The 3′ mRNA I-shaped structure of maize necrotic streak virus binds to eukaryotic translation factors for eIF4F-mediated translation initiation

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Unlike the mRNAs of their eukaryotic hosts, many RNAs of viruses lack a 5′ m7GpppN cap and the 3′ polyadenosine tail, and yet they are translated efficiently. Plant RNA viruses, in particular, have complex structures within their mRNA UTRs that allow them to bypass some cellular translation control steps. In the 3′ UTR of maize necrotic streak virus (MNeSV), an I-shaped RNA structure (ISS) has been shown to bind eukaryotic initiation factor (eIF)4F and to mediate viral translation initiation. A 5′–3′ RNA “kissing-loop” interaction is required for optimal translation. However, the details of how the 3′ ISS mediates translation initiation are not well understood. Here, we studied the binding of the 3′ ISS with eIFs. The eIF4A–eIF4B complex was found to increase binding affinity of eIF4F with the 3′ ISS by 4-fold (from $K_D = 173 \pm 34$ nM to $K_D = 48 \pm 11$ nM). Pre-steady-state analysis indicated that the eIF4A–eIF4B complex increased the RNA association rate and decreased the dissociation rate in an ATP-independent manner. Furthermore, our findings suggest that eIF4F could promote binding of the 3′ ISS with the MNeSV 5′UTR, enhancing the long-distance kissing-loop interaction. However, the association of the 5′ UTR with the 3′ ISS–eIF4F complex did not increase 40S ribosomal subunit binding affinity. These quantitative results suggest a stepwise model in which the first committed step is eIF4F binding to the 3′ ISS, followed by an interaction with the 5′ UTR and subsequent 40S ribosomal subunit binding.

MNeSV is a single-stranded positive sense RNA virus and belongs to the family Tombusvirus (1, 2). After MNeSV infection, maize leaves display pale green or yellow spots and streaks as early symptoms, and the infected leaves later become necrotic (3). MNeSV virus is widespread and can lead to huge economic losses.

In eukaryotic mRNA translation initiation, the first step is recruitment of the 40S ribosomal subunit and translation initiation factors (eIFs) to mRNA (4, 5). eIF4F binding with mRNA is believed to be the first committed step for mRNA activation. In plants, eIF4F consists of two subunits eIF4E and eIF4G. eIF4E has an m7GTP cap binding pocket (6), and eIF4F functions as a scaffold protein, which has several binding sites for other translation initiation factors, including eIF4A, eIF4B, poly(A)-binding protein, and eIF3 (7, 8). The eIF4F–eIF4A–eIF4B complex together with ATP hydrolysis unwinds the structured 5′UTR and facilitates loading of the 43S pre-initiation complex, consisting of the 40S ribosomal subunit, initiator tRNA and eIFs, onto mRNA (9, 10). eIF4A is an ATP-dependent helicase (11, 12). eIF4B associates with eIF4A to promote eIF4A’s helicase activity (13, 14). eIF3 participates in 40S ribosomal subunit recruitment to mRNA.

Unlike canonical eukaryotic mRNA translation initiation, the majority of plant virus RNA genomes lacks a 5′ m7GpppN cap and poly(A) tail (15–17). Plant viruses, including Tombusvirus, Luteovirus, and Umbravirus, utilize 3′ cap independent translation elements (CITEs) in the untranslated regions to recruit translation initiation factors and perhaps the 40S ribosomal subunit to mRNA’s 3′ UTR (18). CITEs, with different structures, conserved sequences, and translation initiation mechanisms, are grouped into, but not limited to, six different categories of which the I-shaped structure (ISS) is one.

MNeSV 3′ UTR is 197 nucleotides long and contains the 3′ ISS, an ~100-nucleotide–long element, close to the 5′ end of this 3′ UTR. As described by its terminology, the 3′ ISS forms an extended stem-loop structure (16). ISS has also been reported in other viral families, including Carmovirus and Aonenvirus. A conserved sequence is located in the central region of the ISS (19). Six single-strand nucleotides in ISS’s apical loop are complementary to an upstream 5′UTR loop and can form a “kissing-loop” interaction (20).

Previous work (21) has shown that the 3′ ISS interacts with both eIF4F subunits, eIF4E and eIF4G. Furthermore, a tripartate 5′UTR–3′ CITE–eIF4F complex was demonstrated to form. eIF4F binding affected translational ability of the 3′ ISS. In this study, we measured quantitative binding affinities, kinetics and thermodynamics.

