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A novel mechanism of eukaryotic translation initiation that is neither m^{7}G-cap-, nor IRES-dependent

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ABSTRACT

Resistance of translation of some eukaryotic messenger RNAs (mRNAs) to inactivation of the cap-binding factor eIF4E under unfavorable conditions is well documented. To date, it is the mechanism of internal ribosome entry that is predominantly thought to underlay this stress tolerance. However, many cellular mRNAs that had been considered to contain internal ribosome entry sites (IRESs) failed to pass stringent control tests for internal initiation, thus raising the question of how they are translated under stress conditions. Here, we show that inserting an eIF4G-binding element from a virus IRES into 5′-UTRs of strongly cap-dependent mRNAs dramatically reduces their requirement for the 5′-terminal m^{7}G-cap, though such cap-independent translation remains dependent on a vacant 5′-terminus of these mRNAs. Importantly, direct binding of eIF4G to the 5′-UTR of mRNA makes its translation resistant to eIF4F inactivation both *in vitro* and *in vivo*. These data may substantiate a new paradigm of translational control under stress to complement IRES-driven mechanism of translation.

INTRODUCTION

Translation initiation in eukaryotes occurs via two principal mechanisms, namely, cap-dependent ribosome scanning and internal ribosome entry. In the former case, the ribosomal pre-initiation complex is attached to a messenger RNA (mRNA) through the interaction of the 5′-terminal m^{7}G-cap structure with the cap-binding protein eIF4E followed by unidirectional movement inwards the mRNA until the initiation codon is found (1). At the same time, a few families of viruses do not possess a cap on their mRNAs and circumvent its absence by directly engaging the ribosome to the close proximity of the initiator codon by virtue of internal ribosome entry sites (IRESs), comprising highly specific binding sites for components of the translation machinery. The key feature of the IRES-driven translation is its 5′ end independence.

Under unfavorable conditions eIF4E is displaced out of eIF4F by means of 4E-binding proteins (4E-BPs) (2), thereby disabling ribosome binding to mRNAs via the cap. Likewise, some viruses induce 4E-BP activation during infection and/or encode proteases, which cleave eIF4G in a way that physically separates the eIF4E-binding site from the functional core of the protein, resulting in the inhibition of cap-dependent translation.

Nonetheless, some cellular mRNAs are translated under conditions of stress, apoptosis or virus infection regardless of eIF4F inactivation. The overwhelmingly predominant explanation of this fact is that they also possess IRESs, but this point of view is currently under dispute. The ongoing controversy about the nature of the so-called cellular IRESs has prompted us and others to reassess the criteria used to demonstrate internal ribosome entry, and many of the reported IRESs do not meet them (3–6). However, maintenance of translation of certain mRNAs under stress conditions is well documented and thus awaits explanation. Our recent hypothesis suggested that mammalian mRNAs might exhibit a relaxed cap dependence and resistance to stress because of the presence of so-called cap-independent translation enhancers (CITE) within their untranslated regions (6). CITEs are elements in the mRNA that attract key initiation factors, thereby promoting the assembly of translation initiation complexes. Originally, CITEs were discovered over two decades ago within untranslated regions (UTRs) of mRNAs from some plant viruses, where they provide high levels of translation of naturally uncapped virus mRNAs (7–9). However, this tempting idea failed to disseminate into the field of mammalian translation because of overwhelming predominance of the concept of ‘cellular IRES elements’, which was used to explain many properties of these mRNAs’ 5′-UTRs that were apparently

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unconventional. Thus, we focused on a principal demonstration of the ability of CITEs to function in mammalian cells and their extracts. If successful, this would provide researchers with a new paradigm of translational control.

There are well-known examples of direct binding of initiation factors to 5′-UTRs of mRNAs in higher eukaryotes. These are the IRES elements of picornaviruses, which bind eIF4G through domains J-K [encephalomyocarditis virus (EMCV) or foot-and-mouth disease virus (FMDV)] or domain V (poliovirus) (10–12). We hypothesized that inserting the J-K domain of the EMCV IRES into the 5′-UTR of a cap-dependent mRNA would affect translational properties of the latter, thereby playing the role of a CITE. Indeed, our results suggest that the direct binding of eIF4G dramatically reduces the requirements for the 5′-terminal m^7G-cap and, therefore, increases the resistance to translation inhibition by the inactivation of eIF4F by treatment with competing m^7GTP, by the activity of 4E-BPs or by cleavage of eIF4G by poliovirus 2A protease. Importantly, we show that binding of eIF4G *per se* is not sufficient to result in internal initiation. Thus, the use of an IRES is not the only way to tolerate eIF4E inactivation, revealing the possibility that certain cellular mRNAs known to be more or less resistant to stress do not bear IRESs, but rather contain CITEs that directly or indirectly bind key components of the translational machinery.

MATERIALS AND METHODS

Plasmids

Plasmids pGL-EMCV (13), pGL-L1 (14) and pGL-βGlo (3) have been described, and pGEX-6p-4EBP1 was a gift from N. Sonenberg. DNA corresponding to poliovirus 2A-protease (gift from M. Niepmann) was amplified with primers 2A-dir and 2A-rev and cloned into pET-28a(+) plasmid (Novagen) between BamHI and XhoI sites. To obtain EMCV mutant lacking eIF4G-binding activity (pGL-EMCV^{mut}), the nucleotides corresponding to $UAAAAA_{769\text{-}774}$ of EMCV mRNA were replaced with the sequence AU using primers JK^{mut}-dir and JK^{mut}-rev. To obtain pGL-L1JK, we inserted the JK domain amplified with primers plasmid JK-dir and JK-rev between NarI (that within L1 sequence) and NcoI of pGL-L1. To insert the J-K domain into the 5′-UTR of β-globin, plasmid pGL-βGlo was digested with SpeI, blunted and ligated with the blunted NheI-BalI fragment from pGL-L1JK. pGL-L1JK^{mut} and pGL-βGlo^{mut} were obtained in a similar way. Correctness of all DNA constructs was confirmed by sequencing. For sequences of the oligos see Supplementary Data.

