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Translational control of gene expression mediated by the 3' untranslated region of Barley yellow dwarf virus

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Translational control of gene expression mediated by the 3' untranslated region of

Barley yellow dwarf virus

by

Elizabeth Lynn Pettit

A dissertation submitted to the graduate faculty
In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Program of Study Committee:
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2005

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iv</td>
</tr>
<tr>
<td>CHAPTER 1. GENERAL INTRODUCTION</td>
<td>1</td>
</tr>
<tr>
<td>CHAPTER 2. SEPARATE DOMAINS IN THE BARLEY YELLOW DWARF LUTEOVIRUS 3’ UTR CONFER CAP-INDEPENDENT AND POLY(A)-INDEPENDENT TRANSLATION</td>
<td>29</td>
</tr>
<tr>
<td>CHAPTER 3. INTERACTION OF CAP-BINDING FACTORS WITH THE 3’ END OF AN UNCAPPED VIRAL RNA</td>
<td>69</td>
</tr>
<tr>
<td>CHAPTER 4. CONSERVED RNA STRUCTURES AND SEQUENCES IN THE 3’ UTR OF BARLEY YELLOW DWARF VIRUS ARE REQUIRED FOR REPLICATION AND TRANSLATION</td>
<td>97</td>
</tr>
<tr>
<td>CHAPTER 5. GENERAL CONCLUSIONS</td>
<td>131</td>
</tr>
<tr>
<td>APPENDIX A. DEVELOPMENT OF TOEPRINTING METHODS TO ASSESS RIBOSOME BINDING TO BYDV RNA</td>
<td>152</td>
</tr>
<tr>
<td>APPENDIX B. EVIDENCE THAT 4E MAY BE RECRUITED TO THE BYDV 5’ UTR</td>
<td>163</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>164</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>167</td>
</tr>
</tbody>
</table>
ABSTRACT

The 869 nt 3' untranslated region (3' UTR) of *Barley yellow dwarf luteovirus* (BYDV) RNA facilitates cap-independent and poly(A) tail-independent translation. A 105 nt cap-independent translation element (BTE, or BYDV TE) within the 3' UTR was previously defined using wheat germ translation extracts (Guo et al, 2000), but additional 3' UTR sequence is required for translation in plant cells. One domain, nt 4918-5008, confers cap-independent translation *in vivo* in a discrete and strong manner. Some of this extra sequence places the BTE on a phylogenetically conserved, long double-stranded “stalk”, making it more accessible to cellular translational machinery. Another sequence in the 3' UTR, nt 5089-5142 downstream of the cap-mimic is functionally replaceable by a poly(A) tail on the 3' end of the mRNA. The influence of this sequence on translation is somewhat weaker than that of the cap-mimic sequence, and may be correlated with length. The requirement for these elements suggests that at least 250 nt of BYDV 3' UTR are necessary for the virus to compete effectively with host cell mRNAs in recruiting the translation apparatus *in vivo*.

The BTE recruits wheat translation initiation factors, including eukaryotic initiation factor 4E (eIF4E), the cap-binding protein, and its isoform eIFiso4E. Together with eIF4G and eIFiso4G respectively, these proteins complex to form eIF4F and eIFiso4F. Pulldown experiments using BTE RNA as bait showed that both eIF4E and eIF4G are associated with the BTE RNA. UV-crosslinking followed by immunoprecipitation with antisera to eIF4F and eIFiso4F shows that wild type BTE RNA associates with these factors while a nonfunctional mutant BTE does not. Depletion of cap-binding factors resulted in a
substantial loss of translation of viral RNA in vitro which was recovered by adding back recombinant eIF4F and eIFiso4F. Thus cap-binding proteins facilitate cap-independent translation of BYDV RNA. Finally, m\(^7\)GTP added in trans inhibits translation in vitro of both uncapped and capped full-length infectious RNA, suggesting that the cap-binding site of eIF4E or eIFiso4E enables recruitment of the factor for BYDV translation.

CHAPTER 1. GENERAL INTRODUCTION

Background

Since Beijerinck's description of necrotic lesions on diseased tobacco plants as *contagium vivum fluidum* in 1898, biologists have sought to understand and develop cures for viruses of many hosts. In the years since, scientists have found out that a virus requires a living host cell to replicate and spread (1928), that viruses consist of protein and either RNA or DNA (1930s), and that virus infection arises from DNA injection by a bacteriophage (1952). Over 3000 virus species and more than 30,000 viral isolates or strains are known today, and they use hosts of nearly every life form including animals, plants, bacteria and fungi (Fauquet et al., 2000). This diversity indicates that viruses are capable of a multitude of unique strategies to infect, and hints at how much remains to be understood about their biology and their interactions with the host.

Viruses by definition are obligate parasites and require a host cell for their existence. They exist as small infectious particles, or virions, that are transmissible between hosts.

Virions consist of capsid proteins surrounding either a DNA or RNA genome. Once inside a host cell the viral genome is capable of replication and expression of its proteins. Finally, progeny virions are formed inside a host cell, a process that spreads the viral infection to other cells. Throughout the course of infection viruses require their hosts for gene expression.

The advent of molecular biology is intertwined with the field of virology, particularly as techniques and instrumentation were advanced in the early 20th century to study the workings inside cells. Following Watson and Crick's proposal of DNA structure in 1953, the
coding problem, how DNA sequence specifies protein, attracted much attention. Development of cell free systems in the 1950s provided a biochemical framework to address questions on protein synthesis and helped lead to the description of tRNA, mRNA and ribosomes in the 1960s (Judson, 1979). In the 1970s, scientists began to use recombinant DNA and nucleotide sequencing, providing the foundation for an explosion in molecular biology in the last 30 years. These advances helped scientists discover viral replication strategies, describe genetic recombination and evolution among viruses, model biochemical structures such as capsid proteins, and sequence viral genomes.

Recent advances

In recent years, biologists have generated DNA sequences in exponentially-growing numbers, and the genome sequence of several organisms including humans have been completed. This has led to the development of depositories for large amounts of biological data, and a need for computers and the worldwide web to access and analyze sequence data. In this milieu, bioinformatics and computational biology were born, enabling scientists to store and analyze data. Sequence data have provided major insights into gene function, yet work remains to transfer sequence information into knowledge about biological function.

Investigators have begun to appreciate the abundance and influence of sequences that are present in genomes and do not encode genes. One example is that genomes are full of transposable elements that exert their influence over other genes. Another example is small RNAs. Considerable efforts have been focused in the past few years on describing the small RNAs, or “dark matter of biology” (Ruvkun, 2001). Small RNAs usually 21 to 25 nucleotides in length, originally found as the product of virus induced gene silencing, are
now known to be both host and viral genome-derived (Pfeffer et al., 2004). siRNAs and microRNAs (two small RNA classes) modify the expression of a gene in the nucleus, target mRNAs for degradation, and influence the translation of mRNAs (Voinnet, 2002). A third example of an important noncoding sequence is the untranslated region of messenger RNAs that modify the stability or translation of the mRNA, often by binding protein factors (Wilkie et al., 2003). The noncoding sequences are not simply “junk DNA” (or RNA) as previously believed, and in fact modify the expression of other entities.

RNA sequence is not the only factor that influences RNA function. RNA structure influences its function, so folding algorithms contribute much to studying structure. With computer prediction programs such as the popular web-based Mfold (Zuker, 2003) and the Vienna RNA package (Hofacker, 2003), we have the ability to model RNA structure in silico. Folding algorithms are based on thermodynamic modeling scoring systems that maximize the number of base pairs (Eddy, 2004). Their capabilities have expanded to include larger sequence lengths, potentially allowing the prediction of global RNA folds. Energy minimization and weighting for specific structures, such as tetraloops, that fit consensus sequences that are known to have low energy, add a layer of complexity to the algorithms. The user must be mindful that the lowest energy structure is not always the most accurate or functional. To find pseudoknot structures, where loop bases pair with bases outside of a specific stem, one must go beyond Mfold. Each pseudoknot folding algorithm has its limitations, but the programs are potentially useful in formulating testable hypotheses. These programs are reported to place 50-70% of the nucleotides in the right positions (Eddy, 2004), meaning experimental verification or phylogenetic studies are required to confirm structural predictions.
More recently, programs have been made available that combine primary and secondary structure data to generate representations of phylogenetic similarities between RNAs. These programs automate covariation analysis, to show how structures from the RNAs of multiple species or isolates remain the same due to changes in primary sequences that make up basepaired sections. One example is Alidot (Hofacker et al., 1998). Many of the programs require input of a CLUSTAL W multiple sequence alignment. The program will then compute secondary structures and align them, and provide a representation such as an energy plot or mountain plot. These programs seem to work better with shorter RNA sequences and with a larger dataset of sequences. One disadvantage for biologists is that the programs usually require some computer programming; thus as graphical representation methods are better developed these programs may become more widely used.

Specialized databases for well-studied RNAs, such as ribosomal RNAs and group I introns hold a plethora of structural and phylogenetic information. An exciting addition to the collected RNA structures is the Structural Classification of RNA (SCOR) database (Klosterman et al., 2004). SCOR enables one to find RNAs with particular structures or functions, and divides the RNAs into discrete categories.

Because of advances in structure prediction software and the growing numbers of deposits into sequence and structure databases, a researcher must spend quality time examining and thinking about computer outputs. In vitro chemical methods inform us of how RNA behaves in solution, and functional studies allow us to study the RNA in a biologically relevant context. These approaches are crucial for characterizing RNA, and to substantiate, modify or refute a result derived from computational modeling. Although in silico work informs experimental design and advances the pace of discovery at the "wet lab"
bench; biology really should direct and drive the informatics. A sum of these methods can be successfully used to describe a particular length of sequence, a functional RNA element, or even a whole genome. Such a combination of modeling and experimental testing was used in the work described in Chapter 4 to better understand a domain in Barley yellow dwarf viral RNA that contributes to translation in plant cells. This approach, "secondary structure-omics" will no doubt prove useful in studying the biology of RNA.

Many cellular processes are carried out in complexes of heterologous material such as ribonucleoprotein complexes (RNPs). RNPs regulate the flow of gene expression information at every level, and a system-wide study of RNP properties will help us to understand cellular behaviors on a global scale (Hieronymus and Silver, 2004; Keene, 2001). In fact, given that many cellular RNAs are associated with proteins, it is likely the biological context of RNAs would alter their secondary structures (Juan and Wilson, 1999). Virus particles, ribosomes, and mRNAs bound to proteins that traffic them are a few examples of RNPs. These complexes constitute many of the workers in the cellular factory, convening and disbanding as the cell programs them. New technologies have been introduced to detect and catalog RNA protein associates, including protein-RNA chips (Gerber et al., 2004).

Beyond cataloging structural data on RNA/protein interactions, bioinformatics enables one to find patterns from the data available. The Nucleic Acid Interaction Library is comprised of web pages where calculated hydrogen bond interactions between nucleotide bases and amino acids can be viewed (Cheng et al., 2003; Walberer et al., 2003). The amino acid-nucleotide interaction database relies on determined structures in the Protein Data Bank, with the aim to enable comparison of diverse interactions. One predicts (and hopes) that
such catalogues are only the beginning, and the representations of nucleic acid interactions contained therein will only grow and improve.

**Eukaryotic Translation**

The template for translation is the messenger RNA (mRNA), and the mRNA has several features that facilitate the recruitment of the translation machinery (Figure 1). A 5' \( \text{m}^7 \text{GpppN} \) cap and a 3' poly(A) tail are present on the majority of cellular mRNAs (Figure 1A). Other strategies, that have been described for a minority of transcripts, include internal ribosome entry sites (IRES) and 3' elements that facilitate cap-independent translation (Figure 1B and 1C, discussed below). The coding sequence specifies the amino acids to be incorporated in the protein product, and is bordered by a start codon and a stop codon that signal the ribosome to synthesize polypeptides. Untranslated regions on the mRNA flank the coding sequence; even though they do not code for protein these sequences influence translation.
Figure 1. Features of mRNAs
A. Features on a typical cellular mRNA, containing a 5’ m7GpppN cap and a 3’ poly(A) tail and B. Features of mRNAs with internal ribosome entry sites in the 5’ UTR (based on Gebauer and Hentze, 2004). C. Cap-independent translation elements found in the 3’ UTR of plant viruses. The BTE cruciform structure is shown the BTE (see Figure 3B).

Figure 2. Initiation factors interacting with the canonical mRNA containing a 5’ cap (based on Gebauer and Hentze, 2004) (eIF = eukaryotic initiation factor) 40S and 60S are ribosomal subunits, and 43S and 48S are 40S subunits combined in either the preinitiation complex or initiation complex respectively. The curved line with 3 notches represents transfer RNA, and the notches are the anticodon loop. Numbered boxes are added to represent points where viruses are known to interfere with the components of the translation machinery as described in the text.
Protein synthesis occurs by the assembly of initiation factors (eIFs in eukaryotes) and ribosomal subunits at the initiation codon on a mRNA, followed by elongation and termination. Translation initiation is a rate-limiting step (Pestova, 2001) in protein synthesis and is highly regulated (Figure 2). As initiation is the primary subject of the research for this dissertation, it is also the focus of this background discussion. Initiation is described by the ribosome scanning model (Kozak, 1991). Briefly, eIF2 and GTP are joined to Met-tRNA, and then combine with the 40S ribosomal subunit to form the 43S preinitiation complex (Figure 2, bottom). The preinitiation complex meets the mRNA via interactions with factor eIF4E bound at the 5' cap structure. eIF4E is also bound by the scaffold protein eIF4G and the helicase eIF4A. The eIF4E and eIF4G complex is called eIF4F in plants; in animals the 4F complex also includes 4A. eIF4G binds to poly (A) binding protein (PABP), that is bound to the poly(A) tail at the 3' end of the mRNA. Binding of eIF4F to cap results in a conformational change that promotes PABP binding. Recruitment of PABP to this complex promotes ATPase and RNA helicase activities eIF4A and eIF4B (Luo and Goss, 2001 Bi and Goss, 2000). Following binding of the required factors to the 5' end of the mRNA, the 40S subunit and associated proteins scan to the start codon of the mRNA, where the 60S ribosomal subunit joins the mRNA initiation complex. The 80S ribosome then catalyzes the formation of the polypeptide. Alternative mechanisms, including internal initiation (discussed below), suggest other ways for messages to recruit the translation machinery to the start codon.

Circularization of the mRNA is considered a prerequisite for translation of the mRNA, although how exactly the yet-unclear process of scanning depends on events at both ends of the mRNA is unknown. Synergy has been observed between the 5' cap and 3'
poly(A) tail (Gallie, 1991; Tarun and Sachs, 1995), and the factors that bind each of the ends. mRNA circularization would provide an advantage in checking the mRNA ends for intactness, before ribosomes and significant energy are committed to translation. If a mRNA stays circularized, the structure might also serve to promote recycling of ribosomes for more efficient translation on a single template (Howard et al., 1970). Communication between the ends of a mRNA also might balance initiation rates with termination. Atomic force microscopy was used to provide visual and physical evidence for circularization in yeast mRNAs (Wells et al., 1998). Although there is good evidence for circularization, it has not unequivocally been demonstrated in all systems (Kozak, 2004). Thus, a researcher must remain open-minded and rigorous in designing experiments to test translational mechanisms.

The 3' end of the mRNA contributes to translation of the mRNA. The majority of mRNAs are polyadenylated; the poly(A) tail provides a critical link in the stability and translatability of a mRNA. The length is not uniform and is dynamic throughout the life (and death) of a mRNA. Poly(A) tail lengthening and shortening plays a role, and is regulated both developmentally and by external cues (Jacobson, 1996). In general, longer poly(A) tail lengths are associated with active translation, and shorter lengths are related to mRNAs targeted for degradation (Jacobson, 1996). The poly(A) tail confers translational advantages both in vitro and in vivo, particularly in the latter case (Jacobson, 1996). PABP is the best studied protein associated with poly(A) function, and there is genetic, molecular and biochemical evidence that PABP is crucial for poly(A) tail function. Although PABP is known to be a promiscuous RNA-binding protein, its activity is regulated at several levels: multiple domains within PABP function together (Gray et al., 2000), by the assembly of multiple PABP molecules and their phosphorylation status (Le et al., 2000), and by
interaction with other proteins that both negatively and positively affect translation (Craig et al., 1998; Khaleghpour et al., 2001). PABP plays a role in both stability and translation of a cytoplasmic mRNA and in combination with the 3’ poly(A) tail, plays a role at other steps in gene expression (Mangus et al., 2003).

Sequences in the 3’ UTR other than the poly(A) tail also influence post-transcriptional gene expression (Wilkie et al., 2003). Obvious examples are the polyadenylation signals and other AU-rich elements that regulate mRNA stability. Conversely, in the case of 15-lipoxygenase mRNAs, proteins bind the 3’ UTR to repress translation, contributing to the temporal regulation of gene expression for this mRNA. Other 3’ UTR signals specify localization codes for mRNA; these have been well studied in Drosophila oocyte development. Some 3’ UTRs are targets for microRNAs that direct the mRNA for degradation or translational repression. For the mRNAs that lack a poly(A) tail, including histone mRNAs in mammals (plant histone mRNAs are polyadenylated) RNA in the 3’ UTR plays a role in gene expression. The terminal stem loop in Xenopus oocytes mRNAs bind a protein, SLBP, to regulate translation (Sanchez and Marzluff, 2002). The influence of the 3’ end does not preclude ribosome scanning, but the knowledge derived from studies on 3’ UTRs sheds light on the as yet unclear process of scanning.

Because mRNA levels and protein levels are not always correlated, the control of translation is an important step in gene expression regulation. Translational control is implicated in developmental programming, response to infection, stress, and environmental conditions. Thus, translational control can provide rapid control or a feedback response. Specific points of translational control include mRNA localization, message stability, translatability, initiation, polysome association, elongation, and termination. These steps of
regulation represent a significant amount of gene expression control, estimated to affect between 1 and 20% of genes (Pradet-Balade et al., 2001). In some instances, translational control is the major contributor to gene expression control (Kahvejian et al., 2001). For RNA viruses, translational control is an especially important way to modulate gene expression.

Translation and Viruses

Viruses have a fascinating collection of translational strategies. This is due to their compact genomes and the need to compete with the host mRNAs for the cellular protein synthesis machinery. Among the translational mechanisms described for viruses are polyprotein synthesis, leaky scanning, ribosomal frameshifting, and readthrough of stop codons. Many viruses bypass traditional translation mechanisms due to a lack of the standard 5' cap or 3' poly(A) tail. For example, the nonpolyadenylated rotaviruses produce NSP3 viral protein, that binds eIF4G, overriding the function of PABP (Padilla-Noriega et al., 2002) (Figure 2, Box 1). During infection, viruses selectively modify host factors to shut down cellular gene expression. Viral proteases from poliovirus, rhinovirus and others cleave eIF4G, preventing its N terminus from binding capped host mRNAs (Enquist et al., 2000) (Figure 2, Box 2). Some viruses change the phosphorylation status of translation factors such as eIF2α (3 Fig. 1B), eIF4E and eIF4EBP (4E binding protein) (Figure 2, Box 4), that affects their ability to interact with other members of the translation apparatus (Enquist et al., 2000). Furthermore, some viral RNAs control their own fate for replication or translation by selectively binding host and viral protein factors (Gamarnik and Andino, 1998). Host shutoff of translation is a common strategy found during infection by mammalian viruses but is not well known as consequence of plant virus infection, although translational shutoff has been

Translational control strategies and features provide examples of the similarities between different viruses regardless of their individual hosts. Barley yellow dwarf virus, the subject of this research, has some features in common with important mammalian pathogens. Long distance basepairing is proposed to facilitate translation between the ends of the BYDV RNA (Guo et al., 2001). This type of communication is also important for the replication of flaviviruses such as West Nile and Dengue viruses (Khromykh et al., 2001; You and Padmanabhan, 1999). BYDV RNA has cis-acting sequences that facilitate cap-independent translation and bind host translation initiation factors. A striking example of potential similarity in translation mechanisms between Hepatitis A virus IRES and BYDV exists in that both RNAs require eIF4E (Ali et al., 2001). Finally, all positive strand RNA viruses have to balance translation and replication of the genomic RNA. Thus, mechanistic details described for BYDV potentially have parallels in medically important virus/host interactions.

**Internal Initiation**

Internal ribosome entry sites (IRESs) on mRNAs enable an alternative mechanism of translation initiation that is cap-independent (Figure 1B). IRESs, situated in the 5'UTR of mRNAs, contain 300 to 500 nucleotides with extensive secondary and tertiary RNA structure to recruit the translation apparatus. The classical test for an IRES is the introduction of the sequence between two reporter genes to look at translation of the uncapped downstream gene (Jackson et al., 1994). Appropriate controls must be performed in tandem.

Several important viral pathogens and cellular mRNAs contain IRESs. Encephalomyocarditis virus (EMCV) and foot and mouth disease virus both bind the C-
terminal portion of eIF4G along with eIF4A, eIF3, and eIF2 (Martinez-Salas et al., 2001). Hepatitis C virus (HCV) contains an IRES that directly binds the 40S ribosomal subunit and eIF3 (Sizova et al., 1998). Surprisingly, the IRES from cricket paralysis virus recruits ribosomes in the absence of any initiation factors, and is able to do so with a noncanonical initiation codon (Wilson et al., 2000a; Wilson et al., 2000b). Among the cellular mRNAs that use an IRES mechanism are growth factors, eIF4G, oncogenes, and other genes activated under stress (Kieft et al., 2001). Thus, IRESs function in diverse ways in eukaryotes.

A few IRESs have been characterized in plants. The 5' leader of Tobacco etch virus confers cap-independent translation in tobacco cells, through a mechanism that appears to depend on eIF4G (Gallie, 2001). In Crucifer-infected tobamovirus (crTMV) a 148nt internal translation element produces the coat protein; this element stimulates translation better than the EMCV IRES (Dorokhov et al., 2002; Ivanov et al., 1997). The crTMV IRES acts through repeats of GAAA, a motif also found in plant genes (Dorokhov et al., 2002).

