Recruitment of the ribosomal 40S subunit to the 3'untranslated region of a viral mRNA, via the eIF4 complex, facilitates cap-independent translation

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by

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Abstract

Recruitment of the ribosomal 40S subunit to the 3’ untranslated region of a viral mRNA, via the eIF4 complex, facilitates cap-independent translation.

by

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Translation of uncapped plant viral RNAs can be facilitated by either an internal ribosomal entry site (IRES) in the 5’ untranslated region (UTR) or a cap-independent translation element (CITE) in the 3’ UTR. Barley yellow dwarf virus (BYDV) mRNA, which lacks both cap and poly(A) tail, has a translation element (3’BTE) in its 3’ UTR that is essential for efficient translation initiation at the 5’-proximal AUG. This mechanism requires binding of the eukaryotic initiation factor 4G (eIF4G) subunit of the heterodimer eIF4F to the 3’BTE and base pairing between the 3’BTE and the 5’ UTR. Here we investigate how this interaction recruits the ribosome to the 5’ end of the mRNA. Using fluorescence anisotropy, SHAPE analysis and toe printing, we found that (i) 40S ribosomal subunits bind to the 3’BTE, (ii) the helicase complex eIF4F-eIF4A-eIF4B-ATP increases affinity of 40S subunit binding to the conserved SL-I of the 3’ BTE.
by exposing more unpaired bases of the 3’BTE and (iii) long-distance base pairing transfers this complex to the 5’ end of the mRNA where translation initiates. These results reveal an utterly novel mechanism of ribosome recruitment to an mRNA.
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## Contents

Abstract ........................................................................................................... iii

1  INTRODUCTION ......................................................................................... 1
   1.1  OVERVIEW OF TRANSLATION ................................................................. 1
   1.2  5’ CAP-DEPENDENT TRANSLATION INITIATION MODEL ...................... 2
       1.2.1  5’ Capped structure recognition ...................................................... 2
       1.2.2  43S pre-initiation complex formation .............................................. 2
       1.2.3  Attachment of 43S complexes to mRNA .......................................... 3
   1.3  INTERNAL RIBOSOME ENTRY SITE (IRES) MEDIATED CAP-INDEPENDENT
        TRANSLATION 5
       1.3.1  Overview: ....................................................................................... 5
       1.3.2  Structural Features in IRESs ............................................................ 8
   1.4  3’ UTR MEDIATED CAP-INDEPENDENT TRANSLATION ................. 13
       1.4.1  Translation in Barley Yellow Dwarf Virus (BYDV) ............................ 13
       1.4.2  Pathogen Biology ........................................................................... 18
   1.5  FACTORS INVOLVED IN BYDV TRANSLATION .............................. 19
       1.5.1  eIF4F .............................................................................................. 19
       1.5.2  eIF4A and eIF4B ............................................................................ 20
   1.6  EUKARYOTIC SMALLER RIBOSOME (40S) UNIT .............................. 23

2  ELEMENTS OF RNA STRUCTURE ......................................................... 27
2.1 OVERVIEW.................................................................................................................27

2.1.1 Primary structure.................................................................................................28

2.1.2 Secondary structure.............................................................................................29

2.1.3 Tertiary structure................................................................................................29

2.2 FUNCTION OF METAL IONS IN RNA STRUCTURE AND FUNCTION..................32

2.3 SECONDARY STRUCTURE DETERMINATION BY STRUCTURE PROBING

EXPERIMENTS ................................................................................................................33

2.3.1 Introduction...........................................................................................................33

2.3.2 Step 1: RNA Modification by SHAPE Reagents................................................34

2.3.3 Step 2. Primer extension of the modified RNA....................................................35

2.4 METHODS AND MATERIALS FOR SHAPE .........................................................38

2.4.1 RNA design..........................................................................................................38

2.4.2 RNA synthesis.....................................................................................................38

2.4.3 RNA- folding........................................................................................................39

2.4.4 RNA modification...............................................................................................39

2.4.5 Primer design and labeling..................................................................................40

2.4.6 Primer extension..................................................................................................40

2.5 RESULTS ..................................................................................................................42

Structural analysis of RNA.............................................................................................48

3 BINDING STUDIES ........................................................................................................49

3.1 OVERVIEW................................................................................................................49

viii
3.1.1 Fluorescence quenching ................................................................. 50
3.1.2 Dynamic or collisional quenching .................................................. 50
3.1.3 Static quenching ........................................................................... 51
3.2 Fluorescence anisotropy ................................................................... 51
3.3 Gel mobility shift assay ..................................................................... 59
3.4 Materials and methods ...................................................................... 60
  3.4.1 End labeling of mRNAs ................................................................. 60
  3.4.2 Fluorescence quenching experiments .......................................... 62
  3.4.3 Fluorescence anisotropy experiments ........................................... 62
  3.4.4 Gel mobility shift assay ............................................................... 63
3.5 Results ............................................................................................... 65
  3.5.1 eIF4F Binding to Fluorescein Labeled 3’BTE and 3’BTEBF RNA .... 65
  3.5.2 eIF4F Binding to Fluorescein Labeled 5’UTR .............................. 66
  3.5.3 40S ribosomes binding to 3’BTE and its translationally inactive mutant 3’BTEBF 67
  3.5.4 40S ribosomes binding to 5’UTR and 5’SL-D RNA ..................... 68
  3.5.5 Effect of eIF4F on Binding of 40S Ribosome to 3’BTE, 3’BTEBF RNA 69
  3.5.6 Effect of eIF4F on Binding of 40S Ribosome to 5’UTR and 5’SL-D RNA 70
  3.5.7 Effect of eIF4F-4B-4A-ATP (Helicase complex) on Binding of 40S Ribosome to 3’BTE, 3’BTEBF mRNAs 71
3.5.8 Binding of 40S Ribosomes to 3’BTE mRNAs in the presence of complex

\( \text{elF4F-4B-4A-non hydrolyzable ATP (ADP-PNP)} \) ................................................................. 72

3.5.9 Binding of 40S Ribosomes to 5’UTR and 5’S3L-D mRNAs in the presence of helicase complex elF4F-4B-4A-ATP................................................................. 73
gel mobility shift assays ........................................................................................................ 76

4 PRIMER EXTENSION INHIBITION BY REVERSE TRANSCRIPTION ............. 77

4.1 OVERVIEW ......................................................................................................................... 77

4.2 METHODS AND MATERIALS ............................................................................................ 83

4.2.1 Primer design and synthesis ....................................................................................... 83

4.2.2 Toe-printing assays ...................................................................................................... 84

4.3 RESULTS ........................................................................................................................... 86

4.3.1 Toe-printing on BYDV RNA ...................................................................................... 86

TWO POSSIBLE MECHANISMS FOR RIBOSOMES RECRUITMENT .............. 88

4.3.2 \( \text{elF4A, 4B, 4F, ATP and 3’BTE are necessary for ribosome binding to the 5’ end of the message} \) ................................................................................................. 89

5 STRUCTURAL PROBING SHOWS HELICASE TREATMENT INCREASES

RNA ACCESSIBILITY ............................................................................................................. 98

6. DISCUSSION ......................................................................................................................... 100

7 FUTURE DIRECTIONS ......................................................................................................... 106

7.1 OVERVIEW ......................................................................................................................... 106

x
List of Figures

Figure 1.1 Model of the canonical pathway of eukaryotic translation initiation ........................................ 4
Figure 1.2 Comparison of cap-dependent translation and four IRES groups .............................................. 7
Figure 1.3 Structures of the Dicistroviridae intergenic region (IGR) IRES RNAs ....................................... 10
Figure 1.4 The pathway of HCV IRES-mediated translation initiation ...................................................... 12
Figure 1.5 Genome organizations of BYDV showing secondary structures of long-distance interacting regions .................................................................................................................. 16
Figure 1.6 Secondary structure of 3’BTE .................................................................................................. 17
Figure 1.7 Life-cycle of BYDV vector aphid ............................................................................................. 18
Figure 1.8 Schematic representation of eIF4G and its mutants showing regions important for eIF4G– 3’BTE interaction .......................................................................................................................... 21
Figure 1.9 Mapping of the eIF4G-binding sites on the BYDV 3’BTE .......................................................... 22
Figure 1.10 Crystal Structure of 40S ....................................................................................................... 25
Figure 2.1 Common bases found in Nucleic acids are shown, adenine (A), guanine (G), cytosine (C), uracil (U). ........................................................................................................................................ 30
Figure 2.2 (A) Primary, secondary and tertiary structure of yeast phenylalanine tRNA (tRNA\textsubscript{Phe}) ....... 31
Figure 2.3 Mechanism of RNA SHAPE chemistry with BzCN .................................................................... 36
Figure 2.4 Primer extension stops due to RNA adduct formation exactly one nucleotide prior to RNA modification generate a structure specific cDNA library ........................................................................ 37
Figure 2.5 The BYDV 5’UTR modification patterns generated using the SHAPE reagent benzoyl cyanide (BzCN) are shown ............................................................................................................... 42
Figure 2.6 SHAPE reactivities as a function of nucleotide position analyzed by SAFA ................................. 43
Figure 2.7 Superposition of absolute BzCN reactivities on a secondary structure model of BYDV 5’UTR. 44
Figure 3.1 Schematic diagram for measurement of fluorescence anisotropies ............................................. 53
Figure 3.2  Excited-state distribution for immobile fluorophores with $r_0 = 0.4$. ............................................ 55
Figure 3.3 Schematic diagram for L-format measurements of fluorescence anisotropy. ................................. 58
Figure 3.4 Enzymatic pathway of 5’ end labeling of any nucleic acid is shown .................................................. 61
Figure 3.5 (A) eIF4F binding to 3’BTE ($\triangleright$) and 3’BTEBF ($\triangleright$). .................................................. 65
Figure 3.6 eIF4F binding to 5’UTR ($\triangleright$) .......................................................................................... 66
Figure 3.7 Ribosome binding to 3’BYDV-RNA ......................................................................................... 67
Figure 3.8 Ribosome binding to 5’BYDV-RNA ......................................................................................... 68
Figure 3.10 eIF4F didn’t show a significant change on 40S binding affinity with 5’UTR and 5’SL-D. ...... 69
Figure 3.9 eIF4F alone didn’t show a significant effect on 40S binding affinity with 3’BTE .................. 70
Figure 3.11 A combination of eIF4A-4B-4F (helicase complex) and ATP enhanced the binding affinity of 40S for 3’BTE-RNA nearly three-fold ......................................................................................... 71
Figure 3.12 Helicase complex didn’t show effect on 40S binding in the presence of non-hydrolysable ATP analog ADP PNP ......................................................................................................................... 72
Figure 3.13 A combination of eIF4A-4B-4F (helicase complex) and ATP showed no or non-significant binding enhancement for 5’UTR BYDV or 5’SL-D mRNAs. ................................................................. 74
Figure 3.14 Helicase treatment of BYDV 3’UTR increases 40S ribosome binding shown in 2% agarose-2% polycrylamide native composite gel .............................................................................................. 74
Figure 4.1 Illustration of one toe-printing experiment .................................................................................. 79
Figure 4.2 The mRNA pathway on 40S subunits showing the entry channel or aminoacylation site (A), the exposed interface surface peptidylation site (P), and the exit channel (E). ......................................................... 80
Figure 4.3 Mechanisms of action of cycloheximide .................................................................................. 81
Figure 4.4 Canonical mechanism of 48S complex formation ..................................................................... 82
Figure 4.5 Plasmid map of BLucB showing different primer binding site ................................................. 83
Figure 4.6 Genomic organization of BLucB mRNA ............................................................................... 91
Figure 4.7  80S complex formation on capped 5‘UTR BYDV mRNAs. 92

Figure 4.8 80S complex formations on 5’UTR BYDV mRNAs depends on the presence of 3’BTE. 93,94

Figure 4.10 Toe-printing in SL-I 95

Figure 4.11 Activities of eIFs 4A/4B/4F, ATP to recruit ribosome in the 3’BTE 3’SL-I region. 96

Figure 4.12 Activities of eIFs 4A/4B/4F, PABP, and iso4F in the presence and absence of 3’BTE in promoting scanning through 5’-UTRs. 97

Figure 5.1 Secondary structure of 3’BTE determined by SHAPE reactivity 99

Figure 6 BYDV translation model 105

Figure 7.1 Schematic representation of the spectral overlap integral. 107

Figure 7.2 Optical setups for single-molecule detection studies using a prism-based TIRF microscope. The inset depicts a surface tethered labelled bio-molecules located in the evanescent field of the system. 111

Figure 7.3 Cy3-Cy5 BLuCB construct hybridized to biotin-oligo. Traces showing Cy3-Cy5 anti-correlated dynamics with only green laser excitation, indicating FRET. 112

Figure 7.4 Cy3-Cy5 BLuCB construct hybridized to biotin-oligo. Traces showing Cy3-Cy5 dynamics with only green laser excitation, no indication of FRET. 113
1 Introduction

1.1 Overview of translation

Translation is messenger (m) RNA directed synthesis of polypeptides, which involves three sequential steps – initiation, elongation and termination (Kozak, 1978, 1989). During the initiation step of translation, initiator t-RNA, 40S and 60S ribosomal subunits assemble into an 80S ribosome at the initiation codon of mRNA in concert with of eukaryotic initiation factors (eIFs) (Kozak, 1999; Pestova et al., 2001). Elongation is a complex process during which, a new amino acid is added to the growing polypeptide chain assembled at the start codon, until a stop codon is reached (Marintchev and Wagner, 2004). Termination of protein synthesis is the process of recognition of an in-frame stop codon in the mRNA, release of the newly synthesized poly-peptide and dissociation of the ribosomal complexes (Marintchev and Wagner, 2004).

In the following sections I discuss in greater mechanistic detail the initiation step of translation, the roles of the individual translation factors and their interaction with the ribosome.
1.2 5’ Cap-dependent translation initiation model

1.2.1 5’ Capped structure recognition

Initiation stage in eukaryotes is highly regulated and requires participation of at least twelve initiation factors (eIFs). Initiation of protein synthesis consists of several interconnected stages that are governed by several initiation factors (eIFs). Translation in the majority of cellular mRNAs begins with the recognition of the 5 ‘m^7G(5’)ppp(5’)N cap structure by initiation factors eIF4E (Fig. 1.1). Factor eIF4E, in turn, is bound to the scaffold protein eIF4G and helicase protein eIF4A (Merrick, 1996). Scaffold protein eIF4G additionally interacts with poly(A) binding protein (PABP) which interacts with 3’ poly(A) tail and the interaction helps to circularize mRNA (Wells et al., 1998) (Fig. 1.1).

1.2.2 43S pre-initiation complex formation.

Translation is a cyclic process. Ribosomal subunits used in the initiation complex are derived from dissociation of a post-termination complex (Post TC: 80S bound to mRNA, deacylated tRNA and release factor1) with the help of eIF3 (3j subunit), eIF1 and eIF1A. Post termination complexes (Post TCs) are dissociated into free 60S and mRNA, tRNA bound 40S subunits (Jackson et al., 2010; Pisarev et al., 2007). Initiation factors eIF3, eIF1 and eIF1A remain associated with recycled 40S and this activity prevents further 40S binding to 60S subunit. Subsequently with the aid of 40S bound eIF3, eIF1
and eIF1A, P-site tRNA and mRNA are detached from 40S subunits and gives free tRNAs and mRNAs. Initiation factor 2 (eIF2) selects initiator tRNA from the pool of elongation tRNAs in the cell and forms a ternary complex (TC) eIF2-tRNA$_{f\text{met}}$-GTP (Asano et al., 1999; Hershey, 1989). 43S pre-initiation complex is formed when the ternary complex (TC) binds to 40S subunit with eIF3, eIF1, eIF1A and probably eIF5 (Chaudhuri et al., 1999; Hashem et al., 2013a; Jackson et al., 2010) (Fig.1.1).

