Interactions of Eukaryotic Translation Initiation Factors and 3' Untranslated Region of Barley Yellow Dwarf Virus Mrna During Protein Synthesis: A Study of Equilibrium Binding, Kinetics and Thermodynamics

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INTERACTIONS OF EUKARYOTIC TRANSLATION INITIATION FACTORS AND 3’ UNTRANSLATED REGION OF BARLEY YELLOW DWARF VIRUS mRNA DURING PROTEIN SYNTHESIS: A STUDY OF EQUILIBRIUM BINDING, KINETICS AND THERMODYNAMICS

by

Bidisha Banerjee

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Interactions of eukaryotic translation initiation factors and 3’ untranslated region of barley yellow dwarf virus mRNA during protein synthesis: a study of equilibrium binding, kinetics and thermodynamics

by

Bidisha Banerjee

Adviser: Professor Dixie J. Goss

Abstract

Eukaryotic initiation factor (eIF) 4F binding to mRNA is the first committed step in cap-dependent protein synthesis. Barley Yellow Dwarf Virus (BYDV) employs a cap-independent mechanism of translation initiation which is mediated by a structural element BTE (BYDV translation element) located in the 3’ UTR of its mRNA. eIF4F bound the BTE and a translational inactive mutant with high affinity; thus questioning the role of eIF4F in translation of BYDV. To examine the effects of eIF4F in BYDV translation initiation, BTE mutants with widely different in vitro translation efficiencies ranging from 5-164% compared to WT were studied. Using fluorescence anisotropy to obtain quantitative data, we show 1) the equilibrium binding affinity (complex stability) correlated well with translation efficiency, whereas the “on” rate of binding did not. 2) other unidentified proteins or small molecules in wheat germ extract (WGE) prevented eIF4F binding to mutant BTE but not WT BTE. 3) BTE mutants-eIF4F interactions were found to be both enthalpically and entropically favorable with an enthalpic contribution of 52-90% to ΔG° at 25°C suggesting hydrogen bonding contributes to stability and 4) in contrast to cap-dependent and tobacco etch virus (TEV) Internal Ribosome Entry Site (IRES) interaction with eIF4F, PABP did not increase eIF4F binding. Further, the eIF4F bound to the 3’ BTE with higher affinity than for either m7G cap or TEV IRES, suggesting that the 3’
BTE may play a role in sequestering host cell initiation factors and possibly regulating the switch from replication to translation.

In another project, we studied the interaction of a deletion mutant of wheat eukaryotic initiation factor 4B (eIF4B\textsubscript{320-527}) with zinc using the biophysical technique of circular dichroism. eIF4B is suspected to be a metalloprotein and it is known that zinc stimulates eIF4B self-association at physiological concentrations. It was found that in the presence of zinc there is significant change in the secondary structure of eIF4B\textsubscript{320-527}. There was approximately a 70% change in the presence of 500 \(\mu\)M zinc and around 38% change in the presence of 500 \(\mu\)M magnesium in alpha content as compared to native protein. There was a change observed in beta sheet content. The changes in secondary structure caused by zinc may be the one of the causes for the eIF4B self-association or enhanced eIF4B-PABP interaction. These results enhance our understanding of the molecular mechanisms by which cell controls translation initiation which is the rate limiting step of cellular protein synthesis.
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CHAPTER 1

1 INTRODUCTION

1.1 BARLEY YELLOW DWARF VIRUS (BYDV)

BYDV represents pervasive groups of economically important plant viruses. BYDV affects cereal crops such as wheat, barley, oats and grasses and is spread by several aphid species. BYDV can severely limit food grain production and cause yield losses resulting in food scarcity. Average yield losses from BYDV infection typically range from 11-33%. Typical symptoms of BYDV infection are yellowing and stunting in barley, and reddening, leaf stiffness in oats (Fig.1) (1). BYDV belongs to the luteoviridae family. BYDV has a genome which is a positive-sense RNA ≈ 5700 nucleotides long, that encodes six open reading frames (ORFs) (Fig.2) and is lacking both a 5’ cap (m^7GpppX) and a poly (A) tail (2). Several unconventional and remarkable methods such as cap-independent translation mechanism and RNA-based transcription methods are used by BYDV to express the six genes from a single genomic RNA (3,4).
Figure 1: A, Infection of barley by BYDV causes yellowing of older leaves and stunting of the plant*. B, BYDV infection causes characteristic reddening of flag leaf in wheat*.

**Figure 2:** Genome organization of BYDV (2). The secondary structures of long-distance interacting regions are shown. The pink colored region show the bases that pair over long distances indicated by dashed, double-headed arrows. Labeled open boxes above the RNA indicate translatable ORFs whereas black boxes indicate ORFs that are not translatable.
1.2 TRANSLATION INITIATION

Protein synthesis or translation in eukaryotic organisms can be divided into four stages: initiation, elongation, termination, and recycling. At the initiation stage, Met- tRNA$_{met}$ is bound in the peptidyl (P) site of the mRNA and assembly of the ribosome at the initiation codon takes place. At elongation stage, decoding takes place at the acceptor (A) site and the ribosome catalyzes the formation of a peptide bond when it encounters tRNAs. During the translocation stage which is an intermediary step, the tRNAs and the mRNA are moved such that the A site has the next codon and there is repetition of the process. On encountering a stop codon, the termination takes place and the finished peptide is released from the ribosome. The ribosomal subunits are dissociated and recycled in the final stage, releasing the mRNA and deacylated tRNA and the whole set up moves to another round of initiation (5). It is known that regulation of protein synthesis occurs at the initiation stage and not during elongation or termination (6).

There are two mechanisms by which translation is initiated:

1. 5’ Cap Dependent Translation
2. Cap Independent Translation

Translation initiation in eukaryotes involves participation of several initiation factors and follows an intricate multi-step process. Research in the last decade has enabled the determination of the structures and activities of various initiation factors. Interactions with ribosomal initiation complexes have been elucidated, and this has enhanced our understanding of the complex translation initiation process. These advancements have opened up new avenues for studying the regulation of translation initiation by initiation factor activity modulation and through sequence-specific RNA-binding proteins. The various initiation factors involved in wheat translation initiation are: eIF$_4$F, eIF$_4$B, eIF$_4$A, PABP, eIF$_3$, eIF$_2$, eIF(iso)$_4$F, eIF$_1$, eIF$_1$A and eIF$_5$. A brief description of some of these initiation factors used in this work are as follows:
1.2.1 EUKARYOTIC INITIATION FACTORS

1.2.1.1 EUKARYOTIC INITIATION FACTOR 4F (eIF4F)

The interaction of eIF4F with mRNA is supposedly the first committed step in the initiation of translation. In plants, eIF4F binds the m^7G cap on the 5′ end of mRNA, and consists of two subunits: eIF4E, a small cap-binding protein and eIF4G, a large scaffolding protein that interacts with other initiation factors (7). eIF4F also interacts with the poly(A) binding protein (PABP) via eIF4G, the larger subunit of eIF4F which leads to circularization of mRNA (1,8). The 5′ cap on mRNAs and the poly(A) tail work synergistically for stability of message and efficient translation(9-15). A second form of eIF4F present in plants is termed eIF(iso)4F, which is not present in other eukaryotes. eIF(iso)4F possesses similar in vitro properties as eIF4F, consists of two subunits, a smaller eIF(iso)4E and a large subunit eIF(iso)4G (16). There is approximately 50% similarity in the amino acid sequences of wheat eIF (iso)4E and eIF4E and both have a molecular mass ≈ 24 kDa. However, eIF(iso)4G and eIF4G subunits differ significantly to molecular mass ( 86 KDa and 180 kDa respectively) or the lack of N-terminal domain sequence in eIF(iso)4G (10). It is now known that the subunits of eIF4F and eIF(iso)4F are functionally interchangeable and are capable of discriminating between different mRNAs in vitro (17).

The structure of eIF4E, the smaller subunit of wheat eIF4F was revealed using x-ray crystallography and nuclear magnetic resonance (NMR) studies (18). eIF4E was crystallized in a dimeric form and studies have shown that there is an intramolecular disulfide bridge between two cysteines (Cys) that are conserved only in plants. The overall fold of eIF4E consists of three alpha-helices, eight beta-strands, and three extended loops (18) (Fig.3).

eIF4E and eIF(iso)4E subunits have been shown to play multiple roles during plant viral infection, primarily through interaction with the viral Vpg (19). Efficient translation of Picornaviruses and many other viruses require additional proteins and these viruses often use...
internal ribosome entry sites (IRES) as translation initiation sites. eIF4G is the target for certain Picornaviral proteases, where it cleaves the N-terminal region which binds eIF4E and PABP (20). Tobacco Etch Virus (TEV) (Potyviridae) mRNA is known to preferentially bind eIF4G rather than eIFiso4G (21). It is now known that BYDV mRNA interacts with eIF4G subunit of eIF4F (22). It is also known that the eIF(iso)4E knockout plants are resistant to infection by potyviruses (23). It has been shown experimentally that eIF4E and eIF(iso)4E interact directly with translation enhancer elements in the 3’- untranslated region (UTR) of satellite tobacco necrosis virus and Pea enation mosaic virus mRNA (24,25). These evidence suggest that viruses have evolved a sophisticated function (or mechanism) that allows them to manipulate the eIF4F/eIF(iso)4F cellular machinery to their benefit.
**Figure 3:** A, Backbone structure of the wheat eIF4E. The two molecules of the dimer are shown in red and blue. Tryptophan side chains (Trp-62 and Trp-108) involved in cap binding are shown in black, as well as Cys-113 and Cys-151, which form a disulfide bond, are shown in yellow (18). B, Ribbon diagram showing the structure of wheat eIF4E. The Trp of the cap-binding pocket are shown in green, the m₇GDP is shown in magenta and the disulfide bond (right) and positions of the reduced Cys (left) are shown in yellow (18).
1.2.1.2 PABP

Poly(A) binding protein (PABP), present only in eukaryotes, belongs to one of the major classes of highly conserved regulatory proteins and interacts with the 3’ poly(A) tail of mRNA (26). PABPs do not show catalytic activity of their own (26) and it was assumed traditionally that the mRNA is protected from degradation by interaction of PABP with the poly(A) tail (27). PABP binds the 3’ UTR of mRNAs during protein synthesis and performs multiple functions during translation initiation. In recent years, evidence has shown that PABP functions in export, polyadenylation, surveillance of transcripts, control of mRNA stability microRNA (miRNA) activity and viral infection (28,29). PABPs are known to play a role in miRNA-dependent regulatory pathways by controlling gene expression through translational repression and/or mRNA deadenylation and decay (26).

The structure of cytoplasmic PABP (PABPC) is highly conserved (28,30). PABPCs consist of four RNA-recognition motifs (RRMs) and a C-terminal region containing a peptide binding region referred to as PABC domain consisting of five α-helices (31,32). PABPCs interact with poly(A) via RRM s, multiple PABPCs can bind to the same poly(A) binding tract with in vitro binding affinities in the range of 2–7 nM and require a minimum of 12 adenosines for binding (26,28,33). PABPCs show specificity for poly(A), have almost undetectable binding to poly(C) and have very low affinity for poly(G) and poly(U) (34,35). Specificity is achieved while RNA binding by stacking interactions of aromatic residues on the β-sheet surface, hydrogen bonding or van der Waals contacts (33).

In the cytoplasm during translation initiation, PABP binds the mRNA poly(A) tail and stabilizes eIF4G binding to the cap (26). PABP–eIF4G interaction is one of the essential requirements for efficient recruitment of the 43S ribosome complex and translation (36). The interaction of PABP, eIF4E, and eIF4G with the cap and poly(A) tail leads to ‘circularization’ by bringing the two ends of the mRNA in close proximity. However, very little is known about the complexity of these
reactions and the synergistic effects caused by these interactions on translation when mRNAs are both capped and polyadenylated (1,37,38). In plants, it is known that the interactions between PABP and eIF4G or eIF4B increase the poly(A) binding activity of PABP by 10-fold and increase the affinity of eIF4F for the 5’ cap by 40-fold (13,26,39). Cooperative interactions between eIF4F and PABP enhance eIF4B, eIF4A and eIF(iso)4F’s RNA helicase and ATPase activities and increase the affinity of eIF4E for the 5’ cap of the mRNA by lowering the dissociation rate in plants (26). Wheat PABP reduces the activation energy for formation of the 5’ cap–eIFiso4F complex inducing a conformational change resulting in enhancement of RNA interactions (40). PABPs also interact with the PABP-interacting proteins PAIP-1 and -2 and eIF4B in wheat germ (13,41,42). It has been previously shown that by interacting with eRF3, PABP can regulate 60S subunit joining and termination in yeast (26).