**Translation in Plants**

In plants, differential translation occurs in response to developmental cues and environmental stimuli. A plant cannot move themselves under stress; thus fine tuning gene expression under stressful conditions may aid the plant. Flooding, light/dark changes and heat shock are all known to affect the translational status inside a plant cell. Heat shock protein (HSP) mRNAs are translated efficiently during heat shock, even though the phosphorylation status of translation factors is altered, and there are diminished interactions between translation initiation factors and between factors and the mRNA (Le et al., 2000; Pitto et al., 1992). Transitions between light and dark cause rapid changes in the
translatability of the ferredoxin-1 (Fed-1) mRNA, working through the 5' leader (Hansen et al., 2001). In fact the leader of the Fed-1 mRNA enhances Fed-1 translation by binding HSP101 protein, as does the Tobacco mosaic virus 5' UTR (Ling et al., 2000). In the case of flooding, the maize ALCOHOL DEHYDROGENASE1 mRNA apparently has a translational advantage in a cell deprived of oxygen (Kawaguchi and Bailey-Serres, 2002). Thus plant stress results in global and mRNA-specific changes in translation, suggesting that the standard interplay between 5’ and 3’ ends of the mRNA are not always the means for translation. Given the contribution from chloroplast and mitochondrial genomes to the pool of plant mRNAs, many possibilities exist for mechanistic variety in posttranscriptional plant gene expression.

The nomenclature for select initiation factors differs in plants. The eIF4F complex in plants only includes eIF4G and eIF4E (in animals eIF4A is included in the 4F complex because it co-purified with eIF4E) (Browning, 1996). Plants have two isoforms each for eIF4E (26 kDa) and eIF4G (165 kDa), named eIFiso4E (28 kDa) and eIFiso4G (86 kDa). The mechanisms underlying the existence and usage of multiple isoforms for these factors are not yet known. In addition eIF4E, eIFiso4E, eIF4G, and eIFiso4G differ in their location, abundance, activity, and posttranslational modification within a plant (Kawaguchi and Bailey-Serres, 2002). A possible difference between the isoforms is their preference for different mRNA leaders: eIF4F seems to support initiation at the 5' end of highly structured mRNAs better than eIFiso4F (Gallie and Browning, 2001). eIF4F is similarly versatile in promoting the translation of nonstandard mRNAs, such as uncapped mRNAs, or those located in a dicistron (Gallie and Browning, 2001). It is possible that during a virus
infection or other stress, the pools of both the pathogen transcripts and host transcripts are adjusted as needed to keep the plant alive and maintain the parasite virus' ability to replicate.

eIF3, the factor that helps mediate the landing of the 40S ribosomal subunit and eIF2-GTP-tRNA\textsuperscript{Met} ternary complex on the 5' cap has eleven subunits, giving it an additional plant specific subunit (Burks et al., 2001). Another cap-binding protein, nCBP, of unknown function exists in plants. nCBP interacts with eIFiso4G, and functionally replaces either of the eIF4E isoforms. One possible function of this protein is in mRNA sequestration (Ruud, 1998). Finally no 4E binding protein has been identified in plants (Browning, 2004), suggesting that regulation of cap-dependent translation in plants differs significantly from that of other eukaryotes.

Plant Viral Translation Strategies

Plant viruses have adopted ways to translate their genomes in the absence of canonical features such as a 5' cap or a poly (A) tail. Some features in the untranslated regions or ends of the viral mRNAs enable the recruitment of translation factors. In the Tobacco etch virus (TEV) 5'UTR a 143 nt element functions in cap-independent translation (Niepel and Gallie, 1999). This element recruits eIF4G to the TEV message and also works as an IRES in dicistronic constructs (Gallie, 2001). The cap-independent translation enhancer of Satellite tobacco necrosis virus (STNV) binds eIF4E (Gazo et al., 2004). The STNV TE is in the 3' UTR; however its sequence and structure are unlike other translation elements, including the BTE (below).

Among the uncapped plant viruses are the potyviruses TEV and Turnip mosaic virus (TuMV), that have VPg (genome-linked protein) modifications at their 5' ends. Turnip
mosaic virus recruits eIFiso4E to its VPg (Léonard et al., 2002). The VPg is not required for translation; however 5’ UTR of TuMV facilitates cap-independent translation (Basso et al., 1994). eIFiso4E was identified in a loss of susceptibility screen for potyvirus infection (Lellis et al., 2002). Natural resistance mutations to potyviruses in pepper (Ruffel et al., 2002), lettuce (Duprat et al., 2002; Nicaise et al., 2003), and pea (Gao et al., 2004) have been linked to eIF4E or eIFiso4E. These results suggest alternative roles beyond translation initiation for translation factors in promoting the survival of a plant virus.

Many plant viruses, like STNV, have sequences in the 3’UTR that contribute to cap-independent translation (Qu and Morris, 2000; Timmer et al., 1993; Wang and Miller, 1995; Wu and White, 1999) (Figure 1C). These 3’ translation elements (3’TE) are similar in function, but different in structure and sequence. Turnip crinkle virus (TCV) contains a 3’UTR element that facilitates increased expression of the viral coat protein over ORFs that are on the genomic RNA of the uncapped carmovirus (Qu and Morris, 2000). This elements acts in coordination with the 5’ UTRs of both the genomic and subgenomic RNAs. CA and CU-rich sequences exist within the UTRs, but no specific structures or sequences have been described that might be part of the mechanism for TCV cap-independent translation. In Tomato bushy stunt virus (TBSV), a 167 nt sequence has been identified in the 3’UTR of the RNA genome that functions only in vivo, the cap-independent translation enhancer (3’ CITE) (Wu and White, 1999). The 3’ CITE is likely to bind host proteins and regulate replication, in addition to facilitating cap-independent translation.

Members of the Luteoviridae and Tombusviridae families have distinctive translation elements in the 3’ UTR that share similarities with one another. In BYDV the cap-independent translation element (BTE) is located in the 3’ UTR and forms a cruciform
structure (Figure 3) (Wang et al., 1997). Structural and sequence elements of the BTE are found in other luteoviruses, dianthoviruses, and necroviruses (Guo et al., 2001). Experimental results have shown that the BTE-like elements mediate cap-independent translation for at least two of these viruses, Tobacco necrosis virus (TNV) (Shen and Miller, 2004) and Red clover necrotic mosaic dianthovirus RCNMV (Mizumoto et al., 2003).

Examples of nonpolyadenylated plant viruses include Tobacco mosaic virus (TMV), Brome mosaic virus (BMV), and Alfalfa mosaic virus (AMV). BMV RNA contains a tRNA-like structure at its 3' end that helps modulate translation, stability, replication, and encapsidation of the virus (Dreher, 1999). An upstream pseudoknot rich domain in TMV functionally substitutes for a poly (A) tail (Gallie and Walbot, 1990). This domain interacts with HSP101 (Tanguay and Gallie, 1996) and with eEF1A (Zeenko et al., 2002). Likewise, when put into a yeast system, BMV requires a host protein for efficient translation of its nonpolyadenylated RNA. BMV requires functional Lsm1, a component of the yeast decapping machinery (Diez et al., 2000) for its translation. In contrast, AMV may require a viral protein for translation via binding to its nonpolyadenylated 3' UTR. It has been suggested that the AMV 3'UTR RNA can fold into alternative structures, with conformational changes caused by coat protein binding to favor translation or replication (Neeleman et al., 2001).

BYDV as a Model System for Studying Translation

Barley yellow dwarf virus (BYDV), described as the “yellow plague” of cereals (Duffus, 1977), is of worldwide economic importance (D'Arcy and Burnett, 1995). It is a member of the family Luteoviridae, but also shares RNA features with viruses in the family

BYDV as a Model System for Studying Translation

Barley yellow dwarf virus (BYDV), described as the “yellow plague” of cereals (Duffus, 1977), is of worldwide economic importance (D'Arcy and Burnett, 1995). It is a member of the family Luteoviridae, but also shares RNA features with viruses in the family
*Tombusviridae* (Miller et al., 2002). BYDV infects oats, barley, wheat, most grasses, maize and rice (D'Arcy and Burnett, 1995). Symptoms include yellowing and stunting, and the disease causes yield losses due to low seed set (Miller et al., 2002). BYDV can be a limiting factor in cereal production everywhere, with natural infections resulting in a 15 to 25 percent yield loss (Lister and Ranieri, 1995). BYDV is transmitted by aphids, vectors that exist worldwide. Because BYDV is phloem limited, occurs in low concentrations, and symptoms are highly variable, diagnosis requires biological assays (French, 1995). ELISA, using antibodies to the coat protein is the preferred method of diagnosing BYDV infection (French, 1995). However, differences in nucleic acid sequences and genome structure inform us that additional characterization of the viral genome and its expression are fundamental.

Upon infection of the cell, translation of the BYDV genomic RNA (5.7 kb) (Figure 3A) must occur to produce the replicase protein encoded by the virus. Thus ORFs 1 and 2 are translated from genomic RNA (gRNA), followed by replication, that produces subgenomic RNAs (sgRNA). Three sgRNAs are made from the genomic RNA; all are co-terminal with the 3' end. sgRNA1 produces protein from ORFs 3, 4, and 5 (coat proteins and movement protein). The sgRNA2 sequence corresponds exactly to the 3'UTR and may have a role in switching from early to late expression of BYDV genes (Wang et al., 1999). A small ORF resides in sgRNA2, but the protein for ORF6 has not been detected *in vivo*. sgRNA3 covers a small 3' portion of the 3'UTR and has no known function. Promoters for these sgRNAs have been mapped and are diverse in sequence and structure, aside from six bases on the genomic RNA at the 5' end of each promoter (Koev and Miller, 2000). The very 3' end of
the genomic RNA serves as the origin of replication, where (-) strand gRNA and sgRNA synthesis is initiated (Koev et al., 2002).

Figure 3. BYDV genome and BTE
A. BYDV genome. Untranslated regions are indicated by dashed lines, and open reading frames are indicated by open squares. The TE105, nt 4814-4918 (gray box) confers cap-independent translation in vitro (Wang and Miller, 1995, Wang, 1997, Wang, 1999 Guo, 2000). B. Cruciform structure of the BYDV 3’ cap-independent translation element Nucleotides 4814 to 4918 are shown for the PAV-Aus isolate, based on structure probing results (Guo et al, 2000).
BYDV is a model system to study diverse translational strategies employed by a virus in the context of the host plant cell. Its RNA is translated in the host cell despite being both uncapped and nonpolyadenylated. Alternative gene expression strategies of the luteoviruses include frameshifting, stop codon suppression/readthrough, and leaky scanning (Miller et al., 1995). The existence of an infectious clone for BYDV (Di et al., 1993) enables easy manipulation and direct functional studies of this pathogen. Much of the knowledge on BYDV expression was made possible by a oat cell culture system established in our laboratory (Mohan et al., 1995).

In order for cap-independent translation to occur in BYDV, a sequence 5 kb downstream from the start codon in ORF 1 is required (Wang and Miller, 1995). The BTE (BYDV translation element, Figure 3B) facilitates cap-independent translation \textit{in vitro} and \textit{in vivo} (Wang et al., 1997). The BTE forms a cruciform structure (Guo et al., 2000). The BTE acts in at least two ways. The loop of stem loop III (3'SL-III) in the BTE interacts with a complementary loop in the 5' UTR of BYDV (5'SL-IV) (Guo et al., 2000). The same complementarity exists for a loop in the 5' UTR of sgRNA1 loop, though the stem loop is located closer to the 5' end on sgRNA1 than on the genomic RNA. The BTE binds plant translation initiation factor eIF4E (Allen, 2001). The evidence for 4E binding includes pulldown experiments, UV-crosslinking in wheat germ extract and filter binding studies. At least three other unidentified proteins are associated with the BTE in wheat germ extract pulldown experiments. Chapter 3 of this dissertation details experiments to characterize the interaction between plant cap-binding proteins and the BTE.
The 105 nt TE (nts 4814-4918) is not sufficient for translation \textit{in vivo} (Guo et al., 2000; Wang et al., 1997; Wang et al., 1999). Though reporter constructs containing a poly(A) tail in addition to TE105 show an increase in translation \textit{in vivo} over TE alone, a cap further stimulates expression (Wang et al., 1997). Deletion of the sequence between nt 4920 and nt 5008 results in a large drop in translation. Restriction enzyme truncation at two sites within the 3’UTR (PvuI at nt 5320 and PstI at nt 5010) also indicated that sequence 3’ to the TE105 is necessary for BYDV translation in a plant cell, and that some of the sequence may mimic a cap (Wang et al., 1999). There is also evidence that sequence upstream of the \textit{in vitro} defined-TE is necessary for cap-independent translation (Allen, 2001). Taken together, these data show that in addition to the TE105 \textit{in vitro}, additional 3’UTR sequence is needed for cap-independent translation \textit{in vivo}. Furthermore, some of this sequence may provide stability and translatability, serving the function of a poly(A) tail (Guo et al., 2000; Wang et al., 1997; Wang et al., 1999). The research presented in Chapter 2, “Separate Domains in the Barley Yellow Dwarf Luteovirus 3’ UTR Confer Cap-Independent and Poly(A)-Independent Translation” focuses on the mapping of RNA needed for translation of BYDV in cells. Chapter 4, “Conserved RNA Structures and Sequences in the 3’ UTR of Barley Yellow Dwarf Virus are Required for Replication and Translation” further characterizes a portion of the sequence required \textit{in vivo}.

A basic model to explain how the virus is translated is shown in Figure 4. The viral RNA, plant proteins, and ribosomes interact in a way that times gene expression to suit the viral lifecycle. The BTE circularizes the viral mRNA via a direct basepairing mechanism, and also binds plant initiation factors eIF4E and eIF4G. In Chapter 3, the interaction between initiation factors and the 3’ end of BYDV is characterized. This arrangement would
enable a unique means for delivering the ribosome to the start codon on the mRNA. How the ribosome finds the start codon through the long, structured 5'UTR of BYDV is also unknown. Toeprinting experiments might address this question (See Appendix). The research described in this dissertation contributes to our understanding of a novel mechanism for translation of plant viral mRNA.

Figure 4. BTE-mediated translation
Model for cap- and poly(A)-independent translation of BYDV. The BTE recruits eIF4E and eIF4G, and basepairing could deliver the factors to the 5' end of the mRNA. Additional sequence is needed in oat protoplasts (teal), that may participate in tertiary interactions within the 3' UTR, bind other proteins or facilitate interactions with the 5' UTR or within the 3' UTR (dotted line). BYDV RNA is depicted by the black line, and proteins are shown by blue ovals. 40S ribosomal subunit and the 80S ribosome are shown by brown ovals.
References


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CHAPTER 2. SEPARATE DOMAINS IN THE 3' UTR OF BARLEY YELLOW DWARF LUTEOVIRUS RNA CONFER POLY(A) TAIL-INDEPENDENT AND CAP-INDEPENDENT TRANSLATION

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Abstract

The 869 nt 3' untranslated region (3' UTR) of *Barley yellow dwarf luteovirus* (BYDV) RNA facilitates cap-independent and poly(A) tail-independent translation. A 105 nt cap-independent translation element (BTE) within the BYDV 3' UTR was defined previously *in vitro* using wheat germ translation extracts, but additional 3' UTR sequence is required for translation in plant cells. Here we map sequence and secondary structure of the additional RNA sequence required for BYDV translation in plant cells (*in vivo*). Deletion of one domain, nt 4918-5008, in the 3' UTR flanking the 105 nt BTE reduced translation to 6% of wild-type. Translatability was restored by the presence of a 5' cap on the mRNA, indicating that the cap-mimic sequence needed *in vivo* is larger than that necessary *in vitro*. We propose that this extra sequence places the BTE on a phylogenetically conserved, long double-stranded “stalk”, making it more accessible to cellular translational machinery. Deletion of other sequence in the 3' UTR, nt 5089-5142 reduced translation but translation was not restored by the presence of a 5' cap. Instead, translation was restored by the presence of a 60 nt poly(A) tail on the 3' end of the mRNA. Thus, separate domains in the BYDV 3' UTR functionally substitute for either a 5' cap or a poly (A) tail function in facilitating viral translation.
Introduction

Upon infecting a cell, viral RNA must compete for the host translation apparatus. Most cellular mRNAs have a 5' m7G(5')ppp(5')N cap and a 3' poly(A) tail, which mediate the recruitment of translation factors (1,2). Initiation factor eIF4E interacts with the 5' cap structure, and poly(A) binding protein (PABP) interacts with the 3' poly(A) tail (3). The large eIF4G protein acts as a scaffold, binding eIF4E and PABP, to bring the ends of the mRNA into proximity (4). This increases the affinity of the translation factors for the RNA and enhances initiation (5). In the cellular environment, a cap and a poly(A) tail provide advantages for translation.

Many viral mRNAs lack a 5' cap and/or a 3' poly(A) tail, but have other features that allow translation (6,7). For example, internal initiation is an alternative form of translation utilized by many viral and cellular mRNAs. These RNAs have long, structured internal ribosome entry sites (IRESes) in their 5' UTRs. The uncapped RNAs of viruses in the Picornaviridae, Flaviviridae, and Dicistroviridae families of animal viruses contain IRESes (8). Tobacco etch potyvirus mRNA has an IRES in its 5' UTR (9,10). The Tobacco mosaic virus 5' leader sequence enhances cap-dependent translation (11,12).

Plant viruses in the Luteoviridae and Tombusviridae families have sequences in the 3' UTR that facilitate cap-independent translation. Barley yellow dwarf virus (BYDV) RNA in genus Luteovirus of the Luteoviridae family, lacks both a 5' cap and a 3' poly(A) tail. Translation of the RNA depends on the BYDV cap-independent translation element (BTE) located in the 3' UTR (13). The BTE functions naturally from the 3' end of the mRNA as a
cap mimic, but it does not facilitate internal ribosome entry (14). The BTE can also function from the 5' UTR of uncapped mRNAs (15). The BTE has a cruciform secondary structure (15). In vitro studies showed the BTE was localized to bases 4814 to 4918 (TE105, Figure 1A, gray box), but additional sequence is required in vivo (13,15,16).

Viruses in the Dianthovirus and Necrovirus genera (Tombusviridae) harbor BTE-like sequences and structures (13,17-20). The RNA of Satellite tobacco necrosis virus (STNV) also harbors a cap-independent translation element (21,22) which is unrelated in sequence and structure to BYDV RNA. It binds initiation factor eIF4F (eIF4E + eIF4G) (23). The mechanism of cap-independent translation for plant viruses in the Tombusviridae and Luteoviridae families may depend on long distance basepairing interactions between the 3' UTR and the 5' UTR (17,24).

While many plant virus families have nonpolyadenylated genomes, it is not well understood how they are translated. Yet plant viral mRNAs must be translated efficiently in host cells where most nuclear-encoded mRNAs (including histone mRNAs) are polyadenylated. Turnip yellow mosaic virus (TYMV), Tobacco mosaic virus (TMV), and Brome mosaic virus (BMV) RNA contain tRNA like structures at their 3' ends which play a role in translation, stability, replication, and encapsidation (25,26). Translation of TYMV RNA may depend on interaction with eEF1A (25). A pseudoknot rich domain in TMV RNA functionally substitutes for a poly(A) tail (27).
Some viruses require a viral protein for poly(A) tail-independent translation. The best characterized example is rotavirus RNA which contains a sequence that binds specifically to the viral protein NSP3A. NSP3A also binds eIF4G, displacing PABP (28). The *Alfalfa mosaic virus* (AMV) 3' UTR RNA possibly folds into a series of stem-loops or an alternative tRNA-like structure. Coat protein binding to this portion of the RNA is necessary for efficient translation (29). BYDV coat protein is not required for its translation (13).

Previous experiments indicated the BYDV 3' UTR may contain a sequence that allows the viral mRNA to be translated in the absence of a poly (A) tail. Addition of a 60 nt poly(A) tail to reporter constructs containing only the BTE in the 3' UTR stimulated translation 50-fold, although translation was still one-fifth of that conferred by the full length BYDV 3' UTR (15). BYDV reporter RNAs with 3' UTRs truncated at restriction sites downstream of the BTE translate in a cap-independent manner to different degrees, depending on how much BYDV 3' UTR RNA is present (13). Thus, the additional 3' UTR sequence is needed for cap-independent translation *in vivo*, and may also serve the function of a 3' poly(A) tail, providing stability or translatability to the viral RNA.

To investigate the mechanism of BYDV translational control mediated by the BYDV 3' UTR, we constructed reporter RNAs with deletions in the 3' UTR and tested their ability to translate in oat protoplasts. For transcripts that had reduced mRNA function, we tested the ability of either a 5' m^7^GpppG cap or a 60 nt poly(A) tail to restore translation. Restoration by a 5' cap revealed sequence needed for cap-independent translation mediated by the BTE.
Sequences that could be replaced by a poly(A) tail to mediate translation are defined as poly(A) mimic domains. In the present study, we report that the cap-mimic sequence and the poly(A) mimic sequences are separate domains in the BYDV 3' UTR, and are required for translation in vivo. With both of these domains in the 3' end of the genomic RNA, we hypothesize that BYDV mRNA uses a novel translation mechanism that differs from known poly(A)-dependent or cap-dependent translation processes.

Materials and Methods

Plasmid construction and in vitro transcription

Reporter constructs were derived from the parent plasmid pLUC869 (16), which contains the T7 promoter, BYDV 5' UTR, firefly luciferase gene, and BYDV 3' UTR nt 4809-5677. From deletion mutants (Figure 1B) generated previously (30,31) in the infectious clone context, the 3' UTR was subcloned into pLUC869 utilizing the restriction sites BamHI and SmaI, which are within the BYDV 3' UTR. For some deletion mutants the full-length BYDV genomic clone pPAV6 (32) was the template in PCR reactions using the ExSite PCR-based method (Stratagene). Using inverse PCR, the regions flanking the deletion desired were amplified with primers containing an NcoI site (30). Following digestion with NcoI, the PCR products were religated. Other deletion mutants are derived from those in Paul et al, (2001), which were made using the Promega Erase-a-Base system. One mutant, Δ5017-5048 was made by two step PCR mutagenesis in which mutagenic primers contained sequences flanking the region to be deleted (31).
The plasmids used in Figures 2, 3 and 4 were constructed by amplifying the desired 3' UTR region with a 5' primer having a KpnI site and the 3' primer containing a SmaI site. DNAs containing nts 5015-5142 and either nts 5350-5677, 5432-5677 and 5537-5677 (Figure 3B) were created by amplifying p5'UTRluc5015-5677 using two step PCR mutagenesis. In the first step primers overlapping the region to be deleted were used in conjunction with either an upstream primer containing the KpnI site, and a downstream primer 3' to the SmaI site. In the second step, the products from the first PCR were combined and amplification with the downstream and upstream primers was carried out. To construct a 3' UTR containing 5015-5142 and 5350-5432 (Figure 3B), a downstream primer, with a SmaI site inserted at nt 5432, was used. PCR products were purified with the Qiagen PCR purification kit, and digested with KpnI and SmaI, and the resulting fragments were ligated into pLUC869 cut with KpnI and SmaI.