1.2.3 Attachment of 43S complexes to mRNA.

48S complexes are subsequently formed by a ‘scanning’ mechanism, where a 43S pre-initiation complex attaches to the capped 5′ proximal region of mRNAs in an unwinding event of the mRNA’s 5′ terminal secondary structure by eIF4A, eIF4B and eIF4F (Kozak, 1978, 1989; Pestova et al., 1998). After binding the capped structure, 43S PIC scans mRNA for an AUG codon followed by base pairing between anticodon of Met-tRNA$^{\text{i}}$ and AUG codon in the P site of 40S subunit. Codon recognition event cause arrest of 43S scanning and release of GDP bound eIF2 from PIC (Lomakin et al., 2003) (Fig.1.1). Finally initiation involves formation of a translational-competent ribosome, 80S when large ribosomal subunit 60S joins pre-initiation complex in the mRNA. Although the scanning mechanism is the most common of those studied, many viral mRNAs follow a cap-independent initiation mechanism where the small ribosomal subunit is recognized by an internal ribosome entry site (IRES) sequence in the 5′-UTR (Kieft, 2008) (Fig.1.1).
Figure 1.1 Model of the canonical pathway of eukaryotic translation initiation.

Source: Richard J. Jackson et al, Nature Reviews Molecular cell Biology, 2010 (Jackson et al., 2010).
1.3 Internal Ribosome Entry Site (IRES) mediated Cap-Independent Translation

1.3.1 Overview:

Although the canonical cap-dependent mechanism accounts for translation in major eukaryotic mRNAs, many viral mRNAs and some cellular mRNAs translate efficiently via a non-canonical cap-independent pathway using a structured RNA sequence located in its 5’ untranslated region (UTR) known as an internal ribosomal entry site (IRES). Since ribosomes interact directly with these sequences they are called internal ribosomal entry sites (IRESs). In the year of 1988, it was reported for the first time that both Polio Virus (PV) and Encephalomyocarditis Virus (EMCV) RNA use a cap-independent internal translation initiation mechanism (Jang et al., 1988; Pelletier and Sonenberg, 1988). Since their discovery, the importance of IRESs in eukaryotic translation initiation and pathogenic viral translation has led us to understand the IRES function. IRESs have been reported in at least 39 viral RNAs (Baird et al., 2006) e.g, hepatitis A virus (HAV) (Glass and Summers, 1992), hepatitis C virus (HCV) (Tsukiyama-Kohara et al., 1992), foot-and-mouth-disease virus (FMDV) (Kuhn et al., 1990), Human Immuno Deficiency Virus (HIV) (Brasey et al., 2003) etc and 85 cellular mRNAs (Baird et al., 2006). All IRESs dependent mechanism bypass the requirement of 5’ capped structure in mRNA and many initiation factors (eIFs), although they vary from each other in many ways. Depending on their structural differences, requirement of eIFs
during initiation step, location of start codon relative to IRES; viral IRESs are categorized into four groups (Fig.1.2).

**Group 1:** IRES RNAs directly interacting with the ribosome do not require protein factors or methionyl-tRNA\textsuperscript{i} for ribosomal interaction. Members of this group include *Cricket paralysis virus* (CrPV) (Wilson et al., 2000), *Plautia stali intestine* virus (PSIV) (Sasaki and Nakashima, 1999) and *Taura syndrome virus* (TSV) (Hatakeyama et al., 2004) etc.

**Group 2:** IRES RNAs that bind to the 40S subunits and also require participation of some eIFs (eIF3, eIF2) as well as Met-tRNA\textsuperscript{i}. Viruses like *Hepatitis C Virus* (HCV) (Tsukiyama-Kohara et al., 1992), *Classical Swine Fever Virus* (CSFV) (Rijnbrand et al., 1997) and Porcine Teschovirus 1 (PTV-1) (Pisarev et al., 2004) belong to this category.

**Group 3:** This IRES group requires some canonical eIFs, Met-tRNA\textsuperscript{i} and some other proteins called IRES trans-activating factors (ITAFs). Translation initiation start point of this group is 3’ end of IRES. *Encephalomyocarditis Virus* (EMCV) (Jang et al., 1988), *Foot-and-Mouth-Disease Virus* (FMDV) (Kuhn et al., 1990) and *Theiler’s Murine Encephalomyelitis virus* (TMEV) (Pilipenko et al., 1994) are the members of this group.

**Group 4:** IRESs need some canonical eIFs, Met-tRNA\textsuperscript{i} and ITAFs for their function. The translation initiation starts at an AUG codon somewhat downstream of the IRES. Members include *Polio Virus* (PV) (Pelletier and Sonenberg, 1988) and rhinovirus (Borman and Jackson, 1992).
Figure 1.2 *Comparison of cap-dependent translation and four IRES groups.*

1.3.2 Structural Features in IRESs

RNA structures play a vital role in viral IRES-dependent translation. Three-dimensional structural information is available mostly for IRESs of group 1 and 2. Group 1 IRESs are found in the intergenic region (IGR) between two open reading frames (ORF) in the Dicistroviridae viruses. These IRESs initiate translation in a highly unusual fashion (Kieft, 2008), major characteristic of this translation mechanism are:

(i) Starting point is a non-AUG start codon.

(ii) It occurs from ribosomes’s aminoacyl tRNA A site rather than P site.

(iii) It occurs place without participation of eIFs or methionyl tRNA.

1.3.2.1 Structures of group 1 IGR IRESs.

Phylogenetic analysis and high-resolution structural studies indicate that all IGR IRESs adopt similar secondary structure containing three pseudo-knots (PK I, II, III) and two conserved stem-loops (SL IV and V) (Kanamori and Nakashima, 2001). There are two major domains present in IGR IRESs, one larger domain (region 1 and 2 in Fig 1.3) containing PK II, PK III and SL IV, V and a smaller domain 3 containing PK I (Jan and Sarnow, 2002; Jan et al., 2001). PK I mimics an anticodon tRNA stem–loop mediating ribosome positioning such that the start non-AUG codon of the IRES occupies the ribosomal A-site (Costantino et al., 2008; Kanamori and Nakashima, 2001; Wilson et al., 2000). IRESs can also fold into a highly stable globular tertiary structure (Fig.1.3). The
larger domain (region 1 and 2) can fold independently and exhibit binding affinity for ribosome (Costantino and Kieft, 2005; Nishiyama et al., 2003) using stem-loops IV and V. High resolution cryo-EM structure of 80S ribosome- *Cricket paralysis virus* (CrPV) IRES portends a better idea of structural parameters of IGR IRES driven cap-independent model (Spahn et al., 2004). The study indicates that conserved stem-loops (SL IV and V) interact with small ribosomal subunit protein (rp) S5 and some unidentified protein rpSX, dynamic loop L1.1 of region 2 contacts large ribosomal subunit L1 stock. PK2 interacts with ribosomal protein rpL11. Domain 3 contacts 18S rRNA helices h18 and 34 (Fig. 1.3).
Figure 1.3 Structures of the Dicistroviridae intergenic region (IGR) IRES RNAs. (a) Proposed secondary structure of the PSIV IRES. (b), (c) and (d) crystal structures of PSIV and CrPV IRESs. (e) Cryo-EM difference density of the CrPV IRES bound to the 80S ribosome. (f) Cryo-EM reconstruction of the CrPV IGR IRES bound to a human 80S ribosome, with the IRES density in magenta, the 60S subunit in cyan and the 40S subunit in yellow.

Source: Jeffrey Kieft, Trends in Biochemical Sciences, 2008 (Kieft, 2008).
1.3.2.2 Structures of Group 2 IRESs.

Structural features of Group 2 IRESs are widely different from Group 1. Group 2 IRESs exhibit a secondary structure with two major domains, II to IV that contain essential parts necessary for their translation (Fig. 1.4) (Kieft, 2008; Lukavsky, 2009). The domain organization and several structural motifs are conserved among related viruses (Lukavsky, 2009). The larger domain III consists of branching hairpin stem–loops (IIIabcdef) where each loop forms three or four way junctions (Brown et al., 1992). The basal part of domain III contains a pseudoknot (IIIf) and a small stem–loop (IIIe) (Rijnbrand et al., 2000) (Fig. 1.4). These RNA sequences fold into an extended tertiary structure containing two domains (i) domain II (iii) domains III and IV together. The basal domains IIIdef, and domain IIIc are responsible for 40S ribosomal subunit binding and the apical domains IIIab provide a platform for eIF3 binding (Kieft et al., 2001; Kieft et al., 1999; Kolupaeva et al., 2000; Lytle et al., 2001; Sizova et al., 1998; Spahn et al., 2001). The conserved pseudoknot structure IIIef also play a critical role in IRESs mediated translation. Mutation in this region abolishes IRES activity but shows little effect on 40S binding. The exact functionality of the region has yet to be verified.
Figure 1.4 The pathway of HCV IRES-mediated translation initiation. (A) Secondary structure of the HCV IRES RNA with individual domains (II–IV) indicated. The 40S interaction site is shown in pink, the eIF3 interaction site in blue, and the AUG start codon in red. (B) Model of HCV IRES translation initiation. The HCV IRES first binds 40S subunits, then recruits eIF3 and the ternary complex to form a 48S complex. Subsequent 80S formation depends on GTP hydrolysis.

1.4 3’ UTR mediated Cap-Independent Translation

1.4.1 Translation in Barley Yellow Dwarf Virus (BYDV)

Instead of containing IRESes, uncapped RNAs of many plant viruses contain a 3’ cap-independent translation element (3’CITE) in their 3’UTR that confers efficient translation initiation. Unlike with IRESes, ribosome scanning from the 5’ end of the mRNA is required for translation of mRNAs relying on a 3’ CITE (Miller and White, 2006; Sarawaneeyaruk et al., 2009). These include RNAs of plus-strand RNA viruses such as the Tombusviridae family and the Luteovirus and Umbravirus genera, all of which lack both a 5’cap and a 3’ poly (A) tail (Miller and White, 2006; Rakotondrafara et al., 2006; Treder et al., 2008). Tobamo and tymoviruses have a 5’cap but translation depends on a tRNA-like structure at the 3’UTR (Dreher, 2009; Matsuda and Dreher, 2004; Miller and White, 2006).

One of the well-characterized CITEs resides in the barley yellow dwarf virus. Such BYDV-like cap-independent translation elements (3’BTE) are also found in Luteovirus, Dianthovirus and Necrovirus genera. 3’BTE of BYDV is ~105 nt long RNA sequence located in the 3’UTR of BYDV mRNA genome. The RNA genome of BYDV encodes six open reading frames (ORFs) that are ~5677-nts in length (Fig.1.5). The ORF1, ORF 2 encodes viral replication protein and RNA dependent RNA polymerase
(RdRp). Proteins from ORF1 and ORF2 are expressed as a fusion protein and translated via ribosomal frame shifting. BYDV generates two subgenomic (sg) RNAs that serve as mRNA for translation of the remaining ORFs. ORF 3 translates to a 22-kDa coat protein. ORF 4, translated by leaky scanning, encodes a 17-kDa protein that is required for systemic infection in plants (15). The protein translated from ORF 5 is part of the coat protein and required for aphid transmission (10). Another subgenomic RNA sgRNA2 serve as the mRNA for ORF 6 translation and encodes a small and highly variable 4.3-6.7-kDa protein of unknown function (Miller and White, 2006).

3’BTEs contain a 17-nucleotide long conserved sequence (17 nt CS) \( \text{GGAUCCUGgAaACAGG} \) that forms stem-loop-I (3’SL-I) due to base pairing of the underlined sequences (Fig.1.6). In addition, the bases in italics are complementary to a sequence near the 3’end of 18S ribosomal RNA (Wang et al., 1997). The bases in lower case are variable among other 3’BTEs, but the 5-nucleotide terminal loop GgAaA always contain the consensus of a GNRNA pentaloop (N is any base, R is a purine) (Molyneux et al., 1998).

Translation initiation factor eIF4G binds the 3’BTE with high affinity (Treder et al., 2008) and protects SL-I from modification by SHAPE reagents (Kraft et al., 2013). The loop of stem–loop III (3’SL-III) of the 3’BTE forms a long-distance RNA-RNA “kissing” stem-loop base-pairing interaction with a loop in the 5’ end of the mRNA (Kneller et al., 2006; Miller and White, 2006) (Fig.1.6). Binding of the 3’BTE to eIF4G
and to the 5’ UTR are required for efficient cap-independent translation. Finally, the 3’BTE does not appear to be an IRES, because this translation initiation requires ribosome scanning from the 5’ end of the mRNA (Guo et al., 2001; Rakotondrafara et al., 2006). Stem–loop III (3’SL-III) of the 3’BTE interacts with the 5’ end of the mRNA (Kneller et al., 2006; Miller and White, 2006) via a long-distance RNA-RNA “kissing” stem loop interaction (Fig.1.6). In BYDV BTE, all the helices protrude from a central hub. At the hub of the helices there are unpaired bases having non Watson- crick interactions that also play a critical role in function. It has been found that mutation of any base in this region reduces BYDV activity (Kraft et al., 2013). The long distance kissing loop interaction delivers eIF4G bound to the 3’BTE (required) and its binding partner eIF4E (not required) (Treder et al., 2008) and facilitate 5’ ribosomal scanning. The 3’BTE binds eIF4G with unusually high affinity (K_D~ 177nM), which is sufficient to assist translation in the absence of eIF4E (57). eIF4E alone has no effect on translation, whereas eIF4G and eIF4E together (i.e., eIF4F) enhance translation level that are 20% to 30% greater (K_D~37nM) than eIF4G alone (Treder et al., 2008). Involvement of any other initiation factor (e.g, eIF4A,4B etc) in BYDV translation initiation remains unknown (Treder et al., 2008). Furthermore, the ribosome recruitment pathway for BYDV and other luteoviruses remains to be elucidated.
Figure 1.5 Genome organizations of BYDV showing secondary structures of long-distance interacting regions. Bold lines represent genomic RNAs on which boxes indicate translational control elements. Labeled open boxes above the RNA indicate translatable ORFs. Black boxes indicate ORFs not translatable from the RNA shown. Colored loops connected by dashed line indicate long distance base-pairing between 5'UTR (SL-D) and 3'UTR (SL-III).
Figure 1.6 Secondary structure of 3'BTE. Nucleotides are numbered according to their positions in viral genome.
1.4.2 Pathogen Biology

BYD virus particles are isometric icosahedral structures that are made up of 180 subunits of a single 21-23 kDa coat protein and are 25-30nm in diameter. Each virion particle contains one positive-sense, single-stranded RNA molecule of about 5.6-5.8 kb and accounts for 28-37% of the virion composition. *Luteoviruses (BYDV)* are dependent on their aphid vectors for their transmission. The viruses don’t replicate within the aphid. They stay in the haemolymph of aphid for weeks and finally are transmitted to the host plant phloem cell during aphid feeding (Rochow and Brakke, 1964).

![Diagram of the life cycle of BYDV vector aphid](image)

**Figure 1.7** Life-cycle of BYDV vector aphid.
1.5 Factors involved in BYDV translation

1.5.1 eIF4F

Plant eukaryotic initiation factor eIF4F is composed of two subunits, i) the smaller cap-binding subunit eIF4E (26 kDa) and ii) the larger subunit of the scaffolding protein eIF4G (165 kDa). In animals helicase protein eIF4A is also a part of eIF4F but in plants eIF4A exists as an individual protein. In BYDV translation 3’BTE functionally replaces the 5’capped structure and eIF4F protein interacts directly with 3’BTE. Protein factor eIF4G binds with stronger affinity to 3’BTE than eIF4E (Treder et al., 2008). In general the N-terminal of eIF4G contains three HEAT domain repeats (Fig. 1.8). The first HEAT domain MIF4G is involved in eIF4A, mRNA and eIF3 interaction (Marcotrigiano et al., 2001). The second HEAT domain, MA3 is the eIF4A-binding site. The last HEAT domain, W2, is found only in mammalian and binds Mnk kinase (Marcotrigiano et al., 2001). In BYDV translation the eIF4G region in between eIF4E binding site and MIF4G (Fig. 1.8). It interacts with 3’BTE and is vital for its translation. A mutation (mutant :p70) in that specific region completely abolished BYDV translation (Kraft et al., 2013). SHAPE probing data has revealed full length eIF4G interacts specifically with the SL-I region of 3’BTE at an internal bulge downstream of the long-distance kissing loop (Fig. 1.9) (Kraft et al., 2013).
1.5.2 elf4A and elf4B

elf4A is a 45kD protein. It belongs to the family of “DEAD box” proteins, an RNA helicase that contains the sequence Asp-Glu-Ala-Asp (Lorsch and Herschlag, 1998). The DEAD box family protein also share sequence similarity with DNA helicases (Lorsch and Herschlag, 1998). The eukaryotic translation initiation factor elf4B is a dimeric protein with a size of 59kD. It accelerates RNA-dependent ATP hydrolysis, and ATP-dependent RNA helicase activity of elf4A and elf4F during translation initiation (Lorsch and Herschlag, 1998).
Figure 1.8 **Schematic representation of eIF4G and its mutants showing regions important for eIF4G–3′BTE interaction.** HEAT domains, MIF4G and MA3, are shown, with their respective amino acid positions below the full-length map. RNA Binding Domains are shown as numbered brackets. The region important for 3′BTE interaction is highlighted as a gray rectangle underlying the region between the N termini of p86 and p70.

