The 5′-leader of tobacco etch virus (TEV) genomic RNA directs the efficient translation from the naturally uncapped viral RNA. The TEV 143-nt 5′-leader folds into a structure that contains two domains, each of which contains RNA pseudoknots. The 5′-proximal pseudoknot (1PK1) is necessary to promote cap-independent translation (Zeenko, V., and Gallie, D. R. (2005) J. Biol. Chem. 280, 26813–26824). During the translation initiation of cellular mRNAs, eIF4G functions as an adapter that recruits many of the factors involved in stimulating 40 S ribosomal subunit binding to an mRNA. Two related but highly distinct eIF4G proteins are expressed in plants, animals, and yeast. The two plant eIF4F isoforms, referred to as eIF4E and eIFiso4E, differ in size (165 and 86 kDa, respectively) and their functional differences are still unclear. Although eIF4G is required for the translation of TEV mRNA, it is not known if eIF4G binds directly to the TEV RNA itself or if other factors are required. To determine whether binding affinity and isoform preference correlates with translational efficiency, fluorescence spectroscopy was used to measure the binding of eIF4E, eIFiso4E, and their complexes (eIF4F and eIFiso4F, respectively) to the TEV 143-nt 5′-leader (TEV1–143) and a shorter RNA that contained PK1. A mutant (i.e. S1–3) in which the stem of PK1 was disrupted resulting in impaired cap-independent translation, was also tested. These studies demonstrate that eIF4G binds TEV1–143 and PK1 RNA with ~22–30-fold stronger affinity than eIFiso4G. eIF4G and eIF4E bind TEV1–143 with similar affinity, whereas eIFiso4E binds with ~6-fold higher affinity than eIF4G. The binding affinity of eIF4G, eIF4E, and eIFiso4G to S1–3 was reduced by 3–5-fold, consistent with the reduction in the ability of this mutant to promote cap-independent translation. Temperature-dependent binding studies revealed that binding of the TEV 5′-leader to these initiation factors has a large entropic contribution. Overall, these results demonstrate the first direct interaction of eIF4G with the TEV 5′-leader in the absence of other initiation factors. These data correlate well with the observed translational data and provide more detailed information on the translational strategy of potyviruses.

The first committed step in protein synthesis is the binding of the 5′ mRNA cap (m7GpppN, where N is any nucleotide) by the eukaryotic initiation factor (eIF)2 4E, the small subunit of eIF4F (1–4). eIF4G, the large subunit of eIF4F, acts as a scaffold for the assembly of other components of the initiation machinery. The participation of numerous proteins is required for the formation of an 80 S initiation complex at the correct AUG initiation codon. eIF4G interacts with eIF4A to facilitate helicase unwinding activity that removes mRNA secondary structure and promotes ribosome scanning to the initiation codon. eIF3 facilitates ribosome binding to an mRNA and also interacts with eIF4G. eIF4G associates with the poly(A)-binding protein, which enhances cap binding and stabilizes RNA. Accordingly, eIF4G acts as an adaptor protein that recruits other initiation factors involved in stimulating 40 S ribosome binding to mRNA. Functional domains of mammalian eIF4G have been identified. The N-terminal domain is responsible for binding eIF4E and poly(A)-binding protein, the central domain interacts with eIF3 and eIF4A, and the C-terminal domain binds a second molecule of eIF4A and Mnk1, the protein kinase responsible for phosphorylating eIF4E (1–4).

Plants express two related but distinct forms of the cap binding complex designated eIF4F and an isoform, eIFiso4F (5). Each isoform is composed of a small cap binding subunit (eIF4E or eIFiso4E, respectively) and a large subunit (eIF4G or iso4G, respectively). The two isoforms are only 30% identical and differ in size. eIF4G has a molecular mass of 165 kDa, whereas eIFiso4G is 86 kDa (5). In comparison, mammalian eIF4G1 and eIF4GII are 46% identical (7) and yeast eIF4G1 and eIF4G2 are 53% identical (6). The two plant eIF4F isoforms have a number of functional similarities in that eIF4F and eIFiso4F support translation in vitro, facilitate ATP-dependent RNA helicase (RNA unwinding) activity, and function as RNA-dependent ATPases (6–9). Recently, functional differences in these two proteins have begun to be elucidated. eIF4F supported translation of an mRNA containing a structured 5′-leader to a greater extent than did eIFiso4F (10). Further studies showed that in vitro translation using the tobacco etch virus (TEV) 5′-leader to direct cap-independent translation preferentially used eIF4G instead of eIFiso4G (11), although a direct interaction between eIF4G and the TEV leader was not demonstrated.