Nonviral 5' UTR sequence (Figure 3C) was synthesized by PCR using the following primers, which contained NotI and BssHII sites (italicized), and the T7 promoter (bold). forward primer: GCA CTT GTA GCG GCC GCT AAT ACG ACT CAC TAT AGG GTA CTC AAG CTT TTG CAT CCA ACC; reverse primer: CGT CTT CCA TGC GCG CTT GGA TCC GGG TCC TCT AGA TCG GTT GGA TGC AAA AGC TTG AG.

Following digestion with NotI and BssHII, the PCR insert was ligated into pLUC869 cut with the same enzymes. This yielded a nonviral 5' UTR and BYDV 3' UTR sequence flanking the firefly luciferase gene (Figure 3C). The resulting nonviral 5' UTR sequence (start codon underlined) is 53 nucleotides in length: G GUA CUC AAG CUU UUG CAU
CCA ACC GAU CUA GAG GAC CCG GAU CCA AGC GCG C AUG. The start codons in these RNAs are thus in the same context as LUC869.

All constructs were verified by sequencing at the Iowa State University DNA Facility. Prior to transcription, the plasmids were linearized with Smal. To obtain transcripts with a 60 nt poly(A) tail, plasmids were linearized with VspI. The linearized plasmid DNA was extracted with phenol:chloroform and precipitated with ammonium acetate and ethanol. mRNAs were synthesized by in vitro transcription using the T7 MegaScript or mMessage mMMachine kits (Ambion, Austin, TX). RNA concentration was determined by spectrophotometry, and RNA integrity was verified by 1% agarose gel electrophoresis. A minimum of two independently transcribed RNAs for each construct was used as the template for independent translation and replication assays.

**In vivo Reporter Assays**

1 pmol RNA was electroporated into oat protoplasts, along with 0.1 pmol control capped, polyadenylated renilla luciferase RNA for normalization. Following four hours culture in MS media with 0.4 M mannitol, cells were harvested and lysed in Passive Lysis Buffer (Promega) by shaking 15 minutes at room temperature. 2-5 μL lysate was assayed using the dual luciferase reporter assay system (Promega) in a Turner Designs TD 20/20 luminometer. All samples in an experiment were tested in 3 to 5 replicates, and a minimum of three independent experiments were performed.
Half-life determinations were made based on time course studies using *in vivo* reporter assays. Curves were generated with the relative light units observed for firefly luciferase at each time point plotted; the rate of change of the slope was used to determine whether functional stability differed between RNAs (33). Using Microsoft Excel, an equation of the form $y=n\times\ln(t)-m$ was derived. By arbitrary setting of $t=90$ minutes, the value of $k$ was derived using the first order kinetics formula $A(t)=A(0)\times e^{kt}$. The $t^{1/2}$ was then calculated by solving for $t=1/2$.

**In vitro Translation**

0.2 pmol RNA was translated in wheat germ extract (Promega) in the presence of $^{35}$S methionine according to the manufacturer's instructions, in a total reaction volume of 25 μl. Following a one hour incubation at room temperature, five μl of translation product was loaded onto a 10% Bis-Tris NuPage gel (Invitrogen). The products were scanned using the STORM 840 phosphorimager (Molecular Dynamics), and analyzed using ImageQuant™ (Molecular Dynamics).

**Viral Replication Assays**

10 μg RNA was electroporated into oat protoplasts, and the cells were cultured in MS media (Invitrogen) with 0.4 M mannitol for 48 hours at room temperature. Cells were harvested and RNA was extracted with Trizol® (Invitrogen) according to the manufacturer's instruction. Northern blot analysis was carried out as described previously (34), using an RNA probe complementary to the 3' 1.5 kb of the BYDV genome.
BLAST Searches

BLAST searches were carried out using 30 nucleotide segments of the BYDV 3' UTR from nt 4801 to 5677 as a query sequence, with sliding windows of 10 nt. Searches were performed online using the NCBI server with blastn and expect values of 10 and 100.

Results

Deletion Analysis

We first sought to define the sequence outside of TE105 (nt 4814-4918) required for translation of BYDV RNA in vivo (in oat protoplasts). We performed deletion analysis using reporter assays in oat protoplasts, the same oat cell system in which full length infectious BYDV RNAs replicate. The positive control, to which all mutant RNAs were compared is LUC869 RNA (16). LUC869 contains the firefly luciferase gene flanked by the BYDV 5' UTR and the complete 869 nt BYDV 3' UTR. The negative control is LUC869BF, a mutant with a four base duplication that inactivates the BTE (13). Previously, we observed that deletion of large portions of the BYDV 3' UTR downstream of BTE dramatically reduced translation in vivo (Wang et al, 1999). In particular, truncation at the endogenous restriction site PstI$_{5010}$ resulted in minimal translation of a reporter RNA, while truncation to a PvuI site at nt 5320 maintained translation activity above 75% of wild type in vivo (16). Internal deletion of nts 5008 to 5479 dramatically decreased translation as well (35).

To map the BTE necessary for cap-independent translation in vivo, we tested reporter mRNAs lacking specific 50-100 nt portions of the BYDV 3' UTR for the ability to express luciferase in plant cells (Figure 1B). Many of these deletions in the BYDV 3' UTR did not
affect translation dramatically, especially compared to effects seen with mutations in the BTE (15). Most deletion mutants affected luciferase expression only modestly, giving relative light units (RLUs) between 50% and 150% of LUC869. We selected two deletion mutants that reduced LUC synthesis by more than 50% for further analysis: Δ4918-5008 and Δ5089-5142. We tested the effect of either a 5' cap or a 60 nt 3' poly(A) tail on these transcripts to restore LUC synthesis in vivo (Figure 1C). Deletion of nts 4918-5008 reduced expression to 6% of wild type levels in vivo (Figure 1B). Addition of a 5' cap restored translation of this transcript to 66% of the wild type LUC869, whereas a poly(A) tail had no stimulatory effect. Presence of both a 5' cap and a 3' poly(A) tail stimulated luciferase expression to levels greater than that of uncapped LUC869. The effects of deleting nt 5089-5142 were more subtle, as Δ5089-5142 yielded 45% of LUC activity as that from LUC869. This mutant also differed from Δ4918-5008 because LUC expression was not stimulated by a 5' cap, but increased nearly 3-fold (to 130% relative to wild type) by the presence of a 3' poly(A) tail. Unexpectedly, a poly(A) tail alone on Δ5089-5142 gave slightly better luciferase activity than both a poly(A) tail and a cap (Figure 1C).

Deletion of BYDV 3' UTR Regions 4918-5008 or 5089-5142 Had Little Effect on mRNA Stability

To determine whether the deletions affected mRNA stability instead of, or in addition to, translation, we calculated the functional stability of the two mutants Δ4918-5008 and Δ5089-5142 in vivo. Given the vast excess of reporter RNA used for transfection and unknown cellular localization via electroporation, physical stability as visualized by northern blot hybridization may not provide a meaningful assessment of the stability of the mRNA.
The functional stability provides a more accurate measure of mRNA accessibility to ribosomes in the cell. Time course studies comparing the reporter RNAs indicated only small alterations in mRNA half-life, compared with LUC869 (Figure 1D). While overall translation was lower in the mutants, the rate of change of active luciferase accumulation was similar to wild type. The calculated mRNA functional half-lives for two independently transcribed RNAs for each mutant, Δ4918-5008 and Δ5089-5142 averaged around 37.2 and 46.9 minutes respectively, compared with the wild type calculated half-life of 45.0 minutes (Figure 1D). Loss of stability may contribute slightly to the loss of luciferase activity in Δ4918-5008, but doesn’t explain the drastic reduction in translation for this RNA. These results indicate that the decrease in translation correlated with the loss of BYDV 3’ UTR RNA sequences between 4918-5008 and 5089-5142 is not attributable to loss of mRNA stability.

The BYDV 3’ UTR Confers Cap-independent Translation in vivo

The above results indicate that nts 4918-5008 are required for cap-independent but not poly(A) tail-independent translation in vivo. To further define only the cap-independent translation sequence as distinct from a poly(A) “mimic”, if possible, we measured in vivo translation of constructs with 3' UTRs extending beyond TE105 using uncapped RNAs containing a 60 nucleotide poly(A) tail. Samples containing TE105 plus various lengths of flanking sequence were compared to the uncapped, nonpolyadenylated wild type LUC869, which has the entire BYDV 3’ UTR. Translation of RNAs containing the TE105 and ten additional bases (4809-4928 and 4809-4928 poly(A), Fig. 2A) was measurable, but low, with or without a poly(A) tail. Capped versions (gray bars) of these RNAs had approximately 15-
fold higher luciferase activities, suggesting that sequence required for cap-independent translation was missing. By extending the BTE as little as 35 additional nt, a very substantial increase in cap-independent translation in vivo was realized (4809-4963A<sub>60</sub> and 4809-4982A<sub>60</sub>, Fig. 2A). More importantly, stimulation by adding a cap was less than two-fold, the same as seen with LUC-869 RNA (13,15). Thus the 3' end of the BTE needed for full cap-independent translation in vivo maps to the region between nts 4929 and 4963.

LUC expression from capped, polyadenylated RNAs varied among constructs. While our primary concern is not total expression, but the amount of stimulation by a cap or a poly(A) tail, to understand the significance of the level of LUC activity measured, we compared the above constructs with capped, polyadenylated RNA lacking any viral sequence. We compared the RNAs containing extended BTEs with RNAs harboring only vector sequence in the 5' and 3' UTRs (vec-luc-vecA, Figure 2B). As in Figure 2A, the extra nucleotides 3' to nt 4928 enhanced cap-independent translation. The uncapped BTE-containing RNAs produced greater luciferase activity than capped vec-luc-vecA RNA.

**BYDV 3' UTR Sequence Functionally Replaces a 3' poly(A) tail**

The results in Fig. 1 indicate that nts 4918-5008 are required for poly(A) tail-independent but not cap-independent translation in vivo. To further map sequences that confer poly(A) tail independence, we tested expression in vivo from constructs containing 3' truncations of the BYDV 3' UTR (Fig. 3A). Transcripts with 3' truncations to nt 5111 or further upstream yielded luciferase activities that were less than 20% of that obtained from LUC869 (positive control). Transcript truncated to nt 5140 expressed LUC at 43% of the LUC869 level. For all the truncation mutant RNAs tested, addition of a 3' poly(A) tail
restored translation *in vivo*, while a 5' cap did not positively affect translation (Figure 3A). From these data, we conclude that sequence upstream of nt 5140 plays a key role in translation of nonpolyadenylated mRNA *in vivo*.

To delineate the 5' border of the poly(A) tail-independent translation domain, we tested luciferase mRNAs containing isolated 3' UTR segments suspected of conferring poly(A)-independent translation *in vivo* (Figure 3B). We used only capped RNAs to avoid the requirement for the BTE and to ensure that all effects observed were not due to variations in cap-independent translation. From RNA containing only BYDV nt 5087-5142 as its 3' UTR, the same nucleotides that are replaceable by a 3' poly (A) tail (Figure 1C), we observed translation of 15% relative to LUC869 (Figure 3B). A series of constructs with various other portions of the 3' UTR revealed: (i) no transcripts translated as efficiently as those with the full 869 nt 3' UTR; (ii) presence of sequence between nt 5015 and 5087 reduced LUC expression compared to 3'-coterminal sequences lacking this region; (iii) nts 5320-5432 may enhance poly(A) tail-independent translation; (iv) all segments of the BYDV 3' UTR containing nts 5087-5142 gave substantial expression (>30%) relative to a capped, polyadenylated reporter containing a 63 nt nonviral 3' UTR (capped vec-luc-vecA) (Figure 3B), which itself gave 27% of luciferase expression as that from LUC869 RNA (v) all segments of the BYDV 3' UTR facilitated translated at levels at least 10-times greater from than capped, nonpolyadenylated vector-derived mRNA; (bottom, Fig. 3B).

To determine whether the BYDV poly(A) mimic sequence is dependent on the viral 5' UTR, we substituted the viral leader with a 53 nt nonviral 5' UTR for select constructs that
contained portions of the viral 3' UTR. The BTE requires the viral 5' UTR for cap-independent translation, so all transcripts were capped in this set of experiments. Although substantial levels of translation were observed in the RNAs containing nonviral 5' UTRs and long BYDV 3' UTRs compared with the blank samples, their translation levels were 9.6% to 25.8% of LUC869 (Figure 3C). This includes the construct that contains the entire BYDV 3' UTR, and which differs from LUC869 only by having the nonviral 5' UTR. Thus, as observed for cap-independent translation, the viral 5' UTR may contribute to poly(A) tail-independent translation.

Effects of 3' UTR Translation elements in the Full-length Viral RNA

Expression of the viral replicase is necessary for viral infection. Sequences that affect viral translation therefore have an impact on viral replication. To determine the effects of the sequences identified above, that play a role in poly(A)-independent and cap-independent translation on replication, we deleted nts 4918-5008 (transcript PAV6-d4918-5008) or 5089-5142 (transcript PAV6-d5089-5142) from full-length infectious clone PAV6. These transcripts were then tested for translation in vitro and for replication in oat protoplasts. Translation of the 39 kDa ORF 1 product from PAV6-d4918-5008 RNA was reduced compared to wild type PAV6 RNA. This indicates that nt 4918-5008 contributes to translation in vitro. The RNA lacking bases required for poly(A)-independent translation (PAV6-d5089-5142) yielded the same amount of the 39 kDa ORF 1 product in vitro as did the wild type PAV6 RNA. This is not surprising given the lack of poly(A) tail dependency in wheat germ extracts (1). Most importantly, neither deletion mutant replicated in oat protoplasts (Figure 4B), as revealed in northern blot hybridization of total viral RNA
accumulated in protoplasts inoculated with mutant or wild type transcripts. These results show that the regions identified as necessary for translation in vivo, are indeed required for BYDV RNA replication.

Discussion

The 3' UTR of BYDV is a master gene expression control region

The 5.6 kb genome of Barley yellow dwarf luteovirus has a complex 869 nt 3' UTR, which comprises 15% of the entire genome. The BYDV 3' UTR elements that influence viral gene expression include a cap-independent translation element (BTE) (15), ribosomal frameshifting elements (31), subgenomic RNA promoters (34), and the 3' origin of replication (36). Here, we have identified and characterized two additional portions of the 3' UTR that facilitate translation of the BYDV mRNA in vivo. One element is an extension of a previously defined cap-independent translation element, and a less well defined region confers poly(A) tail-independent translation. These elements enable the BYDV RNA to compete for the host translation machinery against cellular messages that contain the canonical modifications at the 5' and 3' ends.

We still do not understand how all 869 nt of the BYDV 3' UTR contribute to the viral lifecycle. Many regions of the BYDV 3' UTR are not necessary for translation initiation. The lack of effect on translation of many of the deletion mutants (Figure 1B) could be due to several causes. Internal deletions result in the replacement of the deleted sequence with adjacent sequences, which could provide some functional substitution for the deleted portion. Effects due to unpredicted aberrant folding of the artificial 3' UTR or altered spacing between
functional elements may also have contributed to the results.

Truncations from the 3' end indicate that sequence downstream of base 5432 may play a role in vivo; however translation was recovered by deleting more sequence, back to base 5355 (Figure 1). There may be repression and anti-repression elements in this region of the BYDV 3' UTR that contribute to the viability of the virus in vivo. Whether these results are due to misfolding of the 3' end of the RNA is not known. Alternatively certain features of the 3' end, such as the secondary structure on some truncations but not others may affect translatability. Because the natural 3' end of the BYDV RNA overlaps with the origin of replication, it could control the destination of the RNA template for replication or translation in a manner analogous to the tRNA-like structure of TYMV (37). In the case of TYMV, eEF1 binds the aminoacylated viral RNA, abolishing RNA synthesis (37).

**The sequence necessary for cap-independent translation in vivo, is predicted to form an extended stalk in the BTE secondary structure**

Previously, we showed that the sequence between nts 4819-4918 was sufficient to confer full cap-independent translation in vitro (15), but that additional sequence was necessary for translation in vivo. The 5’ extremity of the sequence required in vivo was shown previously to be nt 4809 (15), which coincides with the 5’ end of subgenomic RNA 2. Here, we identify the 3’ extremity of the sequence required for cap-independent translation in vivo as nt 5012. Adding 35 bases 3’ to the previously in vitro defined BTE (16) results in a 25-fold stimulation of cap-independent translation in vivo (Figure 2B, 2C). This fully functional BTE promotes translation more efficiently than a 5’ cap structure on nonviral
RNA (Figure 2B). After adding a poly(A) tail to truncations downstream of base 5012, we observed levels of cap-independent translation equivalent to wild type LUC869 RNA which contains the complete BYDV 3' UTR (Figure 3A). These results indicate the BYDV 3' UTR itself might be able to recruit most of the factors required for translation.

To determine if the extra sequence participates in structure of the BTE not present in the minimal *in vitro*-defined BTE, the secondary structure of the entire in vivo-active sequence was predicted using MFOLD (38). It is clear that the bases downstream of 4918 contribute to an extension of stem-IV in the BTE; the stem encompasses at least 35 nucleotides downstream of TE105 (Fig. 5A). This extended helix may stabilize the correct structure of the BTE for translation, and perhaps make the essential stem-loops I, II, and III highly accessible to translation factors and loop III available for base pairing to the 5' UTR (Guo et al., 2001). This extended stem is predicted in MFOLD structures of the entire viral genome as well (data not shown), indicating no obvious competing base pairing or alternative structures. Basepairing near the BamHI site is predicted in all of the isolates (Figure 5), although structure probing did not support the existence of such pairing (15). It is noteworthy that some of the extra 5' sequence (nt 4779-4809) that extends stem IV is absent in our reporters, as it has been replaced by the luciferase coding region. Fortuitously, the LUC bases can also participate in formation of the extended stalk by pairing to bases downstream of 4928 (data not shown). Such fortuitous base pairing of nonviral sequences to viral sequences to maintain a functional translation element was observed previously in other BTE reporter constructs (15).
Phylogenetic comparisons of BTE secondary structure to related viruses adds further credence to a biological role for the extended stem IV helix. A similar long stem IV is also predicted in homologous BTE structures in all RNAs known to have a BYDV-like TE, including all members of the Luteovirus, Dianthovirus, and Necrovirus genera (Fig. 5).

**Sequence necessary for poly(A) tail-independent translation is downstream of the BTE**

In contrast to the BTE, which is a clearly defined and powerful stimulator of translation, the sequence(s) that allow translation in the absence of a poly(A) tail were “fuzzy”. One region that caused a large drop in LUC expression when deleted was between nts 5089 and 5142. Evidence for its role in poly(A) tail-independent translation was shown by the ability of a poly(A) tail, but not a 5’ cap, to restore translatability to RNA lacking this sequence (Fig 1C). Also, uncapped BYDV reporter RNAs lacking sequence downstream of nt 5012 translated very poorly, but a poly(A) tail fully restored translation (Fig. 3A). Thirdly, isolated 3’ UTR pieces, such as BYDV nt 5087-5142 confer substantial translation to a nonpolyadenylated RNA and function in the presence of a 5’ cap (Fig. 3B). In this case, the RNAs lack the BTE. The fullest poly(A)-independent translation is conferred by nt 5087-5677, which stimulates translation more than 75-fold over a vector 3’ UTR sequence. This stimulation is comparable to the BMV and TMV 3’ UTRs which provide 63- and 57-fold stimulation over control sequence in carrot protoplasts (39).

Compared to LUC869, RNAs containing only the poly(A)-tail substitute (PAS) sequence translated at 15% (5087-5142) and 40% (5087-5677) (Figure 3B). This result indicates nt 5087-5142 are not sufficient for translation, and the influence by PAS is
correlated with longer lengths 3’ of base 5087. Such effects may be analogous to the range of stimulation a poly(A) tail provides on cellular mRNAs, where longer poly(A) tails provide more translation (40). The long length of luteoviral 3’ UTRs would therefore contribute to the PAS activity. An alternative, but not mutually exclusive, explanation is that RNA containing both BYDV cap-independent and poly(A)-independent translation elements translates better than a mRNA having only one of the elements. Supporting the idea that other viral sequence is required for PAS function is our result that shows the BYDV PAS may be dependent on the viral 5’UTR for its poly(A) independent function (Fig. 3C) and is dependent on the viral 5’ UTR for cap-independent facilitation of translation (13). This contrasts with the dengue flavivirus 3’ UTR which enhances poly(A)-independent translation independent of the 5’ end (41).

Although 5087-5142 does not confer full translation on its own (15%), it clearly plays a key role. Therefore we performed comparative analyses to identify conserved sequences and potential secondary structures that may participate in translation. Sequences homologous to bases 5080 to 5184 are present in all BYDV 3’ UTRs, and predicted stem-loops are conserved (Fig. 6). The existence of the predicted secondary structures are supported by covariations in other BYDV isolates. Therefore the BYDV PAS is a unique domain that supports poly(A) tail-independent translation for which there is phylogenetic evidence within the luteovirus family.