Source: Kraft et al, NAR, 2013 (Kraft et al., 2013)
Figure 1.9 Mapping of the eIF4G-binding sites on the BYDV 3'BTE.

Grey bars in the secondary structure indicate BzCN protection by protein p100 and p100 +4E.
Source: Kraft et al, NAR, 2013
1.6 Eukaryotic smaller ribosome (40S) unit

The ribosome is a complex molecular machine of RNA-protein complex (MW ~4300kDa). Eukaryotes have 80S ribosomes; each has a small (40S) and large (60S) subunit. The smaller ribosomal subunit contains 33 proteins and an 18S rRNA. During initiation of translation, eIF1, 1A, 3 and eIF2 interacts with 40S. Due to the dynamic nature of the eukaryotic ribosome and the 40S-eIF complexes, structural understanding is limited to the shortcomings of cryo-electron techniques. Recent findings report high-resolution crystal structure of *T. thermophila* eukaryotic small ribosomal subunit in complex with eIF1 (Rabl et al., 2011). The structure of the 40S subunit is comprised of, platform, body, beak, shoulder, right foot, and left foot (Rabl et al., 2011). These are primarily defined by the fold of the 18S rRNA (Fig.1.10). The structure of 40S also contains 33 ribosomal proteins (rp), 18 of which are specific to eukaryotes. Ribosome also relies on its intrinsic helicase activity that helps to unwind secondary structure of mRNA during its entry. In eukaryotes, basic residues of protein rpS3e and rpS30e reside in the mRNA entry channel and interact with the phosphate backbone of mRNA (Rabl et al., 2011). Translation initiation in bacteria depends on interactions of two regions of the small ribosomal subunit with the 5′ untranslated region of mRNA, i) the anti–Shine-Dalgarno sequence near the 3′ end of the 16S rRNA (Korostelev et al., 2007; Sengupta et al., 2001) and, ii) ribosomal protein rpS1p (Sengupta et al., 2001). In eukaryotes they
don’t require anti Shine-Dalgarno elements in 18SrRNA or rpS1p homologous protein. Although, in some cases presence of protein rpS28e is found in the mRNA entry channel which has structural similarity with rpS1p protein (Bycroft et al., 1997).
Figure 1.10  Crystal Structure of 40S.  (A) Front and back views of the 3D structure of 40S. 18S rRNA colored according to each domain (5′domain, red; central domain, green; 3 major domain, yellow; 3′minor domain, blue; ESs, magenta), and the proteins as gray. Abbreviations: H, head; Be, beak; N, neck; P, platform; Sh, shoulder; Bo, body; RF, right foot; LF, left foot).

(B) Secondary structure diagram of the *T. thermophile* 18S rRNA.

1.7 Specific Aim

Mechanism of ribosome recruitment to the start codon during BYDV translation.

• *Hypothesis 1: The ribosome is recruited directly to the BTE either via the translation factors that bind the BTE or by direct base pairing to the 18S rRNA. Recruited ribosome is then delivered to the 5’ end by long-distance base pairing.*

• *Hypothesis 2: The ribosome does not bind the BTE. Instead, the ribosome binds the 5’ UTR and does not scan efficiently until the factors are delivered from the BTE by long-distance base pairing.*
2 Elements of RNA structure

2.1 Overview

Ribonucleic acid (RNA) is a biologically important macromolecule, playing a versatile role inside the cell ranging from catalytic processes to complex patterns in gene regulation (Amaral et al., 2008; Fedor and Williamson, 2005; Waters and Storz, 2009). In many RNA viruses and retroviruses RNA molecules carry genetic information, meaning they have the RNA genome. RNAs can also transfer genetic information in the form of messenger (m) RNA from DNA into the language of protein. The process of transfer of genetic information from mRNA to protein is called translation that requires participation of ribosome, transfer RNA (tRNA) and many protein factors (GM, 2000). RNAs participate in diverse biological activities as well other than storage and transmission of genetic information. RNA molecules can act as enzymes; that is, catalyze covalent changes in the structure of substrates (most of which are also RNA molecules). Catalytic RNA molecules are called ribozymes (Cech, 2002; Cech et al., 1981; Guerrier-Takada et al., 1983; Kruger et al., 1982; O'Keefe et al., 1996). Almost all living organisms synthesize ribozymes — called Ribonuclease P (RNase P) that cleaves the head (5') end of the precursors of transfer RNA (tRNA) molecules. Splicosomes, self-splicing introns and ribosomes are examples of ribozymes (Cech, 2000; Emilsson et al., 2003; O'Keefe et
al., 1996; Segault et al., 1999; Valadkhan, 2007). Almost fifteen years ago RNA’s functional repertoire was expanded with the discovery of RNA interference (RNAi) mechanisms in C. elegans (Fire et al., 1998; Grishok et al., 2000). RNA interference pathways are associated with small double stranded (ds) RNAs that act as specificity factors for inactivating complementary messenger RNA sequences in the cell. To date three classes of small RNAs have been identified: 1) short interfering (si)RNAs, 2) micro (mi)RNAs, 3) pi RNAs.

The blend of biological function to molecular structure is a central principle of structural biology. The diverse functions of RNA molecules are embedded in a definite repertoire of structures (Conn and Draper, 1998; Doudna and Cate, 1997). RNA is a macromolecule that consists of four mononucleotide components which are 5’-phosphate esters of the purine nucleosides (i) guanosine (G), (ii) adenosine (A) and the pyrimidine nucleosides (iii) cytidine (C), (iv) uridine (U) (Fig. 2.1). Nucleotides in a RNA molecule are linked via a phosphate ester bond between the 3’-OH end of one nucleotide and the 5’-phosphate end of the following nucleotide (Fig. 2.1).

RNA structure can be classified into three fundamental levels of organization (i) primary (ii) secondary and (iii) tertiary.

### 2.1.1 Primary structure

Primary structure of RNA is just the sequence of nucleotides describing the RNA. The RNA sequence can be obtained from the DNA sequence of the gene encoding the RNA.
Since many RNA molecules are post-transcriptionally modified so DNA sequences may not give the true primary structure. These modifications include methylation of nucleotide bases and 2’-hydroxyl groups of ribose sugars, formation of unusual bases like pseudouracil (Ψ) and dihydrouridine (D), deletion of intervening sequences (introns) from pre-messenger RNAs (Batey et al., 1999). Thus to determine the primary sequence, the RNA must be purified from its native source and characterized by sequencing (). An example of primary structure of RNA is shown below in Fig. 2.2.

2.1.2 Secondary structure

The secondary structure of RNA is the base-pairing pattern formed between different nucleotide bases using hydrogen bonds and intervening unpaired regions (). The canonical base pairs, first described by Watson and Crick (1953), consist of G-C and A-U base pairs. However, non-canonical base pairing is also possible in RNA molecule. Secondary structural elements are: duplexes, single stranded regions, hairpins, bulges, internal loops, and junctions, as illustrated in Fig.2.2 (Chastain and Tinoco, 1991).

2.1.3 Tertiary structure

Tertiary contacts are comprised of the three dimensional structure of the RNA molecule. Tertiary structural contacts are formed due to interaction between distinct secondary structural elements. Tertiary interactions play a dominant role in establishing the global fold of the molecule shown in Fig.2.2 ().
Figure 2.1  Common bases found in Nucleic acids are shown, adenine (A), guanine (G), cytosine (C), uracil (U). They are derivatives of either a purine or pyrimidine. Right panel of the picture shows how nucleotides in a RNA molecule are linked via a phosphate ester bond.
Figure 2.2 (A) Primary structure of yeast phenylalanine tRNA (tRNA\textsuperscript{Phe}). The primary structure contains all the information to determine the three dimensional structure. (B) Secondary structure of tRNA\textsuperscript{Phe}. tRNA forms a cloverleaf structure with four stems (acceptor, D, T, and anticodon arm). (C) The tRNA folds to generate a “L” shaped tertiary structure.
2.2 Function of Metal Ions in RNA Structure and Function

RNAs fold into a variety of complex structures that are essential for their biological functions (Caprara and Nilsen, 2000). Since the strong electrostatic repulsion in the negatively charged phosphate group of RNA backbone tends to disrupt the folded structure of RNA, it is important to understand how these repulsive forces are compensated in the native folded structure of RNA. Although monovalent cations can stabilize and reduce these repulsive forces another unique role of divalent ions have been observed (Burkard, 1999; Draper and Misra, 1998). Magnesium ions (Mg$^{2+}$) strongly stabilize native tertiary structure of most RNAs even when monovalent ions are present (Doudna and Doherty, 1997). Denatured or unfolded (U) state of RNA generally is an extended chain of the molecule with no defined secondary structure. As folding proceeds, RNA forms a compact and disordered intermediate state (I) with no defined tertiary interaction. Then RNA form compact, well defined tertiary structure in their native (N) state. Divalent ion like Mg$^{2+}$ strongly stabilizes the native RNA structure and favors the folding reaction (Brion and Westhof, 1997; Russell et al., 2000; Tinoco and Bustamante, 1999).

Recent studies showed that 3’BTE can form a cation dependent stable native structure in the absence of initiation factors. The study reveals at a low (0.1mM) Mg$^{2+}$
concentration only wild type 3’BTEs show compact folded structure. Non-functional mutants of 3’BTE at that low Mg$^{2+}$ concentration form multiple non-native structures. At a higher Mg$^{2+}$ concentration (10mM) both functional and non-functional mutant adopt a stable native fold (Kraft et al., 2013).

2.3 Secondary structure determination by structure probing experiments using Selective 2’-hydroxyl acylation analyzed by primer extension (SHAPE): quantitative RNA structure analysis at single nucleotide resolution.

2.3.1 Introduction

RNA is an important biological molecule for gene expression. The functionality of RNA depends on its sequence and the native structure it adopts upon folding (Raymond F. Gesteland 1999). Understanding the global structure –function relationships of RNA was a great challenge for many years. RNAs also undergo conformational changes when the molecules adopt their native secondary or tertiary structures. A specific set of nucleotides become conformationally constrained due to higher order interactions or base-pairing. In last few years, many probing techniques involving chemical (eg, DMS) and enzymatic reagents (eg, RNase V, T, A) have been developed to decipher local nucleotide conformations of RNAs in biologically important environments (Ehresmann et al., 1987). Selective 2’-hydroxyl acylation analyzed by primer extension
(SHAPE) is one of them (McGinnis et al., 2009; McGinnis et al., 2012; Merino et al., 2005; Wilkinson et al., 2005, 2006).

Selective 2’-hydroxyl acylation analyzed by primer extension (SHAPE) is a powerful tool for quantitative analysis of the equilibrium structures of various biological RNAs. SHAPE chemistry is based on the fact that nucleophilicity of the ribose 2’-position is very sensitive to the electronic influence of the adjacent 3’-phosphodiester group (Chamberlin et al., 2002; Merino et al., 2005). SHAPE probing technique maps any RNA structure in a simple two-step process, (i) RNA modification by electrophilic reagents like Benzoyl Cyanide (BzCN) and (ii) primer extension by reverse transcriptase enzyme.

2.3.2 Step 1: RNA Modification by SHAPE Reagents

Electrophilic reagents especially anhydrides and acyl cyanides eg, Benzoyl Cyanide (BzCN) (Mortimer and Weeks, 2008) or 1-methyl-7-nitro-isatoic anhydride (1M7) (Mortimer and Weeks, 2007) or N-methyl isatoic anhydride (NMIA) (Merino et al., 2005) form 2’-O adduct with 2’-hydroxyl group of RNA via an acylation reaction (Fig.2.3). Reactivity of a SHAPE reaction depends on the local nucleotide flexibility. Flexible RNA nucleotides are most often located in less constrained or single-stranded regions of RNA. During the reaction time electrophiles also may become inactive via a parallel hydrolysis reaction. So one electrophile either reacts with RNA or become
inactivated by hydrolysis reaction. Generally, the lifetime of a given SHAPE reagent is determined primarily by reagent hydrolysis with \( \sim 55 \) M water (McGinnis et al., 2012; Mortimer and Weeks, 2008). So we can say that due to this competitive hydrolysis reaction of SHAPE reagent, modification reaction of RNA is not dependent on RNA concentration or buffer components, small molecules, or proteins, rather it is a very straightforward approach for RNA structure probing.

2.3.3 Step 2. Primer extension of the modified RNA

Fluorescently or radioactive (\(^{32}P\))-labeled primers are used in a reverse transcription reaction to create a cDNA library (Mortimer and Weeks, 2008; Wilkinson et al., 2006). Stops due to RNA adduct formation exactly one nucleotide prior to RNA modification generate a structure specific cDNA library during a reverse transcription reaction. The length of the cDNA also corresponds to the exact position of modified RNA (Fig.2.4). We always run one control reverse transcription reaction using unmodified RNA to locate the site of full length RNA (natural reverse transcriptase pause) or pre-existing RNA degradation and control experiments involving sequencing ladders which gives idea of RNA sequences.

The labeled cDNAs, which include (i) cDNAs from SHAPE experiments, (ii) cDNAs from sequencing ladder and (iii) cDNAs corresponding to full length RNA, are
then separated using denatured gel electrophoresis or using capillary electrophoresis. The resulting data are then analyzed using software’s like SAFA or SHAPE Finder (Das et al., 2005; Vasa et al., 2008).

Figure 2.3 Mechanism of RNA SHAPE chemistry with BzCN. BzCN reacts both with 2'-hydroxyl groups at flexible positions in RNA to form a 2'-O-adduct and also undergoes inactivation by hydrolysis.

Figure 2.4 Primer extension stops due to RNA adduct formation exactly one nucleotide prior to RNA modification generate a structure specific cDNA library.
2.4 Methods and materials for SHAPE

2.4.1 RNA design

During any SHAPE experiment, some information is always lost both at the 5’ end (8–10 nucleotides) and 3’ end of the RNA (usually 10–20 nucleotides adjacent to the primer-binding site) (Wilkinson et al., 2005). To get most of the information from a SHAPE reaction generally the RNA is embedded within a larger fragment of the native sequence (Wilkinson et al., 2005). In our study we have used a reporter plasmid BLucB (Fig.4.6) as a template for most of the RNA constructs. BLucB is a reporter plasmid containing the firefly luciferase gene flanked by the BYDV genomic 5’- and 3’-UTRs (Wang et al., 1997; Wang et al., 2010). We probed the structure of 5’UTR BYDV RNA, 3’BTE using construct BLucB and the structure of 3’BTEBF using the construct BLucBF.

2.4.2 RNA synthesis

BLucB and BLucBF templates were linearized using SmaI restriction enzyme (NEB) and transcribed using the T7 Megascript kit according to the standard protocol (Ambion). Capping of BYDV 5’UTR mRNAs (BLucB) were done using T7 mScript™ (CellScript) kit. All transcripts were purified by Megaclear kit (Ambion). RNA
concentrations were determined using nano-drop UV/Vis spectrometer and integrity was verified by 8-10% poly-acrylamide gel electrophoresis.

