Eukaryotic Initiation Factor (eIF) 4F Binding to Barley Yellow Dwarf Virus (BYDV) 3’-Untranslated Region Correlates with Translation Efficiency*

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Background: Little is known about BYDV protein synthesis initiation mechanisms.

Results: eIF4F binding correlates with 3’ BTE translation efficiency and is enthalpically and entropically favorable.

Conclusion: Binding of eIF4F to the 3’ BTE is important for efficient BYDV translation.

Significance: Binding of eIF4F to the 3’-UTR to initiate assembly of the protein synthesis initiation complex is a novel mechanism that may be used by other viruses containing a 3’ BTE.

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

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Barley yellow dwarf virus (BYDV)* represents pervasive groups of economically important plant viruses. BYDV affects cereal crops such as wheat, barley, oats, and grasses and is spread by several aphid species severely limiting food grain production and causing yield losses resulting in food scarcity (1). BYDV belongs to the Luteoviridae family, has a genome which is a positive sense RNA ≈5700 nucleotides long, and is lacking both a 5’ cap (m7GpppX) and a poly(A) tail (2). In eukaryotes, the 5’ cap is the binding site for eukaryotic initiation factors (eIFs) eIF4F and eIF4A (isozyme form of eIF4F present in higher plants). The poly(A) tail works synergistically with the cap for stability of the message and efficient translation (3–9). The poly(A)-binding protein (PABP), together with initiation factors, promotes circularization of mRNA, a requirement for efficient translation (10–14). In contrast, BYDV must employ an alternate means of cap-independent translation. Host translation machinery recruitment to the viral mRNA is aided by structures present in 5’- and/or 3’-UTRs of the mRNAs instead of a 5’ cap/poly(A) tail (15). For cap-dependent translation initiation, a significant, central role is played by eIF4F. eIF4F interacts with PABP via the scaffolding protein eIF4G, the larger subunit of eIF4F (16, 17). eIF4F, together with the factors eIF4A and eIF4B, is recruited to the 5’ end of the mRNA by eIF4F to generate the cap-binding complex. The efficient unwinding of secondary structure of mRNA is presumed to be catalyzed by binding of eIF4F, eIF4B, and eIF4A and hydrolysis of ATP (18). At this stage, the 43S preinitiation complex (composed of eIFs 2 and 3, MettRNAi, GTP, and 40S subunit), along with associated eIFs and the 60S ribosomal subunit, forms 80S ribosomes, and protein synthesis commences (19). The role of eIF4F in BYDV translation initiation is not clear.

Instead of a cap and poly(A) tail interacting synergistically, BYDV has a BYDV translation element (BTE) located in the 3’-UTR that interacts with a stem loop in the 5’-UTR of its mRNA (20, 21). The 3’ BTE comprises nucleotides 4814–4918, has a cruciform/trNA-like secondary structure with three major stem-loops (SL-I, SL-II, and SL-III) with stem loop IV forming the base, and is the minimum requirement for successful in vitro translation (see Fig. 1) (20). Cap-independent translation initiation at the upstream AUG is stimulated by 3’ BTE via base pairing (kissing stem loop interaction) of the 3’ SL-III

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2 The abbreviations used are: BYDV, barley yellow dwarf virus; BTE, BYDV translation element; BTEBF, mutant of BTE with a CUAG insert at position 4873; WGE, wheat germ extract; d-WGE, depleted WGE; PABP, poly(A)-binding protein; eIF, eukaryotic initiation factor; PK1, pseudoknot 1.
to the 5′ SL-D loop of the viral genomic RNA (see Fig. 1) (21). Translation was vitiated by single point mutations in any of the five bases of either of the kissing loops because of disrupted interactions both in in vivo and in vitro experiments, whereas base pairing restored by compensatory double mutations restored translation (21).