Our experiments demonstrated that eIF4A and eIF4B together increased ISS–eIF4F binding affinity without the requirement for helicase activity. Pre-steady-state kinetics

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2 The abbreviations used are: MNeSV, maize necrotic streak virus; eIF, eukaryotic initiation factor; ISS, I-shaped structure; CITE, cap-independent translation element; IPTG, isopropyl 1-thio-β-D-galactopyranoside; BYDV, barley yellow dwarf virus; PEMV, pea enation mosaic virus; TE, translation element; AMPNNP, 5′-adenyl-β,γ-imidodiphosphate; BTE, barley 3′-translational enhancer element.
showed that eIF4A and eIF4F not only increased the ISS–eIF4F association rate but also decreased the dissociation rate. Using these data, we formulate a more detailed stepwise mechanism for assembly to the initiation complex. The first committed step in initiation, the binding of eIF4F to the 3’ISS, was facilitated by eIF4B and eIF4A. This is followed by binding of the 5’UTR and subsequent association of the 40S ribosomal subunit. However, the association of the 5’UTR with the 3’ISS–eIF4F complex does not increase 40S ribosomal subunit binding affinity.

Results

In vitro translation efficiencies of WT and mutant 3’ISS were affected by eIF4F-binding efficiency but also required the kissing-loop interaction

Previous experiments have demonstrated that a single nucleotide mutation in 3’ISS stem’s central part dramatically reduced its in vitro translation efficiency in wheat germ extract (21). Three 3’ISS mutants (Fig. 1) with differing translational efficiencies were chosen to examine the correlation between eIF4F binding and translation. All three mutants (Fig. 1) had only one nucleotide mutation in the 3’ISS central region (nomenclature is from Ref. 21). The 3’ISS-CA (18G → A) mfold (22)-predicted lowest energy structure was slightly different from the WT 3’ISS structure. The G18 was predicted to form a guanine–cytosine base pair in the wildtype (WT) 3’ISS secondary structure (Fig. 1D). In 3’ISS-iA/B2 (18G → C), two different conformations (Fig. 1C) with similar ΔG values were predicted by mfold (22). In one predicted conformation the 5’-3’ long-distance kissing-loop interaction was disrupted, although it was maintained in the other. In 3’ISS-C1, G13, which is highly accessible by single-strand–modifying agents, was deleted.

mfold (22)-predicted secondary structure of 3’ISS-C1 was similar to WT 3’ISS (Fig. 1B).

In previous studies, 3’ISS-CA, 3’ISS-iA/B2, and 3’ISS-C1 in vitro translation efficiencies were reported to be 119, 37, and 8% of WT 3’ISS, respectively (21). Fluorescein-labeled 3’ISS and mutants were titrated with eIF4F, and KD values were calculated as described under “Experimental procedures.” 3’ISS-CA had slightly higher binding affinity with eIF4F than WT 3’ISS (Table 1), consistent with in vitro translation efficiencies. Surprisingly, binding affinity of 3’ISS-iA/B2 with eIF4F was as strong as WT 3’ISS. The low translation efficiency of 3’ISS-C1 could be explained by the disrupted 5’–3’ long-distance base pair interaction in one of the two conformations predicted by mfold (Fig. 1C). The disruption of this 5’–3’ long-distance kissing-loop interaction would hinder the transfer of eIFs recruited at 3’ISS to the 5’UTR leading to low in vitro translation efficiency. The 5’–3’ long-distance interaction was not disrupted in the 3’ISS-C1 mutant, but the low translation efficiency could be attributed to its low binding affinity with eIF4F suggesting that eIF4F binding is an essential step in translation initiation.

Table 1

| Temperature-dependent equilibrium dissociation constant (Kd) values for eIF4F with mutants and WT ISS | 30 °C | 25 °C | 20 °C | 15 °C | 10 °C | 5 °C |
|---|---|---|---|---|---|---|
| ISS | 218 ± 2 | 173 ± 2 | 146 ± 2 | 130 ± 2 | ND | ND |
| ISS-C1 | 218 ± 2 | 173 ± 2 | 146 ± 2 | 130 ± 2 | ND | ND |
| ISS-iA/B2 | 229 ± 3 | 169 ± 3 | 75 ± 3 | 50 ± 3 | ND | ND |
| ISS-CA | 108 ± 2 | 89 ± 2 | 81 ± 2 | 56 ± 2 | ND | ND |

a Translation efficiency is normalized to 3’ISS as 100% and is reported elsewhere (21).

b ND indicates the Kd value of the interaction was not determined.