Comparison with poly(A) mimic sequences of unrelated viruses
Based on the data in Figures 1C, 3A and 3B, we conclude that BYDV nt 5087-5142 comprise a core portion of the poly(A)-independent translation sequence. The core sequence (nt 5087-5142) of the BYDV PAS may be novel in its sequence, structure, and function. The BYDV PAS sequence appears to be unlike other plant viral domains implicated in poly(A) independent translation. Sequence alignments of viral isolates in the family Luteoviridae (Figure 5) show that primary sequences homologous to bases 5080 to 5184 are present in several luteoviral 3' UTRs, and that predicted stem-loops are conserved. This region has no predicted resemblance to the TMV pseudoknot domain (27), the tRNA-like structure in TYMV and BMV (26), or the repeats in the AMV 3' end (29). Unlike these viruses, BYDV RNA lacks a 5' cap. We also do not see any obvious homology between BYDV and the TBSV 3' UTR outside of their 3' ends, or with the repeats in rotaviruses that enable recruitment of translation factors (42,43). A search of GenBank for sequences similar to the BYDV core PAS using BLAST (44) did not reveal significant primary sequence homology with other nonviral or viral sequences. Therefore the BYDV PAS is a unique domain that supports poly(A) independent translation for which there is phylogenetic evidence within the luteovirus family.

Both TNV and BYDV have the cap-independent and poly(A)-independent elements in the 3' UTR. Mutations in "poly(A) mimic sequence" of TNV RNA also resulted in a substantial, but incomplete loss of function (Shen, 2004). Two stem loop structures at the 3' end of TNV RNA were found to be necessary for poly(A)-independent translation; however the full-length 3' UTR was required for maximum function (Shen, 2004). Although there is significant homology between the BTE and the TNV cap-independent translation element,
there are no obvious similarities between the poly(A)-independent translation sequences of
the RNAs. Both BYDV and TNV RNAs have a series of tetraloops and a 3' CCC which
appear to be embedded; however there are no other obvious similarities in sequence or
structure between the ends. With a significantly shorter overall 3' UTR length, TNV may be
a simpler model to study the interaction of these elements.

Possible mechanisms of translation

Closely spaced cap-independent and poly(A)-independent RNA elements in the
BYDV 3' UTR may provide an advantage in viral gene expression. The proximity between
these domains might more efficiently recruit, concentrate or retain the translation initiation
factors required, and basepairing between the 5' UTR and 3' UTR would provide the
minimum means needed to assemble the ribosomes at the 5' AUG. Because the BTE binds
several proteins including initiation factors 4E and iso4E (E.P. and E. Allen unpublished
data), it is possible that these or other factors are bound to the poly(A) mimic region. Thus, a
few copies of viral RNA available for translation in the infected cell would better compete
for ribosomes and initiation factors with the large pool of cellular mRNAs. Having both of
the elements in the 3' UTR also enables expression from the subgenomic RNAs (Figure 1A),
which are coterminal at the 3' end with the 3' UTR, and lack the 5' ends that the genomic
RNA has. Finally, these elements are located on either side of the small, non-translatable
ORF6 that resides in the 3' UTR, suggesting a potential regulatory function for the ORF.

Circularization within the 3' UTR through tertiary interactions between the BYDV
PAS and the upstream BTE or between the BYDV PAS and additional downstream elements
may regulate the coordination of translation and replication. There is evidence for a synergistic interaction between the cap-independent and poly(A)-independent elements in the BYDV 3' UTR. The BTE works in conjunction with a poly(A) tail (Figure 2, 3A), and the PAS domain functions better with the BTE in close proximity than it functions with a 5' cap (Fig. 3A vs. Fig. 3B). The cooperation between these domains is a variation on the theme of mRNA circularization (4). Nonetheless, both the BTE and the PAS domains require the viral 5' UTR for optimal function (Figure 3C and (17)), suggesting that there is a requirement for 5' to 3' communication on the mRNA.

Tertiary RNA interactions within the BYDV 3' UTR likely facilitate gene expression. Recently we proposed a model for regulating translation and replication on the same viral RNA (30) where the 3' UTR is involved in long range RNA/RNA interactions. Perhaps the dynamics of folding within the 3' UTR impact the 3' UTR availability for such interactions. The sgRNA promoter (36) overlaps with the cap-mimic region (bases 4918-5008). The poly(A) mimic region contains elements also required for -1 frameshifting, which facilitates translation of the viral RNA dependent RNA polymerase (31). Thus, disruption of this domain would also affect replication, suggesting the correct formation of the 3' UTR architecture would provide the virus with a RNA-based means to regulate multiple modes of gene expression.

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References

Figure 1
Barley yellow dwarf virus genome sequences needed for viral translation in vivo.


B. 3' UTR deletion constructs used to test translation in vivo. LUC869 has the complete BYDV 5' and 3' UTRs, and is uncapped and nonpolyadenylated with nucleotides as marked (Wang, 1999). Deletions in the BYDV 3' UTR for RNAs tested in reporter assays in oat protoplasts are shown at the left. At right are the translation levels relative to LUC869 (wild type). Translation was measured as a percent relative to LUC869. Error bars denote standard deviation above and below the average of at least three independent experiments. LUC869BF is a nonfunctional mutant in the 3'TE, (Guo, 2000).

C. Quantification of luciferase reporter activity in oat protoplasts for d4918-5008 and d5089-5142. The effects of adding a 5' cap (bars with diagonal lines), a 3' poly(A) tail (bars with horizontal lines), and both a 5' cap and 3' poly(A) tail (black bars) were measured relative to uncapped, nonpolyadenylated LUC869.

D. Luciferase activities of 3' UTR deletion mutants over time in oat protoplasts. The functional mRNA half-lives are calculated in minutes based on the slope of the curves.
Figure 2
BYDV 3'UTR sequence needed for cap-independent translation in vivo.
A. RNAs with 3' extensions to the in vitro-defined BYDV TE105 were translated in oat protoplasts and compared with uncapped, nonpolyadenylated LUC869. A60 indicates that a poly(A) tail of 60 nt was present on 3' end of the RNA. RNAs were translated uncapped (white bars) or capped (gray bars). Fold-stimulation of luciferase activity due to a 5' cap is indicated beside the gray bars. The 5' end on the 3' UTR of all reporter constructs tested was at nt 4809.
B. Luciferase activities for uncapped RNAs containing BTE sequence and a poly(A) tail (as in B), relative to capped vec-luc-vecA, which is capped and polyadenylated, and contains nonviral UTRs. Capped vec-luc-vecA, is shown at the top, right, and is set at 100%. Uncapped vec-luc-vecA was also tested as a negative control.
Figure 3
BYDV 3' UTR sequence needed for poly(A) mimic function in vivo.
A. Uncapped reporter RNAs containing the BYDV 3'UTR beginning at nt 4809 and terminating at the base indicated. Translation relative to uncapped, nonpolyadenylated LUC869 is indicated. Unmodified RNA is shown by open bars. A 5' cap (bars with diagonal lines) or a 3' poly(A) tail (bars with horizontal lines), was added to each of the RNAs.
B. Luciferase reporter RNAs containing BYDV 5' UTR and isolated BYDV 3' UTR sequences were translated in oat protoplasts. RNAs tested are shown on the left, below LUC869. Solid lines indicate 3' UTR nucleotides present; dashed lines indicate missing viral 3' UTR sequence. Percent translation is relative to LUC869. For reference a capped, polyadenylated luciferase reporter is shown (capped vec-luc-vecA, second from bottom) which contains a 5'UTR of 66 nt vector sequence and a 3'UTR of 294 nt vector sequence followed by a 60 nt poly(A) tail. Vector sequence is indicated by dotted lines.
C. Reporter activity of capped luciferase RNAs translated in oat protoplasts. The RNAs contained nonviral (NV) 5' UTR sequence (53 nt) and BYDV 3' UTR sequences, and are compared relative to LUC869.
**Figure 4**
Effect of sequences required for translation *in vivo* on the viral RNA in the full length infectious context.

**A.** Translation of BYDV RNA mutants *in vitro* visualized by S35 Methionine incorporation into viral translation products following one hour incubation in wheat germ extract. The 39K product is the putative helicase generated from translation of the viral genomic RNA (Fig. 1A).

**B.** Northern blot analysis assaying replication at 48 hours after inoculation of full-length infectious BYDV RNAs lacking either cap mimic sequence (PAV6d4918-5008) or poly(A) mimic sequence (PAV6d5089-5142) 3' UTRs. PAV6WT is the wild type full-length infectious RNA.
PAV6 WT
PAV6 d4918-5008
PAV6 d5089-5142
BMV Ladder
Mock

Mock
PAV6 d4918-5008
PAV6 d5089-5142
PAV6 WT

36k
Figure 5A. Secondary structure predictions for the extended putative BTEs of luteovirus isolates *Barley yellow dwarf virus* PAV-Aus isolate (X07653): circled nucleotide positions on the BTE indicate 3' ends of 4928 and 4963 used for the assays in Figure 2A and 2B; *Barley yellow dwarf virus* PAS isolate (NC_002160); *Barley yellow dwarf virus* MAV-PS1 (D11028); *Bean leafroll virus* (NC_003369); *Soybean dwarf virus* (NC_003056).
Figure 5B. Secondary structure predictions for the extended putative BTEs of dianthovirus isolates *Carnation ringspot virus RNA 1* (NC_003530); *Red clover necrotic mosaic virus RNA 1* (NC_003756); *Sweet clover necrotic mosaic virus RNA 1* (L07884).
Figure 5C. Secondary structure predictions for the extended putative BTEs of necrovirus isolates *Tobacco necrosis virus* Strain D (NC_003487); *Tobacco necrosis virus* Strain A (NC_001777); *Leek whitestripe virus* (NC_001822); and *Olive latent virus* (NC_001721). Numbers in parentheses indicate GenBank Accession number for each viral sequence.
Figure 6
Sequence alignment of PAS region in luteoviral isolates.
Alignment generated by CLUSTALW (Thompson, 1994) of 3'UTR nucleotides flanking and including the poly(A) mimic region in various luteovirus isolates. Predicted stems generated by Mfold (Zuker, 2003) are indicated by boxes. Basepaired regions are indicated above the alignment by converging arrows that point toward each other to indicate stem-loops. Bulges are indicated by dashed lines on the arrows. Dots indicate no change in sequence, nucleotides in bold indicate base changes that do not disrupt basepairing due to covariations in nucleotides. Italics indicate nucleotides that disrupt a helix. Bold italics indicate a conserved nucleotide, that participates in extended basepaired stem regions. Shown here are nts 5080 to 5184 in the BYDV PAV-Aus isolate (GenBank No. X07653) compared with BYDV Japan, BYDV Purdue, PAS, MAV-PS1, and PAV-GAV isolates (Access. Nos. D85783, D11032, NC_002160, D11028, NC_004666, respectively).
CHAPTER 3. CAP-INDEPENDENT TRANSLATION OF BYDV RNA REQUIRES INTERACTION OF CAP-BINDING FACTORS WITH THE 3' TRANSLATION ELEMENT

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The author contributed all of the data for Figures 1, 2B, 3 and 4-6, and some of the data (blots for 4E and 4G) in Figure 2A

Abstract

The cap-independent translation element (BTE) in the 3' untranslated region (UTR) of Barley yellow dwarf virus (BYDV) RNA facilitates cap-independent translation via kissing stem-loop base pairing to the 5' UTR. We found that BTE-mediated translation is inhibited by cap analog in trans, suggesting that eIF4E or its isoform eIFiso4E may be responsible. Toward understanding this mechanism we used an in vitro pull down assay and immunoprecipitation of a UV-crosslinked complex to identify proteins in wheat germ extract that interact with the BTE. eIFiso4E, eIFiso4G, eIF4E and eIF4G interacted specifically with the BTE. The nonfunctional mutant BTE RNA that differed from the BTE by only four bases, did not bind any translation factors. eIFiso4E and eIF4E bind BTE RNA directly, even though they normally bind only capped mRNA. eIF4A and eIF4B did not interact with the BTE. Recombinant eIF4F and eIFiso4F from wheat stimulated translation of BYDV RNA in extracts depleted of cap-binding factors. Our results indicate that eIF4F is a stronger stimulator of translation activity than eIFiso4F.
Introduction (written with WAM)

For efficient translation initiation, the ends of an mRNA must be brought into close proximity to form a closed-loop structure (Hentze, 1997; Sachs et al., 1997). This closed-loop increases the affinity of translation initiation factors for the mRNA and the ribosome (Pestova et al., 2001). On cellular mRNAs, these interactions are mediated by interaction of initiation factor eIF4E with the 5' m^7G(5')ppp(5')N cap structure, and poly(A) binding protein (PABP) with the 3' poly(A) tail. This complex is circularized by the large adapter protein, eIF4G which binds eIF4E and PABP simultaneously (Wells et al., 1998).

Plants express at least two isoforms of eIF4E and eIF4G: eIF4E pairs with eIF4G to form eIF4F, and eIFiso4E pairs with eIFiso4G to form eIFiso4F (Browning, 1996). The binding affinity of cap-binding proteins to cap and PABP to poly(A) RNA is increased by their association with eIF4G/iso4G (Gross et al., 2003; Sha et al., 1995) resulting in an increased association with mRNAs containing both cap and poly(A) tail. The ribosome is recruited to this complex and then scans by a still-unclear process to the first AUG at which protein synthesis usually begins (Pestova et al., 2001).

Many viral RNAs lack a 5' cap. Translation is usually mediated by an internal ribosome entry site (IRES) that recruits the ribosome (Hellen and Sarnow, 2001). IRESes are usually long, highly structured RNA sequences, that engage the 40S ribosomal subunit, independent of the 5' end. Different IRESes function by very different mechanisms, with
initiation factor requirements ranging from all (Ali et al., 2001) to almost none (Wilson et al., 2000).

Plant viral RNAs in genus *Luteovirus*, and in the related *Tombusviridae* family lack both a cap and a poly(A) tail (Miller et al., 2002). Translation of these mRNAs initiates at the 5'-proximal AUG, but is conferred by sequences residing in their 3' UTRs (Guo et al., 2000; Meulewaeter et al., 1998). The 5,677-nt genome of *Barley yellow dwarf luteovirus* (BYDV, Fig. 1) has a complex 869 nt 3' UTR that contains cap- and poly(A) tail-independent translation elements (Wang et al., 1997) and other regulators of gene expression and replication (Barry and Miller, 2002). The 3' cap-independent translation elements of luteoviruses, necroviruses and dianthoviruses, but not their satellites, have similar BYDV-like cap-independent translation elements (BTE) (Guo et al., 2001; Meulewaeter et al., 2004; Shen and Miller, 2004). The 3' cap-independent translation enhancer domain (TED) of *Satellite tobacco necrosis necrovirus* (STNV) is functionally very similar to that of BYDV but bears no apparent sequence or structural similarity (Wang et al., 1997).

The BTE necessary for cap-independent translation of BYDV RNA in wheat germ extract is a 105 nt tract (TE105) between bases 4814-4918 at the 5' end of the 3' UTR (Guo et al., 2000). The TE105 alone represses translation of viral and nonviral mRNAs when added in *trans*. Addition of exogenous eIF4F restores translation (Wang et al., 1997), suggesting that TE105 binds factors required for both cap-dependent and BTE-dependent translation, although other explanations are possible. Here we provide evidence that the BTE
binds canonical translation initiation factors and that this binding correlates with ability of the BTE to facilitate cap-independent translation.

Materials and Methods

RNA transcription

Plasmids were constructed as described previously (Guo et al., 2000). Plasmid templates were linearized by restriction digestion or amplified by PCR to insure correct RNA length. The RNAs were synthesized by in vitro transcription with T7 or SP6 polymerase using Megascript (for uncapped RNAs) or mMessage mMACHINE (for capped RNAs) kits (Ambion, Austin, TX). RNAs used as probes in dot blot assays were synthesized according to Promega’s small-scale transcription protocol using \( ^{32}\text{P}-\text{CTP} \) as a label. Unincorporated nucleotides were removed on a BioRad P30 spin column. RNA integrity was verified by 1% agarose gel electrophoresis.

Isolation of BTEIPs.

Bait RNAs were biotin labeled at the 3' terminus by modifying the method of von Ahsen and Noller (von Ahsen and Noller, 1995). RNAs were oxidized by adding an equal volume of RNA (3 nmol total) to fresh 100 mM NaIO\(_3\), to a total volume of 100 \( \mu \text{l} \), followed by a one hour incubation in the dark at room temperature. An equal volume of 50% ethylene glycol was added and incubated 15 min in the dark to destroy any remaining periodate. Oxidized RNA was precipitated with ethanol, and dissolved in 80 \( \mu \text{l} \) H\(_2\)O. 20 \( \mu \text{l} \) biotin amidocaproyl hydrazide (Sigma) in DMSO was added to the RNA to a final concentration of 10mM and incubated two hours at 37\(^{\circ}\)C. 100 \( \mu \text{l} \) of 0.2M sodium borohydride was added
with 200 μl 1M Tris-HCl pH 8.2 and the RNA was incubated 30 minutes on ice in the dark. The RNA was precipitated, redissolved in ddH₂O and purified on a BioRad P30 spin column.

Magnetic beads (Promega) conjugated to streptavidin, were washed three times in 0.5X SSC. One nanomole biotinylated RNA was added in 0.5X SSC to beads, and incubated 10 minutes at room temperature. Beads were captured using a magnetic stand, and washed four times in 0.1X SSC. 500 μl wheat germ extract plus 500 μl 2X binding buffer (40mM Tris-HCl pH 7.5, 100 mM KAc, 4 mM DTT, 4 mM MgCl₂, 2 mM EDTA, 10% Glycerol) were added to the beads and incubated 10 min at room temperature. Unbound proteins were removed by 3 to 5 washes in 1X binding buffer plus 5 μg/ml tRNA. Bound protein was eluted by high salt or by heating to 95°C in 1X SDS-PAGE loading buffer (50mM Tris-HCl pH 6.5, 2% SDS, 15% glycerol, 0.72 M BME, 0.01% bromophenol blue) for 5 minutes.

**Protein expression**

eIF4E and eIFiso4E in pET23d vectors were introduced into *E. coli* (BL21 cells) and expression was induced with 100 mM IPTG. Four hours after induction, cells were harvested by centrifugation at 10,000g for 10 minutes. The cells were sonicated in 1X binding buffer and purified using the Novagen Quick 900 His cartridges according to the manufacturer's instructions. Recombinant eIF4F and eIFiso4F were expressed from dicistronic constructs in a pET3D harboring eIF4G and eIF4E or eIFiso4G and eIFiso4E from wheat, respectively (K. Browning, unpublished). The dicistronic plasmids were introduced into *E.coli* (BL21 cells) and induced with 1M NaCl. Four hours post-induction, cells were harvested by centrifugation, and sonicated prior to purification. The lysates were loaded onto a
phosphocellulose column, followed by m$^7$GTP affinity column, and lastly, the protein was purified on a phosphocellulose column. The proteins were either dialyzed against N'·0.1 (20 mM Hepes-KOH, PH 7.16, 100 mM KCl, 1 mM MgCl$_2$, 1 mM DTT) to remove excess m$^7$GTP, or concentrated on Microcon® YM-10 columns with 3 changes of N'·0.1. All proteins were checked using SDS-PAGE and Coomassie staining to verify molecular weight, and by Bradford assay (BioRad Protein Assay) to measure concentration.

Depletion of wheat germ extract

Wheat germ extract was loaded onto a m$^7$GTP affinity column equilibrated in N'·0.1, and eluted in the same buffer. Fractions showing the highest protein concentration at 280 nm, were harvested and pooled, and then re-aliquoted for storage at –80°C prior to use.

In vitro translation

In vitro translation reactions with cap analog added in trans were set up using wheat germ extract from Promega (Figure 1) as described previously (Guo et al., 2000) or with S30 extracts prepared as described previously (Figures 4 and 5) (Lax et al., 1986). 3.2 mM MgCl$_2$ was added to samples containing cap analog. To supplement depleted extracts, protein was diluted to 0.3375 pmol/µl and mixed with N'·0.1 to a total volume of 16 µl. The protein was then added to wheat germ translation mix to a total volume of 50 µl. Luciferase assays were performed using the Luciferase Assay Reporter system from Promega Corporation (Madison, WI). The S35 methionine incorporation assays were performed as described previously (Wang and Miller, 1995).
Western blotting

BTEIPs were blotted onto PVDF membrane and probed using antibodies to known initiation factors. eIFiso4E, eIFiso4G, eIF4A and eIF4B were detected by EMA using the ECF detection kit (Amersham-Pharmacia). For Western blots to eIF4E and eIF4G (done by ELP), protein was electrophoresed in NuPage® 4-12% Bis-Tris gels (Invitrogen) and transferred to Hybond™ P PVDF membranes (Amersham). Membranes were blocked in 3% milk in 1X PBS 0.1% Tween overnight, then probed with primary antibody at a dilution of 1:3000. Blots were washed and incubated with Goat Anti-Rabbit IgG (H+L) HRP conjugate (Bio-Rad) at 1:20000. Chemiluminescent detection was performed with SuperSignal® West Pico substrate (Pierce).

UV cross-linking assay  A wheat germ translation mixture was mixed with \([\alpha-^{32}P]\)-UMP-labeled BTE or TEBF RNA, followed by crosslinking and immunoprecipitation exactly as in Gazo et al (2004). Briefly, 200 µL wheat germ S30 extract was incubated with 24 mM Hepes-KOH, pH 7.6, 2.9 mM MgAc$_2$, 100 mM KAc, 30 mM KCl, 2.4 mM DTT, 0.1 mM spermine, 1 mM ATP, 0.2 mM GTP, 50 µM amino acids, 7.8 µM creatine phosphate, 3 µg creatine kinase, 0.75 A$_{260}$ unit of yeast tRNA and 50 µL $^{32}$P TE or 50 µL $^{32}$P TEBF. The reaction mixture was incubated for 20 minutes at 27°C, followed by irradiation in a StrataLinker (Stratagene) for 4 minutes. The crosslinking reactions were then incubated with 5000 U RNase T1 for 15 minutes at 37°C. The reaction mixture was added to 2.5 mg protein A sepharose (Pharmacia) containing either 10 µL rabbit preimmune, anti eIF4F or anti eIFiso4F serum and incubated at room temperature for 2 hours with mixing. After washing three times with 10 mM Tris·HCl, pH 8, 500 mM NaCl and 0.1% NP-40, the beads were
collected by heating in 50 µL Laemmli sample buffer for 2 minutes at 90°C. The proteins were separated by 12.5% PAGE and detected by autoradiography.