To circumvent host defense mechanisms and to shut-off host cell protein synthesis, the cellular translational initiation factors have always been recognized as viral targets (43). PABP is also implicated to be one of the targets with more complex and unknown roles in viral protein synthesis and replication (44). For instance, cleavage of PABP by certain viruses results in host cell translational shut-off (29). Certain viruses employ additional or alternative strategies such as: recruitment of PABP to the eIF4F complex (e.g., human cytomegalovirus and vaccinia virus), recruitment of PABP to viral 3’UTR (e.g., dengue virus), nuclear relocation of PABP during viral infection and displacement of the PABP from the eIF4G initiation complex (e.g., rotaviruses125) (29,45). PABP also plays a role in the kinetics and stability of eIF4F binding to tobacco etch virus IRES (44,46). It is known that A. thaliana RNA-dependent RNA polymerase (RdRp) and VPg of turnip mosaic virus (TuMV) preferentially interact with class II PABPs such as PABP2, PABP4 and PABP8 (47). All the above evidence shows that PABP is a common viral target. However, very little is known about the specificity, functional role of these interactions and why these methods are advantageous to the virus. It is also unclear how PABPs are exploited by viruses, especially in plants where multiple members of the PABP family are expressed.
1.2.1.3 EUKARYOTIC INITIATION FACTOR 4B (eIF4B)

Wheat eIF4B is a 59 KDa protein that functions to enhance the ATP-dependent RNA helicase activity of eIF4A and eIF4F during translation initiation (48,49). The sequence of eIF4B is the least conserved among the initiation factors, whereas its function or perhaps its structure is highly conserved among plants, yeast and mammals (50). Mammalian eIF4B is a 80 KDa protein that binds the junction of single and double stranded RNA and also promotes ATPase activity of eIF4A and eIF4F (51-53). eIF4B interacts with translation initiation factor eIF3, which along with eIF4G is responsible for association of 43S preinitiation complex to mRNA (54,55). Subsequently, eIF4B along with eIF3 and eIF4F forms the 48S complex and aids mRNA binding to the ribosome (56,57). eIF4B is one of the targets for degradation during apoptosis(58) . The shutdown of protein synthesis of herpes simplex host mRNA is aided by eIF4B (59). eIF4B plays a role in picornaviral internal ribosome entry site-mediated translation (60-62), and eIF4B interferes with ribosome complex formation in Cauliflower mosaic virus (63). Highly phosphorylated forms of both mammalian and wheat eIF4B suggest a role in protein synthesis regulation (64-68). In plants, eIF4B shows higher binding for polypurines, although it binds polypyrimidines as well (56,69). All the above evidence emphasizes the importance of eIF4B in the translation initiation process.

Zinc stimulates homo dimerization of wheat eIF4B in vitro (50) . A region located in the C terminus of eIF4B, connecting the eIF4A and PABP interaction sites is responsible for in vitro dimerization of wheat eIF4B and RNA binding (50). Wheat eIF4B has many RNA binding domains and two tandem PABP/eIF4A binding sites in the C-terminal half of the protein (56). eIF4B interacts with PABP in plants and zinc stimulates this interaction (13,40,49,67,70,71). Specificity of PABP binding to eIF4B over eIF(iso)4G is ensured by zinc (50). The phosphorylation state of both eIF4B and PABP are important for their interaction (67) . The cap-binding affinity of eIF(iso)4F and the poly(A)-binding affinity of PABP are also enhanced by
interaction with eIF4B (13,39,70). Together eIF4B and PABP increase the ATPase and RNA helicase activity of the eIF4A/eIF4B/eIF(iso)4F complex. This suggests that PABP could stimulate mRNA scanning by the eIF4 complex and thereby increase the rate of initiation of translation (49,72). Interactions among PABP, eIF4B, and eIF(iso)4F could provide mRNAs with a competitive translational advantage (70,72).
1.2.1.4 EUKARYOTIC INITIATION FACTOR 4A (eIF4A)

Mammalian eIF4A is a single polypeptide (≈46 KDa), is associated with eIF4F complex, is the prototype DEAD/H helicase, exhibits single-strand RNA-dependent ATPase activity and, in conjunction with eIF4B has double-strand RNA helicase activity (52,53,73-75). eIF4A shares nine highly conserved regions in the DEAD box family, and is involved in multiple cellular processes, such as translation, RNA degradation, RNA splicing, and ribosome biogenesis (76). ATP binding and hydrolysis may produce a cycle of conformational changes in mammalian eIF4A. Such a cycle of conformational changes may be used by DEAD box proteins to transduce the energy derived from ATP hydrolysis into physical work (77,78).

Unlike mammalian eIF4A, wheat germ eIF4A, a 45 kDa polypeptide (not a part of eIF4F complex), exhibits an RNA-independent ATPase activity. Wheat germ eIF4A is functionally equivalent to mammalian eIF4A and can substitute for mammalian eIF4A in translation (49).

In the crystal structure of free eIF4A from yeast, RecA-like domains in its amino and carboxyl terminal are widely separated and there is a functional active site present (79). eIF4A is not a processive helicase and is thought to melt short helices in the mRNA by binding an unpaired RNA strand in its ATP-bound form. Subsequently, ATP hydrolysis either disrupts the neighboring duplex or releases eIF4A for further rounds of RNA binding and melting (80,81). eIF4A undergoes a cycle of conformational and ligand-affinity changes driven by ATP hydrolysis and/or nucleotide binding and release. How these changes result in RNA unwinding is not yet clear, nor is the stoichiometry of events (82-86). A surprising fact is that eIF4A is the most abundant cellular initiation factor; at a concentration of 50 mM in yeast which is almost fivefold excess over ribosomes and at a concentration similar to that of actin (87). Multiple eIF4A molecules can act together during recruitment of a pre initiation complex (PIC) to mRNAs, both in case of mammals and plants (73). The mechanism of action of eIF4A in plant viruses is still poorly understood and will require additional structural and biophysical studies.
Translation Initiation is known to occur by the following mechanisms:

1.2.2 5’ CAP DEPENDENT TRANSLATION INITIATION:

Eukaryotic mRNAs normally contain a cap structure \([\text{m}^\text{7G}(5')\text{ppp}(5')\text{N}]\) at the 5’ end and a poly(A)-tail at the 3’ end (88). Binding of the eIF4E to the cap and PABP to the poly(A) tail simultaneously along with the interaction of eIF4E and PABP with the scaffolding protein eIF4G enables circularization of the mRNA. This “closed-loop” conformation is assumed to be crucial for efficient translation initiation and is the canonical model for 5’ cap dependent translation (89). According to this model (Fig. 4), eIF4 factors and PABP assemble on an mRNA cooperatively and mediate unwinding to produce the activated form. This activated form then binds to the 43S pre-initiation complex and the 5’ end of the mRNA is loaded into the 40S ribosomal subunit. 43S pre-initiation complex formation takes place when the 40S ribosomal subunit is recruited to the 5’ terminus of the mRNA. It is charged by eIF2,GTP, Met-tRNAi and eIF3, and together the complex migrates towards the initiation codon, where it is joined by the 60S ribosomal subunit to form the 80S initiation complex(90). There is genetic and biochemical evidence showing that eIF4A and eIF4F play a role in promoting 43S attachment to mRNA. The movement of the 40S ribosome on mRNA can be hindered by secondary structures in 5’ UTR. eIF4A together with eIF4B, is thought to act as helicase that unwinds secondary structures in the mRNA 5’ UTR. This suggests the translation initiation step in eukaryotes is highly regulated and rate-limiting (80). The reactions involved in directing the 43S PIC to the 5’ end of the mRNA are critical in this scanning mechanism and little is known about these reactions (91). It is known that mRNAs with more structured 5’ UTRs display a greater requirement for initiation factors and this phenomenon needs to be investigated further (92).
Figure 4: Model of canonical eukaryotic translation initiation pathway (92).
1.2.3 CAP INDEPENDENT TRANSLATION INITIATION

Understanding the mechanisms of plant viral RNA translation is vital to providing insights on how to reduce crop losses due to plant virus infection. The RNAs of many plant viruses belonging to diverse families lack a 5’ cap and use cap- independent mechanism as a gene expression strategy (2). 5’ UTRs of many of these viruses contain an internal ribosome entry site (IRES) to circumvent host translational regulation. IRESes obviate the need for a 5’cap, recruiting the ribosome directly to the vicinity of the start codon without ribosome scanning from the 5’ end. Typically, in animal viruses, IRESes structures are present in 5’ UTR and are usually 200–500 nt long. In contrast, plant viral IRESes are less structured, located in the 3’ UTR, and smaller. For instance, Pseudo knot (PK1) of TEV (93).

Efficient cap- independent translation is conferred in conjunction with the poly (A) tail by the 5’ leader (143 nt) of Tobacco etch potyvirus (TEV) (94-96). A 45 nt RNA pseudoknot (PK1) located in the 5’ proximal domain (38–75 nt) of TEV, is essential for cap-independent translation (97). The L3 loop of PK1 is complementary to 1117–1123 nt of 18S rRNA. Disruption of base pairing to the 18S rRNA caused by mutations within L3 substantially reduced translation, while compensatory double mutations that retain complementarity had no effect (43). It is known that even smaller segments of the TEV 5’ UTR, 1–20, 37–65, 67–113 and 110–114 nt can enhance translation (98). Unpublished work done in our lab has shown that recruitment of the eukaryotic initiation factors and the 40S ribosomal subunit occurs through direct base pairing of the PK1 and other smaller fragments to the 40S ribosome.

Further, members of potyviridae family show low sequence conservation among the 5’ UTRs of viral RNAs and the 5’ leader sequence with low GC content lack elaborate secondary structure (99). Typically, with the help from rRNA complementarity, these 5’ leader sequences can function without being structured and have reduced initiation factor dependence (such as helicase group eIF4A and eIF4B) (43). Many 5’ UTRs belonging to  Turnip mosaic virus, Potato
virus Y (PVY) and Plum pox virus (PPV) of Potyviridae stimulate cap-independent translation in many assay systems similar to TEV(100,101). Viruses belonging to potyviridae family and some picornaviruses have a VPg (viral protein, genome-linked) at their 5’ end of mRNA and a poly(A) tail. VPgs of potyviruses are usually several fold larger, their 5’ UTRs are several fold shorter and less structured than picornaviruses (~150 nt for potyviruses whereas 600–1200 nt for picornaviruses). Luteoviridae and tombusviridae viral mRNAs are typically unmodified at the 5’ end, and lack a poly (A) tail. These viruses unlike many animal viruses, have a sequence which is generally a translation element located in the 3’ UTR that facilitates cap-independent translation initiation and facilitates expression of the ORFs located several kilobases upstream near 5’ end of the mRNA (102).
1.2.3.1 SIGNIFICANCE OF 3’ UTR ELEMENTS- BYDV TRANSLATION ENHANCER ELEMENT (BTE):

BYDV and some other members belonging to luteoviridae, necrovirus and dianthovirus genera of tombusviridae, harbor the BYDV like cap-independent translation element (BTE) located in the 5’ end of the 3’UTR (103). BTEs are defined as cap-independent translation elements with two structural features:

(i) All of them contain a conserved 17 nt sequence, GGAUCCUGGGAAACAGG within a stem loop typically SL-I having a GNRNA loop motif (104).

(ii) A loop not located in SL-I that can potentially base pair to a loop in the 5’ UTR of the RNA (105).