Figure 1. Predicted mfold secondary structures of WT 3’ISS (A), 3’ISS-C1 (B), 3’ISS-iA/B2 (C), and 3’ISS-CA (D) by mfold. The relative in vitro translation efficiencies in wheat germ extract were reported elsewhere (21) and are shown below (normalized to a value of 100 for WT 3’ISS).
**Table 2**

|                | 3′ISS   | 3′ISS-CA | 3′ISS-iA/B2 |
|----------------|---------|----------|-------------|
| ∆G° (kJ/mol⁻¹) | 38.4 ± 4.6 | 38.5 ± 5.3 | 39.2 ± 7.2  |
| ∆Δf (kJ/mol⁻¹) | 25.6 ± 2.3 | 24.3 ± 2.7 | 75.6 ± 8.6  |
| ∆S (kJ mol⁻¹ K⁻¹) | 43.3 ± 8.0 | 46.1 ± 9.5 | 122 ± 29    |
| – T∆A/∆G° (%)   | 33.5     | 36.1     | ND§         |

*Free energy change was calculated at 25 °C. §ND means not determined.

**WT 3′ISS and 3′ISS-CA interaction with eIF4F are both enthalpically and entropically favorable**

Thermodynamic parameters of these interactions were determined to further characterize eIF4F binding with 3′ISS and mutants. Steady-state fluorescence anisotropy was used to monitor these interactions as a function of temperature (Fig. 2 and Table 2). The van’t Hoff plots were constructed to calculate the enthalpy and entropy changes in the reactions (Fig. 2 and Table 2). Binding of 3′ISS and 3′ISS-CA with eIF4F had similar favorable changes in entropy and enthalpy. The enthalpy contribution may be attributed to formation of new charge-related interactions between eIF4F and RNA. Releasing water molecules on the complex interface due to eIF4F–RNA association could lead to entropy increases. However, 3′ISS-iA/B2 interaction with eIF4F was only enthalpically driven. This implied that 3′ISS-iA/B2-eIF4F and 3′ISS–eIF4F had different binding mechanisms. These were consistent with the conformational differences between 3′ISS and 3′ISS-iA/B2. The higher enthalpy change suggested that more charge-related interactions, including electrostatic interactions and/or hydrogen bonds, formed between 3′ISS-iA/B2 and eIF4F.

**eIF4F–3′ISS-CA interaction showed a higher association rate than eIF4F–3′ISS**

The quenching of eIF4F protein intrinsic fluorescence upon binding of 3′ISS or 3′ISS-CA was assessed to determine pre-steady-state kinetics. Excess amounts of 3′ISS or 3′ISS-CA were mixed with eIF4F ([eIF4F] ≪ [ISS]) to obtain pseudo-first-order conditions for these interactions. The data were then fitted with both single- and double-exponential functions (Fig. 3, A and C). The double-exponential function did not improve the data fitting. Therefore, observed rate constants were calculated using the single-exponential function. To determine association and dissociation rate constants, the observed rate constants were plotted versus increasing concentrations of 3′ISS or 3′ISS-CA. Observed rate constants were found to be linearly dependent on concentrations of 3′ISS or 3′ISS-CA (Fig. 3, B and C), suggesting a one-step reaction model.

**eIF4A and eIF4B increased binding affinity of eIF4F with 3′ISS**

eIF4A and eIF4B have been known to play essential roles in eukaryotic translation initiation. To determine the effects of
these translation initiation factors on binding of eIF4F with 3′ISS, equilibrium constants of eIF4F–3′-ISS binding in the presence of eIF4A and eIF4B were determined. 3′ISS was labeled with fluorescein at the 5′ end and titrated with increasing concentrations of eIF4F alone or eIF4F–eIF4A–eIF4B complex (Fig. 4). Concentration ratios of [eIF4B]/[eIF4F] and [eIF4A]/[eIF4F] were calculated from previously reported $K_D$ values (23) to ensure that 90% of the eIF4F was complexed with the protein. Binding experiments of 3′ISS to eIF4A or eIF4B were performed to confirm that these two proteins do not bind to the 3′ISS directly. The $K_D$ value of the eIF4F–3′ISS interaction in the presence of eIF4B (169 ± 30 nM) was similar to eIF4F–3′ISS interaction (173 ± 34 nM) indicating that eIF4B had no effect on the interaction. In the presence of eIF4A, eIF4F–3′ISS complex binding affinity improved by less than 2-fold ($K_D = 108 ± 26$ nM) (Fig. 4A and Table 5). Binding affinity of 3′ISS–eIF4F interaction in the presence of eIF4A–eIF4B–ATP ($K_D = 48 ± 11$ nM) was found to be four times stronger than the 3′ISS–eIF4F interaction ($K_D = 173 ± 34$ nM) (Fig. 4 and Table 5).