The sequence and structural elements in the 3′-UTR of the mRNA control the efficiency of translation (22, 23). The 3′ BTE is the binding site of eIF4F (24). The role of eIF4F is unclear because eIF4F binds to the 3′ BTE and the nonfunctional mutant BTEBF with similar affinity in in vitro studies (25). To determine the role of eIF4F and other purified eIFs in the 3′ BTE-mediated translation, biophysical studies were used. The 3′ BTE functionally mimics the 5′ cap and is able to translate efficiently when placed in the 5′-UTR. In this study, WT 3′ BTE and four other mutants BTEBF, SII-m1, SL-III-3, and SL-III SWAP (the mutations/changes in bases are shown in Fig. 1) with in vitro translation efficiencies from 5 to 164% compared with WT BTE (2, 20) were used. Binding of eIF4F and other initiation factors (eIF4B, 4A, and PABP) to the WT 3′ BTE and mutants (BTEBF, SII-m1, SL-III-3, and SL-III SWAP) was characterized using fluorescence anisotropy and stopped flow kinetics, which gave equilibrium binding constant values (Kₐ), a measure of relative complex stability, and kinetic constants.

We show 1) translation efficiencies correlated well with eIF4F binding; 2) as yet unidentified proteins or small molecules in WGE prevented binding of eIF4F to BTEBF mutant but not WT BTE; 3) kinetics experiments showed that the association rate constants were similar for WT and mutants, indicating stability rather than kinetics correlates with translation; and 4) eIF4F interactions with 3′ BTE and mutants were both enthalpically and entropically favorable. The enthalpic contribution to ∆G° was 52–90% at 25 °C. This quantitative information, together with the stability measurements and kinetics, determined the nature of interactions and yielded information on the role of eIF4F in the sequential assembly of the initiation complex and its ability to facilitate translation initiation.

**EXPERIMENTAL PROCEDURES**

GSTrap 4B columns and m⁷GTP-Sepharose 4B were purchased from GE Healthcare. HisPur Cobalt chromatography cartridges were purchased from Thermo Scientific Pierce Protein Biology Products (Rockford, IL). Phosphocellulose P11 cation exchange media was purchased from Whatman (part of GE Healthcare). All chemicals were obtained from Sigma-Aldrich or Thermo Fisher Scientific (Waltham, MA). Millipore Amicon Ultra-15, 10-kDa cutoff centrifugal filter units, Millex (PVDF) 0.22- and 0.45-µm syringe filters, Stericup 250 ml (PVDF 0.22) µm were purchased from Thermo Fisher Scientific. RNAseOUT was purchased from Invitrogen. DNA templates for in vitro transcription were purchased from Integrated DNA Technologies (Coralville, IA).

**Recombinant Protein Expression and Purification**—A dicistronic plasmid construct (vector pET3d) harboring encoding regions for both eIF4G and eIF4E for the expression of the recombinant WT eIF4F complex was a generous gift from Dr. K. S. Browning (University of Texas at Austin, Austin, TX), and protein was purified as described elsewhere (26). The modifications to the protocol are described below.

BL21(DE3) cells were transformed using standard methods and selected with ampicillin antibiotic. A single colony was used for a 50-ml overnight culture and was transferred to 0.8 liter of medium in four 4-liter flasks the next day. All the cultures were incubated at 30 °C and grown to A₆₀₀ = 0.8. Induction of eIF4F expression was accomplished using 0.5 mM IPTG (final concentration). Cells were harvested after 3 h by centrifugation (6000 × g) for 15 min at 4 °C. The composition of Buffer B-0 used in purification was as follows: 20 mM HEPES, 10% glycerol, 1 mM EDTA, and 1 mM DTT with KCl added as required. The cell pellet was suspended in 50 ml of Buffer B-150 (150 mM KCl) containing one protease inhibitor tablet (Roche complete protease inhibitor mixture tablets), 5 mg of soybean trypsin inhibitor, and 500 µl of 10 mg/ml polymethyllsulfonfluoride. The cells were sonicated three times for 30 s at 70% power followed by two times for 30 s at 90% power with intermittent cooling on ice for 2–3 min between sonication bursts. The lysed cells were centrifuged at 40,000 rpm for 1.5 h at 4 °C. The supernatant was diluted to KCl = 100 mM and was loaded on to a 10-ml phosphocellulose column pre-equilibrated with Buffer B-100. eIF4F was washed with buffer B-100 and eluted with Buffer B-300. The fractions containing the highest concentration of eIF4F were pooled and diluted to 100 mM KCl by the addition of Buffer B-0 and loaded on to a 4-ml m⁷GTP Sepharose column equilibrated in Buffer B-100 and washed with the same buffer. eIF4F was eluted with Buffer B-100 containing 100 µM m⁷GTP. The pooled fractions with highest purity and concentration were applied to a 1-ml phosphocellulose column equilibrated in Buffer B-100 and washed with the same buffer. eIF4F was eluted with Buffer B-300. The protein was diluted using B-0 to 100 mM KCl and concentrated using Millipore Amicon Ultra 15-ml, 10-kDa cutoff. The purity of eIF4F was confirmed by 10% SDS-PAGE, and the concentration was determined using the Bradford method with BSA as a standard (27). His-tagged PABP and eIF4A protein were expressed in BL21 (DE3) pLysS *Escherichia coli* strain using pET19b and pET23d vector (a generous gift from Dr. D. R. Gallie, University of California, Riverside, CA), respectively, and were purified as described elsewhere (28, 29).