2.4.3 RNA-folding

The function of RNA molecules is critically dependent on their structure. During isolation of RNA, the structure is usually disrupted by unfolding due to the presence of denaturants, such as guanidinium thiocyanate or the removal of Mg\(^{2+}\) ions by metal ion chelators (mostly EDTA). Thus, refolding or renaturation of the RNA becomes an issue.

RNA is first heated and then snap-cooled in a low ionic strength solution and then a folding solution is added.

*Protocol*

1) Heat the RNA at 90\(^\circ\)C for 2-4 mins in a buffer (20 mM Tris-HCl, pHi 7.8, 140 mM KCl) or water with no Mg\(^{2+}\) or no divalent ion.

2) Slowly cool down to 37\(^\circ\)C. Incubate the sample in 37\(^\circ\)C for 10 to 15 mins. Then add required Mg\(^{2+}\) ion (2mM to 10mM) of buffer with Mg\(^{2+}\) and incubate for another 15 mins. (You can also do the folding step in 50 deg.)

3) Slowly cool down to room temperature.

2.4.4 RNA modification

Benzoyl cyanide (BzCN) is added to the folded RNA for modification reaction.
µL 10X BzCN (600 mM in DMSO) was added to every 10 µL reaction (Wilkinson et al., 2006). No-reagent control reactions were added to 1 µL of DMSO. Modified RNAs were purified by ethanol precipitation and resuspended in 10 µL of RNase free water.

2.4.5 Primer design and labeling

The cDNA primers (a) 5' AGTTGCTCTCCAGCGGTTC 3' and (b) 5' AACGGCGATAACGTGAAG 3' were used for toe-printing assays. The primer (a) 5' AGTTGCTCTCCAGCGGTTC 3' is complementary to the luciferase mRNA in reporter BLucB mRNA (Fig.4.5) while the primer 5' AACGGCGATAACGTGAAG 3' is complementary to the 3' UTR region of BLucB mRNA (Fig.4.5).

2.4.6 Primer extension

The general protocol for primer extension is described previously (Wilkinson et al., 2005, 2006). Briefly, a 32P labeled DNA primer (3 µL) was annealed to the RNA (10 µL, from the folding step) by heating at high temperature (more than melting temperature of the primer, ~65 °C) for 10 min and then incubating the mixture at a lower temperature (annealing temperature, ~35 °C) for another 10 min and then placed on ice for ~2 min. SHAPE enzyme mix (250 mM KCl, 167 mM Tris HCl, pH 8.3, 1.67 mM each dNTP, 17 mM DTT, 10 mM MgCl2) was added. To each sequencing reaction, 1µl ddNTP was added along with enzyme mix. Tubes were heated for 1 min at 48°C. Superscript III were
added and the reactions incubated at 48 °C for 30 min. Primer extension reactions were quenched by adding 4 µL 2mM NaOH and then heated at 90° C for 4min. Each reaction was resuspended in 20 µL gel loading buffer II AM 8547 (Ambion). cDNA fragments were analyzed by denaturing gel electrophoresis.
2.5 Results

Figure 2.5 The BYDV 5’UTR modification patterns generated using the SHAPE reagent benzoyl cyanide (BzCN) are shown. G and U are dideoxy-sequencing lanes, with positions of selected bases indicated.
Figure 2.6 SHAPE reactivities as a function of nucleotide position analyzed by SAFA. Brighter color indicates greater modification. Nucleotides that are not analyzable because they are either close to the 5’end or primer binding site are represented as grey.
Figure 2.7 Superposition of absolute BzCN reactivities on a secondary structure model of BYDV 5’UTR. Color-coded bases indicate BzCN modification with brighter color indicates greater modification. Nucleotides that are not analyzable because they are either close to the 5’end or primer binding site are represented as grey.
Structural probing of the 5’UTR shows the 5’S-L-D is accessible.

SHAPE analysis of the 5’UTR RNA is shown in Fig.2.5-2.7. The SHAPE reactivities by BzCN modification for each nucleotide in the 5’UTR are superimposed on the RNA secondary structure (Fig.2.7). Nucleotides U₃₆–U₄₀ of SL-B loop were highly modified by SHAPE while nucleotides G₃₀–U₃₂ and A₄₃–C₄₅ showed no modification. This modification pattern supports the base-pairing pattern of G₃₀–U₃₂ with A₄₃–C₄₅ of opposite strand while U₃₆–U₄₀ remained in a single stranded loop region. The first three Us (U₆₀–₆₂) of stem-loop C (SL-C) were highly modified by SHAPE. The G₇₀AG had potential to base pair with CUC in the opposite strand and there was less modification for G₇₀AG. The nucleotides of 5’SL-D, U₉₉UGC₁₀₈A were highly modified single stranded region as expected since this region is proposed to base pair with SL-III of the 3’BTE.
Figure 2.8 The BYDV 3’BTE modification patterns generated using the SHAPE reagent benzoyl cyanide (BzCN) are shown. G and U are dideoxy-sequencing lanes, with positions of selected bases indicated.
Figure 2.9 Superposition of absolute BzCN reactivities on a secondary structure model of BYDV 3'UTR. Color-coded bases indicate BzCN modification with brighter color indicates greater modification.
Structural analysis of 3’BTE

SHAPE reaction tells that M-Fold predicted loops of 3’BTE are heavily modified by BzCN than the predicted paired bases. This result also supports the secondary structure prediction of MFold. The SHAPE reactivities for each nucleotide in the 3’ BTE are superimposed on the best fitting RNA secondary structure in Fig.2.9. SHAPE probing of 3’BTE (Kraft et al., 2013) (Fig.2.8 and 2.9) revealed the presence of a stem-loop 3’SIL-I formed by a 17 nt long conserved sequence (CS) at the distal end of a bulged basal helix where the first and second guanidylate were more exposed than the others (Kraft et al., 2013). Conserved GGAUC of 17nt CS which has 18S rRNA complementarity showed low SHAPE reactivity except the first G, which was highly modified by BzCN. Stem-loop 3’SIL-III, possesses six uninterrupted base pairing of GC and CG. It appears that the native fold of 3’BTE is maintained by the functionality of basal helix that helps to form a more accessible 3’BTE (Kraft et al., 2013). SIL-III is the GC rich stem-loop capable of base-pairing to 5’UTR.
3 Binding Studies

3.1 Overview

Elucidation of the molecular mechanism of BYDV translation initiation requires an understanding of the ribosome and protein binding events. Although, it was shown previously that the 3’BTE interacts specifically with eIF4F with a very high binding affinity (\(K_d \sim 37\) nM) (Treder et al., 2008), the mechanism of ribosome recruitment and the role of associated eIFs during this event remain unclear. As the next step in the establishment of a ribosome recruitment mechanism in BYDV translation, we have quantitatively characterized interactions between the 40S subunit with wild type and mutant forms of the BYDV 3’BTE and 5’UTR each and in the presence of different initiation factors (eIFs).

We used fluorescence quenching, anisotropy experiments and a gel mobility shift assays to study the equilibrium binding between different mRNAs and eIFs (mRNA-eIF interactions) or between mRNAs and ribosomes (Firpo et al., 1996; Ray et al., 2006; Yumak et al., 2010). These approaches were used to determine the equilibrium dissociation constants (\(K_d\)'s) for the interactions between 40S-3’BTE, 40S-5’UTR, and 40S-3’BTEBF (3’BTEBF is a translationally inactive mutant of the 3’BTE, containing a four-base duplication of GAUC in the 17CS ,Fig.4.6 (Wang et al., 1997). We also observed the effects of different eIFs on 40S-3’BTE interactions.
3.1.1 **Fluorescence quenching**

Fluorescence quenching refers to any process that causes reduction of the fluorescence intensity of a sample. We usually encounter two quenching processes:

(a) Dynamic or collisional quenching.

(b) Static quenching.

3.1.2 **Dynamic or collisional quenching**

This phenomenon takes place when an excited fluorophore interacts with small molecules or atoms like oxygen, iodide ion which facilitates non-radiative transition of the fluorophore to the ground state. In the simplest case of dynamic quenching (Lakowicz, 2006), the following relation, called the Stern-Volmer equation holds:

\[
\frac{F_0}{F} = 1 + k_q \tau_0 [Q] = 1 + K_D [Q] \]

\[(i)\]

where \( F_0 \) and \( F \) are the fluorescence intensities observed in the absence and presence of the quencher, \([Q]\) is the quencher concentration and \( k_q \) is the Stern-Volmer quenching constant.
3.1.3 Static quenching

In some cases fluorophores can form a stable complex with other molecules. If the ground state of the complex becomes non-fluorescent then the phenomenon of the quenching is called static quenching (Lakowicz, 2006). The dependence of the fluorescence follows the following relation,

\[ \frac{F_0}{F} = 1 + K_s Q \]  

where \( K_s \) is association constant of the complex.

3.2 Fluorescence anisotropy

Fluorescence anisotropy \( (r) \) is used to characterize the extent of linear polarization of fluorescence emission, resulting from photoselection from an optically isotropic sample (Lakowicz, 2006). In fluorescence, molecule absorbs photon and gets excited to higher electronic state. It comes down to lower electronic state by emitting energy. The excitation of electrons can occur only if the electric field of the photons is oriented in a particular axis about the molecule. When a fluorescent molecule is excited with plane polarized light, light is emitted in the same polarized plane, provided that the molecule remains stationary throughout the excited state. The emission will be in a different plane
if the molecule rotates or tumbles out of the plane in the excited state. If the size of the molecule is large then the molecular motion will be less in the excited state, as a result the emitted light will be highly polarized. Similarly, if the size of the molecule is small, the rotation and tumbling will be faster and the emitted light will be depolarized (Lakowicz, 2006).

The measurement of fluorescence anisotropy is described in Fig. 3.1. The sample is excited with a vertically polarized light. The electric vector of the excitation beam is along “Z” axis. The intensity of the emission is characterized using a polarizer. Polarized emission which are parallel (\(\parallel\)) to the excitation electric field are designated as \(I_\parallel\). Likewise, when the emission is perpendicular (\(\perp\)) to the excitation field the intensity (I) is called \(I_\perp\). The mathematical expression of anisotropy \((r)\) is given by,
\[
r = (I_\parallel - I_\perp) / (I_\parallel + 2I_\perp)……………………………………. (iii).
\]
The anisotropy is dimensionless and is independent on total intensity of the sample. This is because the intensity difference \((I_\parallel - I_\perp)\) is normalized by the total intensity \((I_\parallel + 2I_\perp)\). Anisotropy \((r)\) can also be related directly to the angle \(\theta\) using the following equation where \(\theta\) is the angle of the emission dipole relative to the \(z\)-axis,
\[
r = [3\cos^2 \theta - 1] / 2 ……………………………………… (iv).
\]
Polarization \((P)\) of a molecule is expressed as,
\[
P = (I_\parallel - I_\perp) / (I_\parallel + I_\perp) ……………………………………….(v).
\]
Polarization and anisotropy values can be interconverted using the following equation.

\[ P = \frac{3r}{(2+r)} \]  

\text{(vi)}

Figure 3.1 Schematic diagram for measurement of fluorescence anisotropies.

3.2.1.1 Photoselection

When a fluorophore population is excited with polarized light, the molecules having absorption transition moment parallel to the polarized electric field will have the highest probability of excitation. The electric dipole of a fluorophore need not be precisely aligned with the excitation light. The probability of absorption depends on the angle $\theta$, where $\theta$ is the angle the absorption electric field makes with the $z$-axis. Hence, excitation with polarized light results in a population of excited molecules that are partially oriented along the $z$-axis (Fig. 3.2). This phenomenon is called photoselection (Lakowicz, 2006).
Figure 3.2 Excited-state distribution for immobile fluorophores with $r_0 = 0.4$.

3.2.1.2 Measurement of fluorescence anisotropy

Two methods are commonly used for steady state fluorescence anisotropy experiments. These are the L-format method, in which a single emission channel is used, and the T-format method, in which the parallel and perpendicular components are observed simultaneously through separate channels. In the experiments performed in our laboratory, we use L-format method for anisotropy measurements (Lakowicz, 2006).

**L-Format or Single-Channel Method**

In a L-format set up the sample is excited with a vertically / horizontally polarized light and the emission is often observed through a monochromator (Fig.3.3). The monochromator usually has different transmission efficiencies. So the measured emission intensities are actually proportional to the transmission efficiencies of the monochromator. As for example, for vertically polarized excitation the observed polarized emission intensities will be,

\[
I_{VV} = k_S I_\parallel \quad \text{............................................... (vii), where } I_{VV} \text{ corresponds to vertically polarized excitation and vertically polarized emission.}
\]

\[
I_{VH} = k_S I_\perp \quad \text{............................................... (viii), where } I_{VH} \text{ corresponds to vertically polarized excitation and horizontally polarized emission and } k \text{ is a}
\]
proportionality factor to account for the quantum yield of the fluorophore and other instrumental factors aside from the polarization-dependent sensitivity.

Division of (vii) and (viii) yields,

\[ \frac{I_{VV}}{I_{VH}} = \left( \frac{S_v}{S_H} \right) \left( \frac{I_{||}}{I_{\perp}} \right) = \text{G} \left( \frac{I_{||}}{I_{\perp}} \right) \] ............................................... (ix).

Determination of actual intensities is therefore related to “G” factor which is a function of sensitivity of the detection system. Fluorescence anisotropy is normally measured as,

\[ r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \] ........................................... (x)
Figure 3.3 Schematic diagram for L-format measurements of fluorescence anisotropy. MC, monochromators. The shapes on the right are the excited-state distributions.

3.3 Gel mobility shift assay

The gel mobility shift assay is another powerful technique for detecting, quantifying and visualizing protein-RNA interactions. In a gel shift assay, a $^{32}$P-labeled or fluorophore labeled RNA fragment is incubated with a candidate of RNA-binding protein. The protein-RNA complexes are separated from free RNA by electrophoresis through a nondenaturing polyacrylamide gel. The protein or ribosome forms complex with the RNA and delays the movement of the RNA fragments; thus, the free RNA migrates faster than does the complex (Fried and Crothers, 1981; Garner and Revzin, 1981).
3.4 Materials and methods

3.4.1 End labeling of mRNAs

We used 5’ labeled nucleic acids for our binding experiments. RNAs were labeled at their 5’ end with fluorescein 5-maleimide using the 5’end tag nucleic acid labeling system from Vector Laboratories, CA (Cat no. MB-9001). This 5’end labeling for fluorescence studies was performed by an enzymatic metod. Labeling is achieved in two main steps:

a) 5’ phosphorylated ends of nucleic acids are converted to 5’-OH with the use of alkaline phosphatase. Enzyme T4 polynucleotide kinase transfers a thiophosphate from ATPγS to the 5’-OH group of the nucleic acid (Fig.3.4). These reactions take place at 37°C for ~ 30 minutes.

b) Thiolated nucleic acid is chemically coupled to a thiol-reactive fluorophore fluorescein 5-maleimide at 65°C for ~ 30minutes. This step is followed by standard phenol:chloroform extraction and ethanol precipitation of nucleic acid.

End labeling of nucleic acid using radioactive $^{32}$P γATP follows a similar enzymatic mechanism. After dephosphorylation, we utilized radioactive $^{32}$P γATP and enzyme T4 polynucleotide kinase which phosphorylates the 5’-end with $^{32}$P γATP. Purification of radioactive RNAs are done by using micro-bio spin columns (Biorad, Cat No. 732-6223).
Figure 3.4 Enzymatic pathway of 5’end labeling of any nucleic acid is shown.
3.4.2 Fluorescence quenching experiments

Steady state fluorescence was used to monitor protein-RNA interactions (Ray et al., 2006). Fluorescence measurements were carried out using a Horiba Spectra ACQ Fluorolog-3 spectrofluorimeter equipped with excitation and emission filters. The sample temperature was set to 25 °C for all experiments unless otherwise stated. The excitation and emission slits were set on 3 and 4 nm, respectively. Fluorescence changes (quenching or enhancement, depending on the titrations) were monitored using excitation wavelength of 490 nm and emission wavelength of 520 nm (fluorescein 5-maleimide fluorescence). All titrations were performed in a titration buffer (20 mM HEPES-KOH, pH 7.5, 100 mM KCl, 1 mM DTT). The normalized fluorescence difference (F / Fmax) between the RNA-protein complex and the sum of the individual fluorescence spectra was used to determine the equilibrium dissociation constant (Kd) (Ray et al., 2006). Data were fitted using Kaleida Graph (Abelbeck Software).