In vitro transcription

Primers used for amplification of transcription templates are given in Supplementary Data. Preparation of m^7G- or A-capped transcripts was described (3). Briefly, templates for transcription were prepared by polymerase chain reaction (PCR) with appropriate oligonucleotides. As forward primers, we used: T7JK for monocistronic JK-Fluc, T7GL3 for all bicistronic and monocistronic Rluc mRNAs, T7GL4 for all monocistronic Fluc mRNAs and T7GL4-ST for monocistronic Fluc mRNAs with a stem-loop at the very 5′-terminus. As reverse primers, we used FLA50 and GL3r for both bicistronic and monocistronic Fluc mRNAs with or without polyA-tail, respectively. After PCR, we purified all templates from agarose gels with the Wizard® SV Gel and PCR Clean-Up System (Promega).

Transcription was performed with RiboMAX™ Large Scale RNA Production System (Promega). For preparation of m^7G- or A-capped transcripts the 3′-O-Me-m^7GpppG or ApppG (the both from New England Biolabs), respectively, were added to the transcription mix in a proportion of 10:1 to GTP. The resulting RNAs were purified by LiCl precipitation and checked for integrity by polyacrylamide or agarose gel electrophoresis.

For transcription of apt5, we used two overlapping oligonucleotides apt5d and apt5r (or apt5c for a control); overhangs were filled in with Taq DNA polymerase and resulting duplex was used as a template for transcription. Apt5 was purified via gel filtration through Sephadex G-50 columns.

Preparation of HEK293T extract

Cultured cells extracts were performed according to a protocol based on previous reports (15,16). Typically, ten 100-mm culture dishes were grown to ~75% confluency (cells must be in a logarithmic growth phase). Cells were rapidly rinsed with 10 ml of cold phosphate buffered saline (PBS), and then scraped on ice into 1 ml of PBS. The cells were then pipetted to achieve a homogeneous suspension, transferred to a cold 15-ml tube and collected for 5 min at 600 r.p.m. Then, cells were suspended in 1 ml of ice-cold PBS and centrifuged one more time (3000 r.p.m., 5 min). After that, cells were suspended in lysolecithin buffer [1 ml per 8×10^7 cells; 20 mM HEPES–KOH (pH 7.4), 100 mM KOAc, 2.2 mM $Mg(OAc)_2$, 2 mM DTT and 0.1 mg/ml lysolecithin], stored for 1 min on ice and rapidly centrifuged for 10 s at 10 000 r.p.m. Then, rapidly but carefully the supernatant was discarded. It is extremely important to perform this latter step fast, as prolonged incubation with lysolecitin causes cells' lysis. Cells were then suspended in equal volume of ice-cold hypotonic extraction buffer [20 mM HEPES (pH 7.5), 10 mM KOAc, 1 mM $Mg(OAc)_2$, 4 mM DTT and Complete Protease Inhibitor Cocktail (EDTA-free; Roche)], incubated for 5 min on ice and disrupted in a tiny Dounce homogenizer by 20–25 strokes. The lysate was clarified by centrifugation for 10 min at 10 000 r.p.m. Aliquots were frozen with liquid nitrogen and stored at −80°C.

In vitro translation

Translation in mammalian cells extracts

Cytoplasmic extract from mouse Krebs-2 ascites cells was prepared as described previously (17). Translation reactions were performed in a total volume of 10 µl, which

contained 50% v/v S30 extract, translation buffer and 20 nM mRNA at 30°C for 1 h (3). If indicated, translation reactions were pre-incubated for 10 min with different amounts of 4EBP1 or m^7GTP [equilibrated with $Mg(OAc)_2$] as described in the text, or with 0.1 μg $2A^{pro}$ with or without 25 μM elastatinal (Sigma). Then, mRNA was added, and the mixture was incubated further for 50 min.

Translation in plant system

Wheat germ extract (WGE), prepared according to (18), was a gift from E. Alkalaeva. The final translation mixture contained 20% v/v WGE, translation buffer [20 mM HEPES–KOH (pH 7.6), 2.5 mM DTT, 0.25 mM spermidine-HCl, 1.25 mM $Mg(OAc)_2$, 16 mM creatine phosphate, 1 mM ATP, 0.6 mM GTP, 35 mM KOAc, 100 μg/ml creatine phosphokinase and 0.1 mM each amino acid] and two units of Human Placental Ribonuclease Inhibitor (Fermentas) and 20 nM mRNA. Translation was performed at 25°C for 1 h (19).

The luciferase activities were measured using the Dual Luciferase Assay or the Luciferase Assay System kit (both Promega), when appropriate.

Real-time translation

Translation was performed in the essentially the same conditions as described earlier, with 0.1 mM luciferin added to the reactions. Extract and mRNA supplemented with buffer and KOAc were pre-incubated separately for 2 min at 30°C, and then rapidly but thoroughly mixed and placed into the temperature-controlled luminometer cell, and luciferase activity was measured every 15 s. Calculations of full translation time were performed as described in (19). Briefly, curves were differentiated with IgorPro software, and the second derivative was fitted by the numerical solutions of a parameterized differential equation system describing normal Gauss distribution, the Gaussian peak being corresponded to the full translation time.