Full-length GST-tagged pET3d-eIF4F plasmid was also from Dr. Gallie. Protein expression was performed in *E. coli* BL21 (DE3) pLysS, selected using ampicillin, and induced with 1 mM isopropyl 1-thio-β-d-galactopyranoside. Pelleted cells were resuspended in binding buffer (PBS, pH 7.4 along with 140 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) sonicated and ultracentrifuged to remove cell debris. The supernatant was applied to a 1-ml GSTrap 4B column and purified according to manufacturer’s specifications. All buffers used in protein purification were passed through a 0.45-µm filter (Millipore). Purified protein was dialyzed against titration buffer (20 mM HEPES-KOH, pH 7.6, 100 mM KCl, 1.0 mM MgCl₂, and 1.0 mM DTT) and filtered using a 0.22-µm PVDF Millex (Millipore) filter before the anisotropy/stoped measurements were performed.

**Synthesis and 5′ Fluorescein Labeling of RNA**—The RNA oligomers (BTE, BTEBF, SII-m1, SL-III-3 and SL-III SWAP)
were transcribed using dsDNA templates by using T7 RNA polymerase. The transcription reaction was: 1× transcription buffer (stock 10×: 0.4 M Tris, pH 8.0, 100 mM DTT, Triton X-100 0.1%, 10 mM spermidine, filtered using 0.22-μm Millipore sterile PVDF filters); ATP, GTP, CTP, UTP, and GMP (final concentration, 5 mM); 25 mM MgCl₂; dsDNA (final concentration, 200 nM); and T7 polymerase (1:200 of final volume). RNase OUT (Invitrogen) was added to reduce RNA degradation. The volume was made up to the desired transcription volume using diethyl pyrocarbonate-treated water. Pyrophosphatase was added for increased yields. The transcription mix was incubated overnight at 37°C. After incubation, EDTA (50 mM final) was added to stop the reaction by chelating MgCl₂. The transcription mix, along with the loading dye (Life Technologies gel loading buffer II AM8547) in 1:1 ratio was applied onto a 10% denaturing poly acrylamide gel made with TBE and 7 M urea. The gel was prerun for an hour, and then RNA was loaded, and the gel was run for 2 h at 200V. The RNA band was visualized using UV shadowing and cut out. The RNA was eluted from the gel and precipitated using three volumes of absolute ethanol and 0.3 M sodium acetate, pH 5.3, at −20°C. After spinning the precipitate down, the pellet was washed with chilled 70% ethanol and air-dried. RNA was resuspended in RNase-free water and was fluorescein-labeled at the 5′ end using Vector Labs (Burlingame, CA) 5′ end tag labeling kit and purified according to the kit manufacturer’s recommendations. Before titration, the RNAs were refolded by heating to their melting temperature and slow cooling to room temperature in titration buffer containing 1 mM MgCl₂.

Fluorescence Anisotropy Measurements—A Horiba Jobin Yvon Fluorolog-3 FluorEssence™ spectrophotometer was used for fluorescence anisotropy experiments using an L-format detection configuration and a 1-cm-path-length quartz cuvette. Vertically polarized light with wavelength of 490 nm was used for excitation (slit width, 4 nm), and the emission (slit width, 5 nm) was measured at 520 nm in the horizontal and vertical directions. Fluorescein-labeled 3′ BTE (3′BTE) of BYDV mRNA or BTE mutants (50 nM) were titrated in the titration buffer with increasing concentrations of eIF4F, eIF4A, eIF4B, PABP, and eIF4F complexes. An increase in [3′BTE/mutants] RNA anisotropy was used to measure the interaction of initiation factors (eIFs) with 3′ BTE RNA. Temperature was 25°C for all experiments unless otherwise noted (temperature dependence studies). The dissociation equilibrium constant was determined by fitting the anisotropy data to Equation 1 (30).