3.4.3 Fluorescence anisotropy experiments

Fluorescence anisotropy measurements were carried out using the Horiba Spectra ACQ Fluorolog-3 spectrofluorimeter equipped with excitation and emission polarizers. The sample temperature was set to 25 °C for all experiments. Anisotropy experiments were performed using an L-format detection configuration. Direct fluorescence anisotropy titrations were employed to study protein–RNAs (eIFs–BYDV mRNAs), and ribosome–RNAs (40S-BYDV mRNAs) interactions. The fluorescence anisotropy change
was monitored when increasing amounts of eIFs or ribosomes were added to 5′fluorescent labeled mRNAs in 20 mM HEPES Buffer, pH 7.4, 100 mM KCl at 25 °C. The \( K_D \) was determined by fitting the plot of changes in anisotropy vs. ribosome or eIFs concentration using the equation

\[
\text{r}_{\text{obs}} = \text{r}_{\text{min}} + \frac{(\text{r}_{\text{max}} - \text{r}_{\text{min}})}{(2 \text{Fl}[\text{RNA}])} \{b- (b^2 - 4 \text{Fl}[\text{RNA}][\text{eIFs}])^{0.5}\},
\]

where the \( r_{\text{obs}} \) is the observed anisotropy value for any point in the titration curve ; \( r_{\text{min}} \) is the minimal anisotropy value in the absence of protein or ribosome; \( r_{\text{max}} \) is final saturated anisotropy value. \( b = K_D + [\text{FlRNA}] + [\text{eIFs/ribosome}] \) as described elsewhere (Khan and Goss, 2005; Luo and Goss, 2001). Data were fitted using KaleidaGraph (Abelbeck Software).

### 3.4.4 Gel mobility shift assay

Gel mobility shift assays were done following the protocol described previously (Rozen et al., 1990). Initiation factors eIF4A, eIF4B and eIF4F (2.0 µM concentration of each), 5mM ATP, were incubated with 200 ng of \(^{32}\)P labeled 3′BTE RNA or 3′BTEBF RNA for 1hour at 37°C in buffer containing 20 mM (HEPES)-KOH (pH 7.5), 150 mM KCl, 2 mM dithiothreitol, 0.5 mM magnesium acetate, 0.1 mM GTP, 20 U RNAsin (Invitrogen) in a final volume of 10 µl. After 1-hour incubation, 40S ribosomal subunits (final concentration of 5µM) were added to the helicase reaction mixture and incubated at 37°C for another 30 min. As a control experiment, mRNAs were incubated with eIF4F and 40S subunits separately under the similar experimental condition. Reactions were stopped by adding loading dye and each reaction mixture was applied to a native 2%
polyacrylamide-2% agarose gel which had been pre-run at 30 mA for 30 min. Electrophoresis was carried out at 50 mA for 2 h at 4°C. The gel was exposed to phosphorimager (Amersham) overnight and quantified using Imagequant software (GE Healthcare Life sciences).
3.5 Results

3.5.1 eIF4F Binding to Fluorescein Labeled 3’BTE and 3’BTEBF RNA.

Figure 3.5 (A) eIF4F binding to 3’BTE (◊ -) and 3’BTEBF (○ -) was monitored by changes in 5’ fluorescein labeled mRNA fluorescence anisotropy (excitation- 490 nm, emission- 520 nm). 3’BTE and 3’BTEBF show very strong and similar binding affinity ($K_d \approx 35nM$) to eIF4F. Inset is the Scatchard plot of eIF4F and 3’BTE binding which reveals 1:1 binding stoichiometry.

<table>
<thead>
<tr>
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<th>Value</th>
<th>Error</th>
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</tr>
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<td>$n_2$</td>
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<td>0.001</td>
</tr>
<tr>
<td>$R^2$</td>
<td>0.995</td>
<td></td>
</tr>
</tbody>
</table>

Figure 3.5 (B) eIF4F binding to fluorescein labeled 3’BTE using quenching experiment. Quenching experiment also revealed a strong eIF4F: mRNA binding affinity for both 3’BTE (shown in B) and 3’BTEBF (not shown).
3.5.2 eIF4F Binding to Fluorescein Labeled 5’UTR.

Figure 3.6) eIF4F binding to 5’UTR (O-O) was monitored by changes in 5’ fluorescein labeled mRNA fluorescence anisotropy (excitation- 490 nm, emission- 520 nm). 5’UTR show no binding affinity to eIF4F.
3.5.3 40S ribosomes binding to 3’BTE and its translationally inactive mutant 3’BTEBF.

Figure 3.7 Ribosome binding to BYDV-RNA was monitored by changes in 5’ fluorescein labeled BYDV-RNA fluorescence anisotropy (excitation- 490 nm, emission- 520 nm).

Ribosomes bind to 3’BTE with a moderate binding affinity while mutant 3’BTEBF show a very weak non-specific binding with 40S subunits.
3.5.4 40S ribosomes binding to 5’UTR and 5’SL-D RNA.

![Graph showing ribosome binding to BYDV-RNA](image)

Figure 3.8 Ribosome binding to BYDV-RNA was monitored by changes in 5’ fluorescein labeled BYDV-RNA fluorescence anisotropy (excitation- 490 nm, emission- 520 nm). Both 5'UTR BYDV and 5'SL-D show non-specific binding interaction with ribosome.
3.5.5 Effect of eIF4F on Binding of 40S Ribosome to 3’BTE, 3’BTEBF RNA.

Figure 3.9 eIF4F didn’t show a significant change on 40S binding affinity with 5’UTR and 5’SL-D.
3.5.6 Effect of eIF4F on Binding of 40S Ribosome to 5'UTR and 5'SL-D RNA.

Figure 3.10 eIF4F alone didn’t show a significant effect on 40S binding affinity with 3'BTE. Ribosomes bind with a moderate binding affinity to the complex of

\[ K_d \approx 1 \mu M \]

\[ K_d \approx 0.6 \mu M \]
3.5.7 Effect of eIF4F-4B-4A-ATP (Helicase complex) on Binding of 40S Ribosome to 3’BTE, 3’BTEBF mRNAs.

Figure 3.11 A combination of eIF4A-4B-4F (helicase complex) and ATP enhanced the binding affinity of 40S for 3’BTE-RNA nearly three-fold. Non-functional 3’BTEBF didn’t show binding enhancement in a similar reaction.
3.5.8 Binding of 40S Ribosomes to 3'BTE mRNAs in the presence of complex elF4F-4B-4A-non hydrolyzable ATP (ADP-PNP).

Figure 3.12 Helicase complex didn’t show effect on 40S binding in the presence of non-hydrolysable ATP analog ADP PNP.
3.5.9 Binding of 40S Ribosomes to 5'UTR and 5'SL-D mRNAs in the presence of helicase complex eIF4F-4B-4A-ATP.

![Graph showing normalized anisotropy vs ribosome concentration](image)

Figure 3.13 A combination of eIF4A-4B-4F (helicase complex) and ATP showed no or non-significant binding enhancement for 5'UTR BYDV or 5'SL-D.
Figure 3.14  Helicase treatment of BYDV 3’UTR increases 40S ribosome binding shown in 2% agarose-2% polyacrylamide native composite gel.

A) $^{32}$P labeled 3’BTE shows very high 40S binding affinity when the 3’BTE mRNA is treated with eIF4F-4B-4A-ATP (lane 3). 40S ribosomes show weaker binding with 3’BTE (lane 4). 3’BTE shows high binding affinity with eIF4F (lane 2).

B) $^{32}$P labeled mutant 3’BTEBF shows moderate binding affinity with eIF4F (lane 5). 3’BTEBF doesn’t show 40S binding when treated with helicase complex (lane 6 and 7).
Discussion Binding Experiments

Fluorescence anisotropy measurements of purified 40S ribosomal subunits interacting with fluorescein-labeled BTE reflected a moderate binding affinity ($K_D=400\pm30nM$) between 3’BTE and the 40S subunit (Fig. 3.7). In contrast, very weak binding affinities to the 40S subunit were observed for the mutant 3’BTEBF ($K_D=1200\pm50nM$) (Fig. 3.7), the 5’ UTR of BYDV genomic RNA ($K_D=1100\pm50nM$) or for stem-loop D (5’SL-D) of the 5’ UTR ($K_D=700\pm40nM$, respectively) (Fig. 3.8). These weak binding affinities most likely reflect non-specific interactions. In contrast, the highly specific binding of HCV IRES to 40S ribosomes has a $K_D \sim 10$ nM (Maag et al., 2005).

Binding affinity of 40S–3’BTE or 40S-5’UTR was weaker than expected to account for 3’BTE mediated translation, if eIF’s were not required. Because eIFs affect ribosome-binding affinity (Jackson et al., 2010), the effects of different initiation factors in 40S binding were examined. Initiation factor eIF4F alone didn’t show a significant effect on 40S binding affinity with 3’BTE (Fig.3.9 and 3.10). However, a combination of eIF4A-4B-4F (helicase complex) and ATP enhanced the binding affinity of 40S for 3’BTE-RNA nearly three-fold ($K_D =145 \pm 10nM$) (Fig.3.11). In the 17 nt CS, bases 2-7 (GAUCCU) (Fig.1.6) are complementary to a tract near the 3’ end of 18S rRNA at the site where the Shine-Dalgarno sequence is located in prokaryotic ribosomes (Wang et al., 1997). However,
the terminal three bases of this hexamer (underlined) are base-paired within the 17 nt CS (Fig. 1.6) and some of the complementary bases in 18S rRNA are also embedded in a helix. Thus, perhaps unwinding of RNA by the helicase activity of eIF4A (Rozen et al., 1990) made the complementary sequences available to allow base pairing between the GAUCCU and 18S rRNA, enhancing binding of 3’BTE to the 40S subunit. The specificity of this reaction is confirmed in Fig. 3.11, 3.12 and 3.13 where only helicase complex (eIF4A-4B-4F)-treated 3’BTE, in the presence of ATP, showed high affinity binding. None of the controls including a non-hydrolyzable ATP analog (ADP-PNP), nonfunctional BTEBF mutant, and the 5’UTR showed a similar increase in ribosome binding in the presence of the helicase complex.

Binding selectivity of the 40S with 3’BTE and 3’BTEBF in the presence of helicase complex and ATP was verified by gel mobility shift assays in which $^{32}$P-labeled BTE or BTEBF RNAs, treated with the eIF4A-eIF4B-eIF4F-ATP mixture, were incubated with 40S ribosomes and run in a 2% polyacrylamide-2% agarose non-denaturing composite gel (Fig. 3.14). The mobility shift assay revealed that ribosome binding affinity to the BTE increased in the presence of the helicase complex, while no binding increase was detected for the nonfunctional mutant, BTEBF confirming our fluorescence anisotropy measurements.
4 Primer extension inhibition by reverse transcription.

4.1 Overview

Primer extension inhibition assays have been used to examine formation of translation initiation complex. Originally any toe-printing experiments were performed by mixing mRNA with ribosomes/proteins, t-RNA and a cDNA oligonucleotide primer complementary to the part of mRNA followed by reverse transcription. When the reverse transcriptase meets the ribosome bound to the mRNA, polymerization is halted, and a “toe-print” fragment is generated. Typically, the position of the P site of the stalled ribosome is 15–17 nucleotides upstream of the toe-print (Fig.4.1) (Dmitriev et al., 2003; Hartz et al., 1988; Pestova et al., 1998). Nowadays, similar toe-printing assays in crude systems like rabbit reticulocyte (Otto and Puglisi, 2004) or wheat germ extract (Gaba et al., 2005; Sachs et al., 2002) have been successfully performed to know the position of stalled 80S ribosome during protein synthesis initiation.

The mRNA pathway on 40S subunits (Pisareva et al., 2008) comprises three regions: the entry channel or amino acylation site (A), the exposed interface surface peptidyltransferase site (P), and the exit channel (E) (Fig.4.2). Entry of mRNA occurs between the head and shoulder of 40S subunit (Lomakin et al., 2003; Passmore et al., 2007; Yu et al., 2009), then it passes through ribosomal proteins (rp) including rpS2 and rpS3 and next through a layer of rRNA (helices (h) 18 in the body and 34 in the neck). (Passmore
et al., 2007; Yu et al., 2009; Yu et al., 2011) (Fig.4.2). In canonical translation initiation, the initiator tRNA resides in the P-site during initial stages of translation and addition of a tRNA to the A-site leads to peptide-bond formation and translocation. When toe-printing assays are done in reconstituted translation system or in crude translation extract like wheat germ extract (wge), initially the system is treated with elongation blocker like Cycloheximide (CHX) (Obrig et al., 1971; Schneider-Poetsch et al., 2010). CHX binds to ribosomal E site and blocks translocation by inhibiting binding of deacylated tRNA to the A site (Fig.4.3). So a 48S complex is formed in the +15-17 position downstream of start codon AUG (Schneider-Poetsch et al., 2010). When reverse transcription reaction is done, cDNA primer extension inhibition occurs at that position (Fig.4.4).
Figure 4.1 Illustration of one toe-printing experiment. Ternary complex formed by 30S ribosome, t-RNA and mRNA ribosome binding site blocks primer extension in a reverse transcription reaction.

Figure 4.2 The mRNA pathway on 40S subunits showing the entry channel or amino acylation site (A), the exposed interface surface peptidylation site (P), and the exit channel (E).

Figure 4.3 Mechanisms of action of cycloheximide. Cycloheximide binds to the E site of ribosome and blocks translocation by inhibiting binding of deacylated tRNA to the E site.

Source: Tilman Schneider-Poetsch et al, Nature Chemical Biol, vol 6 | march 2010 (Schneider-Poetsch et al., 2010).
Figure 4.4 Canonical mechanism of 48S complex formation that involves unwinding of a stem and produces toe-prints +15-17 nucleotides downstream of the AUG codon.

4.2 Methods and materials

4.2.1 Primer design and synthesis

The cDNA primers (a) 5' AGTTGCTCTCCAGCGGTTC 3' and (b) 5' AACGGCGATAACGTGAAG 3' were used for toe-printing assays. The primer (a) 5' AGTTGCTCTCCAGCGGTTC 3' is complementary to the luciferase mRNA in reporter BLucB mRNA (Fig.4.5) while the primer 5’ AACGGCGATAACGTGAAG 3’ is complimentary to the 3’UTR region of BLucB mRNA (Fig.4.5).

Figure 4.5 Plasmid map of BLuCB showing different primer binding site. The red arrow is the representative of primer “a” and the black arrow is that of primer “b”.
4.2.2 Toe-printing assays

To investigate the nature of ribosome binding during BYDV translation initiation, we performed toe-printing analysis of translation reactions in wheat germ extract (wge) and in the reconstituted translation system. We analyzed mRNAs: (i) 5’-capped version of the BYDV 5’UTR linked to a luciferase reporter gene (BLucB), (ii) uncapped BLucB, (iii) mutant BLucBF, (iv) BLucB-SL-Dm1 (mutated BLucB plasmid where UCGAACA nucleotides of 5’SL-D were mutated to UCCTGAA to disrupt “kissing–loop” interaction) and (v) 5’UTR BYDV containing BYDV 5’UTR region and LUC gene for toe–printing assays.