Proteins purification

Poliovirus 2A protease was expressed with an N-terminal His-tag in *Escherichia coli* at 30°C. Expression was induced at OD_{600} of ~0.3 by addition of 10 μM IPTG. Protein was then bound to Ni-NTA-agarose (Qiagen) under denaturing conditions as suggested by manufacturer, but before elution the column was extensively washed with non-denaturing buffer [100 mM KCl and 20 mM Tris–HCl (pH 7.5)], and protein was eluted with standard elution buffer [100 mM KCl, 20 mM Tris–HCl (pH 7.5) and 250 mM imidazole] and then dialyzed against A100 buffer [20 mM Tris–HCl (pH 7.5), 100 mM KCl, 0.1 mM EDTA, 1 mM DTT and 10% glycerol]. This method of purification was found to yield the most active protease.

Cell culture and transfection procedures

HEK293T or BHK21 cells were grown in DMEM supplemented with 10% FBS. Transfection was performed with Lipofectamine 2000 (Invitrogen) or Unifectin 56 (RusBioLink) as described in (3). Briefly, cells were transfected with 0.2 μg of mRNAs mixture, after 3 h washed with PBS, lyzed with Passive Lysis Buffer (Promega) and assayed for luciferase expression with Dual Luciferase Assay Kit (Promega).

Antibodies

Anti-eIF4G1 antibodies were generated in mice against *E. coli*-expressed polypeptide corresponding to amino acids 682–1085 of human eIF4G1 and used as a serum without further purification. Rabbit polyclonal antibodies against 4E-BP1 were purchased from Millipore (AB3251). Rabbit polyclonal antibodies against human PABPC1 were a gift from L.P. Ovchinnikov.

RESULTS

Direct binding of eIF4G to the 5′-UTR of mRNA renders translation cap independent

To test our assumption that the direct binding of eIF4G to an mRNA should modify translational properties of the latter, we generated several monocistronic mRNAs that contained the J-K domain of the EMCV IRES (corresponding to the nucleotides 680–813 of the virus mRNA). The first one directly started with the J-K domain, and in two others, it was inserted either upstream of the β-globin 5′-UTR, or replacing the 3′ half of long interspersed element 1 (LINE-1) 5′-UTR (Figure 1A). Translation of these mRNAs *in vitro* in Krebs-2 ascites cells or HEK293T extract (Figure 1B and D) or *in vivo* in HEK293T or BHK-21 cells (Figure 1C and E) confirmed our prediction that the insertion of the eIF4G-capturing element reduced the cap dependency of these mRNAs, i.e. the stimulating effect of mRNA capping. Notably, this effect was not dependent on the position of the J-K domain within 5′-UTR.

To prove that it is indeed direct eIF4G binding that makes translation of these mRNAs less cap dependent, we created their mutant variants ($UAAAAA_{769\text{-}774}$ to AU), wherein binding to eIF4G was abolished (20). Indeed, this mutation had a severe effect on EMCV translation (see Figure 2A). Importantly, while the translation of m^7G-capped L1-JK or JK-βGlo mRNAs was only moderately affected by the mutation, their A-capped counterparts only retained 5–10% of their activities, thereby dramatically increasing cap dependences, e.g. from 2,9 ± 0.4 to 23 ± 3 in HEK293T cells in the case of L1-JK (Figure 1C). Similar results were obtained for mRNAs lacking a poly(A)-tail (Figure 1F), indicating that the mRNA circularization does not contribute to the observed effect. It is known that eIF4G from wheat germ does not bind the EMCV IRES (21). Therefore, in such system mRNAs with intact or mutated J-K domain should be translated with similar efficiency and similarly high cap dependence. Indeed, hardly any difference could be observed (Figure 1G), consistent with primary role of eIF4G binding in conferring cap-independent translation of the studied mRNAs. Also, we performed translation in Krebs S30 extract in the presence of aptamer (apt5), which was shown previously to bind eIF4G and inhibit cap-dependent translation (22). This apt5 strongly