$$r_{obs} = r_{min} + \{(r_{max} - r_{min})/(2 \times [3′BTE/mutants])\} \cdot \{(b - (b')^2 - 4[3′BTE/mutants][eIFs])^{0.5}\} \quad (Eq. 1)$$

where $b = K_d + [3′BTE/mutants] + [eIFs]$, $r_{obs}$ is the observed anisotropy for any point in the titration curve, $r_{min}$ is the minimum observed anisotropy in the absence of protein, and $r_{max}$ is the maximum anisotropy at saturation and is fit as a parameter. [3′BTE/mutants] and [eIFs] are the 3′ BTE RNA and its mutants and protein concentrations (eIFs = eIF4F, PABP, eIF4A, eIF4F-PABP, and eIF4F-4A complex), respectively. $K_d$ is the equilibrium dissociation constant assuming one protein-bind-
data fitting (32). The single exponential equation fitted curves correspond to Equation 4,

$$F_t = \Delta F \exp(k_{obs1} t) + F_\infty$$  \hspace{1cm} (Eq. 4)

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

$$F_t = \Delta F_1 \exp(k_{obs1} t) + \Delta F_2 \exp(k_{obs2} t) + F_\infty$$  \hspace{1cm} (Eq. 5)

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

RESULTS

Translation Efficiencies of 3′ BTE and Mutants Correlate Well with eIF4F Binding—Earlier studies (25) and results here showed that eIF4F bound WT 3′ BTE and an inactive mutant BTEBF with high affinity, calling into question the role of eIF4F in translation of this virus. To further examine what, if any, role binding of eIF4F had on translation, a variety of 3′ BTE mutants with widely different translational efficiencies were selected for binding studies. 3′ BTE and mutants with in vitro translation efficiencies from 5 to 164% (WT = 100%) were studied (2, 20). All of the mutants contain a short 17-nucleotide-long sequence (located in SL-1) conserved across viral classes, which is the location for eIF4F binding and is complementary to 18S ribosomal RNA (24, 33). The BTEBF mutant has a four-base duplication (GAUC) introduced into the 17-nucleotide consensus sequence within the BamH11837 site (Fig. 1). This mutation abolished the translation stimulatory activity of the 3′ TE.

SII-m1 was mutated to disrupt the stem base pairing, leaving it with 30% of WT 3′ BTE translation activity. SL-III-3 had mutations in the kissing stem loop. Cap-independent translation is initiated at the upstream 5′-UTR via base pairing of BTE to a complementary loop, SL-D, located in the 5′-UTR shown as dotted lines (24, 25).

As reported for earlier filter binding studies, BTEBF bound eIF4F with high affinity yet was translationally inactive (25). The binding of other 3′ BTE and mutant RNAs was compared with their translational efficiency (Fig. 2B). The translational efficiency was determined by the ability of the 3′ BTE elements to function in the 5′ leader of a luciferase reporter or by the ability to inhibit translation as described elsewhere (2, 20). It was found that the binding of eIF4F to 3′ BTE and mutants expressed as association constants ($K_a$ values) correlated well with the core translation abilities of the mutants with one exception, BTEBF (Fig. 2B). BTEBF was inactive in translation (20), but bound eIF4F with approximately the same affinity as WT 3′ BTE (Fig. 2A). This apparent discrepancy is explained below.

Binding of Other Unidentified Proteins or Small Molecules from WGE with BTEBF Inhibits eIF4F Binding, Explaining Its Inactivity in Translation Assays—Fluorescein-labeled 3′ BTE and mutants (50 nM) were incubated in depleted wheat germ extract (d-WGE) (without ribosomes and cap-binding proteins) prepared by incubating the postribosomal supernatant with m7GTP-Sepharose 4B resin as described elsewhere (34).
and titrated with increasing concentrations of eIF4F. Binding of eIF4F to BTEBF was essentially abolished by the presence of d-WGE (Figs. 2B and 3), whereas eIF4F binding to other 3′ BTE mutants was unaffected. The d-WGE had a small effect on the SL-III-3 mutant. The effect on the SL-IIISWAP mutant was not statistically significant at the 95% confidence level. During previously reported pulldown assays to identify 3′/H11032 BTE/BTEBF interacting proteins in WGE using biotin-labeled RNA, a number of unidentified proteins were also found to be interacting with BTEBF (25). This experiment provides further evidence that binding of other proteins or possibly small molecules from WGE to BTEBF prevents the interaction of eIF4F with BTEBF, thus resulting in its inactivity in in vitro translation assays.