Instead of a cap and poly (A) tail interacting synergistically, 3’ BTE facilitates cap-independent translation of viral proteins from the genomic RNA (gRNA) and sgRNAs in vivo and in vitro (105-107). The 3’BTE comprises nucleotides 4814–4918, has a cruciform/tRNA like secondary structure with three major stem–loops (SL-I, SL-II, SL-III). Stem loop IV forms the base or ‘stalk’ and is the minimum requirement for successful in vitro translation (Fig. 5) (106). CIT initiation at the upstream AUG is stimulated by 3’ BTE via base-pairing (kissing stem loop interaction) of the 3’ SL-III to the 5’ SL-D loop of the viral genomic RNA (Fig. 5) (107). Translation was vitiated by single point mutations in any of the five bases of either of the kissing loops because of disrupted interactions both in in vivo and in vitro experiments, while base-pairing restored by compensatory double mutations restored translation (107). Depleted wheat germ extract (without cap-binding factors and ribosomes) is unable to support 3’ BTE-mediated translation whereas eIF4F addition in small amounts to the extract restores translation of 3’ BTE-containing RNA (22).
**Figure 5:** The predicted secondary structure of the 3’ BTE and mutants. BTE structure is based on SHAPE analysis and mfold data (2,106,108). Purple (SL-I) are the 17 nt conserved bases. Red and blue (SL-III) are the bases complementary to the 5’ UTR BCL. The pink boxes are protected from BzCN modification by eIF4F subunits (109). The BTEBF mutant (green box) has four-base duplication (GAUC). In SL-III SWAP, the SL-III of WT BTE was replaced with the SL-III of isolate PAV-129 which has two 11-base insertions. SL-III-3 has mutations in the kissing stem loop. CIT is initiated at the upstream 5’ UTR via base-pairing of BTE to a complementary loop, SL-D located in the 5’-UTR shown as dotted lines (22,109).
1.3 THE MODEL

In addition to recruiting translation initiation factors that facilitate ribosome recruitment, the 3’ BTE may bind the 40S subunit directly and it must deliver them to the 5’ end of the viral RNA where translation initiates. This is thought to be facilitated by kissing stem loop base pairing (Fig. 6) (22,107). According to the model (2), it is being hypothesized that initially the 3’BTE binds initiation factors and the 40S ribosomal subunit. Additionally, the BCL–BTE long-distance kissing interaction brings the 3’-BTE close to the 5’-end of the mRNA. The recruited translation factors are then delivered, via the BCL–BTE kissing interaction, to the ribosome entering at the 5’-end. Once the ribosome has entered the 5’-end of the mRNA, with associated factors it scans in the 3’ direction toward the start codon. The scanning 43S complex must briefly disrupt the kissing interaction when it reaches the BCL, and then progress to the first start codon. Watson–Crick base pairing may not be the only kind of interaction between the 3’ BTE - 5’ UTR and it is being hypothesized that just like normal cap-dependent translation, BTE-mediated cap-independent translation appears to require ribosome scanning from the 5’ end (43).
1.4 BTE MUTANTS AND SPECIFIC AIMS

For survival, most plant viruses must acquire access to the host translational machinery. Essential to the initiation of protein synthesis and regulation in eukaryotes is 5’ cap recognition on mRNA. Viral mRNAs that translate in the absence of a 5’cap are able to circumvent host regulatory mechanisms and sustain efficient translation. Many unique plant virus translation mechanisms not found in the animal viruses act as novel mechanism of function (43). Further, plant virus-derived sequences are very useful tools for controlling transgene expression in genetically modified plants (93). Plant viruses also serve as elegant model systems and because of their unique evolutionary branch, basic eukaryotic translation processes are better understood, owing to their high titer, lack of human pathogenicity and their small genomes.

For many viruses, the sequence and structural elements in the 3’ UTR of the mRNA control the efficiency of translation (6,110). The TED of STNV and the 3’ BTE are functionally very similar even though they possess no sequence similarity (105). As observed for TED of STNV (25), the pull-down assays, UV cross-linking assays and recent chemical modification studies, the 3’BTE is the binding site of eIF4F (22,109). The role of eIF4F is unclear because eIF4F binds to the 3’BTE and the non-functional mutant BTEBF with similar affinity in in vitro studies (22). The 3’ BTE functionally mimics the 5’ cap and is able to translate efficiently when placed in the 5’UTR. In this study, WT 3’ BTE and four other mutants BTEBF, S-IIIm1, SL-III-3 and SL-III SWAP (the mutations/changes in bases are shown in Fig. 5) with in vitro translation efficiencies from 5-164 % compared to WT BTE (2,106) were used.

The mutants (Fig. 5) were selected with the criteria to evaluate the contribution of the sequences or the secondary structural domains to WT BTE function in terms of ability to stimulate translation (111). SL-I is part of a 17-nt tract (bases 4837–4853) that is conserved across members of luteoviridae with the exception of soybean dwarf virus (SDV), BYDV-PAV 129 variety and Tobacco Necrosis virus (TNV) (105). Within this conserved sequence, the sequence
GAUCCU\textsubscript{4838–4843} can potentially base pair to the AGGAUC sequence located five bases from the 3’ end of 18S rRNA of prokaryotic 16S ribosomal RNA (105). BTEBF mutant (Fig. 5) was selected because it had disrupted potential base pairing to 18S rRNA by duplication of GUAC at BamHI\textsubscript{4837} site. The resultant mutant had an only 5% of the translation ability of WT-BTE (106). S II-m1 mutant (Fig. 5) had disrupted SL-II with two U bases replaced with two A bases resulting in very low cap-independent translation activity (30%) both in 5’ and 3’ UTR contexts. In the case of SL-III SWAP (Fig. 5), the SL-III of PAV6 (wild type) had been replaced with the SL-III of isolate PAV129, containing two 11-base insertions that are predicted to extend the helix. The resulting hybrid TE had 50% of wild-type activity \textit{in vitro} in both the 5’ UTR and 3’ UTR settings (106). SL-3-3 (Fig. 5) mutant was selected because it had disrupted kissing stem loop interactions due to mutations within the five critical bases which disrupted the core cap-independent translation activity of the BTE. When tested in the 5’-UTR context of the luciferase reporter gene, translation of this construct was found to be 164.5 % of the “wild-type” 5’-BTE-LUC RNA, indicating that the mutations within loop III of the BTE did not interfere with the cap-independent translation activity of the BTE (2). However, the SL-III-3 mutant lost the ability to mediate translation from the 3’-UTR due to an inability to interact productively with the 5’-UTR (2).

In this project the mechanisms that modulate protein synthesis efficiency for BYDV viral mRNA were studied to enhance our prior understanding of the regulation of eukaryotic protein synthesis. The details of the IF selection mechanism for mRNAs, the sequence of events for formation of the initiation complex under different physiological conditions and correlation of protein synthesis efficiency and translational machinery recruitment remains a mystery. Also, in the previous studies, it was found that eIF4F binds 3’BTE and the non-functional mutant BTEBF with similar affinity (22) which suggests that eIF4F is not important for translation and brings into question the role played by eIF4F in the BYDV protein synthesis mechanism. For all the studies eIF4F was used as a whole complex instead of using eIF4G and eIF4E separately.
because in plants, the eIF4F is found as one stable complex in contrast to the mammalian system where eIF4F occurs as a tri-meric complex of eIF4A, eIF4G and eIF4E. Unlike the wheat system, these proteins can be readily separated. Also, wheat eIF4G is a labile protein which is very unstable when synthesized separately and has low yields as a result of rapid degradation. This makes eIF4G unfit for use in most of our experiments.

We looked at the following aspects in this project:

(i) In order to determine the role of eIF4F in the 3' BTE mediated translation, we used biophysical studies to see whether binding of eIF4F correlated to protein synthesis efficiency?

(ii) What is the effect of the purified eIFs on the ability of BTE and mutants to translate and ability to bind eIF4F?

(iii) Did kinetics play a role?

(iv) What is the nature of interactions of BTE mutants and purified eIFs?

Binding of eIF4F and other initiation factors (eIF4B, 4A, PABP) to the WT 3' BTE and mutants (BTEBF, SII-m1, SL-III-3 and SL-III SWAP) was characterized using fluorescence anisotropy and stopped flow kinetics which gave equilibrium binding constant values (Kd), a measure of relative complex stability, and kinetic constants.
**Figure 6:** The model, eIF4F binds 3’BTE, 40S ribosomes and subsequent transfer to the 5’UTR.
1.5 FLUORESCENCE SPECTROPHOTOMETRY

Fluorescence spectrophotometry is a class of sensitive techniques that assay the state of a biological system by studying system’s interactions with fluorescent probe molecules by measuring the changes in the fluorescent probe optical properties. Fluorometers are general-purpose instruments designed to measure fluorescence spectrum, polarization and/or lifetime. A typical fluorometer includes a light source, a specimen chamber with integrated optical components, and high sensitivity detectors (Fig.7). The first step involves excitation of the fluorophore to higher energy level (electronic singlet state) by absorption energy from a source such as a xenon arc lamps (Fig.7). These lamps provide a relatively uniform intensity over a broad spectral range from the ultraviolet to the near infrared. The fluorophore remains in the excited electronic state for a period of time on the order of nanoseconds, the fluorescence lifetime. In the excited state, changes can take place in the conformation of the molecule and there is a possibility of interactions with the surrounding molecular environment. The excited molecule partially dissipates some energy yielding relaxed singlet excited state, which decays to the ground state with the emission of light. The energy released during this process produces emission spectra that are characteristic of a particular compound (112).

The use of fluorescence in biology and medicine is very prevalent. In particular, the measurements of fluorescence spectrum and polarization are powerful methods of studying biological structure and function. The fluorescence spectrum is highly sensitive to the biochemical environment of the fluorophore. Fluorescent probes have been designed such that their spectra changes upon interactions with metabolites/ligands as a function of the concentration. Fluorescence spectral changes resulting from solvent relaxation of fluorescent amino acids having aromatic rings, such as tryptophan, phenylalanine and tyrosine, are important intrinsic reporters of protein structure and folding (113).
**Figure 7:** A basic diagram of a fluorometer.*

1.6 FLUORESCENCE ANISOTROPY

Fluorescence polarization is a very useful biophysical tool that measures the rotational diffusion rate of a macromolecule thus providing information about the shape of the proteins. Polarization measurement can quantify diffusional restrictions of molecules in biological macrostructures. Fluorescence anisotropy is commonly used to monitor protein–ligand binding and sometimes oligomerization. When polarized light hits a fluorophore, there is emission of polarized light. The amount of polarized light emitted depends on the location, size and movement of the fluorophore. In commonly used molecular binding assays, the smaller ligand molecules are labelled by a fluorophore. The binding of the small ligand to a larger protein increases the hydrodynamic radius of the composite particle causing a slower rotation or ‘tumbling’ rate resulting in higher polarization signal during the assay (112). The fraction of bound molecules can be found by quantifying the optical signal contributions from the fast and slow diffusers. The binding constant is generally expressed as dissociation constants of the protein–ligand/RNA interaction and can be measured by estimating the fractions of bound and free proteins at different protein–ligand mixing ratios (114). In our experiments, the RNA was fluorescently labeled since RNA is the smaller ligand when compared with the protein. We used a Horiba JobinYvon Fluorolog-3 FluorEssence™ spectrofluorimeter (Fig. 8) for the experiments using an L-format detection configuration and a 1 cm path-length quartz cuvette.
**Figure 8:** A depiction showing L-format fluorescence polarization. V and H represent orientation of each polarizer*.

1.7 STOPPED FLOW KINETICS

Chance (1940) and Gibson & Milnes (1964) first time described the stopped flow method with an aim to probe enzyme kinetics where oscilloscopes were used as first detectors. It is a kind of flow-injection analysis where reactants are rapidly mixed in a mixing chamber, and the flow is stopped with the reactant stream in the flow cell. Detectors are usually some form of spectrophotometry. For instance, UV/Vis & IR, Fluorescence (more sensitive), Chemiluminescence, Circular dichroism, electrical conductivity etc.

To measure the kinetics of a reaction, the right method would be to mix the reactants and periodically sample the mixture in order to analyze the reactant or product. However, for many reactions, the half-life of the reaction is approximately on the same timescale as the mixing. This results in an uncertainty in both initial concentration and time. A stopped-flow method is the method of choice when reactions occur under such conditions. Fig. 8 shows a depiction of a stopped-flow apparatus. In this method, the reactants are injected simultaneously into a mixing chamber, where they begin to react, then moved quickly through a spectrophotometer cell and finally to a stopping syringe. The filling up of stopping syringe and driving the plunger back against a stopping block stops the flow and triggers the activation of data acquisition on a computer. Reaction rates are followed by monitoring a change in fluorescence intensity/absorbance signal of either a reactant or product in the spectrophotometer cell. The time that is required for the reactant and products to travel to the spectrophotometer cell is known as the “dead time.” Typically, the mixing time plus the dead time is on the order of milliseconds, allowing reactions with half-lives of milliseconds or couple of seconds to be measured.
Figure 9: A diagram of stopped flow apparatus
CHAPTER 2

2 EXPERIMENTAL PROCEDURES

2.1 MATERIALS AND METHODS

GSTrap 4B columns and m^7GTP-Sepharose 4B were purchased from GE Healthcare LifeSciences, Pittsburgh, PA. HisPur Cobalt Chromatography Cartridges were purchased from Thermo Scientific Pierce Protein Biology Products, Rockford, IL. Phosphocellulose P11 cation exchange media was purchased from Whatman (part of GE Healthcare, Pittsburgh, PA). All chemicals were obtained from Sigma-Aldrich, St. Louis, MO or Thermo Fisher Scientific, Waltham, MA. Millipore Amicon Ultra-15, 10 kD cutoff centrifugal filter units, Millex (PVDF) 0.22 and 0.45 µm syringe filters, Stericup 250 mL (PVDF 0.22) µm were purchased from Thermo Fisher Scientific, Waltham, MA. RNAseOUT was purchased from Life Technologies, Grand Island, NY. DNA templates for in vitro transcription were purchased from Integrated DNA Technologies, Coralville, Iowa.
2.2 RECOMBINANT PROTEIN EXPRESSION AND PURIFICATION

2.2.1 PURIFICATION OF eIF4F

A dicistronic recombinant plasmid construct was prepared (vector pET3d) into which were spliced in the coding regions for both eIF4G and eIF4E. This construct expressed the recombinant WT eIF4F complex. The bacterial clone was a generous gift from Dr. K.S Browning, University of Texas at Austin, Austin, TX. The protein was purified as described elsewhere (115). The modifications to the protocol are described below:

