Internal Initiation of Translation Directed by the 5'-Untranslated Region of the mRNA for eIF4G, a Factor Involved in the Picornavirus-induced Switch from Cap-dependent to Internal Initiation*

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The eIF4 group initiation factors carry out recognition of the mRNA cap, unwinding of mRNA secondary structure, and binding of mRNA to the 43S preinitiation complex. Infection by picornaviruses results in proteolytic cleavage of one of these factors, eIF4G, an event that severely restricts cap-dependent translation but permits cap-independent initiation to proceed from internal ribosome entry sequences in picornaviral RNAs. The 5'-untranslated region (5'-UTR) of eIF4G mRNA resembles such picornaviral sequences in being unusually long and containing multiple open reading frames and a polypyrrimidine tract. When inserted upstream of a luciferase reporter gene, this 5'-UTR served as a translational enhancer in four different cell lines. Mutation of all four upstream ATG codons to AAG did not alter the translational enhancement. The presence of the eIF4G 5'-UTR between an RNA hairpin and the luciferase cistron stimulated expression 119-fold. Similarly, the presence of the 5'-UTR between the two cistrons of a bicistronic mRNA stimulated expression of the downstream cistron 42-fold. These results indicate that the eIF4G 5'-UTR directs internal initiation. The ability to continue synthesis of eIF4G when the cell is unable to carry out normal cap-dependent translation may represent an autoregulatory mechanism or be part of the cellular response to stresses that interrupt cap-dependent translation.

Initiation of the overwhelming majority of eukaryotic mRNAs proceeds by a cap-dependent mechanism whereby the AUG nearest the 5'-end serves as the initiation codon (1). Yet other modes of initiation codon selection are used in special cases, e.g. leaky scanning, termination-reinitiation, and internal initiation (2). In the latter case, ribosomes are directed to internal AUGs by an internal ribosome entry sequence (IRES) (3, 4). Internal initiation has been demonstrated by both in vivo and in vitro experimentation for picornaviruses, including various members of entero-, rhino-, cardio-, and aphthovirus groups (5), certain other viruses (6–8), and a few non-viral, cellular mRNAs (9–11). For Picornaviridae, the rationale for a cap-independent route is evident: entero-, rhino-, and aphthoviruses encode a protease that cleaves translation initiation factor eIF4G. This action severely inhibits cap-dependent translation (12, 13) but permits IRES-driven internal initiation to continue, actually stimulating it in the case of entero- and rhinoviruses (13–15).

eIF4G forms a heterotrimeric complex termed eIF4F together with eIF4E, the cap-binding protein, and eIF4A, an RNA helicase. The eIF4 group initiation factors (eIF-4A, eIF-4B, eIF-4E, and eIF4G) collectively catalyze the recognition of the mRNA cap, the unwinding of mRNA secondary structure, and the binding of mRNA to the 43S preinitiation complex (16, 17). eIF4G contains binding sites for eIF4E (18, 19) as well as eIF4A and eIF3 (19), the latter being a component of the 43S preinitiation complex. The deavage of eIF4G by picornaviral proteases separates the N-terminal one-third of the molecule, which binds eIF4E, from the C-terminal two-thirds, which binds eIF4A and eIF3, effectively dissociating the cap recognition function from the RNA helicase and ribosome binding functions of the eIF4 factors (19). The cleaved eIF4G molecule is unable to participate in cap-dependent initiation but continues to carry out internal initiation of picornaviral RNAs (2).

The 5'-UTRs of human (20) and yeast (TIF4631 and TIF4632 (21)) eIF4G mRNA are unusual in several respects. First, they are considerably longer (368 for human, 528 for yeast) than the average eukaryotic mRNA 5'-UTR (22). Second, they contain multiple short, open reading frames (4 for human, 11 for TIF4631, 4 for TIF4632). Third, human and yeast TIF4631 mRNAs contain a polyuridymin tract of 9–11 nt located 50–53 nt upstream from the initiation codon. (There is a corresponding U-rich region in TIF4632 mRNA.) All of these features are reminiscent of picornaviral IRES (5). We present evidence here that the 5'-UTR of eIF4G mRNA, like picornaviral IRES, acts as a strong translational enhancer and can efficiently direct internal initiation in vivo.

The luciferase expression vector pGL2-Control (Promega) was either used directly (here designated pGL2/LUC) or modified to contain the 5'-UTR of eIF4G mRNA (pGL2/4G/LUC). cDNA corresponding to nt 1–357 of human eIF4G mRNA was amplified by PCR from pHFC5 (20) with the primers 5'-CCCAAGCTTTCTAGATGGGGGTCC-3 and 5'-CCCCAGCTT- TGATACCTTTCCTCC-3', which created a HindIII site at each end. The product was inserted into the unique HindIII site of pGL2/LUC. The second, third, and fourth upstream ATG codons were changed to AAG by PCR mutagenesis of the above PCR product (23) to create pGL2/4G2-4/LUC, and all four upstream ATG codons were changed to AAG to create pGL2/4G2-4/LUC.