The assay protocol for toe-printing in wheat germ extract (wge) was adapted as described before (Sachs et al., 2002). Wheat germ extract reactions were prepared according to Promega wheat germ in vitro translation kit (L-4380) as they were for use in translation assays, except that a complete amino acid mixture was used and no $^{35}$S-Met was used. Each reaction was treated with 5 mM Cycloheximide (CHX) (Sigma Aldrich, Cat no. C4859-1ML). RNAs were incubated in the wge in a total volume of 10 µL for 30 min at 25°C. Each microliter of the translation reaction was then diluted in primer extension buffer which contains four parts 1X SSIII FS buffer [Invitrogen], one part 0.1M dithiothreitol (DTT), one part 10 mM dNTP mix, 1 U/µL RNaseOUT [Invitrogen] (Wilkinson et al., 2006) and incubated for 2 min at 55°C. $^{32}$P-labeled primer was then annealed with the RNAs at 37°C for 2 min and reverse transcription was done by using
SuperScript III reverse transcriptase (Invitrogen) at 42°C for 20 min. The reactions were stopped by 15 µL gel loading buffer II (Ambion, Cat no AM 8547) and then separated in a 8% polyacrylamide / 7 M urea sequencing gel. The sequencing ladder and toe-prints were visualized by scanning of the dried gel.

Toe-printing reactions using eIFs were done by assembling 40S subunits (16 pmols), mRNAs (6 pmols) with different combinations of eIF4F, eIF4A, eIF4B, eIFiso4F and PABP (6 pmoles each) in 20µl reaction mixture. Each reaction was incubated in a binding buffer (100 mM HEPES at pH 7.8, 6 mM MgCl₂, 100 mM NaCl) at 37°C for 30 min. ³²P-labeled primer was then annealed with the RNAs at 37°C for 2 min and then toe-prints were detected using reverse transcription reaction as described before (Yu et al., 2009).
4.3 Results

4.3.1 The ribosome stalls at the start codon during translation initiation on BYDV RNA.

Although the 3’BTE naturally resides in the 3’ UTR, strong secondary structures or AUG codons in the 5’ UTR can greatly reduce translation initiation (Guo et al., 2001; Rakotondrafara et al., 2006), suggesting that 3’BTE-mediated translation requires scanning from the 5’ end. To further investigate the nature of ribosome binding during BYDV translation initiation, and the role of 5’UTR we performed toe-printing analysis of translation reactions in wheat germ extract (wge). Initially, we analyzed a 5’-capped version of the BYDV 5’UTR linked to a luciferase reporter gene (BLucB) to authenticate the functionality of the system (Fig 4.7). When incubated in wge treated with cycloheximide (CHX), which stalls 80S initiation complexes by inhibiting elongation, primer extension inhibition (toe-prints) were observed 16 nucleotides downstream from the AUG start codon in capped 5’UTR BYDV as expected because the 80S subunit blocks reverse transcriptase access to 16 nt downstream of the P-site codon (Fig.4.6).

We next characterized ribosome recruitment on uncapped BLucB mRNA. When incubated in CHX-treated wge, no significant toe-prints were observed (Figure 4.8, lane 2). However, when 3’BTE was present in the reporter mRNA (BLucB), strong toe-prints were observed 16 nt downstream (+16) of the start codon in the
uncapped message (Figure 4.9, lane 13). We also observed this ribosome loading phenomenon when the 3’BTE was provided to BLuC mRNA in trans (Figure 4.8, lane 3).

As a negative control, we investigated the effect of the nonfunctional BTEBF mutation (BTEBF) on ribosome recruitment to the 5’ end of the reporter mRNA in wge. No corresponding toe-prints were observed when mutant 3’ BTEBF was present in cis on BLucBF mRNA (Figure 4.9, lane 11) or when BTEBF was added to the wge system in trans (Figure 4.8, lane 7). Furthermore, primer extension inhibition of mutant BLucB-SL-Dm1 (Fig. 4.7) containing a mutation in 5’SL-D that prevents the kissing loop interaction between the 5’UTR and 3’BTE gave no prominent toe-print at the +16 or any other 5’ UTR nucleotide position (Figure 4.9, lane 15), indicating the requirement for RNA-RNA interaction in addition to the 3’BTE presence. This observed requirement for long-distance communication to ensure 5’ ribosome binding is consistent with earlier reports of translational inhibition when the 5’-3’ interaction was disrupted (Guo et al., 2001; Rakotondrafara et al., 2006). Our toe-printing assay data confirm that the 3’ BTE is necessary and essential to allow for 5’ ribosomal entrance and delivery to the start codon.
Two possible mechanisms for ribosomes recruitment

Next, we wanted to differentiate between the two possible mechanisms for the ribosome recruitment to the 5’UTR. One mechanism is the ribosome binds the 3’BTE directly, followed by delivery to 5’ end via long-distance base pairing or, alternatively it could bind directly at the 5’UTR only in the presence of eIF4F (or eIF4G) bound to the 3’BTE. We observed toe-prints in the SL-I region of the 3’BTE when the in vitro translation reaction was quenched in a time frame of zero to five minutes in the BLucB mRNA construct, indicating initial loading of translation machinery to the 3’ end (Figure.4.10, lanes 3-5). Additionally, no toe-prints were observed in the SL-I region when the assay was done in wge with mutant BLucBF (Figure 4.10, lane 7). Taken together, these toe-printing assays in wge indicate that the ribosome binds to the 3’SL-I region of the 3’BTE first and then is subsequently delivered to the 5’end of the message requiring the RNA-RNA “kissing loop” interaction for successful transfer.
4.3.2 eIF4A, 4B, 4F, ATP and 3’BTE are necessary for ribosome binding to the 5’ end of the message.

To determine the initiation factor requirements for ribosome binding, toe-printing studies were performed using the purified components of the wheat germ translation system. Toe-prints were observed in the SL-I region when ribosomes were incubated with BLucB in presence of eIF4F, eIF4A, eIF4B and ATP (Figure 4.11, lane 2,3) and a weak toe-print was observed when only 40S subunits were present in the reaction (Figure 4.10, lane 2). A very weak/ no toe-printing was observed when ribosomes were incubated in the presence of eIF4F-4A-4B and a non-hydrolyzable form of ATP (Figure 4.11, lane 1). Efficient stops in the presence of eIF4F/4B/4A or iso4F/PABP were not identified at the 3’SL-I region under the similar reaction condition when individual factors in the absence of ribosomes were used for foot-printing experiments (data not shown).

Similar toe-printing experiments using a combination of different eIFs showed stalled complexes on the uncapped BLuC construct when eIF4F, eIF4A, eIF4B, ATP and the 3’BTE mRNA were delivered in trans (Figure 4.12, lane 4). The other combinations of eIFs and uncapped BLuc mRNA, did not produce a toe-print at +16 region of 5’ UTR (Figure 4.12, lanes 1-3 and 5-7). Factors eIFiso4F and PABP did not produce a toeprint alone (lane 5, 6) or with 40S (lane 10 and 14). Toe-prints were not observed in the 5’
UTR when eIF4F/4A/4B/iso4F were present in the reaction in combination with the 40S subunit in the absence of ATP (lanes 11, 12, 13, 10). In order to obtain a clear toe-print not only were the helicase complex and BTE were required, but also hydrolyzable ATP (lane 9). Most likely, the requirement for ATP and eIF4F-4A-4B in ribosome binding with 5’UTR assisted in unwinding secondary structure of the RNA.

Overall we found a good agreement between the binding of ribosomes observed during primer extension inhibition assays and fluorescence binding assays. Efficient binding of 40S subunits to the 3’BTE was achieved only in the presence of helicase complex and ATP as shown with both assays.
Figure 4.6 Genomic organization of BYDV RNA. SL A, B, C, D represent 5’UTR. SL I-IV represent 3’BTE. Plasmid construct BLuCB contains 5’UTR and 3’UTR flanked by Luc gene.
Figure 4.7  80S complex formation on capped 5’UTR BYDV mRNAs. Capped BLuCB mRNA showed toe-prints in the +16/+17 nucleotides down stream of AUG codon in cycloheximide treated wheat germ extract (lane 5).
Figure 4.8 80S complex formation on 5'UTR BYDV mRNAs depends on the presence of 3'BTE.

Denaturing PAGE showing the products of primer extension generated by reverse transcription of the uncapped BYDV 5' UTR segment with Luc coding region. 5' UTR segments were incubated in wheat germ extracts containing cycloheximide (CHX) for 20 min at 25°C. Stalling of the 80S complex was observed downstream of the AUG codon (+16) only when 3'BTE was present in the reaction (compare lane 1,2 and lane 3). Lane 1 shows uncapped 5'UTR in the absence of wge. No toe-printing was observed when mutant 3'BTEBF was added in the similar reaction (lane 7).
Figure 4.9 Toe-prints of stalled ribosomes were found in ~16 nt downstream (lane 13) of the A of each AUG codon in BLucB mRNA where 3’BTE is present as a part of the mRNA (in cis). No ribosomal foot-prints were observed when mutant 3’BTE was present in the mRNA construct BLucBF (in cis) (lane 11). Toe-printing reaction (lanes 14,15) with BLucB SL-Dm1 mutant in which kissing –loop base pairing between 5’SL-D and 3’BTE is disrupted. No toe-printing or ribosome stalling was observed in 5’UTR (lane 14).
Figure 4.10 When BYDV mRNA (BlucB) was incubated in transnationally active wheat germ extract and the reaction was quenched in 0-15 min time scale, toe-prints were observed in the 3’S-L-I loop. This indicated initially ribosomes bound to the 3’S-L-I loop and were subsequently transferred to the 5’end of the message. Lane 2 shows a weak 40S footprint in the 3’S-L-I region of BLucB mRNA when 6 pmoles of mRNA was incubated with 16 pmoles of 40S and reverse transcription was done. Lane 3-5 indicates toe-prints obtained using wge system when the translation reaction was quenched in 0-15 min time scale. Similar toe-printing assay in wge using mutant BLucBF didn’t produce any prominent toe-print in the 3’S-L-I region (lane 7).
Figure 4.11 Activities of eIFs 4A/4B/4F, ATP to recruit ribosome in the 3’BTE 3’SL-I region.

Toe-printing analysis of stalling of ribosome in 3’BTE in the presence of indicated combinations of factors. Strong 40S toe-prints are observed in the 3’SL-I loop of 3’BTE in the presence of eIF 4A/4B/4F and ATP (lane 2,3). A very weak/ no toeprint was observed when 40S ribosomes were treated with eIF 4A/4B/4F and non-hydrolyzable ATP (lane 1).
Figure 4.12 Activities of eIFs 4A/4B/4F, PABP, and iso4F in the presence and absence of 3’BTE in promoting scanning through 5’-UTRs.

Toe-printing analysis of stalling of ribosome complex in the presence of indicated combinations of factors. When eIF 4A/4B/4F, ATP and 3’BTE are present a prominent 40S toe-print is observed in +16 region of uncapped 5’UTR (lane 4). Any other combination of eIFs (lane 9,10,11,12,13,14) didn’t show a ribosome footprint in the 5’UTR.
5 Structural probing shows helicase treatment increases RNA accessibility.

In order to gain an understanding of the structural aspect of the requirement of ATP dependent eIF4A-4B-4F interaction with 3’BTE for high affinity ribosome binding, 3’BTE RNA structures were probed using SHAPE in the presence and absence of eIF4A-4B-4F-ATP. The chemical benzoyl cyanide (BzCN), which modified flexible and single-stranded nucleotides in a sequence-independent manner (Merino et al., 2005; Wilkinson et al., 2006) was used in SHAPE experiments. Modified residues were mapped by primer extension followed by denaturing gel electrophoresis. Semi-automated footprinting analysis (SAFA) (Laederach et al., 2008) was used to analyze the SHAPE reactivity of each nucleotide. These results were then superimposed onto the Mfold-predicted structure of BTE (Fig. 5.1).

SHAPE studies of helicase complex treated 3’BTE (Fig.5.1) showed more modification of nucleotides in the 18S rRNA complementary region of 3’S-L-I as well as nucleotides (4830-4833) in the 3’S-L-IV; suggesting treatment with the helicase complex resulted in greater accessibility of the 3’BTE in regions complementarity to the ribosomal 18S rRNA.
Figure 5.1 Secondary structure of 3′BTE determined by SHAPE reactivity (Wang et al., 2010).

Secondary structure of 3′BTE determined by SHAPE reactivity (Wang et al., 2010). Bases are color coded based on the level of modification in the SHAPE reaction where red color indicates highest modification. Nucleotides are numbered according to their positions in the viral genome. Bases in bold italics comprise 17 nt the highly conserved sequence in all BTEs. Exposed bases due to helicase activity of eIF4F-4A-4B and ATP are designated by triangles. Interestingly, this helicase-dependent exposed region corresponds to the 18S rRNA-complementary region (GAUCCU, green box, panel B). (B) Circled nucleotides in 3′BTE are protected from SHAPE reagent by eIF4G binding (Kraft et al., 2013). Green box indicates 18S rRNA-complementary region (GAUCCU) in SL-I loop. CAGG sequence (red box) in the 3′SL-I region show a 40S footprint in a helicase complex treated BYDV mRNA.
6. Discussion

In many well-characterized mammalian viruses, 5’UTRs have IRESes (Pfingsten et al., 2006), which recruit the ribosome by either directly interacting with 40S subunits (Hertz and Thompson, 2011; Spahn et al., 2001), or with the help of various initiation factors (Fraser and Doudna, 2007; Reineke and Lloyd, 2011). Here we provide direct evidence of a completely different ribosome recruitment pathway to an mRNA. In this case initiation factors and the mRNA structure (3’BTE) facilitate recruitment of the 40S subunit to the 3’ UTR from which it is delivered to the 5’ end by base pairing. It was shown previously that the 3’BTE facilitates translation by directly interacting with eIF4F and base pairing to the 5’ end of the message (Treder et al., 2008). Here we show that the 40S subunit is also recruited to the 3’BTE.

Using fluorescence anisotropy and gel mobility shift based binding studies, we determined that 3’BTE, which contains a sequence complementary to 18S rRNA (GAUCCU) shows moderate binding affinity with purified 40S ribosomes and that mutation of this sequence to GAUCGAUCU (mutant BTEBF) weakens this binding affinity. Earlier studies showed that this mutation dramatically reduced translation in vitro and in cells (Guo et al., 2000; Rakotondrafara et al., 2006; Wang et al., 1997). The 5’UTR also showed a low affinity binding to 40S ribosomal subunits in fluorescence anisotropy and gel mobility shift assays. These binding affinities appear to be nonspecific based on both the low affinity and inability to toe-print the 40S subunit on the RNAs.
Binding enhancement of 40S ribosomes and 3’BTE was observed in the presence of eIF4F-4A-4B-ATP. This lead us to propose that the helicase activity of this complex disrupts secondary structure of RNA, exposing the 18S complementary sequence and also that of 18S rRNA because in both RNAs some of the complementary bases are in stem-loops, making the complementary sequences accessible for base pairing between the BTE and 18S rRNA.

We found that 80S ribosome stalling occurs close to the AUG start codon in cycloheximide treated wheat germ translation system only when the 3’BTE is present. These studies suggest two possible mechanisms of ribosome loading to the 5’end of the message. One possible mechanistic pathway is the ribosome interacts with 3’BTE first and subsequently is transferred to the 5’end of the message. The second possible mechanism would be that the 3’BTE transfers eIF4F to the 5’ end to which the 40S subunit is recruited as predicted for Tombusviridae genera (Nicholson et al., 2010). In both models, eIF4F is required for 40S recruitment and long-distance base pairing between the 3’BTE and 5’ UTR is required for delivery of host components: either initiation factors, or initiation factors and the 40S subunit. The data presented here support the second mechanism, because toe-printing assays using purified factors and 40S ribosomal subunits showed toe-prints (made by 40S subunit binding) in the 3’BTE SL-I (18S rRNA complimentary) region. Furthermore, we were unable to show significant direct ribosome binding to the 5’end of the message, upstream of the start
codon, with either toe-prints or fluorescence binding assays. Strong toe-prints close to the start codon with purified ribosomes in the presence of initiation factors eIF4F, 4B, 4A, ATP and 3’BTE reflects that recruitment of 40S is possible only in the presence of 3’BTE and it requires participation of eIF4F, 4B, 4A and energy from ATP hydrolysis. Ribosome stalling close to the 5’AUG initiation codon was not observed when the initiation factors were not present nor when the kissing loop between 3’UTR and 5’UTR was disrupted.