BL21(DE3) cells were transformed using standard methods and selected with Ampicillin antibiotic. A single colony was used for a 50mL overnight culture and was transferred to 0.8 L media in four 4-L flasks the next day. All the cultures were incubated at 30 °C and grown to OD$_{600}$ ≈ 0.8. Induction of eIF4F expression was accomplished using 0.5 mM IPTG (final concentration). Cells were harvested after 3 hours by centrifugation (6000 g) for 15 min at 4°C. Composition of buffer used in purification was as follows: Buffer B-0 (20 mM HEPES, 10% glycerol, 1 mM EDTA, 1 mM DTT) and KCl is added as required. The cell pellet was suspended in 50 ml of Buffer B-150 (150mM KCl) containing one protease inhibitor tablet (Roche Complete Protease Inhibitor Cocktail Tablets), 5 mg Soybean trypsin inhibitor and 500 µL of 10mg/mL poly methyl sulfonyl fluoride. The cells were sonicated 3 times for 30 sec at 70% power followed by 2 times for 30 sec at 90% power with intermittent cooling on ice for 2 to 3 min between sonication bursts. The lysed cells were centrifuged at 40,000 rpm for 1.5 h at 4 °C. The supernatant was diluted to KCl ≈ 100 mM, and was loaded on to a 10 mL phosphocellulose column pre-equilibrated with Buffer B-100. Bound eIF4F was washed with buffer B-100 and eluted with Buffer B-300. The fractions containing the highest concentration of eIF4F were pooled and diluted to 100 mM KCl by the addition of Buffer B-0 and loaded on to a 4-mL m$\text{7GTP}$ Sepharose column equilibrated in Buffer B-100 and washed with the same buffer. eIF4F was eluted with Buffer B-100 containing 100 µM m$\text{7GTP}$. The pooled fractions with highest purity
and concentration were applied to a 1-mL phosphocellulose column equilibrated in Buffer B-100 and washed with same buffer. eIF4F was eluted with Buffer B-300. The protein was diluted using B-0 to 100mM KCl and concentrated using Millipore Amicon Ultra-15 mL, 10 kD cut-off. The purity of eIF4F was confirmed by 10% SDS-PAGE and the concentration was determined using the Bradford method with BSA as a standard (116).
2.2.2 PURIFICATION OF PABP AND eIF4A

His-tagged PABP and eIF4A protein were expressed in BL21 (DE3) pLysS E.coli strain using pET19b and pET23d vectors (a generous gift from Dr. D.R. Gallie, University of California, Riverside, CA), respectively and were purified as described elsewhere (62,72).

In brief, a single colony obtained from overnight grown LB fresh plates selected with 100 μg/ml ampicillin and 34 μg/ml chloramphenicol was used to grow 10 mL of LB media overnight at 37 °C. This culture was transferred to a liter of LB media selected with ampicillin and chloramphenicol and was incubated at 37 °C. When the O.D. at λ600 was 0.6, protein expression was induced with 0.19 g/L of IPTG (Isopropyl-1-thio-β-D-galactopyranoside) overnight at 37 °C. After this stage, subsequent steps were performed at 4 °C. The cells were harvested by centrifugation at 6000 rpm for 15 minutes and suspended into binding buffer (50mM sodium phosphate, 300mM sodium chloride, 5 mM imidazole; pH 7.4) containing 0.5 ml of Aprotinin, 1.0 mM Phenylmethylsulfonyl fluoride (PMSF), 100 μg/ml soybean trypsin inhibitor and one Roche cOmplete Protease Inhibitor Cocktail Tablets, EDTA free. The cell suspension was lysed by sonication (at 30% power for 10 minutes using 30s on and 30s off cycle), and the lysate was ultra-centrifuged at 40,000 rpm for 2 hours and supernatant was filtered using 0.45 μm syringe filters prior to application to a 1 ml Pierce HisPur Nickel Resin column equilibrated with binding buffer. Protein was purified using the manufacturer’s specifications/protocol for the column. Protein was washed using PBS buffer, pH 7.4 with 30 mM imidazole and was eluted with 300mM imidazole. Protein elution was monitored by measuring the absorbance of the fractions at 280nm. The pure protein fractions were dialyzed in 20 mM Tris-HCl pH 7.6, 50 mM potassium acetate, 0.1 mM EDTA, 1mM DTT and 10% glycerol to remove excess imidazole at 4 °C and re-dialyzed against a liter of titration buffer. The purity of the proteins was confirmed by 10% SDS-PAGE.
2.2.3 PURIFICATION OF eIF4B

Full length GST-tagged pET3d-eIF4B plasmid was also from Dr. D.R. Gallie, University of California, Riverside, CA. Protein expression was performed in *E.coli* BL21 (DE3) pLysS, and selected using ampicillin. In brief, a single colony obtained from overnight grown LB fresh plates selected with 100 μg/ml ampicillin was used to grow 10 mL of LB media overnight at 37 °C. This culture was transferred to a liter of LB media selected with ampicillin and was incubated at 37 °C and induced with 1 mM isopropyl 1-thio-β-d-galactopyranoside overnight. The cells were harvested by centrifugation at 6000 rpm for 15 min at 4°C. Pelleted cells were suspended in Binding buffer (PBS, pH 7.4 along with 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) sonicated and ultra-centrifuged to remove cell debris. The supernatant was applied to 1mL GSTrap 4B column and purified according to manufacturer’s specifications. All buffers used in protein purification were passed through a 0.45 μM filter (Millipore).

Purified protein was dialyzed against Titration Buffer (20 mM HEPES-KOH, pH 7.6, 100 mM KCl, 1.0 mM MgCl₂, and 1.0 mM DTT) and filtered using a 0.22 μM PVDF Millex (Millipore) filter before the anisotropy/stopped flow measurements were performed.
2.3 SYNTHESIS AND 5’ FLUORESCEIN LABELING OF RNA

The RNA oligomers (BTE, BTEBF, SII-m1, SL-III-3 and SL-III SWAP) were transcribed using double stranded (ds) DNA templates by using T7 RNA polymerase. The transcription reaction was: Transcription buffer 1X (Stock 10X: 0.4M Tris pH 8, 100mM DTT, Triton X-100 0.1%, 10 mM spermidine, filtered using 0.22 µm Millipore PVDF filters), NTPS: ATP, GTP, CTP, UTP and GMP 5mM final concentration, MgCl$_2$ 25 mM, dsDNA final concentration 200 nM, T7 polymerase 1:200 of final volume. RNaseOUT (Life Technologies, Grand Island, NY) was added to reduce RNA degradation. The volume was made up to the desired transcription volume using DEPC treated water. Pyrophosphatase was added for increased yields. The transcription mix was incubated overnight at 37 °C. After incubation, EDTA (50 mM final) was added to stop the reaction by chelating MgCl$_2$.

The transcription mix, along with the loading dye (Life technologies Gel Loading buffer II AM8547) in 1:1 ratio was applied onto a 10% denaturing poly acrylamide gel made with TBE and 7M urea. The gel was pre-run for an hour after which the RNA was loaded and the gel was run for 2 hours at 200V. The RNA band was visualized using UV shadowing and cut out. The RNA was eluted from the gel and precipitated using 3 volumes of absolute ethanol and 0.3M sodium acetate, pH 5.3 at -20 °C. After spinning the precipitate down, the pellet was washed with chilled 70% ethanol and air-dried. RNA was re-suspended in RNase free water and the concentration was calculated using nanodrop 2000. RNA was fluorescein labeled at the 5’ end using Vector Labs, Burlingame, CA 5’end tag labeling kit and purified according to the kit manufacturer’s recommendations. In short, 20 µg of RNA was mixed with 2 µL of universal reaction buffer, 2 µL of T4 polynucleotide kinase and 1 µL of ATPγS. The total volume was brought to 20 µL with Rnase-free water. The mix was incubated for 30 minutes at 37°C. After incubation, 10 µL of Fluorescein maleimide dye (dissolved in DMSO) was added and mixed. The reaction was incubated for 30 minutes at 65°C. The RNA was cleaned by one round of phenol chloroform...
extraction followed by precipitation and re-suspension in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The unincorporated dyes and nucleotides were removed by spinning the sample on BioRad P-30 gel columns. Before titration, the RNAs were refolded by heating to their melting temperature and slow cooling to room temperature in titration buffer containing 1 mM MgCl₂.
2.4 FLUORESCENCE ANISOTROPY MEASUREMENTS

A Horiba JobinYvon Fluorolog-3 FluorEssence™ spectrofluorimeter was used for fluorescence anisotropy experiments using an L-format detection configuration and a 1 cm path-length quartz cuvette. Vertically polarized light with wavelength of 490 nm was used for excitation (slit width 4 nm) and the emission (slit width 5 nm) was measured at 520 nm in the horizontal and vertical directions. Fluorescein labeled 3’ BTE (F1BTE) of BYDV mRNA or BTE mutants (50 nM) were titrated in the titration buffer with increasing concentrations of eIF4F, eIF4A, eIF4B, PABP, eIF4F·4B, eIF4F-PABP and eIF4F-4A complexes. An increase in F1BTE/mutants RNA anisotropy was used to measure the interaction of initiation factors (eIFs) with 3’ BTE RNA. Temperature was 25 °C for all experiments unless otherwise noted (temperature dependence studies). The dissociation equilibrium constant was determined by fitting the anisotropy data to equation (1) (117).

\[ r_{\text{obs}} = r_{\text{min}} + \left\{ \frac{r_{\text{max}} - r_{\text{min}}}{2 \times [F1\text{BTE/mutants}]} \right\} \{b-(b^2-4 [F1\text{BTE/mutants}][\text{eIFs}])^{0.5}\} \]  

where, \( b = K_d + [F1\text{BTE/mutants}] + [\text{eIFs}] \), \( r_{\text{obs}} \) is the observed anisotropy for any point in the titration curve, \( r_{\text{min}} \) is the minimum observed anisotropy in the absence of protein, \( r_{\text{max}} \) is the maximum anisotropy at saturation and is fit as a parameter. \([F1\text{BTE/mutants}]\) and \([\text{eIFs}]\) are the 3’ BTE RNA and its mutants and protein concentrations (eIFs=eIF4F, PABP, eIF4A, eIF4F-PABP and eIF4F-4A complex), respectively. \( K_d \) is the equilibrium dissociation constant assuming one protein binding site/RNA. KaleidaGraph™ software was used to perform nonlinear least squares fitting of the titration data. Uncertainties in \( K_d \) values are reported for one sigma as determined from data fitting and reported as error values. Data values from three independent titration experiments were averaged and used for calculations. The variation among the titrations was less than 20%. The concentrations of protein and RNA were measured using an OLIS HP Diode array (8452A) UV-visible spectrophotometer. Previously reported equilibrium dissociation constant \( (K_d) \) values (13,118) for the interaction of eIF4F-PABP and
eIF4F-eIF4B of 43 nM and 62 nM respectively, calculated by monitoring changes in intrinsic protein fluorescence, were used to calculate the concentrations that had 90% of the eIF4F in complex form (eIF4F·PABP, eIF4F·eIF4B, and eIF4F·4A) at the lowest protein titration point of 50 nM. The samples were incubated for 10 minutes to ensure protein complex formation prior to data collection.
2.5 THERMODYNAMIC ANALYSES OF eIF4F INTERACTION WITH 3’ BTE AND MUTANT RNAs

Temperature dependence of the association equilibrium constants was used to determine the thermodynamic parameters of enthalpy (ΔH), entropy (ΔS), and free energy (ΔG) of eIF4F binding to 3’ BTE RNA using van’t Hoff plots of −ln $K_{eq}$ versus 1/T, according to the following equations:

$$-RT \ln K_{eq} = \Delta H - T \Delta S$$  \hspace{1cm} (2)

$$\Delta G = -RT \ln K_{eq}$$  \hspace{1cm} (3)

where R is the universal gas constant and T is the absolute temperature. $K_{eq}$, the association equilibrium constant, was determined at different temperatures. ΔH and ΔS were determined from the slope and intercept, respectively of the plot of ln $K_{eq}$ vs 1/T and ΔG was determined from Eq. (3).