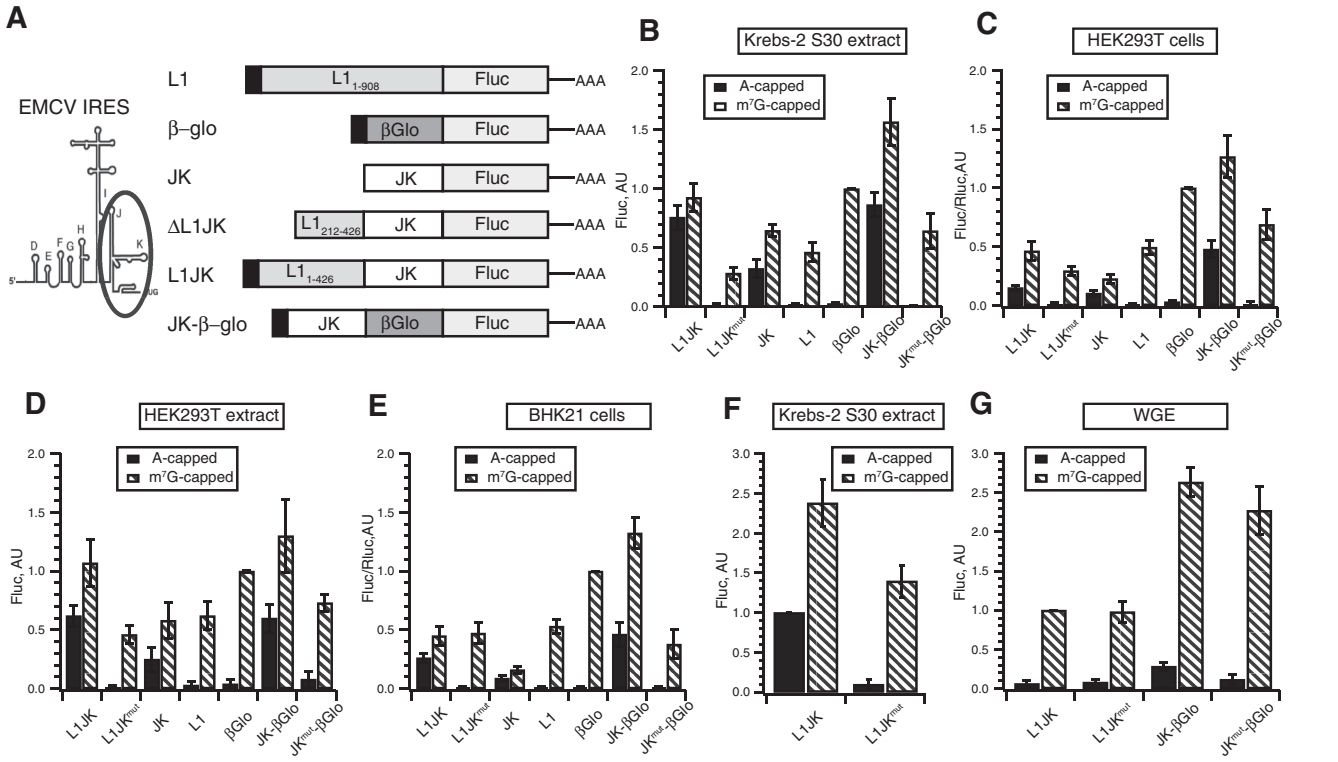

Figure 1. Direct interaction of eIF4G with 5′-UTRs of mRNAs dramatically affects cap dependence of their translation. (**A**) Schematic representation of mRNAs used in the experiments. The black partition represents vector-derived nucleotides. (**B**) Comparison of translation efficiencies of the indicated m^7G- and A-capped monocistronic mRNAs in Krebs-2 cytoplasmic extract. The translation level of m^7G-capped mRNA with the β-globin 5′-UTR is set to 1. (**C**) Results of transfection of the same mRNAs into HEK293T cells. Fluc/Rluc value for m^7G-capped mRNA with the β-globin 5′-UTR is arbitrarily set to 1. (**D**) Translation in HEK293T cells lysate. The translation level of m^7G-capped mRNA with the β-globin 5′-UTR is set to 1. (**E**) Transfection in BHK21 cells. Fluc/Rluc value for m^7G-capped mRNA with the β-globin 5′-UTR is arbitrarily set to 1. (**F**) Indicated A- or m^7G-capped mRNAs lacking poly(A)-tail were translated in Krebs-2 cells S30 extract. The translation level of A-capped mRNA with the L1JK 5′-UTR is set to 1. (**G**) Translation in a WGE. The translation level of A-capped mRNA with the L1JK 5′-UTR is set to 1. All translation and transfection experiments were repeated at least in triplicate.

inhibited translation of m^7G-capped β-globin or LINE1 mRNAs but had even a stimulatory effect on hepatitis C virus (HCV) IRES-driven translation at lower concentrations, most probably because of relieving of competition. However, it only marginally inhibited translation of EMCV, indicating that eIF4G-binding sites of apt5 and EMCV IRES do not overlap or, more likely, just reflecting the difference in binding affinities (22,23). Importantly, translation of L1JK mRNAs was inhibited more strongly than translation of EMCV, but less profoundly than that of β-globin or LINE1 mRNAs (Supplementary Figure S1). This argues against possibility that some other protein could play a key role in cap-independent translation of J-K domain-containing mRNAs.

Translation of mRNA with tethered eIF4G nevertheless requires vacant 5′-termini and is initiated by ribosome scanning

One might argue that high translational activity of L1JK mRNA can be explained by some kind of residual IRES activity of the domain J-K. Notwithstanding the conclusive bulk of mutagenesis reports (24–26), which suggest the J-K domain by itself not being capable of performing IRES function, this however has never been tested directly. Thus, to perform proper controls, we also generated corresponding bicistronic mRNAs. Direct comparison of firefly luciferase expression from the bicistronic mRNA with that from equimolar amounts of monocistronic mRNAs clearly showed lack of IRES activity (Figure 2A). This substantial difference cannot be accounted for by any kind of artificially produced competition with another cistron, inasmuch as it did not vary significantly within three orders of mRNA concentration magnitude (data not shown). Overall, this suggests that the eIF4G-binding translation enhancer strictly requires a free 5′-end for its functioning.

Another distinctive feature of the 5′-dependent translation is its high sensitivity to a secondary structure at the very 5′-end of an mRNA, while the IRES-dependent translation is refractive to such inhibition. Introduction of a 10-bp stem-loop ($\Delta G = -24{,}4$ kcal/mol) to the very 5′-end (no gap between the cap and the stem-loop) of the L1JK mRNA resulted in severe inhibition of translation of both m^7G-capped and A-capped forms (Figure 2B). It should be noted that in mRNAs JK-βGlo-FLuc or JK-Fluc the J-K domain is preceded by 31 and 25 nt, respectively, thus providing a deck for ribosome binding. These results supports the notion that a vacant 5′-end is necessary for CITE to promote translation.