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2 The abbreviations used are: eIF, eukaryotic initiation factor; TEV, tobacco etch virus; EMCV, encephalomyocarditis virus; IRES, internal ribosome entry site; NATA, N-acetyltryptophanamide; VPG, genome-linked virus-encoded protein; nt, nucleotide.
Virtually all eukaryotic cellular mRNAs are capped and most are also polyadenylated. These two sites are critical for the recruitment of the protein synthesis machinery (4). However, a number of viral mRNAs are naturally uncapped and have evolved alternative strategies for translation. Animal picornaviruses, such as poliovirus and encephalomyocarditis virus (EMCV), lack a 5'-cap and instead have evolved a long and structured 5'-leader that promotes internal ribosome entry of the 40 S ribosomal subunit (12–15). For EMCV, eIF4G binds to the internal ribosome entry site (IRES) directly and facilitates binding of the 40 S subunit through interaction with eIF3 (16, 17). In the case of poliovirus, a viral protease cleaves the host eIF4G. The C-terminal fragment of eIF4G is unable to participate in eIF4G-eIF4E complex formation and, as a consequence, cap-dependent translation, but retains the binding sites for the IRES, eIF4A, and eIF3 (18, 19) and therefore is sufficient to support translation of EMCV. Although eIF4G binds the EMCV IRES directly, trans-acting factors are required for eIF4G to bind other IRESs, e.g. that in poliovirus.

Recently, the mechanism by which TEV, a plant member of the picornavirus superfamily, initiates cap-independent translation was reported (11, 20–22). The genomic RNA of TEV is polyadenylated and contains an IRES element in the 143-nt 5'-leader sequence that is sufficient to ensure efficient translation of the RNA. The 5'-leader folds into a complex structure composed of two domains, each of which contains RNA pseudoknots. Zeenko and Gallie (23) reported that mutations in the 5’ proximal pseudoknot, i.e. PK1, and an upstream single-stranded sequence that flanks PK1 reduced translation. A nucleotide sequence of loop 3 in PK1 is complementary to a highly conserved region of 18 S rRNA. Mutations in this region disrupted translation, supporting the idea that base pairing of the two conserved regions is important for cap-independent initiation (23). Although these studies identified the essential structural elements of the TEV IRES required for cap-independent translation and the requirement for eIF4G, they did not examine whether eIF4G binds the TEV IRES directly or if trans-acting factors were required. In this study, we have quantitatively determined the binding affinity of eIF4G and eIFiso4G as well as their complexes (eIF4F and eIFiso4F, respectively) to the TEV 143-nt 5'-leader and a shorter RNA segment that contains only PK1. A mutant that disrupted the stem of PK1 was also tested for binding efficiency. These studies demonstrate that eIF4G binds the TEV leader and the PK1-containing region directly with ~30-fold higher affinity than eIFiso4G. The effect of adding eIF4E or eIFiso4E as well as the thermodynamic parameters are presented. These data provide direct evidence supporting a correlation between eIF4G binding and translational efficiency. They also demonstrate that eIF4G can bind the TEV leader in the absence of trans-acting factors and they show that the PK1 region of TEV IRES is the likely binding site for eIF4G. To our knowledge, no similar finding has been reported for any other potyvirus.