The samples were thermostated at 5, 15, 25, 30 and 35 ± 0.5 °C. Titrations as described previously were performed in 20 mM HEPES-KOH, pH 7.6, 100 mM KCl, 1.0 mM MgCl₂, and 1.0 mM DTT. A thermocouple was used inside the cuvette to monitor temperature. The temperature dependence studies for SL-III-3 and eIF4F were done in the range of 25-35 °C because the tight binding constants made measurement at lower temperatures impractical.
2.6 STOPPED-FLOW FLUORESCENCE KINETICS

An OLIS RSM 1000 stopped-flow system with a 1-ms dead time was used to perform stopped-flow fluorescence experiments. The sample was excited at a wavelength of 280 nm, and the cut-on filter of 300 nm was used to investigate RNA- eIF4F interactions. Flow cell and solution reservoir temperatures were maintained using a temperature-controlled circulating water bath. After rapid mixing of 0.75 µM (0.375 µM after mixing) eIF4F with 3-5 µM (1.5-2.5 µM after mixing) 3’ BTE or mutant RNA in Titration Buffer (20 mM HEPES-KOH, pH 7.6, 100 mM KCl, 10 mM MgCl$_2$, and 1.0 mM DTT), the time course of the fluorescence intensity change was recorded. BTE or mutant RNA binding induced a decrease in eIF4F fluorescence. A reference photomultiplier was used to monitor fluctuations in the lamp intensity. 1000 pairs of data were recorded with 5-7 shots averaged in each experiment. Stopped flow-data from three separate experiment sets were averaged and then fitted to nonlinear analytical equations using Global analysis software provided by OLIS. Single and double exponential functions were used for data fitting. The single exponential equation fitted curves correspond to (119),

$$\text{F}_t = \Delta F \exp(k_{\text{obs}} \cdot t) + F_{\infty}$$

where $\text{F}_t$ is fluorescence observed at any time, $t$, $\Delta F$ is the amplitude, $F_{\infty}$ is the final value of fluorescence, and $k_{\text{obs}}$ is the observed first-order rate constant. For double exponential fits,

$$\text{F}_t = \Delta F_1 \exp(k_{\text{obs1}} \cdot t) + \Delta F_2 \exp(k_{\text{obs2}} \cdot t) + F_{\infty}$$

where $\Delta F_1$ and $\Delta F_2$ are the amplitudes of two exponentials with rate constants $k_{\text{obs1}}$ and $k_{\text{obs2}}$, respectively. The reaction was consistent with a single exponential process as would be expected under the pseudo-first order conditions used in the experiment. Difference between the calculated fit and the experimental data gave the residuals.
CHAPTER 3

3 STUDY OF EFFECTS OF DIFFERENT EUKARYOTIC INITIATION FACTORS ON BINDING OF 3’ BTE AND BTEBF WITH eIF4F

3.1 PABP AND eIF4A HAVE LITTLE EFFECT ON THE BINDING OF 3’ BTE AND BTEBF WITH eIF4F

As reported for earlier filter binding studies, BTEBF bound eIF4F with high affinity, yet was translationally inactive (22). It has been previously found that the presence of eIF4B and PABP enhanced the binding of eIF4F to TEV IRES PK1 (62) and PABP is known to increase the ATPase and RNA helicase activity of eIF4A, eIF4B and eIFiso4F(72). We therefore hypothesized that the presence of other initiation factors such as eIF4A, eIF4B and PABP should affect eIF4F binding to these RNA and thus should be one of the factors contributing to the differential translation abilities of these mutants. The fluorescence anisotropy plots for binding of 3’ BTE with eIF4A, eIF4F, PABP and in complex form are shown in Fig. 10. The fluorescence anisotropy plots for binding of 3’ BTEBF with eIF4A, eIF4F, PABP and in complex form are shown in Fig. 11. The fluorescence anisotropy plots for binding of 3’ BTE and 3’ BTEBF with eIF4B and in complex form with eIF4F are shown in Fig. 12 and 13. The equilibrium binding constants obtained are shown in Table 1. Because binding of eIF4A, eIF4B and PABP alone was very weak, an excess of each of these proteins to eIF4F ensured that at least 90% of the eIF4F was in complex form (eIF4F-PABP, eIF4F-eIF4B, and eIF4F-4A) by using the concentrations predicted using the $K_d$ values (13,118) for the protein–protein interactions. Nonspecific binding was ruled out by using BSA as a control under similar experimental conditions (data not shown).
However in contrast to our hypothesis we found that binding of eIF4F to 3’ BTE was unaffected by PABP and eIF4A as evident from the anisotropy plots (Fig.10, 12) and equilibrium binding constants ($K_d = 32\pm8$ nM for eIF4F.PABP–3’ BTE, $K_d = 40\pm7$ nM for eIF4F·4A–3’ BTE) as shown in Table 1. However, there were minor effects observed on the binding of eIF4F to 3’ BTEBF by the presence of PABP ($K_d = 77\pm15$ nM for eIF4F.PABP–3’ BTEBF) and eIF4A ($K_d = 78\pm12$ nM for eIF4F·4A–3’ BTEBF) (Fig. 11). The binding affinities of eIF4A, eIF4B and PABP to both 3’ BTE and BTEBF RNA were found to be relatively weak (Table 1). The binding affinity of eIF4F for 3’ BTE and BTEBF did not change significantly in the presence of eIF4B (Fig. 12, 13 and Table 1). In contrast, the eIF4F binding to TEV pseudoknot 1 (PK1) was 4 fold stronger in the presence of both PABP and eIF4B (62).
Table 1: Equilibrium binding affinity constants ($K_d$s) for eIF and 4BTE/4BTEBF interactions.

<table>
<thead>
<tr>
<th>Protein/Complex</th>
<th>BTE (nM)</th>
<th>BTEBF (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4F</td>
<td>29 ± 3</td>
<td>33 ± 5</td>
</tr>
<tr>
<td>4B</td>
<td>827 ± 93</td>
<td>806 ± 99</td>
</tr>
<tr>
<td>4F.4B</td>
<td>44 ± 8</td>
<td>45 ± 9</td>
</tr>
<tr>
<td>PABP</td>
<td>415 ± 99</td>
<td>406 ± 67</td>
</tr>
<tr>
<td>4F.PABP</td>
<td>32 ± 8</td>
<td>77 ± 15</td>
</tr>
<tr>
<td>4A</td>
<td>748 ± 68</td>
<td>717 ± 68</td>
</tr>
<tr>
<td>4F.4A</td>
<td>40 ± 7</td>
<td>78 ± 12</td>
</tr>
</tbody>
</table>
**Figure 10:** PABP and eIF4A have little effect on eIF4F binding to FlBTE. Fluorescence anisotropy measurements for the binding of eIF4A· FlBTE (■), eIF4F· FlBTE (●), eIF4F·eIF4A· FlBTE (←), PABP· FlBTE (▲) and eIF4F·PABP - FlBTE (→) are shown. RNA concentration was 50 nM in titration buffer at 25 °C. eIF4F·PABP (1:30) complex was prepared by incubation of 1 μM eIF4F and 30 μM PABP for 15 min at 4 °C. The excitation and emission wavelengths were 490 nm and 520 nm, respectively. The curves were fit to obtain dissociation constants (Kd) values as described in materials and methods.
Figure 11: PABP and eIF4A had a minor effect on eIF4F binding to βBTEBF. Fluorescence anisotropy measurements for the binding of eIF4A- βBTEBF (■), eIF4F- βBTEBF (●), eIF4F-eIF4A- βBTEBF (→), PABP- βBTEBF (▲) and eIF4F·PABP- βBTEBF (△) are shown. RNA concentration was 50 nM in titration buffer at 25 °C. eIF4F•PABP (1:30) complex was prepared by incubation of 1 μM eIF4F and 30 μM PABP for 15 min at 4 °C. Other conditions same as Fig. 10.
Figure 12: eIF4B has negligible effect on eIF4F binding to BTE. Fluorescence anisotropy measurements for the binding of eIF4B- BTE (■), eIF4F- BTE (●) and eIF4F-eIF4B- BTE (→) are shown. RNA concentration was 50 nM in titration buffer at 25 °C. eIF4F•eIF4B (1:10) complex was prepared by incubation of 1 μM eIF4F and 10 μM eIF4B for 15 min at 4 °C. Other conditions same as Fig. 10.
**Figure 13:** eIF4B had little effect on eIF4F binding to $^{31}$BTEBF and is almost similar to binding to $^{31}$BTE. Fluorescence anisotropy measurements for the binding of eIF4B- $^{31}$BTEBF (■), eIF4F- $^{31}$BTEBF (●) and eIF4F·eIF4B- $^{31}$BTEBF (←) are shown. RNA concentration was 50 nM in titration buffer at 25 °C. Other conditions same as Fig. 12.
3.2 DISCUSSION

PABP does not change the affinity of eIF4F for WT 3' BTE significantly, suggesting a lower importance in pre-initiation complex formation, at least in the initial steps of initiation complex formation. BTE and mutants did not interact with strong affinities with PABP, eIF4B or eIF4A suggesting that it is eIF4F which plays the major role in transfer of the pre-initiation complex from 3' to 5' UTR so that translation can initiate. In contrast, eIF4F binding to PK1 RNA of TEV is enhanced multi-fold by the presence of PABP (62) thus showing an alternate mechanism by which BYDV virus assembles its translation machinery. This is in agreement with an earlier predicted trend for IRES RNAs, which correlates to the very well folded and stable 3' BTE structure’s lower requirement for additional factors compared to a single stem loop pseudoknot structure of PK1 RNA (108,120). This is also consistent with studies of deletion mutants (109) which showed the PABP binding site of eIF4G was not necessary for 3' BTE interaction.
CHAPTER 4

4 KINETICS OF BINDING OF eIF4F WITH BTE AND MUTANTS

4.1 KINETICS OF eIF4F BINDING FITS A TWO-STEP MODEL

The presence of other initiation factors had little effect on abilities of BTE and BTEBF to interact with eIF4F. According to earlier studies on neuronal protein HuD and an earlier prediction (22) (121), we thought that kinetics of binding may explain the differential translation abilities of 3’ BTE and BTEBF. Binding of eIF4F to BTE and mutants was investigated using stopped flow kinetics and data were plotted as the normalized fluorescence intensity versus time (Fig. 14). Data were analyzed using nonlinear regression analysis (119) considering a single-exponential and a two-step process as described below. Experiments were performed with excess of RNA for pseudo-first order conditions. The experimentally observed rate constant is predicted to be a linear function of the concentration of RNA under such conditions. However, it was found that the observed binding rates had low RNA concentration dependence within the concentration range of 3–5 µM (before mixing) which is a 4–10-fold excess (Fig 16). A two-step binding process was used to explain the mechanism (70,122):

\[
\text{eIF4F + RNA} \xrightleftharpoons[k_{-1} \text{ (fast)}]{k_1} \text{(eIF4F-RNA)*} \xrightleftharpoons[k_{-2} \text{ (slow)}]{k_2} \text{eIF4F-RNA}
\]

The first step of the reaction is a very fast, almost diffusion controlled association of eIF4F and RNA. The second step is a slow conformational change of first intermediary complex (eIF4F-
RNA)* to the final stable complex, (eIF4F· RNA) and this step results in the fluorescence signal change. The concentration of RNA and binding rates are related by the equation: $1/k_{obs} = 1/k_2 + K_1/k_2[C]$ (119), where $k_{obs}$ is the observed first order rate constant, $k_2$ is the forward rate constant for the second association step, $K_1$ is the equilibrium constant for the first step, and $[C]$ is the substrate concentration (RNA). A plot of $1/k_{obs}$ versus $1/[C]$ of RNA is shown in Fig. 15. The intercept of the linear line generated from this plot gave the $k_2$ values (Table 2). $K_a$, the association equilibrium constant for the reaction as obtained from equilibrium studies, is defined as $k_2/k_2$ and was used for calculation of the dissociation rate constant ($k_{-2}$). Differences between the calculated and experimental kinetic data are the residuals which indicate a good fit using a single-exponential function. The fit was not improved by the use of a double exponential equation as shown by the residuals in Fig. 15.
4.2 THE “ON” RATES (k_2) OF BINDING OF eIF4F WITH 3’ BTE RNAs HAVE LOWER CORRELATION WITH THEIR TRANSLATION EFFICIENCIES THAN EQUILIBRIUM BINDING CONSTANTS

The rate constants for the forward reaction (k_2) for all five mutants were found to be similar, and therefore did not correlate with the efficiency of translation. The off rates (k_-2) show a negative correlation to the translation efficiencies; that is, the lower the k_-2, the more stable the complex and the greater is the translation efficiency (Table 2). Fig. 17 shows a plot of the translational efficiency and K_a values which correlate well. In contrast, for eIF4F and PK1 binding reaction, the rates of binding show linear concentration dependence in the 0.1-1 µM range (46). This suggests that BYDV system does not possess a large kinetic advantage when compared to the TEV system. Our equilibrium binding data shows that the 3’ BTE- eIF4F binding is 5 times tighter than PK1-eIF4F binding and much stronger than eIF4F- cap analog binding which is reported to be in µM range(62,123) and thus BYDV is able to more efficiently sequester eIF4F as compared to host cell.
Figure 14: eIF4F binds BTE and 3’ BTE mutants with similar association rates. The time course represents the intrinsic protein fluorescence intensity decrease of eIF4F caused by binding of 3’ BTE and mutants at 25 °C. A single-exponential fit was used for the data. Excitation wavelength was 280 nm. The signal represents the total normalized fluorescence emission above 300 nm. eIF4F concentration was 0.75 μM and RNA concentration was 3 μM before mixing.
Figure 15: Residuals for the single and double exponential fits for the kinetics data for binding of BTEBF and eIF4F. Differences between the calculated and experimental data are shown by the residuals. Fitting was not improved by using a double exponential fit. A single exponential function is consistent with a single reaction step.
Figure 16: Plots of $1/k_{obs}$ versus $1/[C]$ for the interaction of eIF4F- BTE and mutants were used to calculate $k_{on}$ values from the intercept according to the two step model. Binding of 0.75 µM eIF4F with different BTE and mutants (3, 4 and 5µM, respectively before mixing) is shown. The linear equations generated from the plot were used to calculate the $k_2$ values using the intercept and Kd values from equilibrium binding experiments were used to calculate $k_{-2}$ values.
Table 2: Equilibrium association constants from fluorescence anisotropy experiments and the calculated $k_2$ and $k_2$ rates of binding as obtained from kinetics experiments.