Unquestionably, the translation guided by the J-K domain essentially required a free 5′-terminus, but it was

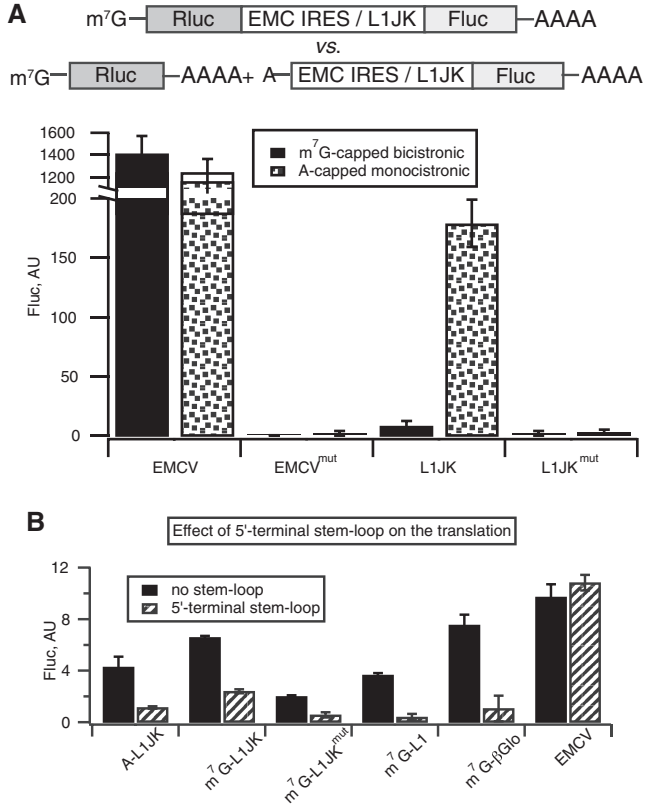

Figure 2. Despite binding of eIF4G at internal positions of mRNAs, the translation is 5′ end-dependent. (**A**) m^7G-capped bicistronic or equivalent equimolar amounts of m^7G-capped Rluc with A-capped Fluc mRNAs were transfected into HEK293T cells. Plotted are relative values of the Firefly luciferase expression. The Fluc value for the bicistronic $EMCV^{mut}$ mRNA is arbitrarily set to 1. (**B**) Results of translation of indicated mRNAs with or without a 5′-terminal stem-loop in Krebs-2 cytoplasmic extract. The Fluc/Rluc value for m^7G-capped mRNA with the β-globin 5′-UTR and the stem-loop is arbitrarily set to 1. Error bars represent standard error of the mean.

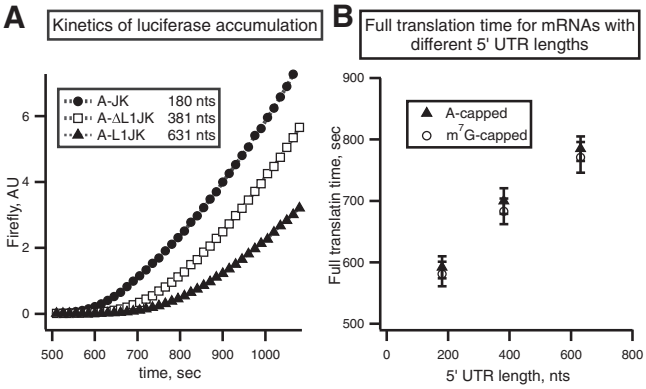

Figure 3. Translation driven by the CITE is initiated by ribosomal scanning. (**A**) Real-time analysis of firefly luciferase accumulation in Krebs-2 extract for A-capped mRNAs with different 5′-UTR lengths (indicated on the plot). (**B**) Full translation times for the m7G- and A-capped mRNAs JK-Fluc, ΔL1JK-Fluc and L1JK-Fluc as calculated from the kinetic curves.

not evident if the translation on the L1JK mRNA involves scanning from the very 5′-end of the mRNA. To address this issue, we took advantage of the ability to directly measure rates of translation initiation in cell-free systems (19). This method is based on real-time evaluation of the synthesized luciferase reporter level. Quantitative dissection of the initial phase of the luciferase accumulation enables one to calculate so-called full translation time, which is the time required for the ribosome to bind mRNA, locate the initiator codon and complete one cycle of protein synthesis. Obviously, if we compare two scanning-dependent mRNAs, which only differ in the lengths of their 5′-UTRs, the difference in their full translation times is accounted for only by the time required for the ribosome to scan the 5′-UTR. We inquired into kinetics of translation of m^7G- and A-capped JK-Fluc (25 nt upstream of J-K domain), L1JK-Fluc (37 nt derived from the vector and 426 nt from L1 and upstream of the J-K domain, 463 in total) and ΔL1JK-Fluc (215 nt from L1 upstream of the J-K domain) mRNAs (Figure 1A). Indeed, as compared with the JK mRNA, we observed a delay in appearance of the luciferase in the case of L1JK-Fluc and ΔL1JK-Fluc mRNAs (Figure 3A), compatible with additional time needed for scanning of their longer 5′-UTRs. There was apparently a linear dependence of full translation time on the length of the 5′-UTR for both A- and m^7G-capped mRNAs (Figure 3B), which is exactly what can be expected for the case of the linear scanning (19). Therefore, these data suggest that despite the ability to bind a component of the translational machinery far from the 5′ end, these mRNAs are scanned by the 40 S ribosome right from their 5′ termini without involvement of internal ribosome entry. Notably, translation of the JK-Fluc mRNA, which possesses a shorter 5′-UTR, resulted initially in a higher Fluc accumulation compared with that of L1JK-Fluc mRNA (Figure 3A). However, translation of the latter significantly increased on a longer incubation (Supplementary Figure S2 and Figure 1), indicating that events other than translation initiation *de novo* contribute to the overall translation potential of a certain mRNA.