**EXPERIMENTAL PROCEDURES**

### Materials

- N-Acetyltryptophanamide (NATA), Tris-HCl, Heps, KCl, KOH, dithiothreitol, phenylmethylsulfonyl fluoride, aprotinin, soybean trypsin inhibitor, diethylpyrocarbonate, and EDTA were purchased from Sigma. Promega RiboMAX™ large scale RNA Production System T7 and Promega RiboMAX™ large scale RNA Production System SP6 were used for in vitro RNA synthesis. Peptone, yeast extract, agar, and NaCl were purchased from Fisher for making LB media and agar plates. The restriction enzymes Sall and NcoI were purchased from New England Biolabs. All other chemicals were of analytical or molecular biology grade. Diethylpyrocarbonate-treated water was used for RNA preparation and all experiments with RNA.

### Expression and Purification of Recombinant Proteins—

- eIFiso4E and eIFiso4G were expressed in *Escherichia coli* strain BL21(DE3) pLys using pET3d and were purified as described (24). Following induction of eIFiso4E, cells were lysed in Buffer B containing 600 mM KCl, centrifuged at high speed to remove the ribosomal fraction followed by dialysis to reduce the KCl concentration to 50 mM. The composition of Buffer B was 20 mM Heps-KOH, 1 mM dithiothreitol, 0.1 mM EDTA, 10% glycerol at pH 7.6, containing 0.5 mM phenylmethylsulfonyl fluoride, 0.5 ml of aprotinin, and 100 μg/ml soybean trypsin inhibitor. The dialyzed sample was applied to a 5-ml HiTrap Mono-Q ion exchange column. The expressed eIFiso4E was initially eluted with a 50 – 400 mM KCl linear gradient and followed by a mGTP-Sepharose affinity column (GE Healthcare) for further purification. eIFiso4E was eluted from the column with the buffer containing 100 mM GTP and 50 mM KCl. For the purification of recombinant eIFiso4G, a HiTrap SP column (Amersham Biosciences) was used (25). The protein was eluted with a 50 – 400 mM KCl linear gradient. The eIFiso4G appeared in the 200 – 300 mM KCl fractions. Purity was confirmed by 10% SDS-polyacrylamide gel electrophoresis. All steps were carried out in a cold box at ~4 °C. Recombinant wheat eIF4F and eIF4G were prepared as described previously (24) and were kindly provided by Dr. Karen Browning (University of Texas, Austin, TX).

### In Vitro TEV mRNA Synthesis—

The pT7-luciferase (*luc*) reporter constructs, in which the firefly *luc*-coding region is under the control of the T7 promoter in a pBluescript-derived vector, have been described previously (23). The PK1-containing region (nt 28 – 77) and the mutant S1 – 3 were introduced into the HindIII and Sall sites within the polylinker and the *luc* reporter introduced into the Sall and BamHI sites. DNA was linearized with Sall, a site immediately upstream of the *luc* open reading frame. The TEV1–143 sequence was positioned next to the SP6 promoter of PTL7SN.3 GUS vector. The restriction enzyme NcoI was used for cleavage to give linear DNA with the TEV1–143 sequence. The plasmid was the gift of James Carrington (Oregon State University). The linearized DNA was treated with Proteinase K (100 μg/ml) and 0.5% SDS in 50 mM Tris-HCl (pH 7.5), 5 mM CaCl₂ for 30 min at 37 °C. DNA was further purified by extraction with phenol:chloroform:i(soy) alcohol (25:24:1) at pH 8.0 followed by ethanol precipitation. Purity was checked in a 1% agarose gel and the concentration was quantified spectrophotometrically following linearization and brought to 0.5 mg/ml. *In vitro* transcription of PK1 and S1–3 was carried out using Promega RiboMAX™ large scale
RNA Production System T7 and TEV1–143 RNA was synthesized using Promega RibomAX™ large scale RNA Production System SP6 following the manufacturer’s protocol. The concentration of RNA was quantified by measuring the optical density at 260 nm using the absorbance at 260 nm of 40 μg/ml RNA as 1. The purity of synthesized RNA was checked by measuring the absorbance ratio, A260/280 nm in diethylpyrocarbonate-treated water. This absorbance ratio varied from 1.9 to 2.2 in different RNA preparations. The folded structure of these TEV RNA constructs was determined by Zeenko and Gallie (23). The structures of the full-length TEV leader, PK1, and the stem mutant S1–3 as described (23) are shown in Fig. 1. The predicted structure of TEV 5′-leader. A, the full-length TEV 5′-leader (nt 1–143); B, the pseudoknot PK1 (bases 28–77); C, the S1–3 mutant of PK1 (bases 28–77).