<table>
<thead>
<tr>
<th>Complex</th>
<th>Translation$^1$</th>
<th>$K_a$ (nM$^{-1}$)</th>
<th>$K_a'$ (nM$^{-1}$)$^2$</th>
<th>$k_2$ (sec$^{-1}$)</th>
<th>$k_2$ (nM sec$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>eIF4F·BTEBF</td>
<td>5.2±0.2</td>
<td>0.030±0.005</td>
<td></td>
<td>3.00±0.08</td>
<td>99±18</td>
</tr>
<tr>
<td>eIF4F·SII-m1</td>
<td>30.0±2.1</td>
<td>0.011±0.002</td>
<td>0.009±0.001</td>
<td>2.16±0.08</td>
<td>205±46</td>
</tr>
<tr>
<td>eIF4F·SL-III SWAP</td>
<td>49.9±0.9</td>
<td>0.016±0.003</td>
<td>0.013±0.003</td>
<td>2.41±0.04</td>
<td>147±22</td>
</tr>
<tr>
<td>eIF4F·SL-III-3</td>
<td>164.5±13.0</td>
<td>0.143±0.018</td>
<td>0.100±0.015</td>
<td>4.28±0.12</td>
<td>30±5</td>
</tr>
<tr>
<td>eIF4F·BTE</td>
<td>100.0±2.2</td>
<td>0.034±0.004</td>
<td>0.035±0.003</td>
<td>3.64±0.06</td>
<td>105±13</td>
</tr>
</tbody>
</table>

$^1$Translation efficiency is normalized to WT BTE as 100% (2,106).

$^2$Equilibrium association constants obtained in d-WGE.
4.3 DISCUSSION

According to earlier predictions (22) and our hypothesis based on earlier studies on neuronal protein HuD (121), we thought that kinetics may explain the similar abilities of 3’ BTE and BTEBF to bind eIF4F. To the contrary, 3’ BTE and mutants did not differentiate in binding with eIF4F in terms of kinetics. This could be attributed to almost similar secondary structures of both WT 3’ BTE and mutants (Fig. 5) in solution resulting in lack of discrimination between 3’ BTE and BTEBF by eIF4F outside of cell or wheat germ extract in in vitro experiments in terms of time taken to reach equilibrium (22).
CHAPTER 5

5 eIF4F BINDING AND TRANSLATION EFFICIENCIES OF 3’ BTE AND BTE MUTANTS CORRELATE WELL

5.1 TRANSLATION EFFICIENCIES OF 3’BTE AND MUTANTS CORRELATION WITH eIF4F BINDING

Earlier studies (22) and results here showed that eIF4F bound WT 3’BTE and an inactive mutant BTEBF with high affinity calling into question the role of eIF4F in translation of this virus. The presence of other initiation factors and kinetics of eIF4F binding to BTE and BTEBF could not explain the differential abilities of these mutants. To further examine what if any role binding of eIF4F had on translation, a variety of 3’BTE mutants with widely different translational efficiencies were selected for binding studies. 3’ BTE and mutants with in vitro translation efficiencies from 5-164 % (WT=100%) were studied (2,106). All of the mutants contain a short 17 nt long sequence (located in SL- I) conserved across viral classes, which is the location for eIF4F binding and is complementary to 18S ribosomal RNA (108,109). The BTEBF mutant has a four-base duplication (GAUC) introduced into the 17 nt consensus sequence within the BamH14837 site (Fig.5). This mutation abolishes the translation stimulatory activity of the 3’TE. S-II m1 was mutated to disrupt the stem base pairing leaving it with 30% of WT 3’ BTE translation activity. SL-III-3 had mutations within the 5 bases believed to be essential for its kissing stem loop interactions with 5’UTR. When tested in the 5’UTR context of a luciferase reporter, SL-III-3 had more efficient translation ability than WT 3’ BTE (164%) (2). SL-III SWAP had two 11-base insertions from the SL-III of isolate PAV129. This hybridized TE had
50% of WT 3’ BTE translation stimulation activity in vitro in both the 5’ UTR and 3’ UTR settings (106,108).

The binding of other mutant 3’ BTE RNAs were compared to their translational efficiencies (Fig. 17). The translational efficiency was determined by the ability of the 3’ BTE elements to function in the 5’ leader of a luciferase reporter or by the ability to inhibit translation as described elsewhere (2,106). Fig.18 shows the fluorescence anisotropy measurements for the binding of eIF4F with 5’-fluorescein labeled 3’ BTE mutants RNA. Fig. 19 shows the Scatchard plot analyses of the binding data as described previously (62) using the Eq. 6:

\[ nK_a - K_aE = E/[eIF4F] = E/(R-E)[BTE]_T \]  

where n is the binding capacity, Ka is the association constant, E is the experimentally determined fractional enhancement of \(^{31}\)BTE RNA anisotropy after saturation with eIF4F, \([eIF4F]\) is the free initiation factor protein concentration, \([BTE]_T\) is the total BTE RNA concentration and R is the eIF4F/BTE molar ratio. The average number of binding sites (n ≈1 for both BTE and BTEBF) was determined from the x-axis intercept of the Scatchard plot E versus E/[eIF4F]. The slope also gives Ka, which is in agreement with Ka values obtained earlier. It was found that the binding of eIF4F to 3’ BTE and mutants expressed as association constants (Kₐs) correlated well with the core translation abilities of the mutants with one exception, BTEBF (Fig. 16). BTEBF was inactive in translation (106), but bound eIF4F with about the same affinity as WT 3’ BTE. This apparent discrepancy is explained below.
5.2 BINDING OF OTHER PROTEINS FROM WHEAT GERM EXTRACT (WGE) WITH BTEBF INHIBITS eIF4F BINDING - EXPLAINING ITS INACTIVITY IN TRANSLATION ASSAYS

All the experiments until now were performed in *in vitro* purified system so we hypothesized what if some component from WGE is playing a role in differential translation abilities of 3’ BTE mutants. Fluorescein labeled 3’ BTE and mutants (50 nM) were incubated in depleted wheat germ extract (d-WGE) (without ribosomes and cap-binding proteins) prepared by incubating the post-ribosomal supernatant with m7GTP-Sepharose 4B resin as described elsewhere (19) and titrated with increasing concentrations of eIF4F. Binding of eIF4F to BTEBF was essentially abolished by the presence of d-WGE (Fig. 17 and 20), whereas eIF4F binding to other 3’ BTE mutants was unaffected. The d-WGE had a small effect on the SL-III-3 mutant. The effect on the SL-III SWAP mutant was not statistically significant at the 95% confidence level. During previously reported pull-down assays to identify 3’ BTE/BTEBF interacting proteins in WGE using biotin labeled RNA, a number of unidentified proteins were also found to be interacting with BTEBF(22). This experiment provides further evidence that binding of other proteins or possibly small molecules from WGE to BTEBF prevents the interaction of eIF4F with BTEBF, thus resulting in its inactivity in *in vitro* translation assays.
In this study, we have shown that the equilibrium binding of eIF4F to 3’ BTE and its mutants correlated well with the translation efficiencies. The weaker the binding of a BTE mutant to eIF4F, the lower was its translation efficiency. This suggests that eIF4F may be the rate-limiting step for BYDV translation as it is believed to be for host cell mRNA translation (22), even though binding is to the 3’ UTR. In *in vitro* studies, the BTEBF mutant bound eIF4F and did not correlate with translation efficiency. Here we show that adding d-WGE inhibits eIF4F binding to BTEBF, while having no effect on WT 3’ BTE binding to eIF4F. The d-WGE had little effect on the stability of the eIF4F with other mutants. Stability under these conditions correlates well with translation for all the mutants tested and WT 3’ BTE. The d-WGE experiments both more closely approximate the in vivo conditions and raise the question as to the nature of the inhibition of BTEBF binding. Earlier pull down experiments (22) and unpublished data from our lab have suggested other proteins bind to the BTEBF, but these are yet to be identified. Similarly, we cannot rule out the possibility that a small molecule or RNA causes the inhibition.
Figure 17: Correlation of eIF4F binding with translation efficiencies. Relative complex stability expressed as association binding constants for binding of eIF4F to 3’ BTE and mutants (gray bars) are shown. Reported translational efficiencies were determined as described elsewhere (2,106). The darker bars indicate the complex stabilities obtained in d-WGE.
**Figure 18:** Equilibrium binding of eIF4F to 3' BTE and mutants. 5' fluorescein labeled 3' BTE and 3' BTEBF binding to eIF4F in absence of d-WGE is shown. The 3'BTE and 3'BTEBF RNA concentration was 50 nM in titration buffer at 25 °C. The excitation and emission wavelengths were 490 nm and 520 nm, respectively. The curves were fit to obtain dissociation constants (Kd) as described in Materials and Methods.
**Figure 19:** Scatchard analyses for binding of 3' BTE and BTEBF with eIF4F showing a one to one binding stoichiometry. The slope also gives $K_a$, which is in agreement with $K_a$ obtained from earlier studies.
Figure 20: d-WGE prevents eIF4F binding to the BTEBF mutant, but does not affect binding to the 3’ BTE and has little effect on other mutants. Fluorescence anisotropy measurements for the binding of eIF4F with 3’ BTE and mutants are shown. The RNA concentration were 50 nM incubated in d-WGE. Other conditions used are same as Fig. 17. K_d values in Table 3.
Figure 21: Pull down assay to identify BTE interacting proteins (22). As can be seen in Lane 8, a number of unidentified proteins were found to be interacting with BTEBF.
**Table 3:** Equilibrium dissociation constants ($K_d$) for the interaction of eIF4F with **β**T RNA and mutants at 25 °C.

<table>
<thead>
<tr>
<th>Complex</th>
<th>$K_d$ (nM) 25°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-WGE</td>
<td>- +</td>
</tr>
<tr>
<td>eIF4F-BTE</td>
<td>29±4 31±3</td>
</tr>
<tr>
<td>eIF4F-BTEBF</td>
<td>33±4 -</td>
</tr>
<tr>
<td>eIF4F-SII-m1</td>
<td>95±18 101±14</td>
</tr>
<tr>
<td>eIF4F-SL-III SWAP</td>
<td>61±8 77±15</td>
</tr>
<tr>
<td>eIF4F-SL-III-3</td>
<td>7.0±0.9 10.0±1.5</td>
</tr>
</tbody>
</table>
CHAPTER 6