eIF4G binding to 5′-UTR renders translation resistant to eIF4F inactivation

The relaxed dependence on the m^7G-cap of the eIF4G-binding mRNAs may have important implications for their resistance to inactivation of eIF4E by means of competition with m^7GTP or the physiologically more relevant displacement from eIF4G by 4E-BP (2). We directly tested this assumption in Krebs-2 cells S30 extract, which is highly sensitive to the aforementioned treatments (3). mRNAs that are unable to directly bind eIF4G ($L1JK^{mut}$ and JK^{mut}-βGlo) behaved just like any other cap-dependent mRNAs, i.e. translation of their m^7G-capped variants was dose-dependently inhibited, and that of their A-capped variants was dose-dependently stimulated by 4E-BP by or m^7GTP competition (Figure 4A and B). On the contrary, both m^7G- and A-capped variants of eIF4G-binding mRNAs that included the intact J-K domain were resistant to the inhibition of cap-dependent translation. Furthermore, their

translation was even stimulated, most probably because of the alleviation of competition of other mRNAs for initiation factors, presumably eIF4F.

Resistance of translation of an mRNA under investigation to cleavage of eIF4G by picornavirus 2A protease is commonly considered as a criterion to confirm the presence of an IRES (27–29). We asked whether translation of our model mRNAs would be at least partially resistant to the protease action and addressed this issue directly by using purified poliovirus 2A protease to cleave eIF4G in Krebs-2 S30 extract. To avoid any secondary effects, we selected such concentration of $2A^{pro}$ that resulted in almost complete cleavage of eIF4G, while integrity of PABP was hardly affected (Figure 4C, left panel). The translation of cap-dependent mRNAs bearing 5′-UTRs of β-globin, β-actin, LINE-1 or Hsp70 5′-UTRs was drastically inhibited, but that of both m^7G- and A-capped mRNAs with intact J-K domain was virtually not affected (Figure 4C, right panel), suggesting that such the tethering of an initiation factor to mRNA can relieve the lack of eIF4G integrity even if the mRNA does not contain an IRES. No effect on translation was observed when elastatinal, which is known to inhibit $2A^{pro}$ (30), was added to translation reactions (data not shown). Also, the activity of c-myc mRNA earlier proposed to harbor an IRES (31) was seriously affected by $2A^{pro}$-mediated eIF4G cleavage, indicating that c-myc mRNA translation is largely dependent on intact eIF4G tethered to the mRNA's cap via eIF4E. Notably, increased sensitivity of c-myc translation to the cleavage of eIF4G may be relevant to the previously reported fact that c-myc mRNA is preferentially associated with eIF4G1 (32), and eIF4G1 is known to be cleaved much faster than another isoform, eIF4G3 (previously named eIF4GII) (33).

Although the aforementioned results look convincing, it was important to reproduce them in cultured cells. To induce 4EBP's dephosphorylation *in vivo,* we used treatments with wortmannin (34), or etoposide (35) or serum deprivation (36). After treatment, HEK293T cells were transfected with mRNAs bearing either the intact or the mutated J-K domain. Although all mRNAs were inhibited, those that contained the functional J-K domain were inhibited less than those that did not (Figure 4D). This indicates that the mRNAs with eIF4G-binding potential are less sensitive to the inactivation of cap-dependent translation than conventional mRNAs.

DISCUSSION

We show here that direct binding of eIF4G to internal positions of the 5′-UTRs of uncapped (A-capped) mRNAs enhanced their translation over an order of magnitude, thereby dramatically reducing their cap dependence both *in vitro* and in living cells. Importantly, such leaders could only provide background levels of the downstream cistron translation when tested in the bicistronic context, indicating that a free 5′-terminus of the mRNA is required for this activity (which by definition excludes