Fluorescence Measurements—Steady state fluorescence experiments were performed using a SPEX spectrophotometer using 10-mm path length quartz cuvettes. Binding experiments were performed using 0.02–0.2 μM eIF4G, eIFiso4G, eIF4F, or eIFiso4F to which increasing amounts of TEV RNA constructs were added. For these experiments at least two independent preparations of initiation factors were used. Data were averages of three or more titrations. Fluorescence emission intensity was measured at 333 nm and excitation was at 280 nm with 4-nm band pass slits for excitation and emission channels. The buffer used in the present study contained 10 mM Tris-HCl, 150 mM KCl at pH 7.5. The sample temperature was maintained at 25 °C for all experiments unless otherwise stated. The fluorescence intensities obtained were corrected for dilution. Inner filter correction for the TEV RNA ligand was performed using NATA as a fluorescent standard as described (26). The observed fluorescence intensity of 10 μM NATA was monitored at 333 nm excitation at 280 nm for each concentration of TEV RNA. The absorption of each TEV RNA concentration was measured at 280 nm. The fluorescence intensity of NATA was normalized and plotted against the absorption of TEV RNA (shown in Fig. 2A). Normalized NATA data versus RNA concentration were generated by fitting with the first-order exponential decay equation using Microlcal Origin software package (version 5.0) from Microlcal Software Inc. (Northampton, MA). For each RNA concentration in the titration, the normalized NATA corrections to the observed intensities were incorporated using the following equation,

\[ F_{corr} = F_{obs}/C_f \]  

(Eq. 1)

where \( F_{corr} \) and \( F_{obs} \) are the corrected and observed fluorescence intensities, respectively, and \( C_f \) is the NATA correction factor.

 Corrections for inner filter effect were also tested using the following equation (27).

\[ F_{corr} = F_{obs} \text{antilog} [(A_{ex} + A_{em})/2] \]  

(Eq. 2)

\( F_{corr} \) and \( F_{obs} \) are the corrected and observed fluorescence intensities. \( A_{ex} \) and \( A_{em} \) are the absorbance of the excitation and emission wavelength, respectively. The quenching of eIFiso4G with PK1 RNA and the inner filter correction is shown in Fig. 2B. The absorbance of the samples were measured using an Ultraspec 1100 Pro UV-visible absorption spectrophotometer.

The dissociation constants (\( K_d \)) of TEV RNA binding to the eIF4G and eIF4G isoforms with their eIF4F complexes were determined by nonlinear curve fitting analysis as described previously (28). A double-reciprocal plot was used for the determination of \( \Delta F_{max} \) and also the association binding constant (\( K_A \)) using Equation 3.

\[ 1/\Delta F = 1/\Delta F_{max} + 1/(K_A \cdot \Delta F_{max} (C_l - C_0)) \]  

(Eq. 3)

\( C_l \) is the concentration of the RNA and \( C_0 \) is the initial concentration of protein. \( \Delta F_{max} \) is the fluorescence change for complete saturation of protein with ligand. The linear double-reciprocal plot of 1/\( \Delta F \) against 1/(\( C_l - C_0 \)) was extrapolated to the ordinate to obtain the value of \( \Delta F_{max} \) from the intercept (28). The double-reciprocal plot of eIFiso4G with PK1 RNA is shown in the inset of Fig. 2B. The approach is based on the assumption that the emission intensity is proportional to the concentration of the ligand and \( C_l \gg C_0 \), i.e. when RNA concentration was in large excess compared with the protein concentration.