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1 The abbreviations used are: IRES, internal ribosome entry sequence(s); 5'-UTR, 5'-untranslated region; BiP, immunoglobulin heavy chain-binding protein; bp, base pair(s); CAT, chloramphenicol acetyltransferase; eIF, eukaryotic initiation factor; HSP, heat shock protein; nt, nucleotides; PCR, polymerase chain reaction.

2 This was formerly known as eIF-4γ, eIF-4Fγ, and p220 but was recently renamed eIF4G by an Expert Panel of the IUBMB convened on April 8, 1995.
4G1–4LUC. For construction of vectors expressing a bicistronic mRNA, a 736-bp fragment containing the CAT coding region, flanked by 5′- and 3′-UTRs of 54 and 23 bp, respectively, was obtained from pCAT-Basic Vector (Promega) by digestion with BanI and Sall and blunt end-ligated into the XbaI site of pGL2/4G/LUC to produce pGL2/CAT/4G/LUC. A similar insertion was made in pGL2/LUC and pGL2/4G/LUC to produce pGL2/CAT/LUC and pGL2/CAT/4G/LUC, respectively. In all cases, constructions were verified by DNA sequencing or restriction endonuclease analysis.

K562, HeLa CCL2, HEL 92.1.7, and Jurkat cells (ATCC, Rockville, MD) were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. Cells were transfected with 30 μg of each luciferase-expressing plasmid and 5 μg of pCMVβ (Clontech Laboratories, Inc.), the latter being a control plasmid that encodes β-galactosidase. Electroporation was performed in triplicate using a Gene Pulsor (Bio-Rad) set at 970 ohms, 960 microfarads, and 0.22 kV. The number of cells electroporated was 8 × 10⁶ for K562, 4 × 10⁵ for HeLa, and 6 × 10⁵ for HEL and J urkat. Cells were cultured for an additional 18 h and collected, and aliquots of 1 × 10⁶ cells were assayed for luciferase activity (24) using a Monolight 2010 luminometer (Analytical Luminescence Laboratory) and β-galactosidase activity (25). Variations in transfection efficiency were corrected by normalizing luciferase activity in each sample with β-galactosidase activity. CAT activity was measured in aliquots of 4.5 × 10⁶ cells using p-threo-[1,2-14C]chloramphenicol (ICN) as described previously (26).

A probe for ribonuclease protection assays was prepared by digestion of pGL2/LUC with XbaI and EcoRI to produce a 540-bp fragment from the luciferase coding region. This was inserted into pBluescript II SK (Stratagene), which had been cut with the same enzymes. Antisense RNA against luciferase mRNA was produced in vitro with T7 polymerase, and ribonuclease protection assays were performed as described (27).

Human eIF4G mRNA contains open reading frames at nt 6–39, 67–88, 90–108, and 165–171, all upstream of the 4188-nt open reading frame that encodes the protein (20). According to the ribosome scanning model, such a 5′-UTR would be expected to down-regulate translation in a manner highly dependent on the presence of the upstream AUGs. To test this, we placed a DNA segment encoding the first 357 nt of eIF4G mRNA 29 bp upstream of the coding region for a luciferase reporter gene, driven by the SV40 promoter (Fig. 1A). The control vector (pGL2/LUC) and the eIF4G 5′-UTR-containing vector (pGL2/4G/LUC) were used to transfect the human chronic myelogenous leukemia cell line K562. Surprisingly, the 5′-UTR enhanced, rather than repressed, luciferase expression 7.3-fold (Fig. 1B).

To determine whether the upstream open reading frames played a role in translation driven by the eIF4G 5′-UTR, we mutated the vector so as to produce mRNA sequences with either the second through fourth AUGs (pGL2/4G–4LUC) or all four upstream AUGs (pGL2/4G–4LUC; Fig. 1A). The expression of luciferase, however, was unaltered (Fig. 1B), indicating that the upstream AUGs do not play a significant role in the translational enhancement.

The presence of the eIF4G 5′-UTR could conceivably alter steady-state levels of the luciferase mRNA. Hence, we performed ribonuclease protection analysis of luciferase mRNA in cells transfected with each of the four constructs (Fig. 1B, inset). The finding that mRNA levels were similar in all cell lines, and not proportional to luciferase expression, indicates that the 5′-UTR acts at the level of translation rather than transcription, splicing, or mRNA stability.