**PABP and eIF4A Have Little Effect on the Binding of 3′ BTE and eIF4F**—The binding of eIF4F to 3′ BTE was unaffected by PABP and eIF4A as evident from the fluorescence anisotropy plots of Fig. 4 and Table 1 ($K_d = 32 \pm 8 \text{ nm}$ for eIF4F-PABP-3′ BTE, $K_d = 40 \pm 7 \text{ nm}$ for eIF4F-eIF4A-3′ BTE). The binding affinities of eIF4A, eIF4B, and PABP to 3′ BTE RNA were found to be relatively weak as shown in Table 1. The binding affinity of eIF4F for 3′ BTE did not change significantly in the presence of eIF4B (Table 1). In contrast, the eIF4F binding to tobacco etch virus pseudoknot 1 (PK1) was 4-fold stronger in the presence of both PABP and eIF4B (28). Because binding of eIF4A, eIF4B, and PABP alone was very weak, an excess of each of these proteins to eIF4F ensured that at least 90% of the eIF4F was in complex form (eIF4F-PABP, eIF4F-eIF4B, and eIF4F-eIF4A) by using the concentrations predicted using the $K_d$ values (7, 31) for the protein-protein interactions. Nonspecific binding was
ruled out by using BSA as a control under similar experimental conditions (data not shown).

Kinetics of eIF4F Binding Fits a Two-step Model, and “On” Rates \( (k_2) \) of Binding of eIF4F with 3’ BTE RNAs Do Not Correlate Well with Their Translation Efficiencies—Binding of eIF4F to BTE and mutants was investigated using stopped flow kinetics, and the data were plotted as the normalized fluorescence intensity versus time (Fig. 5A). The data were analyzed using nonlinear regression analysis (32) considering a single-exponential and a two-step process as described below. Experiments were performed with excess of RNA for pseudo-first order conditions. The experimentally observed rate constant is predicted to be a linear function of the concentration of RNA under such conditions. However, it was found that the observed binding rates had low RNA concentration dependence within the concentration range of 3–5 \( \mu M \) (before mixing), which is a 4–10-fold excess (Fig. 5C). A two-step binding process was used to explain the mechanism (35, 36).

\[
eIF4F + RNA \overset{k_1}{\rightleftharpoons} (eIF4F-RNA)^* \overset{k_2}{\rightarrow} eIF4F-RNA_{fast} \rightarrow eIF4F-RNA_{slow}
\]

**MECHANISM 1**

The first step of the reaction is a very fast, almost diffusion-controlled association of eIF4F and RNA. The second step is a slow conformational change of first intermediary complex (eIF4F-RNA)* to the final stable complex, eIF4F-RNA, and this step results in the fluorescence signal change. The concentration of RNA and binding rates are related by the equation:

\[
\frac{1}{k_{obs}} = \frac{1}{k_2} [C] + \frac{K_1}{k_2} \frac{1}{k_{obs}}
\]

where \( k_{obs} \) is the observed first order rate constant, \( k_2 \) is the forward rate constant for the second association step, \( K_1 \) is the equilibrium constant for the first step, and \([C]\) is the substrate concentration (RNA). A plot of \( 1/k_{obs} \) versus \( 1/[C] \) of RNA is shown in Fig. 5C. The intercept of the linear line generated from this plot gave the \( k_2 \) values (Table 2). \( K_a \), the association equilibrium constant for the reaction as obtained from equilibrium studies, is defined as \( k_2/k_{-2} \) and was used for calculation of the dissociation rate constant \( (k_{-2}) \). Differences between the calculated and experimental kinetic data are the residuals that indicate a good fit using a single-exponential function. The fit was not improved by the use of a double exponential equation as shown by the residuals in Fig. 5B.