Evaluation of Thermodynamic Parameters—Thermodynamic parameters, $\Delta H$ (van’t Hoff enthalpy), $\Delta S$ ( entropy), and $\Delta G$ (free energy), were determined using the following equations (29).

$$
\ln K_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad \text{(Eq. 4)}
$$

$$
\Delta G = -RT \ln K_a \quad \text{(Eq. 5)}
$$

$R$ and $T$ are the universal gas constant and absolute temperature, respectively. $K_a$ was determined at three different temperatures 5, 15, and 25 °C, respectively. $\Delta H$ and $\Delta S$ were determined from the slope and intercept of a plot of $\ln (K_a)$ against $1/T$. $\Delta G$ was determined from Equation 5.

RESULTS

Fluorescence of the eIFiso4G, eIF4G, eIF4F and eIFiso4F-TEV1–143, PK1, and S1–3 RNA Complexes—Upon complex formation with RNA there was a decrease in the protein fluorescence emission intensities. Because of the high absorbance of the RNA (see “Experimental Procedures”), corrections for the inner filter effect were necessary. The fluorescence quenching of eIFiso4G with PK1 RNA is shown in Fig. 2. The correction for the inner filter effect using absorbance and the correction using NATA, a fluorescent analog that does not bind RNA, are shown (Fig. 2B). These results demonstrate that inner filter effects corrected by the two methods give nearly identical results. Corrected quenching data were used to obtain binding equilibrium constants.

The PK1 within the TEV Leader Binds with Stronger Affinity to eIF4G than to eIFiso4G—eIF4G promotes cap-independent translation conferred by the TEV leader to a greater extent than does eIFiso4G (11). Zeenko and Gallie (23) demonstrated that the PK1 within the TEV leader was required for cap-independent translation. PK1 is an H type pseudoknot with two stems, S1 (6 bp) and S2 (5 bp), which are connected by loops L1, L2, and L3. S1 contains a base bulge (C56) (Fig. 1). The two stems are separated by 2 nt of L2. To allow stacking these nucleotides would need to bulge out of the helix or alternatively would produce a bend in the pseudoknot that would also prevent stacking. PK1 is flanked by an unstructured 5′-proximal 37-nt sequence. To correlate the requirement for eIF4G in cap-independent translation with its binding to PK1, we examined the binding efficiency of PK1 with 20 nM eIF4G and 200 nM eIFiso4G. These concentrations of protein were chosen from preliminary studies so that the concentration of protein was less than $K_a$. The binding plots are shown in Fig. 3. The dissociation constants ($K_d$) for eIF4G and eIFiso4G were 0.126 ± 0.01 and 3.85 ± 0.7 μM, respectively, using PK1 RNA. eIF4G bound PK1 with a 30-fold higher affinity than eIFiso4G (see Table 1). These data provide quantitative information to support the preferred binding of eIF4G to PK1.

Binding of PK1 RNA with eIF4F and eIFiso4F—The wheat germ initiation factor eIF4F is a complex of eIF4E, the cap-binding protein, and eIF4G. Similarly, eIFiso4F is a complex of the cap-binding protein eIFiso4E and eIFiso4G. eIF4F and eIFiso4F are stable complexes in plants and therefore are isolated as distinct complexes when purified from wheat germ. The concentrations of eIF4F and eIFiso4F also play a critical role in cap-independent translation. To examine the binding affinity of PK1 RNA with eIF4F and eIFiso4F, 20 nM wheat germ eIF4F or 200 nM eIFiso4F were titrated with PK1 RNA. The binding plots are shown in Fig. 4. eIF4F bound PK1 with a 30-fold greater affinity than eIFiso4F (Table 1). The presence of the cap-binding protein, eIF4E, had little effect on eIF4G binding.
affinity. eIFiso4E, however, increased the affinity of eIFiso4G when present as part of the eIFiso4F complex; eIFiso4F bound to PK1 ~6-fold more strongly than did eIFiso4G (Fig. 5A), whereas the binding of eIF4F and eIF4G were very similar (Table 1 and Fig. 5B). The presence of the cap-binding proteins reduced the discrimination between the two eIF4F isoforms by almost 9-fold.