Our data led us to propose a general model of BTE-mediated cap-independent translation for the ribosome recruitment pathway and delivery of the translation machinery to the 5’ end of the BYDV message. Our model (Fig.6) suggests: i) eIF4F and possibly eIFiso4Fs are recruited directly to the folded 3’BTE ii) helicase complex eIF4F-4A-4B-ATP improves 40S binding affinity with 3’SL-I by exposing more accessible sites of the 3’BTE iii) 40S ribosomes bind to the 3’BTE, then iv) via long distance RNA-RNA interaction between 5’SL-D and 3’SL-III the translation machinery transfers to the 5’end of the message to start scanning.

Other plant viruses utilize various other RNA structures to bind eIF4F, and ultimately deliver the ribosome to the 5’ end of the genome (Nicholson et al., 2010). A tRNA-shaped 3’ CITE of TCV (TSS) was shown to bind directly to the 60S subunit and 80S ribosome, but not the 40S subunit, which leaves the question of how and where it is recruited (Stupina et al., 2008). A TSS-like domain was also found in the 3’ UTR of Pea
enation mosaic virus RNA2 (Gao et al., 2012), adjacent to another 3’ CITE that binds eIF4E (Wang et al., 2011). This TSS was reported to bind the 40S as well as the 60S ribosomal subunits and the 80S ribosome (Gao et al., 2012). Binding of the PEMV RNA2 TSS to the 40S subunit (Kd = 360 nM) was similar to BTE binding to the 40S subunit that we observed in the absence of initiation factors (Kd = 400 nM). However, the roles of translation factors in the function of either of the TSS elements has not been determined.

Our model (Fig. 6) bears interesting resemblance to interaction of Hepatitis C virus RNA with the ribosome to facilitate cap-independent translation, but with different binding sites and factor requirements. Like the 3’BTE, the HCV 5’ IRES binds directly to the ribosomal 40S subunit (Spahn et al., 2001). However it does so in a way that places the start codon directly in the P site without scanning, and it requires no eIF4 factors. Like the BTE, data are consistent with direct base pairing of the HCV IRES to the 40S subunit, but to a different region of the 18S rRNA from that which we propose for the BTE (Hashem et al., 2013b; Malygin et al., 2013). Of particular relevance is that the 3’ UTR of HCV also binds to the 40S subunit. This is not essential for, but greatly enhances activity of the IRES at the 5’ end (Bai et al., 2013). Thus, as in BTE-containing mRNAs, the 5’ and 3’ UTRs can interact simultaneously with the 40S subunit. For the HCV IRES it is proposed that this interaction is for ribosome recycling. In the case of the BTE, the 3’ interaction is much more important, because the 3’BTE is the only 40S
subunit-recruiting domain in BTE-dependent translation, in contrast to HCV in which the IRES in the 5’ UTR is the primary binding site of the 40S subunit. The requirement for eIF4 factors and the proposed base pairing of the BTE to a different portion of 18S rRNA (very near the 3’ end) than that bound by the HCV IRES or 3’UTR, indicate that the interactions are quite different for the BTE, but the end result is the same for both types of viral RNA: efficient cap-independent translation initiation via mRNA circularization.

The interaction of the 3’ UTR with the 5’ UTR to control translation of both BTE-containing RNAs and HCV RNA may indicate that both viruses use this interaction in a mechanism to switch viral RNA from translation to replication. A newly translated replicase would be expected to bind the extreme 3’ terminus (not needed for BTE- or IRES-driven translation), and proceed in the 5’ direction on its template as the template is still undergoing translation. Upon reaching the element in the 3’ UTR required for any (BTE) or maximal (HCV) translation, the replicase would disrupt this structure and shut off (BTE) or reduce (HCV) translation initiation at the 5’ end. This, in turn, would free the coding region of the viral RNA of ribosomes, making it available for full length negative strand synthesis by the replicase. As described previously (Barry and Miller, 2002; Miller and White, 2006) this potentiality provides an elegant feedback loop to assure a productive balance between replication and translation. Our data provide evidence supporting this model. Additionally our model could be applied to other viruses containing BTE -like elements.
Figure 6: BYDV translation model. (A) eIF4F (4G+4E) interacts with 3’BTE. (B) helicase complex eIF4F-4A-4B-ATP improves 40S binding affinity with 3’SL-I by exposing more accessible sites of the 3’BTE. 40S binds to 3’BTE. (c) Long distance RNA-RNA interaction between 5’SL-D and 3’SL-III helps the translation machinery to get transferred to the 5’end to start scanning.
7 Future Directions

Our studies reveal the ribosome recruitment pathway in BYDV. The mechanism involves interaction of 40S ribosomes with 3’BTE in the presence of helicase factors eIF4F-4A-4B and ATP and then possibly via long distance RNA-RNA interaction between 5’S-D and 3’S-III the translation machinery gets transferred to the 5’end of the message to start scanning. The precise function of RNA-RNA interaction hasn’t been characterized yet. We will characterize the involvement of this long distance interaction using ensemble FRET and single molecule FRET studies.

7.1 Overview

7.1.2 Fluorescence Resonance Energy Transfer (FRET)

Fluorescence resonance energy transfer (FRET) is a spectroscopic process between the electronic excited states of two dye molecules in which excitation is transferred from a donor molecule to an acceptor molecule non-radiatively. The relationship between energy transfer and the distance between two fluorophores was first described by Förster (Förster, 1948). The apparent FRET efficiency ($E_{app}$) is dependent on the inverse sixth power of the intermolecular separation (equation xi).

$$E_{app} = \frac{I_A}{I_A + I_D},$$

where $I_A$ and $I_D$ represent acceptor and donor intensities, respectively. ………………….. (xi)
Or $E = \frac{R_0^6}{R^6 + R_0^6}$ with $R$ being the distance between donor and acceptor and $R_0$ is the Förster distance between donor and acceptor at which the FRET efficiency is 50%.

Figure 7.1 Schematic representation of the spectral overlap integral.
7.1.2.3 Preliminary observation using ensemble FRET

We used BLuCB mRNA (5’UTR-Luc-3’UTR) as our primary goal for ensemble FRET experiments. Fluorescent labelled cDNA primers (primer “a” and primer “b”) (Fig.4.5) were hybridized to BLuCB mRNA. Primer “a” is complementary to the 3’end of 5’UTR and primer “b” is complementary to part of 3’UTR. Primer “a” is labeled with donor fluorophore Cy3 (excitation 550 nm and emission maximum 570 nm) while primer “b” is labeled with acceptor fluorophore Cy5 (excitation 650 nm, emission maximum 670 nm). We were expecting to observe resonance energy transfer if 5’UTR and 3’UTR come to the close vicinity (10-90 Å). We observed a FRET with a steady state efficiency of 54% between two UTRs of BLuCB mRNA. Ensemble FRET experiments provide us a FRET based system for studying the structural dynamics of BYDV translation.
7.2 Single–molecule FRET

7.2.1 Overview

Fluorescence resonance energy transfer (FRET) is a prevailing technique for studying dynamics of biological systems. Some conformational changes are difficult to detect using ensemble technique but FRET at single molecular level opens up new avenue to know conformational dynamics or mechanism of a biological system. The properties of individual molecule that would be masked due to the ensemble averaging become available in smFRET technique and also single molecule detection allows us to follow a specific molecule for an extended period of time, uncovering kinetic parameters of conformational changes (Ha, 2001a, b; Joo et al., 2008; Tinoco and Gonzalez, 2011).

7.2.1.1 Prism-based Total Internal Reflection Fluorescence (TIRF) microscope for single-molecule fluorescence imaging.

In a prism-based geometry the aligned, collimated laser beam is focused through a prism onto a microfluidic flow-cell (Joo et al., 2008; Tinoco and Gonzalez, 2011) (Fig.6.3). The prism is then brought into proximity of the specimen and the fluorescence emission is captured on the opposite side by a microscope objective. The principle is based on the total-internal reflection of the excitation laser beam. The laser beam undergoes total internal reflection at the quartz/solution interface of the prism –flow cell at an angle greater that it’s
critical angle. Even though 100% of the excitation light is reflected, an electromagnetic field vector is propagated into the region beyond the flow-cell. This electromagnetic vector decays with distance and is called evanescent field. Fluorescent bio-specimens are tethered on a cover-slip and their fluorescence behavior is detected if they are localized in the evanescent field (Fig.6.3). The fluorescence emission from tethered bio molecules are collected by an objective and then passed towards the sensor of an EMCCD camera that records the fluorescent event as a movie (Dorywalska et al., 2005; Tinoco and Gonzalez, 2011).
Figure 7.2 Optical setups for single-molecule detection studies using a prism-based TIRF microscope. The inset depicts a surface tethered labelled bio-molecules located in the evanescent field of the system.

7.3 Preliminary observation of RNA-RNA interaction in BYDV using sm-FRET

Figure 7.3 Cy3-Cy5 BLuCB construct hybridized to biotin-oligo. Traces showing Cy3-Cy5 anti-correlated dynamics with only green laser excitation, indicating FRET.
Figure 7.4 Cy3-Cy5 BLuCB construct hybridized to biotin-oligo. Traces showing Cy3-Cy5 dynamics with only green laser excitation, no indication of FRET.
Discussion

Preliminary single molecule FRET traces are shown in Fig. 6.3 and 6.4. Anti-correlated behavior in Cy3-Cy5 labeled BLucB was detected in Fig 6.3. This behavior indicates presence of FRET dynamics in the system. Most likely, 5’UTR and 3’UTR are coming into close proximity generating FRET states in BLucB system. Our initial observation also indicated other photo-physical states as found in Fig. 6.4 where FRET states are absent in BLucB system.
7.4 Future Studies

(i) BLuCB Surface Immobilization using a smaller mRNA construct.

We were tethering the BLuCB mRNA molecule using streptavidin–biotin interaction between the slide surface and the mRNA. We synthesized biotin labeled cDNAs complementary to the Luc region of BLuCB which were helping in surface immobilization of mRNA when the surface was coated with streptavidin. Most of our control studies with unlabeled (no biotin) mRNA showed presence of mRNA molecules on the slides. Most probably the negative phosphate backbone of large the mRNA molecule was interacting with streptavidin and helping in surface immobilization. Members of Goss lab will construct a different BLuCB plasmid with smaller Luciferase region to achieve a specific surface immobilization during smFRET experiments.

(ii) Data collection with BLuCB construct

We started collecting smFRET data with BLuCB construct. We observed FRET traces between Cy3-Cy5 molecules indicating possibility of 5′UTR-3′UTR interaction. Statistically significant numbers of data set are still lacking. Members of Goss lab will do similar experiments to achieve a more deterministic model.

(iii) smFRET behavior in presence of different eIFs and 40S

Dynamic behavior of the BYDV translation model in presence of different eIFs and 40S need to be investigated. It is possible that RNA-RNA interaction become more
stable in the presence of eIF4A-4B-4F and 40S. Single molecule technique will be the ideal one for determining the stability of RNA-RNA interaction.
8) Appendix

8.1 BUFFERS AND SOLUTIONS USED IN THE EXPERIMENTS

8.1.1 Cell media

<table>
<thead>
<tr>
<th>1 L Luria-Bertani (LB) media</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td>Yeast Extract</td>
</tr>
<tr>
<td>Peptone</td>
</tr>
<tr>
<td>Dd water</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>1 L LB agar with ampicillin (50ug/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
</tr>
<tr>
<td>Yeast Extract</td>
</tr>
<tr>
<td>Tryptone</td>
</tr>
<tr>
<td>Agar</td>
</tr>
<tr>
<td>Water</td>
</tr>
<tr>
<td>Ampicillin</td>
</tr>
</tbody>
</table>

8.1.2 Buffers for Protein purification and titrations

<table>
<thead>
<tr>
<th>10X HEPES Buffer, pH 7.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEPES</td>
</tr>
<tr>
<td>KCl</td>
</tr>
<tr>
<td>MgCl$_2$</td>
</tr>
<tr>
<td>DTT*</td>
</tr>
<tr>
<td>dd H2O</td>
</tr>
</tbody>
</table>

*Adjust the pH to 7.6 with potassium hydroxide (KOH), then add dd H$_2$O to 1 L.
Add DTT fresh before use to a final concentration of 10 mM.

**Phosphate-buffered Saline (PBS)**

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>8 g</td>
</tr>
<tr>
<td>KCl</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Na2HPO4</td>
<td>1.44 g</td>
</tr>
<tr>
<td>K2HPO4</td>
<td>0.24 g</td>
</tr>
<tr>
<td>dd H2O</td>
<td>ml</td>
</tr>
</tbody>
</table>

*Adjust the pH to 7.4 with Hydrochloric acid (HCl), then add dd H2O 1L.

Buffers for SDS Gel Electrophoresis

10x Tank buffer pH 8.3

<p>| | |</p>
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>Tris Base</td>
<td>30.28 g</td>
</tr>
<tr>
<td>SDS</td>
<td>10 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>144.13 g</td>
</tr>
<tr>
<td>ddH2O</td>
<td>1L</td>
</tr>
</tbody>
</table>

4x Running Gel buffer (1.5 M Tris-Cl, pH 8.8).

4x Stacking Gel buffer (0.5M Tris-Cl, pH 6.8).

10 ml 2x Treatment Buffer
<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>4x Stacking Gel buffer</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>4.0 ml</td>
</tr>
<tr>
<td>Glycerol</td>
<td>ml</td>
</tr>
<tr>
<td>Bromphenol blue</td>
<td>2.0 mg</td>
</tr>
<tr>
<td>DTT</td>
<td>0.31 g</td>
</tr>
<tr>
<td>dd H2O</td>
<td>10 ml</td>
</tr>
</tbody>
</table>

### 8.2 Running Gel

Gel concentration of 15% in 0.25 M Tris-HCl pH 8.8.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer solution*</td>
<td>5 ml</td>
</tr>
<tr>
<td>DD H2O</td>
<td>2.4 ml</td>
</tr>
<tr>
<td>4X Running gel buffer</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>ml</td>
</tr>
<tr>
<td><strong>10% Ammonium Persulfate</strong></td>
<td>50 µL</td>
</tr>
<tr>
<td>----------------------------</td>
<td>------</td>
</tr>
<tr>
<td><strong>TEMED</strong></td>
<td>3.3 µL</td>
</tr>
</tbody>
</table>

*Monomer solution
Acrylamide  60 g
Bisacrylamide  1.6 g
dd H2O to  200 ml
Mix the reagents in a small side-arm vacuum flask leaving out the ammonium persulfate and the TEMED.
Stopper the flask and apply vacuum for several minutes for degassing.
Add the TEMED and ammonium persulfate.
Pour the solution into gel unit.
Overlay gel with n-butanol to ensure a flat surface and to exclude air.
Wash off n-butanol with water after gel has polymerized (about 15 min).

**Stacking Gel**

Gel concentration of 4 % in 0.125 M Tris-HCl pH 6.8
Mix the reagents in a small side-arm vacuum flask leaving out the ammonium persulfate and the TEMED.

Stopper the flask and apply vacuum for several minutes to the solution.

Add the TEMED and ammonium persulfate.

Pour the solution on top of running gel, insert comb, allow polymerizing (about 30 minutes).

Remove comb, and fill each well with 1 X tank buffer.

Put the lid on the gel unit. Fill the assembly with 1X tank buffer.