**TABLE 1**

| Protein/complex | \( K_d \) \( nM \) |
|-----------------|-----------------|
| 4F              | 29 ± 3          |
| 4B              | 827 ± 93        |
| 4F-4B           | 44 ± 8          |
| PABP            | 415 ± 99        |
| 4F-PABP         | 32 ± 8          |
| 4A              | 748 ± 68        |
| 4F-4A           | 40 ± 7          |

**FIGURE 5. Kinetics of eIF4F binding to 3’ BTE and mutants.** A, eIF4F binds BTE and 3’ BTE mutants with similar association rates. The time course represents the intrinsic protein fluorescence intensity decrease of eIF4F caused by binding of 3’ BTE and mutants at 25 °C. A single-exponential fit was used for the data. The excitation wavelength was 280 nm. The signal represents the total normalized fluorescence emission above 300 nm. eIF4F concentration was 0.75 \( \mu M \) and RNA concentration was 3 \( \mu M \) before mixing. B, residuals for the single and double exponential fits for the data. Fitting was not improved by using a double exponential fit. C, plots of \( 1/k_{obs} \) versus \( 1/[C] \) for the interaction of eIF4F-BTE and mutants were used to calculate \( k_{obs} \) values from the intercept according to the two-step model.
**TABLE 2**

| Complex          | Translation efficiency* | $K_a$     | $K_a^{ib}$ | $k_3$   | $k_{-3}$ |
|------------------|-------------------------|-----------|------------|---------|----------|
| eIF4F-BTEBF      | 5.2 ± 0.2               | 0.030 ± 0.005 | 0.009 ± 0.001 | 3.00 ± 0.08 | 99 ± 18   |
| eIF4F-SII-m1     | 30.0 ± 2.1              | 0.011 ± 0.002 | 0.013 ± 0.003 | 2.16 ± 0.08 | 205 ± 46  |
| eIF4F-SL-III SWAP| 49.9 ± 0.9              | 0.016 ± 0.003 | 0.013 ± 0.003 | 2.41 ± 0.04 | 147 ± 22  |
| eIF4F-SL-III-3   | 164.5 ± 13.0            | 0.143 ± 0.018 | 0.100 ± 0.015 | 4.28 ± 0.12 | 30 ± 5    |
| eIF4F-BTE        | 100.0 ± 2.2             | 0.034 ± 0.004 | 0.035 ± 0.003 | 3.64 ± 0.06 | 105 ± 13  |

* Translation efficiency is normalized to WT BTE as 100% (2, 20).

The rate constants for the forward reaction ($k_3$) for all five mutants were found to be similar and therefore did not correlate with the efficiency of translation (Fig. 5A and Table 2). In contrast, Fig. 2B shows a plot of the translational efficiency and $K_a$ values that correlate well.

**DISCUSSION**

In this study, we have shown that the equilibrium binding of eIF4F to 3′ BTE and its mutants correlated well with the translation efficiencies. In *in vitro* studies, the BTEBF mutant bound eIF4F and did not correlate with translation efficiency. Here we show that adding d-WGE inhibits eIF4F binding to BTEBF while having no effect on WT 3′ BTE binding to eIF4F. The d-WGE had little effect on the stability of the eIF4F with other mutants. Stability under these conditions correlates well with translation for all the mutants tested and WT 3′ BTE. The d-WGE experiments both more closely approximate the *in vivo* conditions and raise the question as to the nature of the inhibition of BTEBF binding. Earlier pulldown experiments (25) and unpublished data from our lab have suggested that other proteins bind to the BTEBF, but they are yet to be identified. Similarly, we cannot rule out the possibility that a small molecule or RNA causes the inhibition.