Binding of 143-Nt TEV 5'-Leader with eIF4F, eIFiso4F, eIF4G, and eIFiso4G—To determine whether there were significant differences between binding to PK1 versus the full-length TEV leader, we determined the binding affinity to TEV1–143 with eIF4F, eIFiso4F, eIF4G, and eIFiso4G. The dissociation constant, $K_d$, for eIFiso4G and eIF4G was 2.25 ± 0.33 and 0.10 ± 0.01 μM, respectively. eIF4G bound TEV1–143 with greater than 20-fold higher affinity than did eIFiso4G. These results are similar to the difference in affinity following binding to PK1 alone where the difference was ~30-fold. The smaller difference in affinity between the two proteins is due to increased binding of eIFiso4G to TEV1–143, whereas the binding of eIF4G to TEV1–143 and PK1 is the same within experimental error. The binding affinity of eIFiso4G to TEV1–143 is more difficult to measure because of the large inner filter effect. Consequently, although our data reflect averages of at least three titrations and the error spans the range of values obtained from fitting, these data are less accurate than those for binding RNA with higher binding affinity.

The binding affinity of eIFiso4F and eIF4F to TEV1–143 showed the same pattern as observed for PK1 with binding constants of 0.178 ± 0.03 and 0.079 ± 0.01 μM, respectively. eIF4F binds the TEV1–143 2.3 times more tightly than does eIFiso4F. For PK1, the difference in binding affinity between the two proteins was 3.5-fold. In this case, both proteins show higher binding affinity for TEV1–143, with the larger effect attributable to eIFiso4F. Fig. 6, A and B, show the binding of eIFiso4F and eIF4F with TEV1–143, PK1, and S1–3, the stem mutant of PK1 discussed below.

Mutation of loop 3 within PK1 (i.e. S1–3), which disrupts its potential base pairing with 18 S rRNA, reduced cap-independent translation to ~7.4% of wild-type levels (23). We examined, therefore, the binding of eIF4G or eIFiso4G to S1–3 RNA to determine whether the mutation in S1–3 that substantially reduces cap-independent translation also reduces eIF4G and eIFiso4G binding. When compared with binding to TEV1–143, the binding of eIFiso4G to S1–3 was reduced as least 10-fold (Table 1) although accurate determination of the binding to S1–3 was difficult because of its low affinity and the high concentration of RNA used required large corrections to the data. The value given is a lower estimate of the binding constant and the value may actually be higher. Addition of eIFiso4E to form eIFiso4F resulted in binding that was 10-fold lower than for TEV1–143. The binding affinity, however, was about 10-fold higher than that observed for eIFiso4G alone. Although the presence of eIFiso4E enhanced binding, the level of binding was still relatively low. eIF4G and eIF4F showed similar binding affinity to S1–3, which was reduced 6-fold relative to binding to TEV1–143.

Temperature Effects—A van’t Hoff plot of ln $K_d$ versus the reciprocal of temperature (1/T) was used to calculate the ther-
modynamic parameters of enthalpy ($\Delta H$) and entropy ($\Delta S$). Fig. 7 shows the van't Hoff plots for the various proteins and RNA constructs. The values of $\Delta H$ and $\Delta S$ were obtained from the slope and intercept, respectively. These data are shown in Table 2. The $\Delta G$ value is for 25 °C. Interestingly, the interaction of eIFiso4G with both TEV1–143 and PK1 is enthalpically driven with a relatively small entropic contribution. In contrast, the interaction of eIFiso4F with TEV1–143 is entropically driven and the interaction of eIFiso4F with PK1 has a significant entropic contribution. eIF4F interaction with TEV1–143 is also entropically driven and enthalpically favorable. The interaction of eIF4F with PK1 has nearly equal contributions from enthalpy and entropy. The relatively large, favorable, entropic contributions to the eIFiso4F and eIF4F binding to TEV leader RNA suggest that hydrophobic residues are less solvent exposed in the combined structure. These interactions may be either base stacking interactions with hydrophobic amino acids or could be the result of conformational rearrangements of the proteins themselves. Without more detailed structural information, it is not possible to further define the source of these contributions to protein-RNA complex stability.