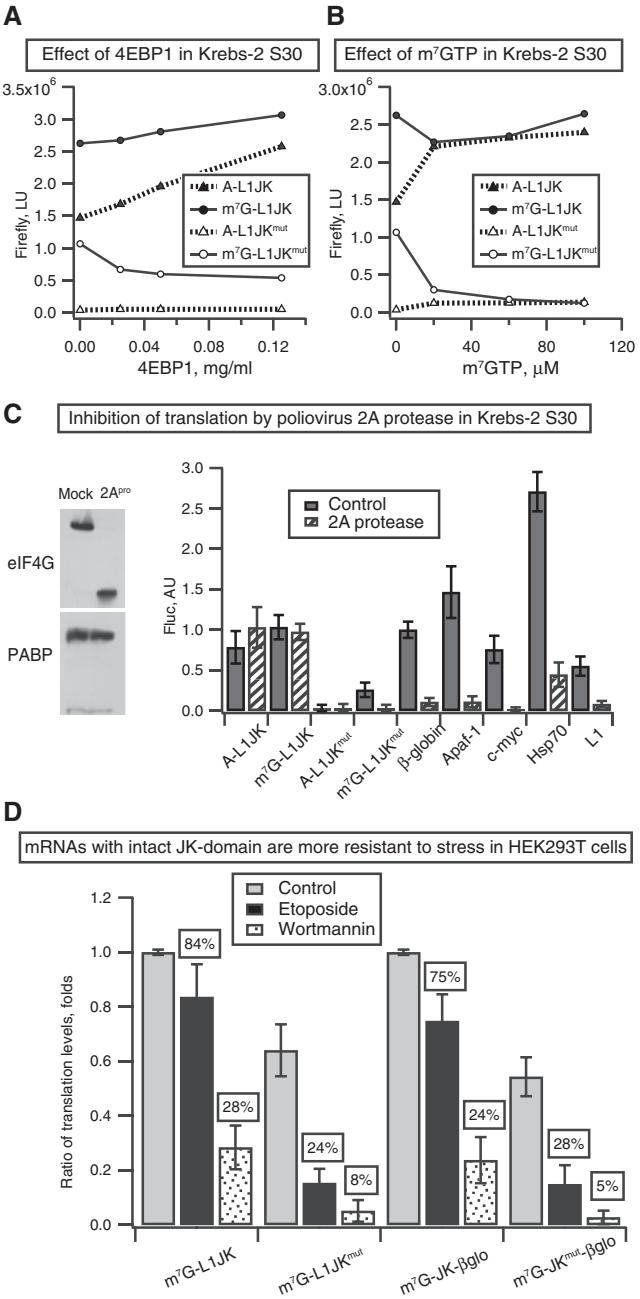

Figure 4. Effect of eIF4E inactivation on the translation of various mRNA constructs *in vitro* in Krebs-2 extract and HEK293T cells. (**A**) An example of the effect of exogenous 4E-BP1 on the translation of the m^7G- and A-capped monocistronic L1JK-Fluc mRNAs containing either an active or disabled binding site for eIF4G. (**B**) Effect of the addition of m^7GTP on the translation of the same mRNAs. (**C**) Effect of eIF4G cleavage by poliovirus 2A protease on the translation of mRNAs with various 5′-UTRs. The translation level of m^7G-capped β-globin mRNA is set to 1. The left panel shows western blot analysis of eIF4G without or with cleavage by 2A protease. (**D**) HEK293T cells were depleted of serum (24 h before transfection), treated with wortmannin (2 h before transfection) or treated with etoposide (36 h before transfection). Then, cells were transfected with the indicated mRNAs, and the expression of reporters was analyzed 3 h later. The data are presented as a ratio of expression from the mRNA with the intact JK-domain to that of the mRNA with the mutated J-K domain for the L1JK-Fluc mRNA or JK-βGlo-Fluc mRNA. The ratio of Fluc/Rluc values of mRNAs with or without intact J-K domain in untreated cells is set to 1. Error bars represent standard error of the mean.

an IRES). Further experiments have shown that although the initial binding of eIF4G within such 5′-UTRs occurs close to the initiator codon, the ribosome nevertheless enters the mRNA at its 5′-terminus (Figure 5). The finding that translation of uncapped mRNAs occurs in a 5′-end-dependent fashion is not unprecedented. However, this was shown either in RRL (37,38), which is intrinsically not much sensitive to m^7G-cap, or in living cells (39), but in the latter case, efficiency of uncapped mRNA translation was low. Thus, we show here for the first time that the translation of uncapped mRNA in animal cells may occur in a 5′-end-dependent fashion with efficiency comparable with that of m^7G-capped mRNA. It is known for two decades that a similar mechanism operates in the case of some plant viruses (7,40); therefore, we show that this phenomenon is more general and not limited to plant cells.

These findings emphasize the notion that we should discriminate between the IRES-dependent translation, which is 5′ independent by definition, and the cap-independent translation, which may be either IRES dependent or CITE dependent, the latter being 5′ dependent. Unfortunately, sometimes cap-independent translation is headlong interpreted as an IRES dependent one without a meticulous proof. Notably, the most challenging crux of the reported cellular IRESs is their inability to provide significant expression level of a downstream cistron when tested in bicistronic mRNAs (3,4), strikingly resembling CITE's behavior.

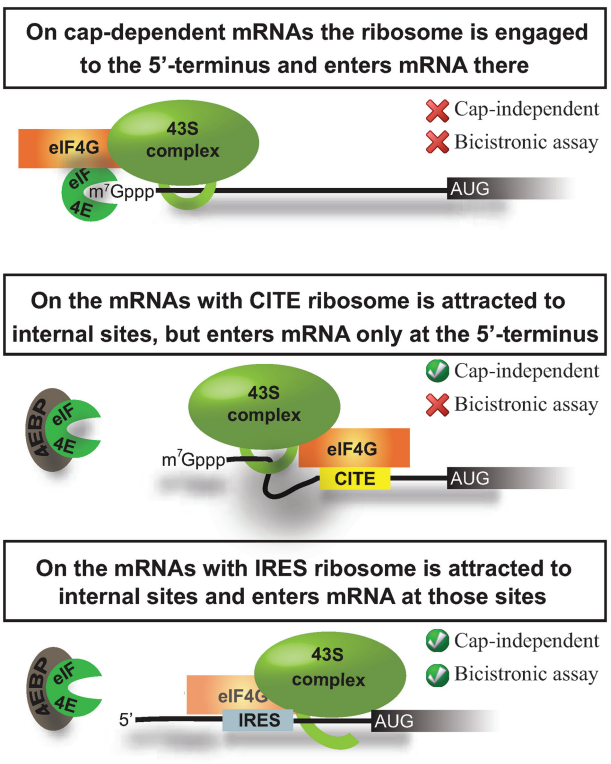

Figure 5. Model of translation mediated by enhancers of cap-independent translation. There are two ways how a 43 S pre-initiation complex can enter the 5′-terminus of an mRNA. The first way is mediated by interaction of the m7G-cap with eIF4E and can be inhibited in various ways. The second one is mediated by an internal element that binds eIF4G. The captured 43 S pre-initiation complex irrespective of the presence of the m7G-cap is then either actively transferred to the 5′-terminus of the mRNA or being snapped in the internal position catches the 5′-end, where it can initiate scanning. In the third mechanism, IRES forces a ribosome to enter mRNA directly into the internal positions of the latter.