The weaker the binding of a BTE mutant to eIF4F, the lower was its translation efficiency. This suggests that eIF4F may be the rate-limiting step for BYDV translation, just as it is believed to be for host cell mRNA translation (25), even though binding is to the 3′-UTR. Although there is no high resolution three-dimensional structure of the 3′ BTE available, the sequence and secondary structure of the 3′ BTE mutants provide some interesting insight. The secondary structure suggests a tRNA-like folding. On examining the various mutants, the SL-III-3 has quite different properties. SL-III-3 had mutations in the five bases, which form the kissing stem loop interaction with 5′ SL-D, and when placed in the 5′ context of the reporter gene, SL-III-3 translated more efficiently (164%) than WT (2) and also bound eIF4F tighter than WT. The fact that the sequence change, which does not alter the secondary structure, increases binding almost 2-fold reveals the possibility that one or more of these bases may be involved in hydrogen bonding to the eIF4F. This is consistent with the distinctly lower entropy obtained for this mutant-eIF4F interaction. Only ~10% of the binding free energy, $\Delta G$, comes from the entropy term, whereas for WT 3′ BTE, almost 50% of the binding $\Delta G$ is attributable to entropy (at 25 °C). Recently published benzyl cyanide footprinting data for eIF4F subunits indicate that the bases 4841–4853 on SL-I and bases 4895–4898 on SL-III of 3′ BTE are protected from benzoyl cyanide modification (24). This suggests that eIF4F is binding 3′ BTE on its central junction from where all the stem loops emerge (Fig. 1) (24). S-II m1 was mutated to disrupt the

![FIGURE 6. van’t Hoff plots for the interaction of 3′ BTE and mutants with eIF4F.](image-url)
stem base pairing (20). This region is also close to the junction and may alter the RNA folding, resulting in lower eIF4F binding affinity and, consequently, translation. The eIF4F binding to S-IIm1 was ~3-fold lower than binding to WT 3' BTE, and the translation was ~30% (Table 3). SL-III SWAP had an extended SL-III with a 22-base insertion from SL-III of isolate PAV129, which translates efficiently. This construct maintained the eIF4F contact sites and likely has a similar three-dimensional structure as WT 3' BTE. The translation and binding efficiencies were ~50% of WT 3' BTE. This reduction may be due to changes in the SL-III loop, which is complementary to the 5' SL-D. By moving the loop further from the junction region, subtle contacts could be disrupted. The SL-III-3 mutant suggests that these contacts play some role in eIF4F binding. The enthalpic contribution to ΔG° is (52–90%) at 25°C for eIF4F-3' BTE and mutants binding. This is much more favorable than eIF4F interaction with m7GTP as shown in Table 4. In contrast, we know that cap binding is entropically driven because of large conformational changes in the eIF4F subunit. However, PK1 (internal ribosome entry site of tobacco etch virus) and 3' BTE RNA bind to the eIF4G subunit of eIF4F (25, 38) with similar thermodynamic properties, suggesting analogous mechanisms of interaction.

PABP does not change the affinity of eIF4F for WT 3' BTE significantly, suggesting a lower importance in preinitiation complex formation, at least in the initial steps of initiation complex formation. BTE and mutants did not interact with strong affinities with PABP, eIF4F, or eIF4A, suggesting that it is eIF4F that plays the major role in transfer of the preinitiation complex from 3'- to 5'-UTR so that translation can initiate. In contrast, eIF4F binding to PK1 RNA of tobacco etch virus is enhanced multifold by the presence of PABP (28), thus showing an alternate mechanism by which BYDV virus assembles its translation machinery. This is in agreement with an earlier predicted trend for internal ribosome entry site RNAs, which is consistent with the lower requirement for additional factors of the very well folded and stable 3' BTE structure compared with a single stem loop pseudoknot structure of PK1 RNA (33, 39). This is also consistent with studies of deletion mutants (24) showing that the PABP-binding site of eIF4G was not necessary for 3' BTE interaction.

Although it has become evident that the interaction of mammalian eIF4G with mRNA is required for efficient mRNA recruitment and translation initiation (40, 41), the role of eIF4G binding to the 3'-UTR in the plant virus system is much less understood. Our data show that eIF4F binding to the 3'-UTR plays a similar role in efficient BYDV mRNA translation. Interestingly, binding of the eIF4F to BYDV 3' BTE is much more stable than binding to either the tobacco etch viral internal ribosome entry site or the 5' mRNA cap. These findings suggest that binding of eIF4F to the 3'-UTR may serve the dual purpose of recruiting ribosomes and sequestering host cell eIFs for viral translation. Further, binding to the 3'-UTR may playwe in the viral switch from replication to translation by blocking the polymerase to favor translation of the mRNA. The mechanism of transfer from the 3'-UTR to the 5'-UTR for translation remains to be investigated.

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