**DISCUSSION**

Many viruses lack a 5′ cap and use a highly structured RNA leader sequence (IRES) to initiate translation. IRES elements recruit the translational machinery in the absence of a 5′ cap, the binding site for eIF4E. TEV, a potyvirus, contains an IRES
TEV Binds eIF4G Preferentially

FIGURE 7. van’t Hoff plots of eIFiso4G, eIFiso4F, and eIF4F with TEV1–143 and PK1 RNA. InKₐ versus 1/T × 10⁻³ for eIFiso4G (0.2 μM) with TEV1–143 (●) and PK1 (○); eIFiso4F (0.2 μM) in the presence of TEV1–143 (▲) and PK1 (◆); eIF4F (0.2 μM) with TEV1–143 (■) and PK1 are shown. Temperature dependent binding measurements were performed at 5, 15, and 25 °C. The thermodynamic parameters, enthalpy (ΔH) and entropy (ΔS), were calculated from the slope and intercept.

TABLE 2
Thermodynamic parameters of enthalpy (ΔH), entropy (ΔS), and Gibb’s free energy (ΔG) for the interactions of eukaryotic initiation factors with TEV1–143 and PK1 RNA

| System          | ΔH       | ΔS       | ΔG       |
|-----------------|----------|----------|----------|
|                 | kJ mol⁻¹ K⁻¹ | J mol⁻¹ K⁻¹ | kJ mol⁻¹ K⁻¹ |
| eIFiso4G + TEV1–143 | -21.18 ± 1.1 | 37.25 ± 3.7 | -32.22 ± 2.2 |
| eIFiso4G + PK1   | -22.03 ± 0.7 | 29.76 ± 2.3 | -30.89 ± 1.4 |
| eIFiso4F + TEV1–143 | -12.97 ± 5.5 | 85.97 ± 11.9 | -38.51 ± 7.1 |
| eIFiso4F + PK1   | -19.54 ± 6.3 | 52.05 ± 21.6 | -35.17 ± 12.7 |
| eIF4F + TEV1–143 | -16.71 ± 4.2 | 80.06 ± 14.4 | -40.52 ± 8.5 |
| eIF4F + PK1      | -19.46 ± 2.9 | 63.35 ± 10.2 | -38.29 ± 5.9 |

ΔG values were calculated using the equation ΔG = -RT lnKₐ at 25 °C.

within the leader sequence that promotes internal initiation and cap-independent translation (30, 31). In addition to the IRES, a virus-encoded protein (VPg) is covalently attached to the 5’ terminus of the TEV genomic RNA, which is likely removed after infection (13). The VPg interacts with eIFiso4E specifically in Arabidopsis and Arabidopsis lacking eIFiso4E shows resistance to TEV infection (33). The consequence of the eIFiso4E and VPg interaction for translational regulation has not been determined. One possibility is that VPg binds eIFiso4E following viral infection and this recruitment of eIFiso4E is only involved in the initial round of translation of the viral RNA, perhaps during virion disassembly. Recently interactions among eIF4G, eIF4E, and VPg have been demonstrated (34, 35), supporting this notion. The TEV leader, however, functions to promote cap-independent translation in the absence of the VPg, thus demonstrating that the TEV IRES can recruit eIF4G without assistance of the VPg (23). Similarly, no viral protein was present in our assays so that the interaction between the TEV leader and the translational machinery could be assessed directly. It is also possible that the interaction between VPg and eIFiso4E is involved in other processes, such as transport during the viral life cycle or cell to cell movement as suggested by recent work (36, 37).

eIF4G has been shown to play a central role in the function of the TEV IRES as it does for animal picornaviruses (11). The EMCV IRES binds eIF4G to a defined region and promotes ribosomal binding (16, 17). Following infection by poliovirus or related viruses, eIF4GI and eIF4GII are cleaved (38), removing the N-terminal eIF4E binding site and preventing eIF4G from recruiting the translational machinery rather than a requirement for eIF4G to bind the IRES. Other viruses, such as hepatitis A do not cleave eIF4G and the virus utilizes intact eIF4G for its function (39). Whether eIF4G or eIFiso4G is cleaved in cells infected by plant viruses is unknown.