Results

Cap analog inhibits BYDV translation

To better understand whether cap-binding proteins are required for the translation of BYDV RNA, we used *in vitro* translation extracts, to which either m$_7$GTP or nonmethylated cap analog was added prior to addition of mRNA. We used two reporter RNAs: one containing the BYDV 5' UTR and 3' UTR flanking the firefly luciferase gene, and as a control, capped RNA containing nonviral vector UTRs (cap’d veclucvec A). This experiment differs from previous experiments (Wang and Miller, 1995) in that a cap-dependent nonviral transcript was tested, and that higher concentrations of cap analog were used to see how completely it would abolish translation. At 0.04 mM cap analog, LUC869 has a luciferase activity that is 27.7% of the LUC869 0 mM control (Figure 1B). Cap’d veclucvecA has activity that is 12.6% of the cap’d veclucvecA 0 mM control (Figure 1B). Higher concentrations of cap analog further inhibited the activity of LUC869 to levels comparable to cap’d veclucvec A.

We also tested how the full-length infectious RNA translates when cap-binding proteins are sequestered by cap analog added *in trans*. We added a 5.7 kb transcript containing the full BYDV sequence (PAV6), or a capped version of this transcript (capped PAV6), to wheat germ extracts in the presence of $^{35}$S methionine. By visualization of either the 39 kDa ORF1 viral translation product or the 61 kDa luciferase product, we observed cap
analog inhibited all the RNAs (Figure 1C). Similar to the reporters, 0.04 mM cap analog inhibited translation somewhat, and the 0.4 mM and 0.8 mM cap analog severely inhibited translation. Capped PAV6 and the capped nonviral RNA were inhibited to a lesser extent than uncapped PAV6. Inhibition of the viral RNA translation occurred regardless of the moiety at the 5' end of the mRNA.
Figure 1. A. Genome organization of BYDV and response to cap analog. Numbered in boxes indicate open reading frames, translatable ORFs are open boxes. Shaded ovals indicate sequences required for cap-independent translation. The BamHI₄₈₃₇ site (nt 4837) in which the GAUC duplication abolishes BTE function (mutant BTEBF) is shown. The RNA is circularized by direct Watson-Crick base pairing between the 3’ BTE and the 5’ UTR (Guo et al., 2001). B. Translation of BYDV (diamonds) and nonviral reporter RNAs (squares) in wheat germ extracts, with m³GTP added in trans. Firefly luciferase activity was measured in relative light units, and the relative activity was determined based on the activity of each RNA where no cap analog was added. LUC869 contains BYDV 5’ and 3’UTR sequences flanking the firefly luciferase reporter. Cap’d veclucvec A is a capped, polyadenylated nonviral firefly reporter RNA. C. In vitro translation of full-length infectious BYDV RNA and nonviral reporter RNA. 35S methionine was included in the translation, and products were determined by SDS-PAGE followed by autoradiography. Along with the indicated amounts of cap analog, 3.2 mM MgCl₂ was added. PAV6 is the full-length BYDV infectious RNA, cap’d PAV6 has a 5’ cap, and Cap’d veclucvecA is the nonviral RNA used in B.
eIFiso4E, eIFiso4G, eIF4E and eIF4G interact with the BTE

To determine whether host proteins that interact with the 105 nt BTE include translation factors, we used TE105 as bait to pull down specific BTE-interacting proteins (BTEIPs) from wheat germ extract (wge). We also used a nonfunctional mutant of TE105, TEBF, which differs only by the presence of a four base GAUC insertion in the BamHI site. TEBF is completely inactive in facilitating cap-independent translation \textit{in cis}, and in inhibiting translation \textit{in trans} (Guo et al., 2000). We also tested as bait the BYDV 5' UTR and the STNV 3' TED RNA, which is functionally similar to the BTE and is known to bind eIF4E and eIFiso4E (Gazo et al., 2004).

To test for the presence of known initiation factors among the BTEIPs, we used factor-specific antibodies as probes in immunoblots of the BTEIPs (Figure 2B). eIFiso4E, eIFiso4G, eIF4E and eIF4G but not eIF4A or eIF4B were identified among the BTEIPs (Figure 2B). As a negative control, we also tested proteins pulled down by a nonviral RNA, vector sequence flanking the firefly luciferase reporter (pGEMluc, Promega) (Fig. 2C). Thus, the BTE interacts with eIFiso4F and eIF4F.

Binding of initiation factors to the BTE but not the nonfunctional TEBF suggests a role for these factors in BTE function. This indicates that the bases in this stem interact with the proteins, or that the mutation that knocks out cap-independent translation function also disrupts the RNA structure required to bind eIFiso4F and eIF4F. The STNV TED-interacting proteins were identified as eIFiso4E, eIFiso4G, eIF4E and eIF4G (Fig. 2B), in agreement with previous observations (Gazo et al., 2004).
germ extract (wge). A. Western blots using antibodies to known initiation factors on proteins pulled down by the indicated RNAs, as in panel A. Each panel represents a different gel and blot. No pure eIF4B was used as positive control (left-most lane), but efficacy of antisera was evident by detection of eIF4B in the low salt wash. Cleavage products of the labile eIF4G are visible. B. Western blots against proteins eluted from nonviral RNA (166 nt)-biotin-bead complexes. Lane 1 (vec): Proteins interacting with vector sequence flanking the firefly luciferase reporter, W1, W2, and W3: Washes 1, 2, and 3 respectively.
Figure 3. A. UV cross-linking between BTE RNAs and cap-binding proteins in wheat germ extracts. Radiolabeled TE105 or TEBF were UV cross-linked to wheat germ extract proteins and immunoprecipitated with either preimmune antisera (PI) or antisera to eIF4F (4F) or eIFiso4F (i4F). RNA-protein mixtures were then loaded on a 12.5% polyacrylamide SDS gel, stained with Coomassie, and visualized by autoradiography. Arrows at left indicate mobility of pure factor on the same gel.
To verify close association of the BTE with eIF4F and eIFiso4F, a uv-crosslinking experiment was performed. TE105 and TEBF were mixed with wheat germ extracts and subjected to UV crosslinking to stabilize protein:RNA interactions. The complexes were immunoprecipitated using antisera specific for eIF4F and eIFiso4F and visualized via SDS-PAGE and autoradiography (Fig. 3A). As a negative control, preimmune antisera were used for each RNA. Antiserum to both eIF4F and eIFiso4F immunoprecipitated BTE-crosslinked proteins, but not TEBF-crosslinked proteins. One additional band BTE-crosslinked protein migrates at approximately 70 kDa. Like the other bands, this band is stronger in the eIF4F lane than the iso4F lane.
Figure 4. Activity of recombinant A. eIF4F and B. eIFiso4F in m7GTP-depleted extracts. Recombinant protein was added to extracts prior to in vitro translation of LUC869 or cap’d veclucvec A.
BYDV Translation is aided by recombinant eIF4F and eIFiso4F

To test how eIF4F and eIFiso4F influence BTE-mediated translation and to verify the protein for functional activity, we depleted wheat germ extracts of cap-binding factors and then supplemented recombinant protein. To deplete the extracts, we applied wheat germ lysate to a m7GTP sepharose column, and used the eluted extracts in translation assays. This process was shown previously to deplete nearly all of eIFiso4E, eIFiso4G, eIF4E, eIF4G, along with some eIF4A, eIF4B and PABP (Gallie, 2001). The depleted extracts showed such low activity compared with undepleted wheat germ, that we were unable to measure luciferase readings at the identical luminometer sensitivity settings. However, both recombinant eIF4F and eIFiso4F restored translation in depleted extracts to 1 pmol BYDV reporter RNA (LUC869) as well as the nonviral reporter RNA (cap’d veclucvec A) (Figure 4). The amount of protein necessary to increase translation for both RNAs was as low as 0.4 pmol. At the highest concentration of added recombinant eIFiso4F protein we supplemented with (5 pmol), luciferase activity was 3.4-fold and 2.7-fold greater for LUC869 and cap’d veclucvec A, respectively than when no protein was added (Figure 4). When we added 5 pmol recombinant 4F, we observed a 63-fold and 56-fold increase in luciferase activity over the control lacking added protein for LUC869 and cap’d veclucvec A, respectively (Figure 4).

We then examined the difference in stimulation by factors of viral RNA translation by comparing recombinant eIF4F and eIFiso4F in the same experiment (Figure 5). Either recombinant eIF4F or eIFiso4F was added to depleted wheat germ extract programmed with LUC869 RNA. At both concentrations of protein tested (1 pmol and 2.7 pmol), eIF4F
strongly stimulated translation of LUC869, 5.9-fold over the activity that eIFiso4F provided. These data indicate that BTE-mediated translation is eIF4F-dependent, and that BYDV RNA uses eIF4F more efficiently than it uses eIFiso4F. It is also evident that eIF4F is a stronger stimulator in general of translation in vitro, at least for the recombinant forms of these proteins.

![Graph showing comparison of 4F and iso4F stimulation of BYDV translation](image)

**Figure 5.** Comparison of 4F and iso4F stimulation of BYDV translation. Wheat germ extract depleted of cap-binding factors was supplemented with either recombinant eIF4F or eIFiso4F. The extracts were then programmed with LUC869 RNA. The x-axis indicates the amount of protein added, and the y-axis indicates the firefly luciferase activity observed for each.
Figure 6. Closed loop models showing arrangement of translation factors that recruit the ribosome to mRNA. For simplicity, only the factors relevant to this report are shown. 

A. Model for conventional capped, polyadenylated mRNAs (Sachs et al., 1997). B. Permuted arrangement of translation factors in BYDV RNA initiation complex. Initiation factor eIF4E (or eIFiso4E) binds directly to the BTE. eIF4G (or eIFiso4G) is bound to eIF4E. The 40S ribosomal and associated factors are recruited interact with eIF4G (or eIFiso4G), via the eIF3 bridge, as is the case for normal 40S subunit binding (Pestova et al., 2001), possibly enhanced by host proteins and additional downstream viral sequence in vivo. This brings the 40S ribosomal subunit in proximity with the 5' end which it enters the RNA and scans by normal processes (Guo et al., 2001) to the 5’-proximal AUG.
Discussion

Recruitment of cap-binding factors to the 3' end

The eIF4F-BTE interaction provides a natural example of tethering initiation factors to an mRNA to facilitate translation. De Gregorio et al. (De Gregorio et al., 1999) artificially tethered eIF4E and eIF4G to the 5' end of an uncapped mRNA by fusing them to the iron regulatory protein which bound the iron regulatory element in the 5' UTR. Here, we show eIF4E/iso4E tethering occurs by direct binding of a natural RNA and is not limited to the 5'UTR. The eIF4F-BTE interaction resembles the interaction of cap-binding factors with the STNV 3' cap-independent translation element (Gazo et al, 2004), in that it promotes translation. These examples contrast with that of the cad mRNA, which is bound by Bicoid protein at the 3' UTR, which in turn recruits eIF4E-related proteins to repress translation (Cho et al., 2005).

BYDV translation differs from that of most cellular mRNAs. A model to explain these differences is illustrated in Figure 6. On typical cellular mRNAs, eIF4E is recruited to the 5' cap structure on cellular mRNAs, and eIF4G is a large scaffold connecting eIF4E with PABP at the 3' end (Figure 6A). Thus protein-protein interactions mediate circularization of the mRNA. For BYDV, eIF4E and eIF4G are recruited to the BTE in the 3' UTR. In BYDV RNA, long distance basepairing would bring the ends into close proximity (Guo et al, 2001), and to allow ribosome recruitment. Thus, the arrangement of factors on BYDV RNA is permuted with respect to that of the cellular mRNA.
The interaction between pure eIF4E and the BTE is present for functional BTE, though relatively weak, and nonexistent for the nonfunctional BF mutant (Figures 2, 3B). This interaction may be stronger in the context of the complete BTEIP complex of proteins. Affinity of eIF4E for the BTE may be enhanced by eIF4G, because eIF4G enhances affinity of eIF4E for cap by about two orders of magnitude (Haghighat and Sonenberg, 1997; von Der Haar et al., 2000). The STNV TED binds with much higher affinity to eIF4F and eIFiso4F than to free eIF4E and eIFiso4E (Gazo et al, 2004). In fact eIF4F and eIFiso4F are crosslinked to functional BTE, suggesting that the complex may bind. Whether Poly(A) binding protein or other proteins that may interact with the additional BYDV 3' UTR sequence contribute to cap-independent translation in vivo remains to be investigated.

Cap-binding protein interactions with other viruses

eIF4E/eIFiso4E binds the genome linked protein (VPg) at the 5' end of potyviruses (Leonard et al., 2000; Schaad et al., 2000), and is necessary for virus replication (Lellis et al., 2002). VPg-Pro interacts with the eIFiso4F complex (Plante et al., 2004) and co-purifies with PABP (Léonard et al., 2004). A role for eIF4E and eIFiso4E in potyviral translation has yet to be elucidated. The VPg is unnecessary for potyviral cap-independent translation (Niepel and Gallie, 1999). Furthermore, TEV 5' UTR-mediated cap-independent translation is not inhibited by cap analog in rabbit reticulocyte extracts (Carrington and Freed, 1990); however TEV 5' UTR activity is enhanced by eIF4G alone, and not by eIF4E or eIFiso4E (Gallie, 2001) indicating the large subunit of the eIF4F complex is responsible for translation of TEV RNA. Mutations in eIF4E (Ruffel et al., 2002) or eIFiso4E (Duprat et al., 2002; Gao et al., 2004; Kanyuka et al., 2005; Lellis et al., 2002; Nicaise et al., 2003; Stein et al., 2005)
can prevent replication of potyviruses, perhaps reflecting disruption of the interaction between eIF4E/iso4E and VPg. It has been suggested that the link between eIF4E and replication involves virus movement (Gao et al., 2004). VPg also competes with m7GTP for eIF4F, indicating that perhaps the interaction serves to inhibit host translation (Plante et al., 2004). We suggest that eIF4F or eIFiso4F recruitment by BYDV and STNV (Gazo et al, 2004) by the 3’UTRs mediates cap-independent translation.

eIF4E is required by the IRES of Hepatitis A virus (Ali et al., 2001), hinting that HAV and BYDV sequences may share some mechanistic similarities in ribosome recruitment. Cap analog also inhibits HAV-driven translation. In reticulocyte lysates, 0.025-0.066 mM of cap analog is required to inhibit translation of HAV RNA to less than 50% (Ali et al 2001; Borman et al, 2001). eIF4F, and in particular an intact cap-binding pocket is thus necessary for optimum translation of HAV and BYDV RNA. Cap analog binding to eIF4E may also negatively affect the recruitment of eIF4G to the complex, rendering eIF4F incompetent. However, if TEV RNA were able to recruit eIF4G under conditions where the cap-binding pocket on eIF4E is occupied (see above), this may not always be the case. Along these lines, STNV RNA translation also is not inhibited significantly by cap analog, compared with other RNAs, at least at concentrations up to 0.05 mM (Fletcher et al., 1990; Smith and Clark, 1979). In contrast, both BYDV and HAV appear to require the cap-binding pocket of eIF4E, although the exact mechanism of how the eIF4F complex is assembled for these RNAs remains unknown. Unlike HAV, however, the BTE is not an IRES (Allen et al., 1999) and it is located in the 3’ UTR.
Translational activity of plant eIF4F/iso4F

The plant isoforms of eIF4F and eIFiso4F are different from one another, especially between eIF4G and eIFiso4G in size and the presence of domains that recruit other factors (Browning, 1996). The function of these differences is not well understood. We found that eIF4F stimulates translation better than eIFiso4F does (Figures 4, 5). Gallie (2001) showed that eIF4F preferentially enables cap-independent translation mediated by the TEV 5' UTR under conditions where the factor is limiting. Perhaps this is due to the structured nature of the two viral leaders, as eIF4F supports translation of a leader with secondary structure in the 5' UTR (Gallie and Browning, 2001). However, our results in Figure 4 indicate eIFiso4F stimulates less efficiently than eIF4F for a nonviral RNA containing a shorter 5' UTR. eIF4F is actually limiting in wheat germ extract, as there is five-fold more eIFiso4F than eIF4F (Browning et al., 1990). If more viral RNAs are found to preferentially use eIF4F, it is possible that viral RNAs have evolved to use mainly eIF4F, leaving eIFiso4F for the cellular mRNAs and reducing competition. Alternatively, each virus-host interaction requires a different isoform, or each isoform contributes to different points in the viral lifecycle. The mRNA distribution (Rodriguez et al., 1998), but not protein distribution, of the isoforms is known: it would be interesting to see if virus localization and expression in the plant corresponds with the location of particular isoforms.

Recognition of eIF4F on uncapped RNA

Regardless of the presence or absence of cap on the 5' end of the viral RNA, cap analog inhibited translation of the viral RNA (Figure 1C). How eI4F recognizes RNA
without a m^7G moiety is a mystery, considering that the 7-methyl group forms key hydrogen bonds and electrostatic interactions in the cap-binding pocket of eIF4E (Marcotrigiano et al., 1997). The nonfunctional BTE mutant, TEBF did not bind any of the initiation factors, indicating that the BTE could be responsible for the recruitment of eIF4F. The mutation in TEBF overlaps with a portion of stem-loop I of the BTE; thus stem-loop I is a potential protein binding site. It is highly conserved in all viruses that harbor a BTE, and it fits the consensus of a GNRNA pentaloop (Legault et al., 1998). This structure forms similar intra-loop interactions as a GNRA tetraloop, but the fourth base protrudes from the loop. Such a structure recruits specific host and bacteriophage λ proteins involved in anti-termination of transcription in *E. coli* (Legault et al., 1998).

Another example of an uncapped RNA that binds eIF4E are aptamers that were discovered by SELEX (Mochizuki et al., 2005). That RNA appears unrelated in sequence and structure to the BTE. However, eIF4E mutants tested with this aptamer indicate that recognition by the protein may be outside of the core cap-binding pocket. Among the 4E mutants that did not bind were mutants in the C-terminal loop region on eIF4E. Given that this region in 4E may be responsible in part for the recognition of downstream nucleosides in the RNA (Tomoo et al., 2003), it is possible this region participates in binding other uncapped RNAs.

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References


CHAPTER 4. PHYLOGENETIC, STRUCTURAL, AND FUNCTIONAL CHARACTERIZATION OF 3' UTR SEQUENCE NEEDED FOR BYDV EXPRESSION IN VIVO

Abstract

The 3' UTR of Barley yellow dwarf virus RNA confers cap-independent and poly(A) tail-independent translation. Much of this 3' UTR sequence is required for poly(A)-independent translation, and the substantial length and structure of this sequence may contribute to its activity. We have predicted the structure of the entire 3'UTR using computer folding methods, with an emphasis on a domain in nt 5089-5142 which is replaceable by a 3' poly(A) tail. In vitro structure probing shows that the predicted structure is mostly accurate, and that there are three stem-loops included in nt 5089-5142. Phylogenetic analysis shows that these stem-loops are conserved in other BYDV isolates, and functional analysis shows that these stem-loops are required for viral replication. Many of the structures, however are not required for translation in vivo, while structures at the 3' end of the RNA are required. We speculate that tertiary interactions within the BYDV RNA also facilitate folding of the RNA to enhance translation.

Introduction

The majority of eukaryotic mRNAs have a 5' m7GpppNG cap and a 3' poly (A) tail. The 5' cap interacts with initiation factor eIF4E, and the 3' poly (A) tail interacts with poly(A) binding protein (PABP). The large scaffold protein, eIF4G binds both eIF4E and PABP, which is thought to bring the mRNA ends into close proximity. The mRNA is thus thought to be circularized in order for initiation to occur. The protein:protein and
protein:mRNA interactions are synergistic with each other in promoting recruitment of the 40S ribosomal subunit to the 5' end of the mRNA. Once the 40S subunit is recruited, scanning to the 5' AUG occurs by a process which is still unclear, resulting in translation initiation. The 5' cap and 3' poly(A) tail not only recruit these factors but also stabilize the mRNA. While many viral RNAs, including those of the Picornaviridae, Flaviviridae, Dicistroviridae, and families, lack either a 5' cap or a 3' poly(A) tail (Jackson, 2000), they are still translatable in their host cells owing, at least partly, to specific viral RNA features.

Barley yellow dwarf luteovirus (BYDV) is a member of the Luteoviridae family of plant viruses. The BYDV genomic RNA is 5.6 kb in length and encodes three subgenomic RNAs (Figure 1). BYDV RNA lacks both a 5' cap and a 3' poly(A) tail, that are present on most cellular mRNAs. A 3' translation element (BTE) confers cap-independent translation both in vitro and in vivo (Guo et al., 2000; Wang et al., 1997; Wang and Miller, 1995). This element resides in the 3' UTR, and is cruciform in structure (Guo et al., 2000). The BTE is thought to act by binding plant initiation factors (Chapter 3 herein), and by interacting with a complementary sequence in the BYDV 5' UTR via long distance basepairing (Guo et al., 2001). Like the other members of genus Luteovirus, BYDV has a long (869 nt for BYDV) 3' UTR.

The BYDV 3' UTR contains multiple elements that are required for several viral expression processes. The BTE (above) facilitates cap-independent translation. Other sequences in the 3' UTR are required for poly(A)-independent translation (Chapter 2 of this dissertation). The BYDV 3' UTR also contains elements that modulate ribosomal
frameshifting to control expression of the viral RNA dependent RNA polymerase (RdRP) (Barry and Miller, 2002; Paul et al., 2001). At least two other 3' UTR sequences serve as promoters for the synthesis of subgenomic RNA (Koev and Miller, 2000). Finally, the very 3' end of the BYDV RNA is the origin of replication, from which viral RNA dependent RNA polymerase begins synthesis of additional viral RNA (Koev et al., 2002). Thus, the 3' noncoding region of BYDV impacts viral gene expression at multiple levels.