Obviously, the lack of a strict m^7G-cap requirement suggests that mRNAs with CITEs should be resistant to inactivation of the cap-binding factor eIF4E. This is indeed the case on the score of the both *in vitro* and *in vivo* data. Disruption of the eIF4E-cap or eIF4G-eIF4E interactions by competition with m^7GTP or with 4E-BP1, respectively, hardly affects translation of the mRNAs that contain intact J-K domain (Figure 4A and B). Activation of endogenous 4E-BP in HEK293T cells under various stress conditions similarly results in a less pronounced inhibition of translation (Figure 4D).

Clearly, the binding of canonical initiation factors or the ribosome itself to internal positions of the 5′-UTRs of mRNAs is required but not sufficient to promote internal initiation. Thus, the most important hallmark of true IRESs may be their ability to promote a correct positioning of the 5′-end-distant regions of mRNA into the RNA-binding cleft of the 40 S ribosome, all other properties being corollary to this one. Indeed, a recent study has shown that the domain IIb of the HCV IRES, which does not contribute to the 40 S or eIF3 binding, is strictly required for accommodation of the IRES's AUG-proximal region to the 40 S ribosome (41). As a consequence, virus IRESs do not require eIF1 or eIF1A (42), unless mechanism of their action involve extensive scanning, e.g. in the case of FMDV (43). Therefore, it is important to note that the conformational changes in the 40 S ribosome that are induced by the HCV IRES resemble those induced by eIF1 and eIF1A (44,45). All this suggests that a bona fide IRES is a functional entity rather than an assembly of independent redundant modular domains. The only notable exception is 5′-terminal IRES of *Rhopalosyphum padi* virus, which can be accommodated by the ribosome because of its single-stranded nature, but in turn, it requires eIF1 and eIF1A (46).

When the domain J-K was tested in bicistronic mRNAs, its mutation reduced translation of the downstream cistron ~3-fold (Figure 2A). These data are reminiscent of those obtained in M. Hentze's laboratory about a decade ago. These authors showed that tethering eIF4E or eIF4G to the intercistronic position resulted in increased translation of the downstream cistron (47,48). However, the level of translation was negligible compared with the cap-dependent translation of the upstream cistron (~2.5%), just as observed in our experiments. In the experiments depicted in Figure 2A, mutation of the domain J-K in the context of bicistronic mRNAs also resulted in appreciable reduction (~2-fold) of the first cistron expression. This indicate that eIF4G-binding sites may also stimulate translation when placed within the 3′-UTR, a conclusion that is in accordance with the finding that a eIF4G-capturing site in a bicistronic mRNA stimulates upstream ORF translation (49). Our preliminary

data indicate that positioning of the J-K domain into the 3′-UTR of the A-capped Renilla luciferase mRNA indeed significantly improves its translation (Supplementary Figure S3). However, it is not legitimate to imply that 3′-CITE and 5′-CITE behave in essentially similar ways. For example, some deletion mutants of CITE from barley dwarf yellow virus, which fail to operate within 3′-UTR, are nevertheless fully functional in the context of 5′-UTR (50).

Virus IRESs provide one more case of initiation factor binding. IRESs of HCV-like type are capable of both eIF3 and 40 S ribosomal subunit binding (51). We mutated the HCV IRES so that it was unable to bind either eIF3 or 40 S, or both. However, these mutants were translated in extremely cap-dependent fashion (data not shown). These data are in line with previously reported inability of the HCV IRES to stimulate translation of the upstream cistron, in contrast to picornavirus IRESs from poliovirus or FMDV (49). Such dissimilitude may have several origins. First of all, it may reflect fundamental difference between the modes of action of these factors in a process of translation initiation. Second, it is possible that binding site for eIF4G provides more flexibility for initiation machinery to interact with mRNA than coiled up HCV IRES.

Recent studies suggest that depletion of eIF4G results in differential effects on the translation of particular mRNAs both in mammalian cells (52) and in yeast (53–55). In this regard, one should recall the old concept of 'weak' and 'strong' mRNAs (56), which predicts that mRNAs with higher rates of ribosome binding (containing CITEs in our case) are preferentially translated under conditions of limited activity of the translational machinery. Our concept does not necessarily require that all stress-tolerant mRNAs bear highly specific sites for initiation factors. Rather, they may contain several sites with lower affinities, which act together to provide a high local concentration of translation machinery components, or the same effect could be achieved by co-localization of a certain mRNA with active initiation factors in specialized cell foci. However, our aforementioned failure to make HCV IRES to function as CITE argues against such simplified interpretation. It is alluring to speculate further that *trans*-acting protein factors may modulate activity of CITEs in mammalian mRNAs, as it occurs in the case of the true IRESs (11,57). They may be represented by mRNA-binding proteins from the same pool as IRES trans-activating factors (ITAFs): PTB, La, unr, PCBP, etc., as many of them were found to associate with 'cellular IRESs' (58).

To summarize, we show here that CITE can confer cap-independent translation in mammalian cells, thus complementing IRES-driven mechanism of translation initiation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Figures 1–3 and Supplementary Methods.

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