The TEV IRES confers a translational advantage only under conditions of competitive translation when eIF4F and eIFiso4F are partially depleted or at higher mRNA concentrations (11). Similar observations were made for the stimulatory effects of the 5’ cap (10). These results imply that the IRES recruits factors required for translation and that when these factors are present in excess the TEV leader or cap loses the competitive advantage that they provide to an mRNA. Gallie (11) showed by supplementing factors to depleted translation lysate that eIF4G was responsible for TEV IRES activity. However, it was not clear if eIF4G bound directly to the IRES or whether other factors were required, e.g. eIF4A, eIF4B, or other proteins. EMCV requires eIF4A and eIF4B for efficient binding of eIF4G to the IRES and these factors are required for 48 S complex formation (16, 17). Gallie (11) showed that eIF4A and eIF4B stimulated the effect of eIF4G on TEV IRES activity, but it was not clear if these factors were required because some endogenous eIF4A and eIF4B remained in the depleted lysate. Here we present direct evidence for the preferential role of eIF4G compared with eIFiso4G in binding to the TEV leader and the PK1 element within the leader. The binding affinity of eIF4G to the TEV leader was ~22-fold higher than the affinity of eIFiso4G for the same RNA (Fig. 8). These results are consistent with earlier data showing that eIF4G was sufficient to overcome the competitive advantage of TEV leader when initiation factors were present in limiting amounts (11). These data also show that eIF4G has a very high binding affinity for the TEV leader in the absence of other factors. The Kₐ at 25 °C is 79 ± 1 nM, which is comparable with the Kₐ of 56.7 ± 0.6 nM obtained for eIFiso4E binding to m7GTP, indicating that under limiting concentration conditions, the TEV leader competes well for eIF4G. Whereas other initiation factors will probably increase the binding affinity of eIF4G as was observed for the binding of initiation complexes to a cap (25, 40–42), they are not essential for efficient binding. These data also suggest that other transacting factors, such as polypyrimidine tract-binding protein required for Theiler murine encephalomyelitis virus, are not
required for recruitment of eIF4G to the TEV leader (43). Such trans-acting factors are thought to be important in maintaining IRES structure. The TEV IRES, however, is shorter and less structured than those of animal picornaviruses, suggesting that this may be a simpler system with fewer interactions.

The addition of the cognate cap-binding protein to each of the eIF4G isoforms produced an interesting result. The addition of eIF4E to eIF4G had little effect on TEV binding. However, addition of eIF iso4E to eIF iso4G increased its binding affinity to the TEV leader more than 12-fold reducing the difference in binding affinity between eIF4F and eIF iso4F to little over 2-fold. eIF iso4F alone had little affinity for any of the RNAs tested (data not shown). Whereas there are no reports of whether eIF4G or eIF iso4G is cleaved in plants following viral infection, such cleavage to remove the cap-binding protein would clearly give a greater competitive advantage to eIF4G in binding the TEV IRES. Similar results were obtained for PK1, the 5'-proximal pseudoknot in the TEV leader. In this case, eIF4G and eIF iso4G had an even larger difference in binding affinity with eIF4G having greater than 30-fold tighter binding compared with eIF iso4G. This almost 10-fold increase in binding discrimination was due to the reduced binding affinity of eIF iso4G, whereas the binding affinity of eIF4G remained essentially unchanged. This suggests that eIF iso4G may bind to a slightly different region of the TEV leader, perhaps including a region not present in PK1. It is also possible that the smaller size of PK1 relative to the full-length TEV leader may lack conformational elements recognized by eIF iso4G. eIF iso4F has been shown to translate an mRNA with an unstructured leader sequence more efficiently than an mRNA containing structured leader (10). The full-length TEV leader contains more single-stranded regions than does PK1 (Fig. 1). The addition of the cognate cap-binding protein also reduced the difference in binding affinity to PK1. Again, the addition of eIF4E had little effect on eIF4G binding, but the addition of eIF iso4E increased the affinity of eIF iso4G for PK1. These differences cannot be attributed to differences in protein preparation. Different prepar-
TEV Binds eIF4G Preferentially

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