Several plant viruses have elements that confer translation in the absence of a 3' poly(A) tail. Turnip yellow mosaic virus (TYMV), Tobacco mosaic virus (TMV) and Brome mosaic virus (BMV) contain tRNA like structures at their 3' ends that facilitate translation (Dreher, 1999; Matsuda and Dreher, 2004). With TMV RNA, a pseudoknot rich domain upstream of the tRNA-like structure (UPD) functionally substitutes for a poly (A) tail (Gallie and Walbot, 1990). The UPD binds eEF1A (Zeenko et al., 2002) and hsp101 (Tanguay and Gallie, 1996; Wells et al., 1998), but how exactly these interactions facilitate translation is not understood. The 3' UTR of Alfalfa mosaic virus (AMV) possibly folds into a series of stem-loops and an alternative tRNA-like structure, where coat protein binds (Neeleman et al., 2001). However the tRNA like structure may not be required, at least for replication (Petrillo et al., 2005). Tobacco necrosis virus (TNV) has a double stem-loop structure which functionally replaces a poly(A) tail (Shen, 2004). Thus, many plant viruses are nonpolyadenylated, and there are several plant viral 3' UTR sequences which might serve the purpose of a poly(A) tail. Yet their mechanisms of action are poorly understood.
Secondary structure prediction of the RNA in viral genomes aids in our understanding of viral gene expression. A detailed survey of secondary structures in the genomes of the Picornaviridae showed that internal ribosome entry sites (IRESes) are conserved at fewer nucleotides than originally thought, and that there are several structural elements within coding regions (Witwer et al., 2001). Phylogenetic analysis of secondary structures in Flaviviridae genomes has also shed light on the differences between viruses with no known vectors and those with insect vectors (Charlier et al., 2002). Another survey of the Flaviviridae provided information on IRESes, 3’ UTR motifs, and cyclization sequences (Thurner et al., 2004). Recently, these types of analysis have been extended to include other virus families, including plant viruses (Hofacker et al., 2004; Simmonds et al., 2004). Although these analyses give insights into the structural features and their phylogenetic conservation, comprehensive functional analysis would tell us even more about the biology of viral RNAs.

To better understand the function of BYDV 3’ UTR sequence, we undertook an approach based on RNA secondary structure. We made secondary structure predictions of the BYDV 3’ UTR using RNA folding algorithms, and then performed phylogenetic comparisons on the structures of multiple BYDV isolates. We also examined the structure using in vitro chemical probing, and finally testing the effects of mutating structure in vivo. We focused primarily on nt 5089-5142, as they were previously shown to be involved in poly(A) tail-independent translation (Chapter 2 herein). Additional sequences at the very 3’ end of the mRNA also contributed to poly(A)-independent translation in vivo. Our findings indicate that tertiary interactions in the RNA of the 3’ UTR might also be important.
Materials and Methods

GenBank Accession Numbers

The accession numbers used for phylogenetic analysis are as follows. PAV-Aus isolate, X07653; Purdue, D11032; BYDV Japan, D85783; PAS, NC_002160; MAV-PS1, D11028; and PAV-GAV, NC_004666.

Plasmid construction

The wild type PAV6 plasmid, the infectious clone for BYDV Aus isolate was used as the template for plasmids tested in replication assays (Di et al., 1993). Mutations were introduced into pPAV6 by PCR based mutagenesis with overlapping primers containing a mutation in the site of interest. Outside forward primers were used either upstream of the Acc65I site in PAV6 (4152F) or upstream of the BsmI site in pPAV6 (4524F); the reverse primer was in the vector 3' to the viral 3' end. The mutants created by PCR with 4152F were: A5096U, G5097C; U5099A, G5102C; A5116U, U5117A; G5122C; G51255C, G5126C; C5142G, C5143G; A5154U, A5155U A5156U; C5162G, G5163C; 5165 add C; and 5165dUAU. All others were created by PCR with 4524F. The sequence of primer 4152F is 5' GGA ATT CGG TAC CAT ATC TGT GAC 3', and the sequence of 4524F is 5' GGA CAT ACC AAG GAC AG A AC 3'. The sequence of the reverse primer (UnivF) is: 5' CGC CAG GGT TTT CCC AGT CAC GAC 3'. Following the first round of PCR, the two products of the first PCR were combined and used as template in a PCR reaction with the two outside primers. The product was gel purified and digested with Acc65I and Smal or BsmI and Smal. pPAV6 was also digested with either combination of enzymes as appropriate, and all digestion products were electrophoresed and gel purified. The digested
inserts were then ligated into pPAV6 using T4 DNA ligase (NEB) and transformed into TOP10 cells (Invitrogen).

Luciferase reporter constructs were derived from the viral replication plasmids constructed above. The viral plasmid was digested with *BamHI* and *SmaI*, gel purified, and ligated using T4 Quick DNA ligase (NEB) into pLUC869 (Wang et al., 1999). Mutations to the 3' end of BYDV (Figure 8) were derived from Koev et al (2002). The 3' UTR was excised using *BamHI* and *SmaI* sites, and ligated into pLUC869. All plasmids were verified by sequencing (Iowa State University DNA Facility) across the entire length of the insert.

**RNA modification and primer extension**

pPAV6, which contains the entire BYDV genome, was linearized with *SmaI* followed by phenol: chloroform extraction. Full length infectious RNA was transcribed using the T7 MegaScript kit (Ambion). After verification of RNA integrity by 1% agarose gel electrophoresis, and UV spectrometry, RNA was diluted to 0.4 pmol/µl and used as template for kethoxal or DMS probing as in (Merryman and Noller, 1998), except that DMS probing was performed on ice for two hours. For imidazole probing, the RNA was treated exactly as in (Vlassov et al., 1995) followed by precipitation as in Guo et al, (2000).

**Replication assays**

10 µg RNA was electroporated into oat protoplasts, and the cells were cultured in MS media (Invitrogen) with 0.4 M mannitol for 48 hours at room temperature. Cells were harvested and RNA was extracted with Trizol® (Invitrogen) according to the manufacturer's
Northern blot analysis was carried out as described previously (Koev et al., 2002), using an RNA probe complementary to the 3' 1.5 kb of the BYDV genome.

**Translation Assays**

1 pmol RNA was electroporated into oat protoplasts, along with 0.1 pmol control capped, polyadenylated renilla luciferase RNA for normalization. Following four hours culture in MS media with 0.4 M mannitol, cells were harvested and lysed in Passive Lysis Buffer (Promega) by shaking 15 minutes at room temperature. 2-5 μL lysate was assayed using the dual luciferase reporter assay system (Promega) in a Turner Designs TD 20/20 luminometer. All samples in an experiment were tested in 3 to 5 replicates, and a minimum of three independent experiments were performed.

**Figure 1.** BYDV genome and the 3' elements that confer poly(A) independent translation. The PAM domain, nts 5089-5142 and 3' end (nts 5574-5677) are marked by rectangles in the 3' UTR. Open reading frames are depicted by boxes above the genome, with protein product sizes indicated, and the untranslated regions are depicted by dashed lines.
Results

Nucleotides 5089 to 5180 of BYDV RNA are conserved in secondary structure

BYDV nt 5089-5142 (PAV-Aus isolate) are needed for translation in vivo, and are replaceable by a 3’ poly (A) tail (Chapter 1 herein). To examine the secondary structure in this region of BYDV RNA, we analyzed the predicted secondary structure of the 3’ UTRs from several luteovirus isolates. Using MFOLD (Zuker, 2003) we predicted this region to have at least three stem-loops (Figure 2A). We followed this analysis with alignments of primary sequence in this region, to determine whether each stem-loop is conserved (Figure 2B-D). Finally, we folded each BYDV isolate using MFOLD (Zuker, 2003), to confirm the stem-loops predicted for each isolate. We named each of the stem-loops by the nucleotide number from the PAV isolate at the 5’ end of the stem-loop, that is, where the 5’ stem starts.

We found that SL5095, which was predicted to have a UNCG tetraloop in PAV-Aus, was strongly conserved in PAV-Aus, PAV-Jpn, and PAV-Purdue isolates (Figure 2B). However the loop sequence differed significantly in other related isolates. For the other stems, all were closed by C-G pair near the loop and a G-C pair at the base of the stem, with covariations in the stem sequences occurring for the MAV and GAV isolates. For Bean leafroll virus (BLRV), which is more distantly related, a UNCG tetraloop was present, but the stem was predicted to be quite weak, and the primary sequence had very little overlap with the other isolates. We were unable to find a homologous structure to SL5095 in another distantly related luteovirus, Soybean dwarf virus (SbDV).
The stem-loop which starts at nt 5109 is quite strong, with five G-C pairs. For SL5109, we found homologous structures in all the luteovirus isolates examined, except SbDV and BLRV (Figure 2C). For several isolates, there are covariations from G-C to C-G, U-A, A-U, and G-U pairs, and for MAV, PAS, and GAV, the helix is extended. Every isolate has at least one purine bulged on the 5' stem, except for Jpn, which has purines flanking the ends of the stem on both sides. Some isolates also have at least one bulged nucleotide on the 3' part of the stem. The loop sequence for SL5109 varied slightly in length and composition, with most isolates having either a seven or eight nucleotide loop, and MAV having a four nucleotide loop. The consensus loop is $PYUAACNP$, with UAAC (italicized) being common to all of the isolates.
Figure 2. A. Predicted secondary structure of the BYDV PAM domain for the type isolate, PAV-Aus. SL5095, SL5104, and SL5140 green are boxed. B-D. Secondary structure predictions for individual stem loops for BYDV isolates. See Materials and Methods for GenBank Accession numbers for each isolate. B. Predictions for SL5095 by isolate. C. Predictions for SL5104 by isolate, and D. Predictions for SL5140 by isolate.
A third stem-loop is predicted starting at nt 5140, with covariations in the stem for every isolate (Figure 2D). SL5140 is G-C rich. In this stem, all isolates have a bulged UA on the 3’ portion of the stem, flanked by a G-U pair. The loop is 11-12 nucleotides long. Its sequence is more highly conserved than the SL5109 loop. The consensus loop sequence of SL5140 is A C U/A U R A A U R C/A C.

**Nucleotides 5089 to 5180 of BYDV RNA are structured *in vitro***

We performed chemical modification analysis on this region of the viral RNA, to determine whether the structures could exist *in vitro*. To probe the structure, we incubated full-length viral RNA with either DMS, which modifies Cs and As, kethoxal, which modifies Gs, or with imidazole which modifies any base whose backbone is accessible and flexible. Following reverse transcription of the RNA and incorporation of radiolabeled ATP, we observed the modification patterns on polyacrylamide gels (Figure 3A and 3B). Strong bands appeared across all lanes, including for unmodified RNA. These K-bands indicate places in the structure that the reverse transcriptase did not entirely process through, and reveal locations of stems in the RNA (for example the 3’ base of SL 5109). The results of the structure probing analysis are summarized in Figure 3C.

The 5099UNCG5102 tetraloop in SL5095 was not readily modified; except G5103 was modified by both kethoxal and imidazole (Figure 3A and data not shown). It is possible that interactions among the nucleotides of the tetraloop make them inaccessible to the chemicals. K-bands (see above) were observed for the bases on the 5’ helix of SL5095, suggesting there may be some structure in that region. The 20 bases upstream of SL5095 are
likely to be single-stranded, and the LDFE stem-loop (Barry and Miller, 2002) was also identified in this analysis.

The other two predicted stem-loops between 5080 and 5180, SL5109 and SL5140 were clearly identified by the structure probing (Figure 3A,B). The U-A pair at the base of SL5095 was also somewhat accessible to modification. This suggests that some of the 5' bases on the predicted helix of SL5095 are exposed. Strong K-bands were observed for the 3' bases, showing that the stem is likely to exist. Many of the loop bases were at least weakly susceptible to modification by imidazole and either of the base-specific chemicals, with the exception of A5116, U5117, and G5122. These data suggest that SL5109 is very likely to exist \textit{in vitro}. Between nt 5111 and 5138, most of the bases are moderately accessible, suggesting that the region between SL5109 and SL5140 is mostly single-stranded (Figure 3B). The probing data supported the presence of SL5140, including the bulged bases 5165-5167.
Figure 3. Chemical modification analysis on the BYDV PAM domain. RNA was modified, reverse transcribed, and the product was electrophoresed as described in the Materials and Methods. A. Structure probing gels for nts 5050-5142 (kethoxal, DMS) and B. 5110-5190 (kethoxal, DMS and imidazole). For A and B, GAUC indicate sequencing lanes, and the chemical used is indicated above the other lanes. Numbers to the left of the gel indicate nucleotide positions in PAV-Aus. Interpretation of stem-loop structures is indicated to the right of each gel. C. Summary of structure probing results. Nucleotides colored in orange and red, indicate imidazole cutting weakly, or strongly, respectively. Triangles indicate kethoxal modifications resulting in termination of reverse transcription (large for strong stops small for weak stops). Arrows indicate DMS modification sites (large for strong, small for weak). K-bands, indicated in gray, are sites of reverse transcriptase stops or pauses, which occur in unmodified as well as unmodified RNA.
RNA between 5089 and 5180 is necessary for viral replication, but not translation

To test whether the secondary structures of BYDV nt 5089-5180 are functional in vivo, we studied the effects of introduced mutations in the structures on viral replication in oat protoplasts. We examined three types of mutations: i) Stem disruptions at the central bases in the stem on both sides, ii) deletion of bulged nucleotides, and iii) loop mutations to selected bases (Figure 4).

First we examined SL5095, to see whether changes in the tetraloop sequence UNCG affected replication. A change to the loop (U5099A, G5102C) which resulted in the loop sequence aUCc (lower case bases indicate nucleotides mutated) was very deleterious to replication while a change (U5100A, C5101G) to UagG was not deleterious (Figure 4A). Likewise, one mutation to the stem, A5096U, G5097C caused no change in genomic subgenomic RNA (sgRNA) accumulation, while the C5104G, U5105A abolished replication (Figure 4B). Thus, primary sequences in addition or instead of secondary structure, may be important for SL5095.

To investigate the role of SL5109, we deleted the bulged A5111 (5111ΔA) and altered nucleotides 5116 and 5117 from AU to UA (A5116U, U5117A). These changes were not well tolerated (Figure 4A). Because mutations of 5116-5117 alone abolished replication, it is not possible to determine whether the loss of A5111 also contributed. Based on the structure probing results (Figure 3C), it seemed possible that G5122 may interact with some other base, since it was mostly inaccessible to the probes. Therefore, we also tested whether altering G5122 to C would change replication, and found sgRNA2 accumulation may be
slightly affected; however the other RNAs accumulated to high levels (Figure 4B). Thus, this base may not necessarily be involved in a crucial tertiary interaction. Both disruptions to the stem of SL5109 resulted in a loss of a viral replication (Figure 4B). Thus, the sequence and/or possibly the structure of SL5109 are important for BYDV replication. Finally we examined how changes to SL5140 affect viral replication. In fact, both changes to lower portion of the stem, C5142G, C5143G and G5169C, G5170 prevent replication (Figure 4A). The bulge for nucleotides 5165-5167 was also important for replication (Figure 4A).

Figure 4. Northern blots against RNA extracted from oat protoplasts 48 hours after inoculation. The accumulation of genomic and subgenomic RNAs is measured by hybridization to a radioactive probe corresponding to the 3' 1.5 kB of the genome. Above each lane, the mutation position is indicated by nucleotide number and the identity of the bases introduced; the mutations are also on the secondary structure of individual stem-loops.
Because several of these mutations negatively affected viral replication, we next sought to determine whether the defects were at the level of translational control. The 3' UTRs from the replication constructs were subcloned into LUC869, a luciferase reporter construct containing the BYDV 5' UTR and 3' UTR (Wang et al., 1999). Figure 5 shows the results of reporter assays performed following electroporation of mutant RNAs with 3' UTRs into oat protoplasts. Most mutations in SL5095 and SL5140 had no substantial negative effect on translation. All the mutations to SL5109 had a negative effect on translation in vivo. The mutations in SL5109 translated between 27.9% to 70.8% of LUC869, and translation was recovered to near wild type levels when a poly(A) tail was added to the 3' end. This suggests that SL5109 might contribute to poly(A) independent translation. For SL5142, a mutant where the stem was restored in structure (RestoreSL5142) was the only mutant that had significantly lower translatability; SL 5142 had activity of 46.0% relative to LUC869 (Figure 5C).
Figure 5. Results of in vivo translation assays. Reporter RNAs were electroporated into oat protoplasts, and firefly luciferase activity was measured relative to the wild type LUC869 RNA. A. SL SL509 mutations. B. SL5104 mutations. The effects of adding a poly(A) tail to the 3' end of the RNA were also tested (gray bars). and C. SL5140 mutations.
A

% Translation Relative to U1269

A5096U G5097C  C5104G U5105A  Restore SL5096  U5099A G5102C

B

% Translation Relative to U1269

G5113C C5125G Restore A5116U S111dA  G5114C C5126G SL5113 U5117A

C

% Translation Relative to U1269

C5142G C5143G  G5169C G5170C  Restore SL5142  A5154U A5155U A5156U  5165 dUAU
Figure 6. Predicted secondary structure of the entire 3' UTR, based on MFOLD (Zuker, 2003) and structural analysis (Guo et al, 2000; Koev et al, 2002). Lines connect adjacent nucleotides.
Modeling of entire 3' UTR

Because the entire BYDV 3' UTR confers more efficient poly(A)-independent translation than isolated segments of the BYDV 3' UTR (Chapter 2 herein), we also sought to understand the RNA secondary structure of the entire 869 nt 3' UTR. Using MFOLD (Zuker, 2003), we predicted many stem-loops throughout the 3' UTR. (Figure 6).

Downstream of the PAM domain (nt 5089-5142) discussed above, we found several G-C rich stems in and adjacent to a region which enhances frameshifting including a stem beginning at nt 5228, 5290, and 5372 (Paul et al., 2001). We were also able to find a series of stem loops between nts 5416 and 5545; it is unknown what role, if any, these structures play in the function of the RNA.

![Diagram of RNA secondary structure](image)

**Figure 7.** Potential long distance RNA-RNA interactions within the BYDV 3' UTR for several isolates between the 3' end of the BTE and SL5095.
Long distance interactions are possible within the BYDV 3' UTR

We also searched for other possible RNA/RNA interactions in this region of the BYDV 3' UTR. Such interactions might explain where some loop nucleotides could fold in the overall RNA genome. The tetraloop in SL5095 has complementarity to nucleotides in the BTE (Figure 7). This potential interaction involves four G-C pairs in the middle of the interacting bases, with several G-U and A-U pairs flanking them. This basepairing is well-conserved between isolates, and several covariations support the possibility of basepairing (Figure 7). Mutations to the AUG nt 4920 resulted in a loss of replication, with no change to translation (A. Rakotondrafara, pers. comm.). Mutations to the loop involving bases U5099 and G5102 also abolished translation, but did not negatively affect translation (Figures 4 and 5).

The 3' end of the BYDV RNA contributes to poly (A) independent translation

We also examined whether the 3' end of the BYDV RNA is necessary for translation. Koev et al (2002) found that the 3' end is crucial for replication. Mutations in the 3' end were introduced into the reporter construct by subcloning from Koev et al's collection, and tested for translational activity in oat protoplasts (Figure 8). Deletion of three of the stems in the 3' end resulted in significantly decreased translational activity (Figure 8B). For one of the deletions (SL2), a poly(A) tail restored translation to levels which were above that of wild type LUC869. A poly(A) tail stimulated translation only slightly in the other to deletion mutants, SL1 and SL3. None of the other mutations significantly affected translation. In general the translation results agreed with the replication results (Koev et al., 2002); however some mutants that translated did not replicate (Figure 8B), as above.
Figure 8. Translation of reporter RNAs contain mutations in the 3’ end of BYDV 3’ UTR.  
A. Secondary structure of the 3’ end (Koev et al, 2002), with positions of mutations shown.  
B. Results of replication assays (left column, summarized from Koev et al, 2002), and translation in oat protoplasts of mutants with (middle column) or without a poly(A) tail (right column). n/d=not determined.
Discussion

In this study, we characterized a region of the BYDV 3' UTR previously mapped as a poly(A)-independent translation element. We found that the 3' UTR secondary structure is well-conserved in all the BYDV isolates studied, and that the predicted structure is in fairly good agreement with in vitro structure probing. Individual stem-loop structures as well as other key residues in this region were found to be necessary for BYDV replication; however few mutations in this region, aside from those in SL5109 negatively affected translation in a reporter assay system.

Why replication, not translation?

Most of the mutations to the 3' UTR severely impaired replication of BYDV RNA; however many had no or weak effects on translation (Figure 4 vs. Figure 5). Mutations to disrupt the structure of SL5109 resulted in somewhat poor translatability of reporter RNAs in vivo (Figure 5B). Most of these mutants still translated at approximately 50% of wild type LUC869, similar to the deletion of a larger portion of this region, Δ5089-5142, although mutations made to disrupt the stem of SL5109 resulted in even lower levels of translation. Nonetheless, the replication data and phylogenetic conservation of these structures suggests are important.

Replication is an end result indicating the sum of effects from other viral processes, and is a good measure of the outcome of modifications to the virus. Defects in replication could result from an inability to recruit protein related to replication, destabilization of a crucial portion of the whole infectious RNA, disrupt long-distance interaction in the full-
length RNA. Since the elements interact partially with frameshifting enhancers, replicase production could be negatively altered. Viral fitness might be related to other processes we did not study, for example movement, or ability to suppress a host defense. The collection of mutants generated here would aid in future studies if these possibilities were explored.

One intriguing possibility is that microRNAs (Voinnet, 2002) homologous to the 3′ UTR or other viral sequence might play a role in replication and pathogenicity of BYDV in the host. Because sgRNA2 is in such high abundance during infection, and is thought to regulate BYDV expression (Shen and Miller, 2004; Wang et al., 1999), it could be a target of microRNAs. A search of the microRNAs registry (Griffiths-Jones, 2004) using the entire BYDV 3′ UTR as the query sequence with BLASTN on the microRNAs registry website indicated there are no microRNAs homologous to the BYDV 3′ UTR. Using BYDV 3′ UTR sequences that were 60-70 nt in length as the query sequence resulted in multiple matches for almost every sequence queried. However, none of the matches was greater than 10 nt in length, and the matches came from diverse organisms. Thus, the BYDV 3′UTR RNA is not complementary to, nor is it known to be a target of microRNAs. Other portions of the viral RNA might be complementary to a microRNA, or a micro RNA complementary to the BYDV 3′ UTR may yet be discovered. However, it may be more likely that virus-derived siRNAs, if they exist, rather than host-derived microRNAs, play a role.

