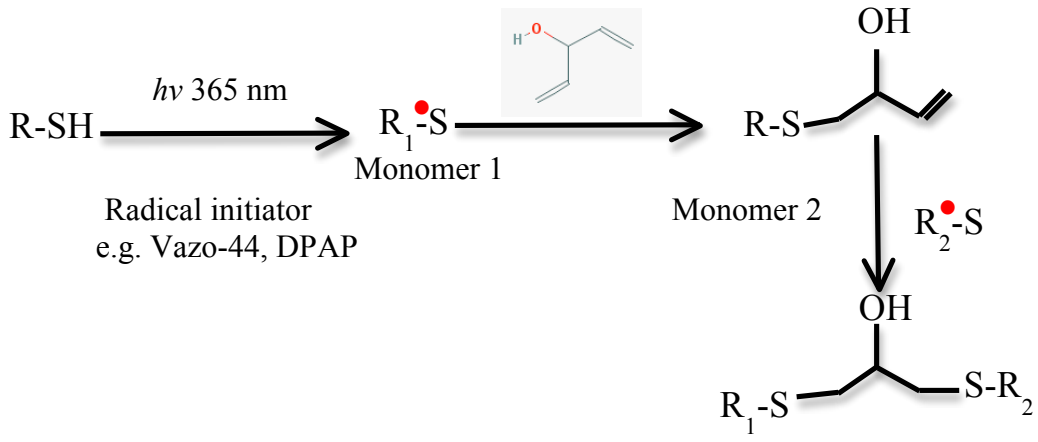



**Fig 5.4. General Scheme for thiol-ene crosslinking**

### 5.3.2 Crosslinking protocol

RevN70 was immobilized on cOmplete Resin™ via the N-terminal His-Tag. The immobilized protein was washed with a buffer containing 5M GdmHCl, 200 mM NaPi, 1 mM TCEP, pH 6 to exchange the buffer. Vazo-44 (0.2 eq) and 1,4-pentadiene-3-ol (30-fold mole excess) was added and irradiated under UV 365 nm for 1 h. 10 uL of the sample was set aside for MS analysis to confirm the presence of the crosslinker. The resin was then washed extensively to get rid of the unreacted crosslinker and a 2-fold mole excess of His-tag cleaved RevN70 was added in the presence of the photoinitiator (Vazo-44) and irradiated for 5 h with intermittent shaking. At the end of 5 h, the flow-through, which contains the unreacted His-tag cleaved RevN70 was collected. The buffer was exchanged to a buffer containing 5M Gdm HCl, 8M Urea, 40 mM Tris, pH 7.4. The column was then washed in a buffer containing 8M urea. The bound protein, which contains the crosslinked dimers, was eluted in a buffer containing 8M urea and 1M imidazole, 10 mM NaCl, 40 mM Tris, pH 7.4.

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# Conclusions and Future Directions

We have successfully installed a fluorescent probe at the C-terminal end of Rev and demonstrated the binding of RevL with chimeric sIIB- $tRNA^{Phe}$ RNA molecule. Based on the size-exclusion chromatography data and analytical ultracentrifugation data we hypothesized that the Rev: sIIB-$tRNA^{Phe}$ complex may contain two monomers of sIIB-$tRNA^{Phe}$ mediated by Rev. This enabled us to determine the number of Rev molecules that are present in the complex which eluted at 6.98 mL on the SEC using Shodex-803. In the context of sIIB-$tRNA^{Phe}$, we determined the stoichiometry to be 4:1 RevL: sIIB-$tRNA^{Phe}$ in the discrete complex seen in the SEC data.

Rev dimer structure with Rev monomers interacting via the A:A interface with ARM region protruding from each monomer in a prong-like manner at a 140º angle from each other (DiMattia et al. 2010). Two monomers of sIIB were successfully docked on to the ARM regions of the two Rev monomers without any steric hindrance. Also, the elution volume as calculated by Hydropro using this model came to be 7.09 mL, which is in close agreement with the experimentally obtained value of 6.98 mL.

We intend to perform similar stoichiometry determination studies on $tRNA^{Phe}$-T2 and the full-length $tRNA^{Phe}$-RRE using RevL. Recent SAXS structural studies on the RRE suggest that the RRE (240 bases) adopts an A-shape (Fang et al., 2015). The $tRNA^{Phe}$-T2 RNA contains a 273 base insert consisting of bases 45-311 of the RRE. This RRE truncation contains the natural hairpin regions of the RRE and the IIB Rev tight binding site. $tRNA^{Phe}$-T2 is as long as the length of RRE used in the SAXS studies, this molecule

will allow us to explore the bridging model of Rev assembly as proposed by Fang et al (2015). We aim to cleave the $tRNA^{Phe}$ scaffold using RNase H technology and study the interactions of Rev and T2 in the presence and absence of the $tRNA^{Phe}$ scaffold. This also allows us to further validate the interference from the tRNA scaffold, if any.

Studies done on the full length RRE indicated that the long Stem I of the RRE makes tertiary interactions folding back towards the core of the 3-way junction of the RRE making the overall fold more compact (Bai et al., 2015). Does Rev mediate a dimer of the full-length RRE? Or does it bridge between RNA helix legs of the A-shape structure? Full length RRE-$tRNA^{Phe}$ will be purified using the selective PEG precipitation that yielded great results with T2-$tRNA^{Phe}$ RNA. RNase H will be used to cleave the tRNA scaffold from the RRE and complexes using RevL will provide us a more reliable stoichiometry between Rev and the RRE.

Our lab has developed a cleavage reagent (EPD-$Fe^{2+}$) that can be attached to a protein via a disulfide linkage. When the modified protein is treated with ascorbate in the presence of oxygen or hydrogen peroxide, reactive oxygen species are formed that can cleave the protein or the nucleic acid if in complex with the protein. We have demonstrated the effectiveness of this reagent and successfully used this technique to cleave proteins, protein-DNA complexes and protein-RNA complexes.

In the light of the published crystal structures of Rev dimers and the co-crystal of Rev bound to a fragment of the RRE, we hope to validate the correctness of these crystal

structures by employing EPD-Fe2+ to sites on RevN70. The cleavage reagent has a footprinting radius of about 15Å, therefore by simulating the cleavage of RRE bound to Rev and comparing with the data obtained by experimental methods can provide us with information about the extent of Rev binding to the RRE.

In conclusion, we have shown that the stoichiometry of Rev in context of the sIIB-$tRNA^{Phe}$ to be 4:1. The obligate RevN70 crosslinked dimers can further provide us whether it is the A:A or B:B interactions of Rev that help in the dimerization of RNA. With the chemically modified Rev and the future studies proposed one can hope to better fully understand the dynamic nature of Rev and its interactions with the RRE. Most importantly, we may be able to obtain a high-resolution structure of a biologically relevant Rev:RRE complex from all the information one can garner from the proposed studies.