Because eIF4A–eIF4B–ATP complex has helicase activity, which can unwind mRNA secondary structure during 40S ribosomal subunit loading (13, 24); the binding affinity of eIF4F for 3′ISS was examined in the absence of ATP and in the presence of AMP-PNP (nonhydrolyzable ATP) which prevents the helicase reaction. The data showed that $K_D$ for this interaction in the absence of ATP (54 ± 14 nM) was similar to that with ATP present ($K_D = 48 ± 11$ nM) (Fig. 4B). As expected, AMP-PNP has a similar effect ($K_D = 52 ± 16$ nM). This indicated that the eIF4A–eIF4B complex improved the binding affinity of 3′ISS to eIF4F without the requirement for unwinding 3′ISS. This increased binding affinity is probably due to a conformational change of eIF4F after eIF4A–eIF4B associates with eIF4F (25).

**eIF4A–eIF4B complex increased stability of the eIF4F–3′ISS complex mainly through decreasing the dissociation rate**

Pre-steady-state kinetics studies of eIF4F interaction with 3′ISS were also performed in the presence of the eIF4A and eIF4B complex. Excess amounts of 3′ISS were rapidly mixed with the eIF4F–eIF4A–eIF4B complex to obtain pseudo-first-order interaction conditions, and the time course of eIF4F–eIF4A–eIF4B complex intrinsic fluorescence change was measured. Association and dissociation rate constants were 42.7 ± 5.9 M$^{-1}$s$^{-1}$ and 2.3 ± 0.9 s$^{-1}$, respectively (Table 4). Our results demonstrated that, in the presence of eIF4A and eIF4B, the association rate for eIF4F–3′ISS increased slightly, and the dissociation rate decreased by more than 2-fold when compared with kinetics for the eIF4F–3′ISS interaction (Fig. 5), thus stabilizing this complex. The $K_D$ value of this interaction calculated from $k_1$ and $k_{-1}$ was consistent with $K_D$ value determined by steady-state binding studies ($k_{-1}/k_1 = 53$ nM and $K_D = 54 ± 14$ nM) indicating a simple one-step association process (Table 5).

**eIF4F facilitated binding of 5′UTR with 3′ISS**

Long distance kissing-loop interactions between the 5′UTR and 3′ISS have been shown to be necessary for translation initiation (Fig. 6A) (21). To better understand the role of this interaction in translation initiation, we tested the binding affinity of 5′UTR with 3′ISS using steady-state fluorescence anisotropy. We titrated fluorescein-labeled 5′UTR with increasing concentrations of unlabeled 3′ISS. No binding of the two RNAs was observed, even after adding a 20-fold excess of the 3′ISS. In another set of experiments, fluorescein-labeled 5′UTR was titrated with increasing concentrations of preincubated unlabeled 3′ISS. Using steady-state fluorescence anisotropy, we performed another set of experiments, fluorescein-labeled 5′UTR was titrated with increasing concentrations of preincubated unlabeled 3′ISS.
beled 3’ISS–eIF4F complex. The dissociation equilibrium constant, $K_D$, for this interaction was 202 ± 30 nM (Fig. 6, B and C). We also tested the binding of 5′UTR with eIF4F alone, but the binding was found to be weak and probably nonspecific with $K_D > 2 \mu M$. These results indicated that eIF4F facilitated the binding of 5′UTR with 3′ISS. Our data showed that the 5′UTR did not bind with 3′ISS without the aid of eIF4F. This implies that eIF4F binding with 3′ISS is a necessary precursor to the 5′UTR–3′ISS interaction rather than the hypothesis that eIF4F stabilizes the 5′UTR–3′ISS complex. The effects of eIF4A and eIF4B on the interaction were determined. However, no difference was observed in the $K_D$ values in the absence and presence of eIF4A–eIF4B–ATP, indicating that RNA unwinding is not necessary for the formation of this complex (Fig. 6B).