How does this region compare with other plant virus poly(A)-independent elements?

The RNA structures of other poly(A)-independent elements fall into several categories: i) pseudoknot structures (TMV) (Leathers et al., 1993), ii) series of organized
repeats which bind a viral protein (AMV, like rotaviruses) (Neeleman et al., 2001), iii) tRNA-like structures (TYMV, BMV) (Gallie and Kobayashi, 1994; Matsuda and Dreher, 2004; Noueiry et al., 2003) iv) those with long, structured 3’ UTRs (TNV, BYDV) (R. Shen unpublished, and herein). Thus, we suggest that BYDV and TNV belong to a new category of poly(A)-independent viral RNAs, and that each virus has unique sequence elements to confer translation in the absence of a poly(A) tail. Outside the BTE, sequence alignments on TNV and BYDV 3’ UTRs fail (not shown), so there are apparently not large overlaps in primary sequence similarity between the two. More extensive mutation analysis on all of these elements might shed light on how each works and whether there are commonalities between them. In sum, there is significant variety in the poly(A)-independent translation sequences and structures, and much remains to be understood about their functions.

Interestingly, we found that SL5095 has exact homology to another plant viral 3’ UTR sequence, Arm B in the the 3’ end of Barley stripe mosaic hordeivirus (BSMV) (Solovyev et al., 1996). The sequence is conserved in Poa semilatent hordeivirus (PSLV), but only for the first six nucleotides. This region in BSMV is required for viral replication (Zhou and Jackson, 1996); however nothing is known about its function, if any, in translation (A. Jackson, pers. comm.). Given that mutations to SL5095 did not affect translation significantly (Figure 5A), this role of this stem is also ambiguous for BYDV.

3’ end of the Viral RNA

The 3’ end of the RNA may be important for poly(A) independent translation. The 3’ end of the mRNA plays an important role in stability of the mRNA. For example, histone
mRNAs, which are nonpolyadenylated in most eukaryotes (they are polyadenylated in plants), have a 3' stem loop which recruits factors to facilitate translation (Ling et al., 2002; Sanchez and Marzluff, 2002). We are unaware of a similar example in plant mRNAs, other than for viral RNA, the AMV CP (Neeleman et al., 2001), that binds the 3' end of plant mRNAs. Given the number of nonpolyadenylated plant viruses with significant 3' RNA structures, it will be interesting to see which factors if any interact at the 3' end of the RNA to stabilize the RNA and/or recruit the translation apparatus.

The 3' end of TNV RNA also plays a role in poly(A)-independent translation and replication. Two stem-loop structures of the TNV RNA are necessary, but not sufficient for poly(A)-independent translation: the full length of the TNV 3' UTR is required (R. Shen unpublished). Aside from the presence of tetraloops and the 3' CCC on the BYDV and TNV 3' ends, there are no obvious similarities in sequence or structure between the ends. Each viral RNA has a stem-loop with bulged purines, these were not mutated for either. Also, SL-II in the BYDV 3' end is similar to one of the stem-loops implicated in TNV translation, yet the loop sequence varies within necroviruses, suggesting the primary sequence may not be crucial. It is possible that the 3' end is also embedded for TNV, tombus virus, an comovirus genera as it is for BYDV RNA (Koev et al., 2002), because there is potential basepairing between the end nucleotides and other 3' end nucleotides (not shown). Whether any factors bind the 3' ends of these RNAs is still unknown; however if viral replicase is bound, it could prevent the RNA from degradation.
Other Potential Interactions in BYDV 3' UTR Sequence

Tetraloops make up all of the loops in the 3' end of BYDV RNA (nts 5574 to 5677), and are present in several other places in the BYDV 3' UTR (Figure 6). Many large RNA molecules fold into compact structures, where the overall structure is a result of tertiary interactions, many of which involve tetraloop interactions (Batey et al., 1999). So-called tetraloop receptors are nucleotides which docking with tetraloops (Batey et al, 1999). We examined the BYDV genome for tetraloop receptor sites. Using results from in vitro selection experiments performed by (Costa and Michel, 1997), we queried the BYDV genome sequence for possible tetraloop receptors. No possible tetraloop receptors were found; however some tetraloop motifs are quite simple and would not have been found by this method. For BYDV RNA, the stability of the tetraloops might lead to the stems' formation in the 3' end. This would maintain a 3' end which is structured overall, and is resistant to exonuclease attack. Additional mutations, including some that would expose the 3' end of the BYDV RNA would help us understand how the 3' end contributes to translation.

Because SL5109 was found to be important in both replication and translation, we wondered if its sequence is homologous to any known functional RNA sequence, including sequences that would bind proteins. We searched the SCOR database (Klosterman et al., 2004), which is a compilation of RNAs by structural and functional classification, for similar sequences. When we searched the entire loop sequence of the Australian isolate (AUUAACGA), or the consensus loop (PYUAACNP), we found no matches. However, the sequence YUAACN in the middle of the loop did have several matches. These matches
include: i) the 8 nt phage antitermination loop that binds a bacteriophage antitermination protein (Cilley and Williamson, 2003); ii) in the Tetrahymena Group I P4-P6 interaction (Cate et al., 1996); iii) in the S2(S8) hairpin in 16S rRNA (H. Zhang, M. Culyba, H. Volkman, T. R. Krugh, unpublished); and iv) in GNRA loops and U-turns as well. There were no matches to the stem sequences of SL5109 in the SCOR database; however that does not rule out that the secondary structure of the stem is homologous to other stem-loops. If future experiments reveal tertiary interactions or proteins bound to this region, it would be interesting to see if there is similarity between the architecture of SL5109 and the examples above.

Future work includes identifying additional long-distance interactions in the viral genome, and developing methods to test the predicted long distance interactions. Given that there are significant secondary structures predicted within the coding regions (not shown), it would also be interesting to discover the roles, if any of those structures. This is particularly true of the replicase ORF, which also harbors elements required for ribosomal frameshifting. In addition, it would be interesting to see whether there are any proteins that bind the PAM domain or the 3' end, particularly if there are any shared proteins with those that bind the BTE.

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References


CHAPTER 5. GENERAL CONCLUSIONS

Small portions of this text are excerpted from part of my contribution to a manuscript submitted to Virus Research entitled "Cap-independent translation of Plant Viral RNAs" by Elizabeth L. Pettit, Aurelie Rakotondrafara, and W. Allen Miller

RNA in the BYDV 3' UTR

Our laboratory previously found that BYDV RNA contains a translation element in the 3' UTR, the BTE, that confers cap-independent translation (Wang et al., 1997; Wang and Miller, 1995). The BTE folds into a cruciform structure, and 105 nt is sufficient to facilitate cap-independent translation \textit{in vitro} (TE105) (Guo et al., 2000). The BTE has the potential to participate in a long distance basepairing interaction with the viral 5' UTR (Guo et al., 2001), which would circularize the mRNA for translation. It was noted that additional sequence beyond the TE105 was required for BYDV translation \textit{in vivo}. However, exactly which sequence is needed, and how it functions was not understood. In Chapters 2 and 4, I describe the RNA sequences and structures in the BYDV 3' UTR, and how they play a role in viral translation, in more detail. The results of these studies add to the caution that in mapping functional RNAs, "less isn't always more" (Uhlenbeck, 2003).

The 3' UTR contains domains that function \textit{in vivo} to facilitate cap-independent translation

From mapping BYDV 3' UTR sequence needed \textit{in vivo}, I determined that nt 4918-5008 act as a cap-mimic (Chapter 2). The effects of this sequence are quite strong. When this sequence is deleted, translation in oat protoplasts is quite low. By extending the BTE by 35 nt 3' to the \textit{in vitro}-defined TE105 a large increase in translation was realized. In
agreement with previous results generated using the TE105 (Guo et al.), I found that the in vivo cap-mimic sequence functions well in conjunction with a 3' poly(A) tail directly adjacent to it. I also showed that this sequence is needed in vitro as well as in vivo.

The extra cap-mimic sequence is predicted to fold the BTE so that SL-IV is on an extended stalk. This structure is conserved across multiple viral genera. To understand how this structure functions, additional experiments are needed. Experiments to determine whether the other viruses containing BTE-like structures also require the stalk and this length in vivo would provide broader understanding of plant viral cap-independent translation elements. Mutations to disrupt the long stalk should be made and tested in vivo, for their effects on translation. This would help to demonstrate the functional significance of the stalk. Also, whether this stalk is required for sgRNA2 synthesis is unknown, as the BTE overlaps with the sgRNA2 promoter. Finally, because the BTE is associated with host translation factors, it would be interesting to see whether the longer BTE RNA binds translation factors with higher affinity than TE105. In at least one experiment, I observed that using a shorter BTE to pull down associated factors in wheat germ extracts, resulted in poor binding by the eIF4F complex, suggesting a longer BTE recruits eIF4F more efficiently.

3' UTR contains sequences that facilitate poly(A) independent translation.

Most mRNAs have a 3' poly(A) tail. BYDV RNA has a long 3' UTR that contains sequences that compensate for the lack of a poly(A) tail. The data in Chapter 2 show that nt 5089-5142 confer poly(A)-independent translation to BYDV RNA. The effects resulting from deletion of this sequence are weaker than those resulting from deletion of the cap-
mimic sequence. Nonetheless, several conclusions can be made about the poly(A) mimic function conferred by the BYDV 3' UTR. Small segments of this poly(A) mimic sequence (PAM) function in isolation when placed in the 3' UTR of reporter RNAs. However, a longer 3' UTR sequence, everything downstream of nt 5089, confers maximum translation. Like a poly(A) tail, the PAM sequence functions in vivo only and is not needed in vitro. The poly(A)-mimic sequence works in conjunction with a 5' cap and it also works with the BTE nearby in the 3' UTR, indicating that its function is synergistic with the cap-mimic function. The sequence between nt 5089 and 5142 is phylogenetically conserved in primary sequence and secondary structure. It is also a unique sequence, in that it shows no homology to other viral elements which control poly(A)-independent translation. This work is the first attempt that I know of to characterize the mechanism of poly(A)-independent translation in the Luteoviridae.

Sequence in the 3' end of BYDV RNA, that also serves as the origin of replication (Koev et al., 2002), contributes to poly(A)-independent translation (Chapter 4). The 3' end structure may prevent exonuclease attack, maintaining the integrity of the BYDV mRNA. The structured 3' end could also function in communicating the replication status of the viral RNA to the translational machinery, because this is where the replicase is presumably bound. Individual bases in both the 3' end and in the PAM domain between nt 5089-5142 are required for replication (Koev et al., 2002). Thus, however weak the poly(A) mimic function, individual bases in the regions of BYDV 3' UTR that have this function are required for virus survival. Perhaps tertiary interactions between these two 3' UTR portions
or elsewhere within 3' UTR provide a higher level of coordination of gene expression. Possible tertiary RNA interactions are proposed in Chapter 4, hinting at this possibility.

The location of cap-independent and poly(A)-independent translation elements next to each other may be advantageous for viral gene expression. Closely spaced elements would provide synergy between the protein/RNA interactions, analogous to the synergy observed between factors that interact with the 5' and 3' ends of mRNAs (Gallie, 1991; Tarun and Sachs, 1995). This spacing would also enable the viral RNA to more efficiently recruit the factors and concentrate them to the viral RNA. It may also avoid the need for viral proteins during translation, allowing translation to occur efficiently at the earliest stages in the infection cycle. The fact that these elements overlap with other control elements, such as frameshifting (Barry and Miller, 2002; Paul et al., 2001) and sgRNA promoters (Koev and Miller, 2000), suggests that their positioning might enable the coordination of multiple processes at once.

**Translation of mRNAs lacking a poly(A) tail**

The RNAs of many plant viruses are nonpolyadenylated. It is not known whether most other plant virus families have a poly(A) "mimic sequence", although it has been suggested there is some dependence on 3' UTR length in some cases (Tanguay and Gallie, 1996a; Tanguay and Gallie, 1996c). Experiments to determine the effects, if any, of the 3' UTR sequences of other plant viruses in poly(A)-independent translation are needed. In particular, it would be interesting to see whether substituting vector sequence of varying types and lengths for these 3' UTRs gives comparable stimulation of translation. Finally,
more complete genome sequencing will provide an accurate view of the length and composition of plant viral 3’ UTRs, as the actual length of viral genomes may differ from the current database due to incomplete sequencing of the viral RNA ends.

Is a poly(A) tail really required for translation? The association of PABP with the poly(A) tail stabilizes the mRNA, participates in mRNA export from the nucleus, and promotes translation, and there are multiple forms of the factor that are distributed in different cellular locations (Kühn and Wahle, 2004). However it has been suggested the contribution of PABP to translation may not be equal to that of the 5’ m7G(5’)ppp(5’)NG (Kozak, 2004). In fact, my results show that the poly(A) mimic portion of the 5’ UTR is neither strong in activity, nor discrete in boundary (Chapter 2). Also, PABP phosphorylation (Le et al., 2000) and regulation by other proteins (e.g. paipl and paip2 at least in mammals (Khaleghpour et al., 2001; Roy et al., 2002)) would play a role. It is not known whether PABP phosphorylation status is changed during plant viral infection, or whether the factor is cleaved as in a enterovirus infection (Joachims et al., 1999; Kerekatte et al., 1999). A long 3’ UTR could simply protect the functional portions of the RNA from exonuclease degradation.

For some viruses, a viral protein enables poly(A)-independent translation. In the case of rotaviruses, the NSP3 protein is bound to the viral 3’ UTR and eIF4G, evicting PABP from the complex (Piron et al., 1998; Vende et al., 2000). AMV RNA is capped, but nonpolyadenylated. The viral coat protein binds the 3’ UTR (Neeleman et al., 2001), and recruits the eIF4G subunit of eIF4F (Krab et al., 2005). Cellular proteins could be involved as in the case of TMV, where hsp101 binds a pseudoknot rich domain in the viral 3’ UTR.
(Tanguay and Gallie, 1996b). Unlike those viruses, BYDV RNA is uncapped. Also, viral proteins are dispensable for efficient translation of BYDV RNA (Wang and Miller, 1995). It has been proposed that long distance basepairing enables translation of the nonpolyadenylated (and uncapped) BYDV and TBSV RNAs (Fabian and White; Guo et al., 2001), although this mechanism is poorly understood.

**Is circularization of the mRNA necessary?**

In the field of translational control of gene expression, it is widely thought that mRNA circularization occurs, where the 5' and 3' ends interact synergistically to facilitate initiation. Circularization is facilitated on a eukaryotic cellular mRNA by the interaction of the large eIF4G protein that binds eIF4E and PABP, which are bound at the 5' and 3' ends of the mRNA, respectively. The rationale for circularization is that communication with the 3' UTR provides insurance that only intact mRNAs will be translated. With a circular mRNA, ribosomes could more easily be recycled once a round of translation is completed (Howard et al., 1970).

The direct evidence for circularization is limited (Alvarez et al., 2005; Wells et al., 1998), and there are variations on the theme. For example, with BYDV and TBSV RNAs, long distance basepairing is proposed to facilitate circularization (Fabian and White; Guo et al., 2001). Whether the basepairing is strong, or occurs for a long enough period of time during the initiation process is unknown. On BYDV RNAs, synergy between a 5' cap and a 3' poly(A) tail is not always observed (Chapter 2 herein). This is likely due to the presence of the BTE in the 3' UTR. On the natural BYDV RNA, cap-independent and poly(A)-
independent elements are both found in the 3' UTRs. This suggests the purpose of circularization might only be needed to recruit the ribosome to the 5' UTR via the eIF4F interaction with the BTE. If eIF4E were able to at least transiently interact with the 5' UTR (see Appendix), then the ribosome could be directly recruited by the RNA in the 5'UTR.

The ribosome is recruited directly to IRESes, where the requirement for protein factors varies depending on the mRNA. Whether all IRES-containing mRNAs have to circularize is unknown. In the case of Hepatitis C virus, it was reported that a region in the viral 3' UTR, the X region may influence translation very mildly (Ito et al., 1998). However subsequent studies showed that the effects, if any, of the X-mediated stimulation may be related to quasispecies variations (Kean, 2003; Michel et al., 2001). The exact nature of the ribosome scanning process, even for model cellular mRNAs, is unclear. It would be interesting to see if the relationship between scanning and circularization is a direct one.

**BYDV and Initiation Factors**

**Plant Viruses Usurping Translation Factors**

The eIF4F complex, comprised of eIF4E and eIF4G, and its isoform eIFiso4F (eIFiso4E and eIFiso4G), play a role in the infection cycle of many plant viruses (Table 1). The emerging picture shows: i) different viruses use different isoforms of the complex, and ii) different viruses require the interaction for diverse stages during the viral lifecycle.
Potyvirus VPg binds Host Translation Factors

Potyvirus RNAs lack a 5' cap, but the VPg at the 5' end of potyviral RNA binds the host cap-binding translation factors, eIF4E and eIFiso4E. The TuMV VPg binds eIFiso4E in vitro and in the yeast two-hybrid system (Wittmann et al., 1997), and in Arabidopsis plants (Léonard et al., 2000). PABP co-purifies with the complex of VPg-Pro and eIF4E or eIFiso4E (Léonard et al., 2004). VPg-Pro has also been shown to interact with the eIFiso4E-eIFiso4G (eIFiso4F) complex (Plante et al., 2004). Mutations in the domain on VPg necessary for interaction with eIFiso4E reduced TuMV infectivity (Léonard et al., 2000).

There is also substantial genetic evidence for a role of eIF4E in virus infection. Recessive resistance genes to potyviruses in pepper (Ruffel et al., 2002), lettuce (Duprat et al., 2002; Lellis et al., 2002; Nicaise et al., 2003), and pea (Gao et al., 2004a), and loss-of-susceptibility mutations to potyviruses in Arabidopsis (Lellis et al., 2002) all proved to be caused by mutations in eIF4E or eIFiso4E, or by reduced expression of one of these factors.

However, I am unaware of any direct evidence that the eIF4E/eIFiso4E interaction with VPg facilitates translation. In fact, some evidence suggests that this interaction serves other function(s) in the virus life cycle. First, as discussed above, the VPg is unnecessary for efficient cap-independent translation. The 5’ UTR alone of several potyviruses confers this function (Basso et al., 1994; Carrington and Freed, 1990; Gallie et al., 1995; Levis and Astier-Manifacier, 1993). Secondly, potyviral proteins other than the VPg also interact with eIF4E. The TEV nuclear inclusion protein, Nla, binds eIF4E from tomato and tobacco in a strain specific manner (Schaad et al., 2000). The role of eIF4E in the infection cycle of PSbMV is postulated to be in cell-to-cell movement (Gao et al., 2004b). Mutations in eIF4E
(resistance gene cum1) or eIF4G (resistance gene cum2) prevent replication of Cucumber mosaic cucumovirus (CMV) that has capped RNAs (Yoshii et al., 2004). Thus, even capped plant viral RNAs apparently use the factors for processes other than (or in addition to) translation. Finally, a screen of Arabidopsis mutant lines showed that mutations of 4E (and not iso4E) are associated with susceptibility to Clover yellow vein potyvirus, while mutations in iso4E (and not 4E) are associated with susceptibility to TuMV (Sato et al., 2005).

Translation factors are often commandeered by the viral replicase for RNA replication, suggesting a possible role for factor recruitment. Replicase holoenzymes of RNA viruses such as bacteriophage Qβ (Blumenthal and Carmichael, 1979) to Brome mosaic virus (Quadt et al., 1993) require translation factors. Zucchini yellow mosaic virus RdRP interacts with PABP in yeast two hybrid assays (Wang et al., 2000). The ability of potyvirus VPg-Pro to bind eIF4E may recruit translation factors to the replicase complex rather than facilitate translation.

These observations suggest there are alternative roles for translation factors in the life cycle of potyviruses. Given that eIFiso4G binds microtubules (Bokros et al., 1995), by binding viral proteins or RNAs it could facilitate movement by localizing viral RNA to the ER that spans the plasmodesmatal connections. Thus, viral recruitment of translation factors might include intercellular trafficking of the viral RNA to the correct location for either replication, translation, or packaging and movement.
Table 1. Relationships between plant translation initiation factors and plant viruses. Natural resistance indicates situations where plant varieties naturally differ in their susceptibility to virus infection, and there are mutations in eIF4E or eIFiso4E that correlate with the difference in susceptibility. Loss of susceptibility applies to plants that have differences in eIF4E or eIFiso4E which were generated in the laboratory.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Viral Element</th>
<th>Host Factor</th>
<th>Binding?</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TuMV</td>
<td>Vpg-Pro</td>
<td>eIFiso4E, eIFiso4G</td>
<td>✓</td>
<td>replication (iso4E)</td>
<td></td>
</tr>
<tr>
<td>LMV</td>
<td>?</td>
<td>eIFiso4E</td>
<td>?</td>
<td>loss of susceptibility</td>
<td>Duprat et al</td>
</tr>
<tr>
<td>CIYVV</td>
<td>?</td>
<td>eIF4E</td>
<td>?</td>
<td>loss of susceptibility</td>
<td>Sato et al, 2005</td>
</tr>
<tr>
<td>TEV</td>
<td>?</td>
<td>eIFiso4E</td>
<td>?</td>
<td>loss of susceptibility</td>
<td>Lellis et al, 2002</td>
</tr>
<tr>
<td>TEV</td>
<td>5′ UTR</td>
<td>eIF4G-depend</td>
<td>?</td>
<td>translation</td>
<td>Gallie, 2001</td>
</tr>
<tr>
<td>TEV</td>
<td>Nla protein</td>
<td>eIF4E</td>
<td>✓</td>
<td>replication?</td>
<td>Schaad et al, 2000</td>
</tr>
</tbody>
</table>
Table 1, continued. Relationships between plant translation factors and plant viruses.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Viral Element</th>
<th>Host Factor</th>
<th>Binding?</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>ToRSV</td>
<td>VPg-Pro</td>
<td>eIFiso4E</td>
<td>✓</td>
<td>?</td>
<td>Léonard et al, 2004</td>
</tr>
<tr>
<td>AMV</td>
<td>viral CP</td>
<td>eIF4G, eIFiso4G</td>
<td>✓</td>
<td>poly(A)-independent translation</td>
<td>Krab et al, 2005</td>
</tr>
<tr>
<td>STNV</td>
<td>3' TED</td>
<td>eIFiso4F, eIF4F</td>
<td>✓</td>
<td>cap-independent translation</td>
<td>Gazo et al, 2004</td>
</tr>
<tr>
<td>BYDV</td>
<td>3' BTE</td>
<td>eIFiso4F, eIF4F</td>
<td>✓</td>
<td>cap-independent translation</td>
<td>Chapter 3 (herein)</td>
</tr>
</tbody>
</table>

E. Allen, 2001
Viruses with 3' RNA elements that bind eIF4F

Viruses with uncapped genomes apparently bind eIF4F for different purposes, and the mechanism of translation for each virus is likely to be different (Table 2). The uncapped potyviral RNAs have 5' UTR elements that confer cap-independent translation, yet their VPg binds eIF4E or eIFiso4E (above). In contrast to the potyviruses, 4E binding the BTE might not be required for BYDV replication. This is because the BTE is apparently not necessary for RNA synthesis, at least in studies using ectopically expressed BTE in combination with mutants (J. Jackson, unpublished). BYDV and STNV appear to be unlike the above examples. First, the translation factors are recruited directly to the RNA. Also, binding eIF4F is mediated by cap-independent translation elements in the 3' UTR. eIF4F reverses translation inhibition caused by adding the BTE or STNV TED in trans (Wang et al., 1997). Thus, the function of binding to eIF4F and its isoform by BYDV and STNV RNAs is in translation. However, BYDV and STNV RNAs differ in sequence and structure, suggesting the mechanism of recognition of the RNA elements by the proteins may differ between BYDV and STNV.