6 THERMODYNAMICS OF BINDING OF 3’ BTE AND MUTANTS WITH eIF4F: TEMPERATURE DEPENDENCE OF EQUILIBRIUM BINDING CONSTANTS

6.1 THE BINDING OF eIF4F WITH WT AND MUTANT 3’ BTE RNAs IS ENTHALPICALLY AND ENTROPICALLY FAVORABLE

Next, we investigated the temperature dependence of equilibrium binding constants for 3’ BTE mutants and eIF4F interaction both in purified system as well as in d-WGE. Thermodynamic parameters of enthalpy (ΔH) and entropy (ΔS) were calculated from van’t Hoff plots of ln K_{eq} and the reciprocal of temperature (1/T) as shown in Fig. 27. Eq. (3) and Table 3 values were used to calculate the ΔG_{25°C} values, the slope and the intercept determined ΔH and ΔS values, respectively (Table 5). Fig 22-27 and Table 4 show the temperature dependence plots for 3’ BTE and mutants binding to eIF4F and the corresponding equilibrium binding constants (K_{d}) values as obtained. The van’t Hoff analyses showed that the eIF4F interaction with 3’ BTE and mutants is enthalpically and entropically favorable with the enthalpic contribution of 52-90 % to ΔG° at 25°C and is much more favorable than m⁷ GTP binding to eIF4F (123) and more favorable than binding to the pseudoknot PK₁ of the 5’UTR of TEV. 3’ BTE and mutants showed similar thermodynamic properties with the exception of SL-III-3 which had a much lower entropy contribution to binding. The higher enthalpic value suggests additional hydrogen bonds between the eIF4F and 3’ BTE mutant.
Figure 22: Temperature dependence of binding of BTEBF with eIF4F. BTEBF interacts with eIF4F only in absence of d-WGE. The ψBTEBF RNA concentration was 50 nM in titration buffer at different temperatures. The excitation and emission wavelengths were 490 nm and 520 nm, respectively. The curves were fit to obtain dissociation constants (K_d) values as described in materials and methods.
Figure 23: Temperature dependence of binding of 3' BTE with eIF4F. A, in absence B, in presence of d- WGE. The F1BTE RNA concentration was 50 nM in titration buffer at different temperatures. The RNA was incubated in depleted WGE for at least 15 minutes. Other conditions same as Fig. 22.
Figure 24: Temperature dependence of binding of SII- m1 with eIF4F. A, in absence B, in presence of d-WGE. The experimental conditions were same as stated in Fig. 22.
Figure 25: Temperature dependence of binding of SL-III-SWAP with eIF4F. A, in absence B, in presence of d-WGE. The experimental conditions were same as stated in Fig. 22.
**Figure 26:** Temperature dependence of binding of SL-III-3 with eIF4F. A, in absence B, in presence of d- WGE. For SL-III-3 mutant, the studies were done at temperatures higher than 25°C because of uncertainties associated with measuring Kd values lower than 5 nM. The other conditions were same as stated in Fig. 22.
Figure 27: Van't Hoff plots for the interaction of βBTE and mutants with eIF4F. A, in absence B, in presence of d-WGE. The lnK_{eq} values were plotted against different temperatures (1000/T). Enthalpy (ΔH) and entropy (ΔS) were calculated from the slope and intercept, respectively using Equation 2 and 3.
Table 4: Equilibrium dissociation constants ($K_d$) for the interaction of eIF4F with $^3$BTE RNA and mutants.

<table>
<thead>
<tr>
<th>Complex</th>
<th>$K_d$ (nM)</th>
<th>5°C</th>
<th>15°C</th>
<th>25°C</th>
<th>30°C</th>
<th>35°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-WGE</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>eIF4F·BTE</td>
<td>15±3</td>
<td>16±2</td>
<td>23±4</td>
<td>23±3</td>
<td>29±4</td>
<td>31±3</td>
</tr>
<tr>
<td>eIF4F·BTEBF</td>
<td>16±3</td>
<td>-</td>
<td>23±3</td>
<td>-</td>
<td>33±4</td>
<td>-</td>
</tr>
<tr>
<td>eIF4F·SII-m1</td>
<td>49±9</td>
<td>56±8</td>
<td>77±14</td>
<td>75±12</td>
<td>95±18</td>
<td>101±14</td>
</tr>
<tr>
<td>eIF4F·SL-III SWAP</td>
<td>28±5</td>
<td>31±7</td>
<td>44±3</td>
<td>46±4</td>
<td>61±8</td>
<td>77±15</td>
</tr>
<tr>
<td>eIF4F·SL-III-3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>7.0±0.9</td>
</tr>
</tbody>
</table>
Table 5: Thermodynamic parameters for the interaction of eIF4F with $^{53}$BTE RNA and mutants.

<table>
<thead>
<tr>
<th>RNA-eIF4F</th>
<th>$\Delta G^\circ$ (kJ mol$^{-1}$)</th>
<th>$\Delta H$ (kJ mol$^{-1}$)</th>
<th>$\Delta S$ (J mol$^{-1}$ K$^{-1}$)</th>
<th>$\Delta S^\circ/\Delta G$ percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>d-WGE</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BTE</td>
<td>-43.0±0.1</td>
<td>-42.9±0.1</td>
<td>-22.3±2.5</td>
<td>-22.7±0.7</td>
</tr>
<tr>
<td>S-II m1</td>
<td>-40.1±0.2</td>
<td>-39.9±0.1</td>
<td>-23.4±3.9</td>
<td>-20.6±1.1</td>
</tr>
<tr>
<td>SL-III SWAP</td>
<td>-41.2±0.1</td>
<td>-40.6±0.2</td>
<td>-25.8±1.2</td>
<td>-31.1±3.7</td>
</tr>
<tr>
<td>SL-III -3</td>
<td>-46.6±0.1</td>
<td>-45.6±0.2</td>
<td>-42.1±1.2</td>
<td>-40.6±2.9</td>
</tr>
<tr>
<td>PK1(62)</td>
<td>-38.2±0.2</td>
<td>-</td>
<td>-15.5±1.5</td>
<td>-</td>
</tr>
<tr>
<td>m$^7$GTP(123)</td>
<td>-30.6±0.8</td>
<td>-</td>
<td>28.7±0.7</td>
<td>-</td>
</tr>
</tbody>
</table>
6.2 DISCUSSION

While there is no high resolution three dimensional structure of the 3’ BTE available, the sequence and secondary structure of the 3’ BTE mutants provide some interesting insight. The secondary structure suggests a tRNA like folding. On examining the various mutants, the SL-III-3 has quite different properties. SL-III-3 had mutations in the five bases which form the kissing stem loop interaction with 5'SL-D and when placed in the 5’ context of the reporter gene, SL-III-3 translated more efficiently (164%) than WT (2) and also bound eIF4F tighter than WT. The fact that the sequence change, which does not alter the secondary structure, increases binding almost 2-fold reveals the possibility that one or more of these bases may be involved in hydrogen bonding to the eIF4F. This is consistent with the distinctly lower entropy obtained for this mutant-eIF4F interaction. Only ~10% of the binding free energy, ΔG, comes from the entropy term, whereas for WT 3’ BTE, almost 50% of the binding ΔG is attributable to entropy (at 25°C). Recently published BzCN footprinting data for eIF4F subunits indicate that the bases 4841-4853 on SL-I and bases 4895-4898 on SL-III of 3’ BTE are protected from BzCN modification (109). This suggests that eIF4F is binding 3’ BTE on its central junction from where all the stem loops emerge (Fig. 5) (109). S-II m1 was mutated to disrupt the stem base pairing (106). This region is also close to the junction and may alter the RNA folding, resulting in lower eIF4F binding affinity and consequently translation. The eIF4F binding to S-IIm1 was about 3-fold lower than binding to WT 3’ BTE and the translation was about 30% (Table 4). SL-III SWAP had an extended SL-III with 22 base insertion from SL-III of isolate PAV129, which translates efficiently. This construct maintained the eIF4F contact sites and likely has a similar three dimensional structure as WT 3’ BTE. The translation and binding efficiencies were about 50% of WT 3’ BTE. This reduction may be due to changes in the SL-III loop which is complementary to the 5’ SL-D. By moving the loop further from the junction region, subtle contacts could be disrupted. The SL-III-3 mutant suggests these contacts play some role in eIF4F binding. The
enthalpic contribution to $\Delta G^\circ$ is (52-90%) at 25°C for eIF4F-3’ BTE and mutants binding. This is much more favorable than eIF4F interaction with m$^7$GTP as shown in Table 5. In contrast, we know that cap binding is entropically driven due to large conformational changes in the eIF4E subunit. However, PK1 (IRES of Tobacco Etch Virus) and 3’ BTE RNA bind to the eIF4G subunit of eIF4F (21,22) with similar thermodynamic properties, suggesting similar mechanisms of interaction.

While it has become evident that the interaction of mammalian eIF4G with mRNA is required for efficient mRNA recruitment and translation initiation (124,125), the role of eIF4G binding to the 3’ UTR in the plant virus system is much less understood. Experiments are underway in our lab using eIF4G deletion mutants to map the binding site of BTE on the eIF4F. Our data show that eIF4F binding to the 3’ UTR plays an important role in efficient BYDV mRNA translation. Interestingly, binding of the eIF4F to BYDV 3’ BTE is much more stable than binding to either the TEV IRES or the 5’ mRNA cap. These findings suggest that binding of eIF4F to the 3’ UTR may serve the dual purpose of recruiting ribosomes and sequestering host cell eIFs for viral translation. Further, binding to the 3’ UTR may play a role in the viral switch from replication to translation by blocking the polymerase to favor translation of the mRNA. The mechanism of transfer from the 3’ UTR to the 5’ UTR for translation remains to be investigated.
CHAPTER 7

7 BIOPHYSICAL CHARACTERIZATION OF WHEAT eIF4B\textsubscript{320-527} MUTANT WITH ZINC USING CIRCULAR DICHROISM

7.1 INTRODUCTION

7.1.1 WHEAT eIF4B\textsubscript{320-527}

eIF4B (320-527) is a deletion mutant of full-length GST-eIF4B- (1-527), and contains the C-terminal RNA binding domain and is GST tagged. It is known that the region located between the amino acid residues 320 and 360 contains the domain which is essential for eIF4B homodimerization (56). This region also includes C-terminal RNA-binding domain. The map of various eIF4B deletion mutants used in the project are shown below in Fig. 28. For this set of experiments, only eIF4B\textsubscript{320-527} was used.

<table>
<thead>
<tr>
<th>eIF4B Deletion Mutant</th>
<th>RNA Binding</th>
<th>eIF4A Binding</th>
<th>eIF504G Binding</th>
<th>Tryptophan residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-527</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>9</td>
</tr>
<tr>
<td>69-527</td>
<td>+++</td>
<td>+++</td>
<td>+</td>
<td>7</td>
</tr>
<tr>
<td>320-527</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3</td>
</tr>
<tr>
<td>69-360</td>
<td>+++</td>
<td>+++</td>
<td></td>
<td>4</td>
</tr>
</tbody>
</table>

Figure 28: Different eIF4B mutants and their properties (71) .
7.1.2 CIRCULAR DICHROISM (CD)

CD spectroscopy is a form of light absorption spectroscopy that measures the difference in absorbance of right- and left-circularly polarized light by a substance. Circular dichroism technique is very sensitive to the secondary structure of proteins and polypeptides (126). CD spectra between 260 -≈180 nm is required for analysis of different secondary structural types such as alpha helix, parallel and antiparallel beta sheet, turn, or random coil etc. Traditionally, ellipticity is the unit of circular dichroism and is defined as the tangent of the ratio of the minor to major elliptical axis. The most commonly used CD units in current literature are mean residue ellipticity (degree cm²dmol⁻¹) and the difference in molar extinction coefficients called the molar circular dichroism or delta epsilon (liter mol⁻¹cm⁻¹). Modern CD instruments are capable of milli-degree precision. Several methods have been developed which analyze the experimental CD spectra using a database of reference protein CD spectra containing known amounts of secondary structure (127,128). There are two essential requirements to successful secondary structure analysis. First is that the CD spectra should be recorded from 260 nm - 184 nm at least or if possible lower up to 178 nm (129) and the other is to have accurate protein concentration (essentially < 10% error) is important. (129). Modern secondary structure determination by CD are reported to achieve accuracies of 0.75 for beta sheet, 0.97 for helices, 0.50 for turns, and 0.89 for other structure types (127).