**Presence of the 5′ UTR did not affect eIF4F–3′ISS–40S ribosomal subunit association**

The role of the 3′ISS in ribosome recruitment is not well understood. We investigated the association of the eIF4F–3′ISS complex to the 40S ribosomal subunit. Fluorescein-labeled 3′ISS was premixed with excess eIF4F to ensure that 90% 3′ISS was in complex with eIF4F. Fluorescence anisotropy was monitored after mixing with eIF4F to confirm the formation of the eIF4F–3′ISS complex. This eIF4F–3′ISS complex was then titrated with increasing concentrations of 40S ribosomal subunits. The binding affinity was found to be moderate with $K_D = 617 ± 34 \text{ nM}$ (Fig. 7). As a comparison, 3′ISS–40S ribosomal subunit interaction in the absence of eIF4F is nonspecific with $K_D > 2 \mu M$. Adding eIF4A and eIF4B did not increase the binding affinity of the eIF4F–3′ISS complex with the 40S ribosomal subunit. Loading of the 40S ribosomal subunit on the 5′UTR is a key step in translation initiation. To prepare the 3′ISS–5′UTR–eIF4F complex, 5′UTR and eIF4F were added in excess. The resulting complex was then titrated with 40S ribosomal subunits. A $K_D$ value of 572 ± 27 \text{ nM} was obtained indicating that the 3′ISS–5′UTR–eIF4F complex did not increase the binding affinity as compared with the 3′ISS–eIF4F complex showing that the presence of the 5′UTR itself did not increase 40S ribosomal subunit affinity (Fig. 7). Because binding of the 40S ribosomal subunit to the labeled 3′ISS is near the maximum anisotropy, the addition of the 5′UTR to this complex could not be directly determined. However, this value could be calculated via the thermodynamic cycle (calculated $K_D = 187 \text{ nM}$) (Fig. 7B). These data showed that the long-distance base-pairing interaction was not blocked by eIF4F and 40S ribosomal subunit binding to the 3′ISS. However, eIF4F and 5′UTR association with the 3′ISS did not enhance 40S ribosomal subunit binding.
Increase in entropy. In contrast to eIF4E–m7GTP interactions, of the water molecules in the cap binding pocket led to the acid, not in the same position, was mutated. However, 3
Discussion
In both 3’ISS-C1 and 3’ISS-CA mutants, only one nucleic acid, not in the same position, was mutated. However, 3’ISS-C1 showed low binding affinity to eIF4F and also a low translation efficiency, whereas 3’ISS-CA showed tighter binding to eIF4F than WT 3’ISS. Interestingly, these two mutants predicted secondary structures are very similar. Similar results were reported for barley yellow dwarf virus (BYDV), where a 3’SL-III-3 mutant with few bases changes in the primary sequence and no change in secondary structure had a translation efficiency of 164%, more than WT (26). It was proposed that the mutated bases might be involved in hydrogen bonding with eIF4F. Here, we also suggest that the mutated G13 residue in 3’ISS-C1, which is highly accessible to chemical modification (16), can participate in hydrogen bonding with eIF4F and possibly directly locate in the eIF4E cap-binding site. This proposed hydrogen bonding is consistent with the enthalpy change observed for the eIF4F–RNA interaction.

eIF4F interactions with both 3’ISS and 3’ISS-CA were found to be both enthalpically and entropically favorable. Binding interactions of eIF4F to other translation elements (TEs) such as tobacco etch virus PK1 and BYDV 3’BTE have also been shown to be enthalpically and entropically favorable (26, 27). However, eIF4E–m’GTP binding is reported to be only entropically driven (28), and the entropy change was more than twice the entropy changes for the interactions cited above. Stacking interactions have been reported between m’GTP and two tryptophan residues in the cap binding pocket of eIF4E (29). Release of the water molecules in the cap binding pocket led to the increase in entropy. In contrast to eIF4E–m’GTP interactions, more charge related interactions, such as salt bridges and electrostatic forces, probably form between eIF4F and RNA TEs. This implies that eIF4F–3’ISS may utilize a different binding mechanism not primarily involving stacking interactions inside the eIF4E cap binding pocket.

Our laboratory had shown that eIF4A–eIF4B–ATP could catalyze 3’BTE unwinding and in turn increase 3’BTE binding affinity with eIF4F (26). Data here show enhanced binding affinity of eIF4F to 3’ISS with the help of eIF4A–eIF4B, but this improved binding affinity was not dependent on the presence of ATP. This ruled out the possibility that the improved binding affinity was due to 3’ISS RNA unwinding. Additionally, 3’ISS requires both eIF4G and eIF4E subunits of eIF4F for binding (16), but 3’BTE bound with eIF4G alone (25). In both MNeSV and BYDV systems, eIF4A–eIF4B increase viral RNA ability to compete with host mRNA to sequester eIF4F, but the processes are different. Based on our data, we propose the following mechanism for eIF4F binding with 3’ISS. The eIF4A and eIF4B bind with eIF4F at the eIF4G-binding site. This association causes a conformational change of eIF4F as shown for eIF4G binding to BTE (25) and in turn may cause eIF4F to be more accessible to 3’ISS and/or stabilize the eIF4F–3’ISS binding complex.

Unlike the 3’BTE, which has an 18S rRNA complementary sequence (30), the 3’ISS does not have complementarity to the RNA. In the 3’BTE, unwinding can expose this sequence, but it is not necessary for 3’ISS binding to 40S ribosomal subunits. However, compared with other well-studied 40S–TEs systems, including BYDV, pea enation mosaic virus (PEMV), and hepatitis C virus, eIF4F–3’ISS binding affinity to 40S ribosomal subunits had lower affinity (30–32). 3’BTE binding with 40S ribosomal subunits in the presence of eIF4A, eIF4B, and eIF4F is 4-fold tighter than that of 3’ISS. 3’ISS of PEMV binding with the 40S ribosomal subunit was 2-fold tighter than 3’ISS with 40S ribosomal subunit. This suggests that other translation initiation factors may be required for efficient MNeSV RNA binding and translation initiation.