As complexes, eIF4F and eIFiso4F promote translation of BYDV more efficiently than the individual subunits alone do (Chapter 4 and unpublished observations). This agrees with what occurs on a capped mRNA as well: cap binding and thus translational activity are promoted by the recruitment of the eIF4F complex (Haghhighat and Sonenberg, 1997; von Der Haar et al., 2000). Binding of individual subunits to STNV was much lower than binding of eIF4F or eIFiso4F alone than for the complexes (Gazo et al.). Recent experiments indicate that eIF4G is responsible for a greater amount of translation stimulation
Table 2. Plant Viruses that recruit eIF4F: translation mechanisms and possible functions of factors recruitment.

<table>
<thead>
<tr>
<th>Interacting Factors</th>
<th>Potyviruses</th>
<th>STNV</th>
<th>BYDV (-like)</th>
<th>AMV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Element</td>
<td>4E, iso4E</td>
<td>4F, iso4F</td>
<td>4F, iso4F</td>
<td>4G, iso4G</td>
</tr>
<tr>
<td>rRNA complementarity</td>
<td>5' VPg</td>
<td>TED in 3' UTR</td>
<td>BTE in 3' UTR</td>
<td>viral CP</td>
</tr>
<tr>
<td></td>
<td>5' UTR</td>
<td>5' UTR</td>
<td>3' UTR</td>
<td>unknown</td>
</tr>
<tr>
<td>Possible factor function</td>
<td>Replication</td>
<td>Translation</td>
<td>Translation</td>
<td>poly(A)-independent translation</td>
</tr>
</tbody>
</table>
on BYDV RNA than eIF4E (K. Treder, unpublished). However it is unknown whether this factor can bind directly to the BTE, although eIFiso4G binding is not detectable (E. Allen, unpublished). Thus, the mechanism of BTE-mediated translation and that of the BTE-like elements may require recruitment of eIF4G first; for STNV the contribution of individual subunits to translational activity is not known. TNV has a BTE-like element (Meulewaeter et al., 2004; Shen and Miller, 2004b). Perhaps eIF4G binds these elements through its RNA-recognition motif, or the plant viral RNAs use eIF4G in a manner analogous to the way animal virus IRESes do (Prévôt et al., 2003). It would be interesting to see if and how TNV RNAs and STNV RNAs are both recruiting these factors at the same time during an infection: would the viral RNAs compete against each other or work together?

**eIF4F vs. eIFiso4F**

Finally, the roles of the different isoforms of eIF4E and eIF4G in viral translation are unknown. One functional difference between the isoforms is their ability to support translation on a mRNA containing a structured 5' UTR leader (eIF4F stimulates more than eIFiso4F) (Gallie and Browning, 2001). With its highly structured 5' UTR leader, BYDV translation was also more responsive to eIF4F, at least with recombinant protein (Chapter 4 herein). Also, UV crosslinking to STNV and BYDV RNAs followed by immunoprecipitation also indicates that the association with eIF4F may be stronger than with eIFiso4F (Gazo et al, 2004; Chapter 4 herein), although it is not possible with this method to make a direct comparison in binding. It is possible that some feature on the viral RNAs preferentially recruits eIF4F, that is available in more limited quantities in wheat cells than is eIFiso4F (Browning et al., 1990). But these results also beg the question whether eIF4F is
simply overall a better factor for translation. Thus, it would be interesting whether the same would be true for other mRNAs, particularly cellular mRNAs. Perhaps a susceptible interaction enables the viruses to exploit these differences.

**Unexplored Territory**

As obligate parasites, viruses rely on many host processes. For viral gene expression, host factors play a major role. During virus infections in animals, the host translational machinery is known to be modified several ways. Shutoff of host translation is a well-known viral strategy in many organisms, although it is not known if this is a widespread phenomenon during plant virus infection. The BYDV sgRNA2 is a regulator of viral gene expression (Shen and Miller, 2004b; Wang et al., 1999), and may also affect host gene expression (R. Shen and W. A. Miller unpublished). Whether the domains identified in this study contribute to any effects caused by sgRNA2 accumulation is an interesting question. If the recruitment of eIF4F to the BTE competes with eIF4F recruitment by capped RNAs, it is possible that availability of the factor for host mRNAs is altered.

Many 3’ cap-independent translation elements that resemble the BTE have been predicted *in silico* (Chapter 2 herein), yet the function has been tested for just three of these elements: TNV-D (Meulewaeter et al., 2004; Shen and Miller, 2004a), TNV-A (Meulewaeter et al., 2004), and RCNMV (Mizumoto et al., 2003). Functional experiments to determine whether these elements work in the context of other plant viruses and their host systems will shed light on the mechanism of cap-independent translation. It would also be interesting to see whether all of these elements recruit eIF4F, and if or how the observed 18S rRNA
complementarity in these and other elements facilitates translation (Table 2). With a better understanding of these elements, it will be possible to use them to design vectors for transgenic expression. Although homologous elements have not been found in host genomes, there may be mechanistic similarities between viral translation and host expression. Thus, an understanding of the BTE-like elements and the cellular factors they require will shed light on both viral and host gene expression.

Other aspects of the viral lifecycle that may relate to the translational control of gene expression remain to be understood. For example, whether there are siRNAs present during a luteovirus infection and if they affect gene expression remain to be seen. Cymbidium ringspot tombusvirus infection results in the accumulation of siRNAs which are complementary to sites throughout the genome, particularly to structured regions of RNA (Molnár et al., 2005). Given that CymRSV is a member of the closely related Tombusviridae, it is possible that plants infected with members of Luteoviridae also generate virus-derived siRNAs associated with infection, that would in turn influence viral gene expression.

In many systems, it is also known that phosphorylation affects the translation status in host cells. The evidence for altered phosphorylation of translation factors during a plant viral infection is limiting so far (Bilgin et al., 2003). However, plant eIF2 is affected by virus infection, and also by a variety of abiotic stresses (Bailey-Serres, 1999; Bilgin et al., 2003), suggesting this factor and others may play a role in viral gene expression. Also, the phosphorylation status of plant initiation factors affects mRNA cap-binding (Khan and Goss,
A better catalog of which proteins bind the viral RNAs, and which other proteins are subsequently associated with the viral RNA:host proteins complex will shed light on both viral gene expression and the signaling involved during a plant virus infection. The work described herein represents a sliver of the contribution made to our understanding of viral translation strategies, and the RNA features that operate in controlling gene expression.

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.


APPENDIX A.
DEVELOPMENT OF A TOEPRINTING STRATEGY TO ASSESS THE POSITION OF THE RIBOSOME ON BYDV RNA DURING TRANSLATION INITIATION

The objective of these experiments was to optimize methods for determining how the ribosome is recruited to BYDV RNA. Using in vitro wheat germ extracts, and a combination of translation inhibitors, we sought to understand where the 48S complex and the 60S complex assemble on the viral RNA. The basic procedure has been described in a handful of other examples, several of which were in reconstituted systems, and none of which were in plants. Hopefully it will be possible to further develop its applications to understand translation in plant systems. Thusfar, a few other methods have been used to understand the ribosome’s position on viral RNAs (Browning et al., 1980; Konarska et al., 1981; Tyc et al., 1984).

The overall procedure involves using mRNA on which translation has been initiated in vitro as the template for reverse transcription. As a positive control for extension, naked RNA in the absence of translation extract is used. Translation reactions are prepared separately with different translation inhibitors. m\textsuperscript{7}GTP cap analog prevents eIF4E from binding the 5' cap, and thus prevents the recruitment of the 40S ribosomal subunit; thus m\textsuperscript{7}GTP is a negative control. GMPPNP is a GTP analog which prevents joining of the 60S subunit with the 48S complex—when GMPPNP is present, the 48S complex should be arrested at the initiation codon. Cycloheximide is added after a brief period to stop translation. The translation mix is combined with a reverse transcription mix and a radiolabeled oligonucleotide that anneals 3' to the initiation codon is then added. The primer
is allowed to anneal to the mRNA template before reverse transcriptase is added. The primer extension products are precipitated and resolved on a denaturing polyacrylamide gel, and visualized by autoradiography.

To begin, we followed procedures described by Dmitriev et al. (2002) and Sachs et al. (2002) in rabbit reticulocyte lysates and cell free extracts of the fungi *Neurospora crassa* and *Saccharomyces cerevisiae*, respectively. A protocol has also been described by Kozak (1998); however Kozak’s protocol has an annealing step before the translation mix is added, and it was previously determined that this protocol did not work as well (O. Namy, pers. comm.). We followed Dmitriev’s suggestions for inhibitor concentrations, and ultimately used Sach’s reaction mixes with some changes suggested by Olivier Namy (Brierley Lab). As mRNA templates in all the experiments, we used LUC869 (Wang et al., 1999) and we used the Sflucas oligonucleotide which anneals within the 5’ portion of the firefly luciferase gene. In a few experiments we used a oligonucleotide which anneals downstream of the BTE; however our extension results were difficult to interpret. Thus, we chose to optimize the conditions for mapping the position of the ribosome in the 5’ UTR, since this is where it is likely to scan from to the start codon.

We took several steps to optimize the procedure. Adding an annealing step after the primer is added, 2 minutes at 50°C followed by 5 minutes at 37°C improved our results. We also experimented with different reverse transcriptases and found that the Invitrogen SuperScript II enzyme gave the best results. The limiting factor in the success of the extension reaction was wheat germ extract. Presumably wheat germ extract has endogenous
RNAses that affect template quality. Thus, the less wheat germ used in the reaction, the better. Also, BSA is included in the reverse transcription mix which may help.

We found that the toeprint on BYDV RNA did not change significantly when the inhibitors were added. This is somewhat surprising given that \( m^7 \)GTP should inhibit the recruitment of the 40S subunit, and that \( m^7 \)GTP is known to inhibit translation of BYDV RNA (Wang and Miller, 1995). Our preliminary experiments indicate the toeprint is 3' to SL-IV of the 5'UTR, about 15 bases upstream of the start codon. Interestingly, this is the loop that is proposed to be involved in the long distance basepairing interaction with the BTE. Thus, the basepairing would put the initiation factors that bind the BTE in close proximity to the location in the 5'UTR where the ribosome is recruited, or perhaps even deliver the ribosomal subunits to the 5'UTR. These experiments need to be repeated, and the effects of the inhibitors need to be re-examined.

We need to check again whether high concentrations (0.8 mM) \( m^7 \)GTP cap analog change the position of the toeprint or not. Chapter 3 indicates that this concentration should affect ribosome recruitment, since the addition of high concentrations abolishes BYDV translation. We also could try this procedure on a nonviral RNA, to confirm whether our reaction conditions provide a reliable result in comparison with published work. And we could test whether there is a difference in recruitment between the wild type and nonfunctional BF mutant, and whether a toeprint occurs on the BTE. Our early results indicated little change between the two BTEs. To obtain clearer results, we may want to change the start codon context for the mRNA template to the optimal RCCAUGG, and use a
viral template instead of the reporter mRNA. The multiple AUGs in the luciferase gene downstream of the start codon may also have complicated our results; it would be useful to use a viral RNA instead of the reporter RNA in future experiments. To rule out effects specific to wheat germ, future experiments could be performed using reticulocyte lysates.

References


Figure 1. Results of primer extension inhibition experiment on LUC869 RNA (Lanes 1-13) and nonfunctional LUC869 containing the BF mutation in the BTE (Lanes 14-18). Lanes 1-4 Sequencing lanes (using PAV6 as the template); Lanes 5-10 Sachs’ mix (see Protocol): Lane 5 48S, Lanes 6-8 no initiation (cap analog added to 0.8 mM, 0.4 mM, and 0.04 mM, respectively), Lane 9 80S, Lane 10 m7GTP; Lanes 11-13 Namy’s mix (see Protocol): Lane 11 48S, Lane 12 0.4 mM cap analog, Lane 13, 80S; Lanes 15-17 Sachs’ mix on mutant RNA: Lane 15 Naked RNA, Lane 16, 48S, Lane 17 0.4 mM cap analog, Lane 18 80S.
Figure 2. Secondary structure of the BYDV 5' UTR.
## TOEPRINTING REVISED STRATEGY

Worked out with Dr. Ian Brierley in his laboratory at Cambridge University, Cambridge, UK.

Adapted from Sachs et al. (2002) Methods 26, 105-114, with some modifications suggested by Olivier Namy.


**Per sample:**

<table>
<thead>
<tr>
<th>For 48S:</th>
<th>16mM GMPPNP</th>
<th>1.25 μl</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>56mM MgOAc₂</td>
<td>0.35 μl</td>
</tr>
</tbody>
</table>

| For 80S: | water | 0.6 μl |
|          | 10mg/ml CHX | 1 μl |

| For no assembly: | 20mM m⁷GTP | 0.4 μl |
|                 | 16mM MgOAc₂ | 0.4 μl |
|                 | 12.5mg/ml CHX | 0.8 μl |

CHX = cycloheximide

### 2. Prepare annealing/reverse transcription mixes (Sachs et al, 2002).

Two reaction solutions are shown for testing purposes; magnesium added in both cases as per Olivier Namy’s suggestion)

**A. Per reaction:**

<table>
<thead>
<tr>
<th>H₂O</th>
<th>.5 μl</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X Invitrogen SSII 1st strand buffer</td>
<td>2 μl</td>
</tr>
</tbody>
</table>
DTT (0.1M) 1 µl

\textit{dNTPs (2.5mM)} 1 µl

RNAGuard 0.25 µl

MgCl\textsubscript{2} (140mM) 0.5 µl

\textit{OR B. per reaction:}

Olivier Namy’s reverse transcription mix (minus translation inhibitor)

H\textsubscript{2}O 2 µl

5X Invitrogen SSII 1\textsuperscript{st} strand buffer 2 µl

MgCl\textsubscript{2} (140mM) 0.5 µl

RNAGuard 1 µl

\textit{dNTPs (5mM)} 1 µl

DTT (0.1M) 1 µl

BSA (1:10 dilution) 0.5 µl

3. \textbf{Assemble translation reactions on ice.}

\textit{Wheat Germ} (for 200µl)

Wheat germ extract 100 µl

1mM aa-met 16 µl

1mM aa – cys 16 µl

1M K\textsubscript{OA}c 15 µl

Water 53 µl

Total 200 µl

For RRL, use lab. aliquots. Add 1.25µl aa-cys per 47.5µl aliquot.
4. Combine inhibitors with translation mix.

48S, 80S or no translation  
WGE

5. Ribosome: mRNA complex assembly

Add 1-3 µl RNA per 25 µl translation mix.

Translate for 10 minutes.

Add 1 µl cycloheximide (10 mg/mL) to stop translation.

Place on ice for 10 minutes.

6. Reverse transcription set-up

A. If using Sachs’ annealing solution prepared in 2A.

RT mix 5.5 µl
Translation mix 3 µl

B. If using Namy’s RT mix prepared in 2B.

RT mix 8 µl
Translation mix 0.5 µl

Incubate 3 minutes at 50°C and place immediately on ice.

7. Anneal primer

Add 1 µl ³²P Toeprint primer*

Anneal at 50°C for 2 minutes

Place at 37°C for 5 minutes

8. Reverse transcribe

Add 0.5 µl Superscript II Reverse transcriptase (Invitrogen)
Incubate 37°C 10-15 minutes.

9. **Clean up and gel electrophoresis**

Add 80 µl water, 100 µl phenol/chloroform. Vortex, spin, keep 90µl aqueous phase add 10µl 3M NaOAc, 250µl EtOH. Mix, -20°C for 30 mins or -70°C storage.

Spin 10min, wash 95% EtOH, dry, resuspend in 4µl water. Add 6µl FDLB (formamide dye loading buffer which is the same as Ambion buffer II). Heat 80°C, 3 minutes, load onto a suitable % denaturing PAGE, probably 6% or so.

Run at 35W for about 1.5 to 2 hours, fix (10% acetic acid, 10% methanol). Dry down, expose to film. Develop.

*SUPPLEMENTAL PROTOCOL: Labeling the Primer*

5 µl primer (50 pmol/µl)
5 µl 10X PNK buffer
5 µl $\gamma^{32}$P-ATP (50 µCi)
5 µl T4 PNK
30 µl H$_2$O

Incubate one hour at 37°C, and then 15 minutes at 65°C.

Purify on spin column (Sephadex G50).

Store at -20°C in a radioactive storage container or use immediately.
APPENDIX B.
EVIDENCE THAT EIF4E, BUT NOT EIF4G, COULD INTERACT WITH THE BYDV 5' UTR.

In the course of optimizing the western blot procedure used in Chapter 3, I found that primary antibody concentration played a role in determining the specificity of the detection of BTE-interacting proteins. For eIF4E, a 1:2500 dilution of antibody resulted in the detection of a protein that interacted with the BYDV 5' UTR bait.

**Figure 1.** Western blot on BTE and BYDV 5' UTR-interacting proteins. Antibody to wheat eIF4G (left) or eIF4E (right) were used to probe the blots. The procedure for isolating proteins that interact with BYDV RNAs is described in Chapter 3.
Abbreviations

AMV  Alfalfa mosaic alfamovirus
BLRV  Bean leafroll luteovirus
BMV  Brome mosaic bromovirus
BSA  Bovine serum albumin
BTE  *Barley yellow dwarf virus* cap-independent translation element
BYDV  *Barley yellow dwarf luteovirus*
BYMV  *Barley yellow mosaic bunyavirus*
CHX  Cycloheximide
CLYVV  Clover yellow vein potyvirus
CMV  Cucumber mosaic potyvirus
CRSV  Carnation ringspot dianthovirus
DTT  Dithiothreitol
eIF4E  eukaryotic initiation factor 4E (cap-binding protein)
eIFiso4E  eukaryotic initiation factor iso4E (cap-binding protein isoform)
eIF4G  eukaryotic initiation factor 4G
eIFiso4G  eukaryotic initiation factor 4G isoform
eIF4F  eukaryotic initiation factor 4F complex (eIF4E + eIF4G)
eIFiso4F  eukaryotic initiation factor iso4F complex (isoforms, eIFiso4E + eIFiso4G)
GMPPNP  Guanosine 5-[[β, γ-imido] triphosphate trisodium salt
HAV  *Hepatitis A virus*
HCV  *Hepatitis C virus*
IRES  Internal ribosome entry site
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMV</td>
<td>Lettuce mosaic potyvirus</td>
</tr>
<tr>
<td>Luc</td>
<td>Luciferase, fluc for firefly luciferase and rluc for renilla luciferase</td>
</tr>
<tr>
<td>LWSV</td>
<td>Leek whitestripe necrovirus</td>
</tr>
<tr>
<td>OLV</td>
<td>Olive latent necrovirus</td>
</tr>
<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>PABP</td>
<td>Poly (A)-binding protein</td>
</tr>
<tr>
<td>PepMoV</td>
<td>Pepper mottle potyvirus</td>
</tr>
<tr>
<td>PsbMv</td>
<td>Pea seedborne mosaic potyvirus</td>
</tr>
<tr>
<td>PVY</td>
<td>Potato virus Y potyvirus</td>
</tr>
<tr>
<td>RCNMV</td>
<td>Red clover necrotic mosaic dianthovirus</td>
</tr>
<tr>
<td>SCNV</td>
<td>Sweet clover necrotic mosaic dianthovirus</td>
</tr>
<tr>
<td>SDV</td>
<td>Soybean dwarf luteovirus</td>
</tr>
<tr>
<td>SLBP</td>
<td>Stem-loop binding protein</td>
</tr>
<tr>
<td>STNV</td>
<td>Satellite tobacco necrosis virus</td>
</tr>
<tr>
<td>TBSV</td>
<td>Tomato bushy stunt tombusvirus</td>
</tr>
<tr>
<td>TE105</td>
<td>Translation element required for in vitro cap-independent translation of BYDV defined as bases 4814 to 4918</td>
</tr>
<tr>
<td>TED</td>
<td>Translation enhancer domain, bases 621 to 741 of STNV</td>
</tr>
<tr>
<td>TEV</td>
<td>Tobacco etch potyvirus</td>
</tr>
<tr>
<td>TMV</td>
<td>Tobacco mosaic tobamovirus</td>
</tr>
<tr>
<td>TNV</td>
<td>Tobacco necrosis necrovirus</td>
</tr>
<tr>
<td>ToRSV</td>
<td>Tomato ringspot nepovirus</td>
</tr>
<tr>
<td>TuMV</td>
<td>Turnip mosaic potyvirus</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>TYMV</td>
<td><em>Turnip yellow mosaic tymovirus</em></td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>VPG</td>
<td>viral genome linked protein</td>
</tr>
<tr>
<td>WGE</td>
<td>wheat germ extract</td>
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</tbody>
</table>
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