CD spectroscopy can also be used for the determination of tertiary structure of globular proteins. The method proposed by Venyaminov & Vassilenko (130) claims very high accuracy for predicting all alpha, alpha/beta, and denatured proteins, 85% for alpha & beta and 75% for all beta proteins.
CD spectroscopy is very often used for structural characterization of peptides and proteins. The application of CD for conformational studies in peptides can be categorized into following applications

i. Estimation of secondary structural content under any given conditions. For instance, a peptide is 50% helical.

ii. Monitoring conformational or structural changes e.g., monomer-oligomer, substrate binding, denaturation, etc.
7.2 MATERIALS AND METHODS

7.2.1 PURIFICATION OF eIF$_{4B}^{320-527}$

eIF$_{4B}^{320-527}$ GST-tagged pET3d-eIF4B plasmid for the protein was a generous gift from Dr. D.R. Gallie, University of California, Riverside, CA. Protein expression was performed in *E.coli* BL21 (DE3) pLysS, selected using ampicillin. In brief, a single colony obtained from overnight grown LB fresh plates selected with 100 μg/ml ampicillin was used to grow 10 mL of LB media overnight at 37 °C. This culture was transferred to a liter of LB media selected with ampicillin and was incubated at 37 °C, grown until A$_{600}$= 0.6 and was induced with 1 mM IPTG (isopropyl 1-thio-β-d-galactopyranoside) overnight. The cells were harvested by centrifugation at 6000 rpm for 15 min at 4°C. Pelleted cells were resuspended in Binding buffer (PBS, pH 7.4 along with 140 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$, pH 7.4) sonicated and ultra-centrifuged to remove cell debris. The supernatant was applied to 1mL GSTrap 4B column and purified according to manufacturer’s specifications. All buffers used in protein purification and the supernatant prior to loading on the column were passed through a 0.45 μM filter (Millipore). Purified protein was dialyzed against Titration Buffer (10mM Na-phosphate and 100mM NaCl, pH=7.4) and was filtered using a 0.22 μM PVDF Millex (Millipore) filter, concentration calculated using Bradford’s method before the CD measurements were performed.
7.2.2 CD SPECTROSCOPY

A Jasco model J730 spectropolarimeter equipped with Peltier temperature controller was used for CD measurements at 25 °C. Spectra were acquired from 190 to 260 nm using a bandwidth of 1 nm, path length of 1mm and at a scan speed of 100 nm/min with 10 acquisitions per sample. The protein concentration was 0.4mg/ml for all spectra acquisition. Spectra were corrected for buffer contribution and the CD signal was converted to mean residue ellipticity (MRE) in degcm²dmol⁻¹, MRE = (θ/10*n*Cp*l), where θ is the observed ellipticity in mdeg, n is number of peptide bonds, Cp is the molar concentration and l is the pathlength in cm. The helical content of proteins was calculated from the MRE value at 222nm using the following equation 7:

\[ \% \alpha \text{ helix} = \left\{ \frac{(- \text{MRE}_{222} - 2340)}{30300} \right\} \times 100 \quad (7) \]

CD spectra were also analyzed using K2d3 software(131)
7.3 RESULTS

7.3.1 CD ANALYSIS OF INTERACTION OF eIF4B\textsubscript{320-527} AND Zn & Mg

To better understand how the binding of zinc affects eIF4B\textsubscript{320-527} secondary structure, a CD analysis experiment was conducted. The addition of zinc (from a stock 10 mM ZnCl\textsubscript{2} solution) to eIF4B\textsubscript{320-527} resulted in a change in CD spectra (Figure 29). The circular dichroism analysis using K2D3 algorithm revealed that addition of zinc to eIF4B\textsubscript{320-527} results in a change in alpha helical content of eIF4B\textsubscript{320-527}. eIF4B\textsubscript{320-527} contains approximately 38% alpha helix in its secondary structure (Table 6). The addition of increasing amount of zinc resulted in ≈ 70% decrease in alpha helical content of the protein (Table 6). After addition of 500 μM of zinc, no further significant changes were observed in CD spectra of eIF4B\textsubscript{320-527} (Figure 29). As a control, titrations were repeated by addition of magnesium (from a stock 10 mM MgCl\textsubscript{2} solution) to eIF4B\textsubscript{320-527} which also resulted in a change in CD spectra and almost 37% decrease in alpha helical content (Figure 30) and Table 7.
Figure 29: CD spectra for the titration of eIF4B<sub>320-527</sub> and different concentrations of Zn<sup>2+</sup>. The y axis shows the CD signal which is observed ellipticity θ in mdeg.
Figure 30: CD spectra for the titration of eIF4B$_{320-527}$ and different concentrations of Mg$^{+2}$. The y axis shows the CD signal which is observed ellipticity $\theta$ in mdeg.
Table 6: Secondary structure calculations for titrations of eIF4B$_{320-527}$ and different concentrations of Zn$^{2+}$.

<table>
<thead>
<tr>
<th>Zn$^{2+}$ [µM]</th>
<th>Theoretical</th>
<th>K2D3 analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% α</td>
<td>% α</td>
</tr>
<tr>
<td><strong>Protein only</strong></td>
<td>23</td>
<td>39</td>
</tr>
<tr>
<td>100</td>
<td>17</td>
<td>33</td>
</tr>
<tr>
<td>200</td>
<td>13</td>
<td>27</td>
</tr>
<tr>
<td>500</td>
<td>8</td>
<td>12</td>
</tr>
</tbody>
</table>
Table 7: Secondary structure calculations for titrations of eIF4B\textsubscript{320-527} and different concentrations of Mg\textsuperscript{2+}.

<table>
<thead>
<tr>
<th>Mg\textsuperscript{2+} [µM]</th>
<th>Theoretical</th>
<th>K2D3 analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% α</td>
<td>% α</td>
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<tr>
<td>Protein only</td>
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<td>38</td>
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<td>32</td>
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<td>200</td>
<td>23</td>
<td>30</td>
</tr>
<tr>
<td>500</td>
<td>17</td>
<td>24</td>
</tr>
</tbody>
</table>
Zinc is known to have a preference for the imidazole ring of histidine residues in proteins just like other divalent ions Ni\(^{+2}\) and Co\(^{+2}\) which are used in metal affinity chromatography (132). Full length eIF4B has seven histidine residues that could potentially interact with zinc and it is suspected to be a metalloprotein (50). Using sequence analysis it was found that out of seven histidine residues, six are present in the C-terminal region of the protein from residue number 320. Interaction with these six His residues may explain why the effect of zinc is very prominent (almost 70% change in alpha helical content) on the mutant eIF4B\(_{320-527}\). There is some alpha content change observed in presence of magnesium as well but the change is not so pronounced when compared to effects of zinc. The changes in secondary structure may be one of the causes for the eIF4B self-association stimulation caused by zinc. It is known that eIF4B is not a zinc finger protein, yet in our experiments we could see significant change is secondary structure of eIF4B by presence of zinc. Also zinc enhances eIF4B-PABP interactions (50). This suggests that zinc may be playing an important role during cellular protein synthesis and these interactions modulated by zinc can be important therapeutic targets.
APPENDIX 1 RECOMBINANT PROTEIN PURIFICATION AND DETECTION

1.1 eIF4F PURIFICATION

The buffers used in the purification are:

Buffer B-0: 20 mM HEPES, 10% glycerol, 1 mM EDTA, 1 mM DTT and KCl is added as required.

Columns used:

1. Phosphocellulose column
2. mGTP Sepharose column

FLOWCHART OF PURIFICATION:

Wheat eIF4F pET 3d plasmid is transformed into BL21(DE3) cells. Fresh transformations work well. Do not use a plate that is more than a few days old for this clone.

Grow 50 mL (4 No.s) overnight cultures at 30°C. Also the large (uninoculated) media flasks should be left pre-warming and shaking overnight at 30°C.

Use ~45ml ON culture to induce each of 4@1.2 liters (of 1.5X LB) in 6 L flasks. Use dry air shaking baths. Grow at 30°C to OD600 of 0.8-1.
Induce with 0.5mM IPTG. Harvest after 3 hours by centrifuging at 6000 rpm for 15 min.

Resuspend cells in 50 ml B-0 + 0.15 M [KCl] with 5 mg STI, 500ul of 10mg/ml PMSF in IPA, and 1 lg Roche protease inhibitor tablet. Keep on ice and sonicate as described earlier.

Centrifuge for 45 min @ 40,000 RPM in ultracentrifuge.

Dilute (batchwise) to [KCl]~0.1M and load onto 2 @ 10 ml PhosphoCellulose columns equilibrated in B-0 + 0.1 M [KCl]. Pool columns to wash.

Elute with B-0 + 0.3 M [KCl].

Dilute desired fractions batchwise to [KCl]~0.15. Load onto 4 ml m7GTP Sepharose column. Wash with B-0 + 0.1 M [KCl]. Raise baseline using ~10ml B-0 + 0.1 M [KCl] + 100µM GTP.

Elute with ~20 ml B-0 + 0.1 M [KCl] + 100µM m7GTP.

Concentrate and remove m7GTP using 3 ml Phosphocellulose column. Load/wash in B-0 + 0.1 M [KCl]. Elute with B-0 + 0.3 M [KCl].

eIF4G is VERY susceptible to degradation until it is clean on the PC. All freezes are fast freezes. Protein never gets above ice cold. Columns run at 4°C. Keep feed flasks on ice. After purification, a SDS-PAGE Gel (Fig. 31) was run to check the purity of the protein.
**Figure 31:** SDS Gel showing purified eIF4F and its comparison to purified eIF4F at Dr. Browning’s lab (115).
APPENDIX 2 RNA PURIFICATION AND 5’ FLUORESCEIN LABELING

STEP1: It is recommended that before setting up 1 mL Transcription mix, a 20 µL transcription should be run to ensure transcription success and minimization of reagents wastage. The transcription success can be verified by running a 10% PAGE 8.3 X 7.3 cm, 0.75 mm gel after treating the sample with DNase as shown in Fig. 31. This will also ensure that the position of expected RNA band is known for later use during excision using UV shadowing of the bigger 18 X 15 cm, 3 mm PAGE. The following protocol can be used for setting up 20 µL transcription:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume added (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 X transcription buffer</td>
<td>2 (1X)</td>
</tr>
<tr>
<td>ATP (Stock 100 mM)</td>
<td>1 (5 mM)</td>
</tr>
<tr>
<td>GTP (Stock 100 mM)</td>
<td>1 (5 mM)</td>
</tr>
<tr>
<td>CTP(Stock 100 mM)</td>
<td>1 (5 mM)</td>
</tr>
<tr>
<td>UTP(Stock 100 mM)</td>
<td>1 (5 mM)</td>
</tr>
<tr>
<td>GMP(Stock 100 mM)</td>
<td>1 (5 mM)</td>
</tr>
<tr>
<td>MgCl₂(Stock 2 M)</td>
<td>0.25(25 mM)</td>
</tr>
<tr>
<td>dsDNA (Stock 5 µM)</td>
<td>0.8 (200nM)</td>
</tr>
<tr>
<td>T7 polymerase (1:200)</td>
<td>1</td>
</tr>
<tr>
<td>RNase free water</td>
<td>10.95</td>
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</tbody>
</table>
Mix and incubate for 2 hours at 37 °C. Add 1 μL of DNase (NEB) and incubate for another 15 minutes at 37 °C. Mix 5 μL of the transcription mix + 2.5 μL of the Ambion 2X loading dye and run the gel in 1X TBE for 2 hours at 100 V. Stain with ethidium bromide solution to visualize. The DNA is prepared by annealing the single strands at 90°C for 2 minutes in TE buffer and then slow cooling to room temperature. The composition of 10X Transcription buffer is listed in Chapter 2 under section 2.3. After dissolving required amount of NTPs in RNase free water, the final pH is adjusted to pH 5 using 1M NaOH with pH paper as an indicator.

**STEP2: SETTING UP 1 mL Transcription Mix**

After 20 μL transcription reaction is successful, the following reagents were mixed in the given proportions and incubated overnight at 37 °C.
**STEP 3:** The reaction is stopped by adding 50 mM EDTA and rest of the steps are followed as listed in section 1.3 of Chapter 2. In some of the transcription reactions, the RNA precipitated out of the gel particles was first filtered using 0.45 µm filter and then concentrated by spinning at 6000 rpm using Amicon ultra 15 mL10 KD cutoff filter prior to ethanol precipitation.

**STEP 4:** The concentration of RNA was calculated using Nanodrop 2000. A spectra of pure SII-m1 RNA sample after transcription is shown in Fig.33. The RNA was labeled as per manufacturer’s directions of Vector Labs, Burlingame, CA 5’end tag labeling kit as described in Chapter 2. After labeling and purification, the absorbance spectra is shown in Fig.34.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume added (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X transcription buffer</td>
<td>100 (1X)</td>
</tr>
<tr>
<td>ATP (Stock 100 mM)</td>
<td>50 (5 mM)</td>
</tr>
<tr>
<td>GTP (Stock 100 mM)</td>
<td>50 (5 mM)</td>
</tr>
<tr>
<td>CTP (Stock 100 mM)</td>
<td>50 (5 mM)</td>
</tr>
<tr>
<td>UTP (Stock 100 mM)</td>
<td>50 (5 mM)</td>
</tr>
<tr>
<td>GMP (Stock 100 mM)</td>
<td>50 (5 mM)</td>
</tr>
<tr>
<td>MgCl₂ (Stock 2 M)</td>
<td>12.5 (25 mM)</td>
</tr>
<tr>
<td>dsDNA (Stock 5 µM)</td>
<td>40 (200nM)</td>
</tr>
<tr>
<td>RNase OUT</td>
<td>1</td>
</tr>
<tr>
<td>Pyrophosphatase</td>
<td>3</td>
</tr>
<tr>
<td>T7 polymerase (1:200)</td>
<td>10</td>
</tr>
<tr>
<td>RNase free water</td>
<td>696</td>
</tr>
</tbody>
</table>
Figure 32: 10% Polyacrylamide gel showing purified BTE RNA and corresponding marker.
Figure 33: A spectra of purified SII-m1 RNA as read by Nanodrop 2000.
Figure 34: A spectra of purified fluorescein labeled SII-m1 RNA as read by Nanodrop 2000.
BIBLIOGRAPHY


70. Khan, M. A., and Goss, D. J. (2005) Translation initiation factor (eIF) 4B affects the rates of binding of the mRNA m7G cap analogue to wheat germ eIFiso4F and eIFiso4F.PABP. *Biochemistry* **44**, 4510-4516