This study showed that eIF4F facilitated 5’UTR–3’ISS interaction in MNeSV. Binding of 5’UTR with 3’ISS in the absence of factors, reported previously, was not detected (16). We employed solution fluorescence anisotropy, which represents an equilibrium condition, whereas the RNA–RNA EMSA technique represents somewhat more static conditions (33). eIF4A–eIF4B–ATP, as a helicase complex, unwound RNA, but this complex had no effect on association of 5’UTR–3’ISS–eIF4F complex, suggesting that long distance 5’–3’ interaction did not require unwinding of structured 5’UTR and 3’ISS.

Our studies also suggest some possibilities for replication regulation in MNeSV. Moderate binding affinity of 40S ribosomal subunits on 3’ISS would lead to stalling of the complex on the 3’ end, which could in turn prevent replication from disrupting ongoing translation. Additionally, our data showed

**Figure 7.** A, normalized anisotropy change for 3’ISS–eIF4F complex binding to 40S ribosomal subunits (red circle) and the effect of the 5’UTR (blue box) (excitation = 492 nm and emission = 519 nm). B, thermodynamic cycle for formation of 5’UTR–3’ISS–eIF4F–40S ribosomal subunit complex. *, Kd value was calculated based on the thermodynamic cycle.
that eIF4A and eIF4B stabilized the eIF4F–3′ISS complex by decreasing the dissociation rate. A lower dissociation rate would increase 3′ISS–eIF4F-bound time and in turn give more chance to initiate translation rather than replication.

Combining previous research results (21) and new conclusions from this study, we propose the following MNeSV translation initiation model (Fig. 8). In step 1, elf4F, including both elf4G and elf4E subunits, binds to the 3′ISS with the aid of elf4A and elf4B. elf4A and elf4B increase eIF4F binding affinity with 3′ISS by inducing an eIF4F conformational change. In step 2, binding of elf4F with 3′ISS facilitates the 5′–3′ long-distance kissing-loop interaction between the 5′UTR and the 3′ISS. The presence of elf4A and elf4B did not affect this long-distance interaction. In step 3, the 40S ribosomal subunit preferentially bind the 3′ISS rather than the 5′UTR. However, the 3′ISS–5′UTR–elf4F complex could be associated with 40S ribosomal subunits. This indicated that the 5′–3′ long-distance kissing-loop interaction and 40S ribosomal subunit binding with 3′ UTR are compatible. Finally, this complex is transferred to the 5′UTR initiation site where translation begins.

**Experimental procedures**

**Materials and methods**

HisTrap™ HP column and Sephadex G-25 media were purchased from GE Healthcare. Cation-exchange cellulose P11 resin was purchased from Whatman. m7GTP-agarose column was purchased from Jena Bioscience. The elf4F recombinant protein expression construct was a generous gift from Dr. Karen Browning (University of Texas, Austin). elf4A and elf4B recombinant protein expression constructs were generous gifts from Dr. D. R. Gallie (University of California, Riverside). All DNA templates were purchased from Integrated DNA Technologies (Coralville, IA). HiScribe™ T7 high-yield RNA synthesis kit was purchased from New England Biolabs. 5′EndTag nucleic acid labeling kit was purchased from Vector Laboratories.

**Expression and purification of recombinant proteins**

Recombinant proteins eIF4F, eIF4A, and elf4B were expressed and purified as described previously, with slight modifications (34). Untagged elf4F was expressed in *Escherichia coli* BL21 (DE3) using a bicistronic pET3d vector containing wheat elf4F and elf4E cassettes. Bacterial cells were grown at 37 °C until the absorbance (A600) reached 0.8 and then were induced for 2 h at 30 °C after adding 0.5 mM IPTG. The collected cells were lysed by sonication. The cell debris was removed with centrifugation. The supernatant was applied to a 20-ml Whatman phosphocellulose column. Bound elf4F was eluted with elution buffer B-300 (20 mM HEPES-KOH (pH 7.6), 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 300 mM KCl). The collected samples from the phosphocellulose column were then applied to a 1-ml m7GTP-agarose column (Jena Bioscience), and elf4F was eluted using elution buffer (20 mM HEPES-KOH (pH 7.6), 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 100 mM KCl, 30 mM GTP). The eluted sample was then applied to a second 1-ml phosphocellulose column and eluted with elution buffer B-300. The eluted fractions were analyzed on SDS-polyacrylamide gels, pooled, and dialyzed against equilibrium buffer B-100 (20 mM HEPES-KOH (pH 7.6), 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 100 mM KCl).

elf4A and elf4B were also expressed in *E. coli* BL21 (DE3). Expression of the protein was induced for 3 h at 37 °C after adding 0.5 mM IPTG. The His-tagged proteins were purified using a 1-ml HisTrap™ HP column according to the manufacturer’s instructions. Eluted proteins were dialyzed against PBS buffer without imidazole.