### 8.3 Protocol for *in vitro* transcription of RNA using T7 RNA polymerase

This is a protocol for transcribing RNA oligomers using double stranded (ds)DNA template by using T7 RNA polymerase. The DNA template must include a 17nt promoter segment:
Top (sense) DNA sequence (5’-3’):

5’ -TAA TAC GAC TCA CTA TA…

Bottom (template) DNA sequence (5’-3’):

...TA TAG TGA GTC GTA TTA -3’

The RNA oligomer must start with a single G. Up to 3-4 G can be used and they increase the yield, but with 4 and more Gs, cruciform structures have been known to form that inhibit transcription. The following ranges of concentrations are typical. For a given DNA template, the transcription reaction is optimized by varying reaction conditions. PEG 8000 can be used for transcribing longer RNA oligomers, but I would try with and without. Most of the time it helps the yield if I use PEG. Water used should be RNase free, so DepC-treated or 5000 MWCO-filtered. RNaseOUT is optional and can be added to protect RNA from degradation if RNases are a concern, usually due to longer incubation time. Pyrophosphatase (PPase) increases the yield. Glycerol contained in T7 storage buffer (glycerol and DTT) inhibits transcription reaction, so no more than 10ul of T7 should be used for 1ml of total transcription volume.
The list contains ingredients in the order they should be added in. Mix all non-enzyme components first, vortex and add enzymes last, T7 being the very last.

<table>
<thead>
<tr>
<th><strong>Reagent:</strong></th>
<th><strong>Final Concentration:</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$O</td>
<td>As needed to increase to desired volume</td>
</tr>
<tr>
<td>10X (0Mg buffer)</td>
<td>1X</td>
</tr>
<tr>
<td>ATP</td>
<td>3-5 mM</td>
</tr>
<tr>
<td>CTP</td>
<td>3-5 mM</td>
</tr>
<tr>
<td>GTP</td>
<td>3-5 mM</td>
</tr>
<tr>
<td>UTP</td>
<td>3-5 mM</td>
</tr>
<tr>
<td>GMP</td>
<td>5 mM</td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>15 or 25 mM</td>
</tr>
<tr>
<td>PEG</td>
<td>0 or 8% (from 40% stock)</td>
</tr>
<tr>
<td>RNaseOUT (invitrogen) 40U/ul (optional)</td>
<td>1ul/1 ml of transcription</td>
</tr>
<tr>
<td>PPase, inorganic E.coli (0.4units/1ul)</td>
<td>1 unit/1 ml of transcription</td>
</tr>
<tr>
<td>dsDNA (annealed beforehand in 1X TE buffer)</td>
<td>200 nM</td>
</tr>
<tr>
<td>T7</td>
<td>1:200</td>
</tr>
</tbody>
</table>
**10X (0Mg) Transcription Buffer:**

- 0.4 M Tris pH 8.0
- 100 mM DTT
- Triton X-100 0.1%
- 10 mM spermidine
- 0.2um sterile filter

**T7 Storage Buffer:**

- 50 mM Tris pH 7.5
- 50% glycerol
- 200 mM NaCl
- mM DTT

**1X TE**

- 10 mM Tris pH 8.0
- mM EDTA

Incubate for 4 hours or longer (only if necessary) at 37°C. If no PPase is used cloudiness usually indicates a successful transcription. Check by running 2ul aliquots sampled at different times on a denaturing PAGE gel to determine optimum time. Following
incubation transcriptions may be frozen. For large scale transcriptions, after incubation add EDTA to stop the reaction by chelating MgCl$_2$ (2X amount of MgCl$_2$ used).

8.3.1 RNA purification

RNA in sodium acetate (final concentration .3M), 100% ethanol (3times the volume) → Dissolve and precipitate RNA at -20 for ~ 1 hour → Centrifuge at 4º C for 30 -40 mins → Remove supernatant, add 70% ethanol (don’t vortex), centrifuge it for another 30 mins → remove the supernatant and dry the pellet → dissolve the pellet in denaturing dye (enough) & heat it to 90ºC for 3min → PAGE → RNA band visualized using UV → Mark ad cut the gel band → take the gel in a microfuge tube → add NaOAc (final conc .3M) → shake it overnight so that all the RNA diffuses into the buffer → give a quick spin and remove the supernatant → add some more 0.3M sodium Acetate and vortex, centrifuge and remove the supernatant and add to the previous supernatant solution → Now to the final solution add 2.5-3times the volume of ethanol → incubate it on dry ice or -20ºc for 30 min and centrifuge at 4ºC at 12500 rpm for 30mins → remove supernatant and add 70% ethanol and centrifuge it for 20-30 min and remove supernatant and dry the pellet (can store the pellet at room temperature for one /two weeks).
8.4 Isolation of 40S ribosome from wheat germ

- Grind 120 g of wheat germ with 120 g of powdered alumina in a cold mortar.

- Mix the ground wheat germ with 300 ml of extraction buffer (20 mM HEPES-KOH, pH 7.6, containing 100 mM KCl, 1 mM (MgOAc)$_2$, 2 mM CaCl$_2$, and 5 mM 2-mercaptoethanol) and centrifuge for 10 min at 15,000g.

- Apply the supernatant (about 150 ml containing 450-550 $A_{260}$ units/ml) to a 1.2-liter Sephadex G-25 column equilibrated in Buffer A (20 mM HEPES-KOH, pH 7.6, 5 mM Mg(OAc)$_2$, 6 mM 2-mercaptoethanol, and 10% glycerol) containing 120 mM KCl and develop the column with the same buffer.

- Pool fractions with greater than 90 $A_{260}$ units/ml and centrifuge for 20 min at 25,000g.

- Centrifuge the supernatant from this centrifugation (about 200-250 ml containing approximately 150 $A_{260}$ units/ml) for 3 h at 170,000g.

- Suspend the ribosomes in Buffer A containing 120 mM KCl at a concentration of about 45 mg/ml (500 $A_{260}$ units/ml).

- Dilute the ribosomes (45 mg/ml) with an equal volume of Buffer A containing 1.2 M KCl to give a final KCl concentration of about 0.6 M.
• Allow the ribosomes to sit in high salt for 30 min in an ice bath and then centrifuge for 5 h at 150,000g.

• Suspend the ribosomes in Buffer A containing 50 mM KCl and store in small aliquots at a concentration of 45 mg/ml at -70°C.

• Dilute 3.5 ml ribosomes (containing 45 mg/ml) with 35 ml of room temperature buffer I (50 mM Tris-HCl, pH 7.7, 2 mM dithiothreitol, 0.1 mM EDTA, 0.6 M KCl, 3 mM MgCl₂, and 5% sucrose by weight) and incubate at 30°C for 5 min.

• Separate the ribosomal subunits by centrifugation through a linear 10-30% sucrose gradient buffer I for 4 h at 150,000g at 10°C.

• Collect 15 ml fractions and pool those fractions with the highest absorbance at 260 nm in the 40S and 60S regions of the gradient.

• Dialyze the pooled fractions for 4 h against 1 liter of buffer II containing 50 mM Tris-HCl, pH 7.7, 5 mM MgCl₂,, 50 mM KCl, 0.1 mM EDTA, 1.0 mM dithiothreitol, and 10% glycerol.

• Collect the 40S ribosomal subunit by centrifugation for about 12 h at 113,000g.

• Suspend the 40S ribosomal subunit at a concentration of approximately 80 A₂₆₀ units/ml in Buffer A containing 50 mM KCl, divide into 0.05-ml aliquots, fast froze in dry ice-acetone, and store at -70°C.
Buffer E (Extraction Buffer) pH 7.6

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM HEPES-KOH</td>
<td>1.19 g</td>
</tr>
<tr>
<td>1 mM Mg(OAc)$_2$</td>
<td>0.0536 g</td>
</tr>
<tr>
<td>2 mM CaCl$_2$</td>
<td>0.0735 g</td>
</tr>
<tr>
<td>6 mM DTT</td>
<td>0.15 ml</td>
</tr>
<tr>
<td>120 mM KCl</td>
<td>2.237 g</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>to 250 ml</td>
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Buffer A pH 7.6

<table>
<thead>
<tr>
<th>Component</th>
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</thead>
<tbody>
<tr>
<td>20 mM HEPES-KOH</td>
<td>4.76 g</td>
</tr>
<tr>
<td>5 mM Mg(OAc)$_2$</td>
<td>1.07 g</td>
</tr>
<tr>
<td>10% Glycerol</td>
<td>100 ml</td>
</tr>
<tr>
<td>6 mM DTT</td>
<td>0.6 ml</td>
</tr>
<tr>
<td>120 mM KCl</td>
<td>8.95 g</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>to 1000 ml</td>
</tr>
</tbody>
</table>
Buffer I pH 7.7

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mM Tris-HCl</td>
<td>3 g</td>
</tr>
<tr>
<td>0.1 mM EDTA</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>3 mM MgCl$_2$</td>
<td>0.3 g</td>
</tr>
<tr>
<td>2 mM DTT</td>
<td>0.2 ml</td>
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<tr>
<td>0.6 M KCl</td>
<td>22.37 g</td>
</tr>
<tr>
<td>5% Sucrose</td>
<td>25 g</td>
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<tr>
<td>ddH$_2$O</td>
<td>to 500 ml</td>
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</table>

Buffer II pH 7.7

<table>
<thead>
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<th>Component</th>
<th>Amount</th>
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</thead>
<tbody>
<tr>
<td>50 mM Tris-HCl</td>
<td>6 g</td>
</tr>
<tr>
<td>0.1 mM EDTA</td>
<td>0.4 ml</td>
</tr>
<tr>
<td>5 mM MgCl$_2$</td>
<td>1 g</td>
</tr>
<tr>
<td>1 mM DTT</td>
<td>1 ml</td>
</tr>
<tr>
<td>50 mM KCl</td>
<td>3.73 g</td>
</tr>
<tr>
<td>10% Glycerol</td>
<td>100 ml</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>to 1000 ml</td>
</tr>
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</table>
Luciferase Activity Assay Buffer

<table>
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<tr>
<th>Component</th>
<th>Volume</th>
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</thead>
<tbody>
<tr>
<td>25 mM Tricine pH 8</td>
<td>1 ml</td>
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<tr>
<td>0.1 mM EDTA</td>
<td>0.008 ml</td>
</tr>
<tr>
<td>5 mM MgCl$_2$</td>
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</tr>
<tr>
<td>1 mM coenzyme A</td>
<td>5 ml</td>
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<tr>
<td>10 mM ATP</td>
<td>5 ml</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>to 20 ml</td>
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</tbody>
</table>

Helicase Activity Buffer

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<th>Component</th>
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</thead>
<tbody>
<tr>
<td>20 mM Tris-HCl pH 7.5</td>
<td>2 ml</td>
</tr>
<tr>
<td>1 mM MgOAc</td>
<td>0.022 g</td>
</tr>
<tr>
<td>2 mM DTT</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>70 mM KCl</td>
<td>0.52 g</td>
</tr>
<tr>
<td>2u/µl RNAse inhibitor RNAsin</td>
<td>4 ml</td>
</tr>
<tr>
<td>ddH$_2$O</td>
<td>to 100 ml</td>
</tr>
</tbody>
</table>
8.5 Effect of metal ions on plant initiation factor eIF4B(69-527) using Circular Dichroism

CD measurements made at 25 °C on a Jasco model spectropolarimeter (J730) equipped with Peltier temperature controller. 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 accumulations per sample. All the spectra were measured at protein concentration of 0.4mg/ml. Spectra were corrected for buffer contribution and the CD signal was converted to mean residue ellipticity (MRE) in degcm$^2$dmol$^{-1}$,

$$\text{MRE} = \left( \frac{\text{Θ}}{10*n*Cp*l} \right),$$

where $\text{Θ}$ 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:

$$\%\alpha\text{-helix} = \left\{ \frac{(-\text{MRE}_{222} - 2340)}{30,300} \right\} \times 100$$

Far UV-CD spectra were also analyzed using K2d software.
Buffer

10mM Na-phosphate, 100Mm NaCl, pH=7.4

Titration with ZnCl₂.

Secondary Structure Calculation

Secondary structure analysis using k₃d software.

<table>
<thead>
<tr>
<th></th>
<th>Alpha (%)</th>
<th>Beta(%)</th>
<th>Random(%)</th>
<th>Max Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>28</td>
<td>17</td>
<td>55</td>
<td>0.227</td>
</tr>
<tr>
<td>10 uM Zn²⁺</td>
<td>27</td>
<td>18</td>
<td>55</td>
<td>0.227</td>
</tr>
<tr>
<td>20 uM Zn²⁺</td>
<td>27</td>
<td>19</td>
<td>54</td>
<td>0.227</td>
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<td>50 uM Zn²⁺</td>
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<td>0.227</td>
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<tr>
<td>100 uM Zn²⁺</td>
<td>20</td>
<td>28</td>
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</tr>
<tr>
<td>200 uM Zn²⁺</td>
<td>10</td>
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<td>48</td>
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<td>300 uM Zn²⁺</td>
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<td>48</td>
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<td>500 uM Zn²⁺</td>
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<td>45</td>
<td>48</td>
<td>0.227</td>
</tr>
</tbody>
</table>

Theoretical calculation of % of alpha helix.
Protein 12.73
10 uM Zn$^{2+}$ 9.15
20 uM Zn$^{2+}$ 7.64
50 uM Zn$^{2+}$ 6.33
100 uM Zn$^{2+}$ 4.19
200 uM Zn$^{2+}$ 3.24
300 uM Zn$^{2+}$ 1.62
500 uM Zn$^{2+}$ 1.82

Titration with MgCl$_2$

Secondary Structure Calculation using k$_2$d software.

<table>
<thead>
<tr>
<th></th>
<th>Alpha (%)</th>
<th>Beta(%)</th>
<th>Random(%)</th>
<th>Max Error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>30</td>
<td>15</td>
<td>55</td>
<td>0.227</td>
</tr>
<tr>
<td>10 uM Mg$^{2+}$</td>
<td>29</td>
<td>17</td>
<td>54</td>
<td>0.227</td>
</tr>
<tr>
<td>20 uM Mg$^{2+}$</td>
<td>28</td>
<td>18</td>
<td>54</td>
<td>0.227</td>
</tr>
<tr>
<td>Mg$^{2+}$ Concentration</td>
<td>% of α Helix</td>
<td>% of β Sheet</td>
<td>% of Turn</td>
<td>% of Random</td>
</tr>
<tr>
<td>--------------------------</td>
<td>--------------</td>
<td>--------------</td>
<td>-----------</td>
<td>-------------</td>
</tr>
<tr>
<td>50 uM Mg$^{2+}$</td>
<td>27</td>
<td>19</td>
<td>54</td>
<td>0.227</td>
</tr>
<tr>
<td>100 uM Mg$^{2+}$</td>
<td>25</td>
<td>24</td>
<td>51</td>
<td>0.227</td>
</tr>
<tr>
<td>200 uM Mg$^{2+}$</td>
<td>17</td>
<td>34</td>
<td>48</td>
<td>0.227</td>
</tr>
<tr>
<td>300 uM Mg$^{2+}$</td>
<td>15</td>
<td>37</td>
<td>48</td>
<td>0.227</td>
</tr>
<tr>
<td>500 uM Mg$^{2+}$</td>
<td>8</td>
<td>44</td>
<td>48</td>
<td>0.227</td>
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</tbody>
</table>

Theoretical calculation of % of α helix.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Theoretical Calculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 uM Mg$^{2+}$</td>
<td>14.99</td>
</tr>
<tr>
<td>20 uM Mg$^{2+}$</td>
<td>10.80</td>
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<tr>
<td>50 uM Mg$^{2+}$</td>
<td>9.82</td>
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<td>3.59</td>
</tr>
<tr>
<td>1000 uM Mg$^{2+}$</td>
<td>3.44</td>
</tr>
</tbody>
</table>
Conclusion.

Absorption in 240 nm and below is due to the peptide bond; there is a weak but broad n →Π* transition around 220 nm and a prominent Π →Π* transition around 190 nm. The absorbance at 222 nm and 190 nm is due to alpha helical structure of the protein. Alpha helix has negative bands at 222 nm and 208nm and a positive one at 190nm. A number of algorithms exist which use the data from far UV CD spectra to provide an estimation of the secondary structure composition of proteins. In this study we used K2d algorithm to analyze structural changes in protein in presence of metal ions. Alpha helical contribution dropped significantly nearly 20% both in presence of Zn$^{2+}$ and Mg$^{2+}$ ions.
9) References


Guo, L., Allen, E., and Miller, W.A. (2000). Structure and function of a cap-independent translation element that functions in either the 3' or the 5' untranslated region. RNA 6, 1808-1820.


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.