Purity of the recombinant proteins was checked on SDS-polyacrylamide gels, and protein concentrations were determined using Bradford assay method.

**Purification of 40S ribosomal subunits from wheat germ**

Wheat germ ribosomal subunits were purified using methods reported previously, with minor modifications (35, 36). Wheat germ (Bob’s Mill) was homogenized with powdered alumina in extraction buffer (20 mM HEPES-KOH (pH 7.6), 100 mM KCl, 100 mM MgCl2). The collected supernatant was applied to a 1-ml m7GTP-agarose column, and eIF4F complex was eluted using elution buffer (20 mM HEPES-KOH (pH 7.6), 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 300 mM KCl). The eluted sample was then applied to a second 1-ml phosphocellulose column and eluted with elution buffer B-300. The eluted fractions were analyzed on SDS-polyacrylamide gels, pooled, and dialyzed against equilibrium buffer B-100 (20 mM HEPES-KOH (pH 7.6), 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 100 mM KCl).

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**Purification of 40S ribosomal subunits from wheat germ**

Wheat germ ribosomal subunits were purified using methods reported previously, with minor modifications (35, 36). Wheat germ (Bob’s Mill) was homogenized with powdered alumina in extraction buffer (20 mM HEPES-KOH (pH 7.6), 100 mM KCl, 100 mM MgCl2). The collected supernatant was applied to a 1-ml m7GTP-agarose column, and eIF4F complex was eluted using elution buffer (20 mM HEPES-KOH (pH 7.6), 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 300 mM KCl). The eluted sample was then applied to a second 1-ml phosphocellulose column and eluted with elution buffer B-300. The eluted fractions were analyzed on SDS-polyacrylamide gels, pooled, and dialyzed against equilibrium buffer B-100 (20 mM HEPES-KOH (pH 7.6), 0.1 mM EDTA, 1 mM DTT, 10% glycerol, 100 mM KCl).
mm KCl, 1 mM MgAc₂, 2 mM CaCl₂, and 2 mM DTT, and 10% glycerol) on ice using a cold mortar and pestle. The homogenized samples were centrifuged at 15,000 × g for 15 min at 4 °C, and supernatants were applied to 1.2-liter Sephadex G-25 column. Fractions with A₂₆₀ > 150 units/ml were pooled and centrifuged for 3 h at 170,000 × g. Pellets were suspended with high-salt buffer (0.6 M KCl) and kept in an ice bath for 30 min. The high-salt washed ribosomes were centrifuged at 4 °C for 5 h at 150,000 × g. The pellets were resuspended in storage buffer (20 mM HEPES-KOH (pH 7.6), 50 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 2 mM DTT, and 10% glycerol), aliquoted, and stored at −80 °C. 40S ribosomal subunits were then isolated using a linear 10−30% sucrose gradient. During fractionation, the absorbance at A₂₆₀ was monitored, and fractions with 40S ribosomal subunits were pooled together and dialyzed against storage buffer (20 mM HEPES-KOH (pH 7.6), 5 mM MgCl₂, 50 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 10% glycerol). The quality of 40S ribosomal subunits was assayed on 1% agarose gels, and the concentration was determined using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific).

In vitro transcription and labeling of 3’ISS and mutant RNAs

The WT and mutant 3’ISS RNAs (3’ISS-CA, 3’ISS-C1, and 3’ISS-iA/B2) were transcribed in vitro from DNA oligonucleotides containing the T7 promoter, purchased from Integrated DNA Technologies, Inc. The complementary DNA oligonucleotides were annealed by heating to 94 °C and then slow cooling to 25 °C. RNAs were transcribed in vitro using HiScribe™T7 high-yield RNA synthesis kit (New England Biolabs) and were purified using phenol extraction and ethanol precipitation. Free nucleotides were removed from the purified RNAs using NucAway™ spin columns (Ambion). The purities were determined on denaturing urea-polyacrylamide gels (urea-PAGE), and concentrations were determined using a Nanodrop 1000 spectrophotometer. The RNAs were labeled with fluorescein on at the 5’ end with 5’ EndTag nucleic acid labeling kit (Vector Laboratories) and purified following the manufacturer’s protocol.

Steady-state fluorescence studies

Anisotropy changes of protein binding to the 5’ end-labeled RNAs were monitored on an L-format Spex Fluorolog r2 spectrofluorometer. Fluorescein excitation wavelength was 492 nm, and emission wavelength was 519 nm. Excitation and emission slit widths were 4 and 5 nm, respectively. Slit widths were optimized to decrease light scattering. Fluorescein-labeled RNAs were refolded by heating at 94 °C followed by cooling to 25 °C before starting the reactions. Refolded WT and mutant 3’ISS RNAs were incubated with increasing concentrations of proteins or 40S ribosomal subunits in titration buffer (20 mM HEPES-KOH (pH 7.6), 2.0 mM MgCl₂, 1.0 mM DTT, and 100 mM KCl) at 25 °C, and the change in anisotropy was monitored. Raw anisotropy values were normalized and plotted versus each protein or 40S ribosomal subunit concentration. K_D values were then determined by fitting the normalized data to Equation 1 (37).

**Equation 1:**

\[
r_{ob} = r_{min} + (r_{max} - r_{min}) \frac{(b - 4 \cdot [RNA] \cdot [proteins/40s])}{2 \cdot [RNA]}
\]

where \( b = K_D + [RNA] + [protein/40S] \); \( r_{ob} \) is observed anisotropy; \( r_{min} \) is minimum anisotropy value for RNA alone, and \( r_{max} \) is the maximum anisotropy value obtained at saturation. \( K_D \) is dissociation constant of each interaction. The data were fitted with nonlinear least-squares fitting software KaleidaGraph version 2.1.3 (Abelbeck Software).

**Thermodynamic analysis**

eIF4F interactions with WT and mutant 3’ISS RNA were studied over a temperature range from 5 to 30 °C to determine thermodynamic parameters. Association equilibrium constants were then calculated and plotted versus T⁻¹. Enthalpy change (ΔH), entropy change (ΔS), and free energy change (ΔG) were determined with van’t Hoff Equations 2 and 3,

\[
\ln K_{eq} = \frac{\Delta H}{RT} + \frac{\Delta S}{R}
\]

\[
\ln K_{eq} = \frac{-\Delta G}{RT}
\]

where \( K_{eq} \) is the association equilibrium constants, T is the absolute temperature, and R is the gas constant.

**Stopped-flow fluorescence studies and analyses**

Stopped-flow protein intrinsic fluorescence quenching studies were conducted with an OLIS RSM1000F spectrophotometer. Excitation was from a 450-watt xenon arc lamp at 280 nm. Emission was monitored with a 320-nm cut-on filter. The dead time of the stopped-flow was ~1 ms. Sample mixing chamber temperature was maintained at 25 °C with a circulating water bath and monitored with a thermoprobe. eIF4F and RNAs were prepared in titration buffer (20 mM HEPES-KOH (pH 7.6), 5 mM MgCl₂, 100 mM KCl, and 2 mM DTT). Data were averaged from at least 10 shots and were fitted to both single- and double-exponential functions (38). Single-exponential function is shown in Equation 4,

\[
F_{obs} = F_{min} + A \cdot e^{-k_{on}t}
\]

where \( F_{obs} \) is observed fluorescence at time point t; A is the fluorescence intensity change amplitude; \( F_{min} \) is fluorescence intensity when the reaction reaches equilibrium; and \( k_{on} \) is the observed rate constant. Double-exponential function is shown in Equation 5,

\[
F_{obs} = F_{min} + A_1 \cdot e^{-k_{on1}t} + A_2 \cdot e^{-k_{on2}t}
\]

where \( A_1 \) and \( A_2 \) are amplitudes for the first and second components of reactions with different observed rate constants \( k_{on1} \) and \( k_{on2} \), respectively.

Binding mechanisms were considered by one- and two-step binding processes. For a one-step process, \( k_1 \) and \( k_{-1} \) are forward and reverse rate constants, respectively. Under pseudofirst-order conditions, the observed rate constant and ISS con-
centration were predicted to be linearly related as shown in Equation 6,

$$k_{\text{obs}} = k_1 [\text{RNA}] + k_-1$$  \hspace{1cm} (Eq. 6)\\

For a two-step binding process, the first step is assumed to be a very fast association of eIF4F with RNA followed by slow complex conformation change to a more stable state. The second step causes fluorescence intensity quenching. $1/k_{\text{obs}}$ is linearly related to $1/[\text{ISS}]$ as described previously (26) and as shown in Equation 7,

$$\frac{1}{k_{\text{obs}}} = \frac{1}{k_2} + \frac{K_1}{k_2 [\text{RNA}]}$$  \hspace{1cm} (Eq. 7)\\

with the assumption of $k_2 \gg k_-2$. $K_1$ is the equilibrium constant for first step. $k_2$ and $k_-2$ are the forward and reverse rate constants, respectively for the second step.

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**Bindings of ISS with eukaryotic translation factors**