To determine whether the translational enhancement was unique to K562 cells, we tested other human cell lines of diverse origins. HeLa cells, derived from a cervical carcinoma, HEL 92.1.7 cells, an erythroblast leukemia line derived from malignant peripheral blood, and Jurkat cells, derived from an acute T cell leukemia, were transfected with pGL2/LUC and pGL2/4G/LUC (Fig. 2). Even though the level of luciferase expression was considerably lower in all three cell lines compared with K562 cells (note scale change), the presence of the 5′-UTR of eIF4G mRNA stimulated rather than inhibited expression in...
between the palindrome and the luciferase cistron, expression sequences encoding the 5'-UTR of eIF4G mRNA have good sequence contexts for initiation (20); the second AUG in the 5'-UTR is out of frame with the luciferase coding region; and there was no significant change in expression when these AUGs were altered (Fig. 1B). We tested directly for an internal entry mechanism by two different approaches. First, we placed a palindromic sequence 45 bp upstream of the luciferase coding region (pGL2/H/LUC; see Fig. 3A). The free energy of formation of the transcribed 78-nt RNA hairpin was calculated to be −78.9 kcal/mol using the computer program OLG0 (National Biosciences), considerably more stable than the −50 kcal/mol previously shown to be sufficient for preventing movement of the scanning small ribosomal subunit (28). The hairpin reduced luciferase expression to 2.5% of the control vector containing no palindrome (pGL2/LUC; Fig. 3B). However, when sequences encoding the 5'-UTR of eIF4G mRNA were placed between the palindrome and the luciferase cistron, expression was stimulated 119-fold (pGL2/H/4G/LUC versus pGL2/H/LUC). The presence of the palindrome in the eIF4G 5'-UTR-containing vectors caused only a slight drop (26%) in luciferase expression (pGL2/4G/LUC versus pGL2/H/4G/LUC).

Bicistronic mRNAs have been effectively used in vivo to demonstrate the existence of IRES in both picornaviral (3, 29, 30) and non-picornaviral (9, 10) RNAs. As most ribosomes fail to continue through the intercistronic region, the relative translational efficiency of the downstream cistron is greatly reduced unless preceded by an IRES. As a second approach to test for internal initiation, we inserted DNA encoding the eIF4G 5'-UTR into the 68-bp spacer separating CAT and luciferase cistrons (Fig. 4A). Expression of the downstream cistron was stimulated 42-fold compared with the bicistronic construct containing the spacer alone (Fig. 4C). CAT was synthesized in cells containing both constructs (Fig. 4B), although the presence of the eIF4G 5'-UTR depressed its expression. This has also been observed for in vivo expression driven by both BiP and polio virus IRES (9) and is thought to represent competition between cap-dependent and IRES-dependent translation of bicistronic mRNA.

The foregoing results suggest that eIF4G mRNA is translated in vivo by a cap-independent mechanism involving internal initiation. The presence of multiple upstream open reading frames would normally diminish mRNA translation severely (31), but the 5'-UTR of eIF4G mRNA actually stimulates translation (Figs. 1 and 2). An upstream RNA hairpin drastically inhibits translation, presumably by stopping the scanning small ribosomal subunit (28), but insertion of the 5'-UTR of eIF4G rescues translation (Fig. 3). Finally, the dramatic increase in translation of the downstream cistron of a bicistronic mRNA (Fig. 4) is difficult to explain without invoking internal initiation. Yet it should be noted that all of the experiments described here involve stimulation of a reporter gene and not eIF4G itself.

Although there seems to be a clear rationale for the use of internal initiation by viruses, the advantage of this mechanism of initiation for the cellular mRNAs encoding BiP (9), Antennapedia (10), and basic fibroblast growth factor (11) is more difficult to understand. In the case of eIF4G, which is essential for cap-dependent translation but not for internal initiation, at least in its intact form, there are some intriguing possibilities why its mRNA would utilize internal initiation.

First, this may represent a normal homeostatic mechanism for the maintenance of intracellular levels of eIF4G. If eIF4G levels decreased, the capacity of the cell to carry out cap-dependent initiation would also decrease. The requirement of eIF4G for internal initiation may be less than that for cap-dependent initiation, although this is yet to be demonstrated. If so, the ribosomes and other components of the translational machinery would then become more available for increased
Fig. 4. Expression of CAT and luciferase from bicistronic mRNAs in K562 cells. A, construction of vectors. Vectors encoding bicistronic mRNAs containing CAT and luciferase cistrons were constructed as described in the text. In one case, a DNA segment encoding the 5′-UTR of elf4G mRNA was inserted between them. B, expression of CAT activity. K562 cells were transfected with the plasmids shown in A, and CAT activity was measured as described in the text. The autoradiogram obtained from a thin-layer chromatogram is shown, with positions of the origin (O), chloramphenicol (C), acetylated chloramphenicol (AC; two isomers), and diacetylated chloramphenicol (DAC) indicated. C, expression of luciferase activity in the same transfected cells.

translation of mRNAs that can utilize a cap-independent route. This would in turn increase elf4G levels, completing an autoregulatory loop.

Second, there are physiological events that suddenly switch the cell's predominant mode of initiation from cap-dependent to cap-independent (32). The best understood of these is infection by enterovirus, rhinovirus, and aphthovirus, in which cap-dependent translation decreases drastically while cap-independent translation persists. If elf4G synthesis continues during infection, it may provide a host defense mechanism whereby viral takeover would be slowed, or recovery from infection accelerated, by replenishment of intact elf4G in order to restore cap-dependent initiation. An alternative view is that the virus actually take advantage of the continued synthesis of elf4G during infection. Since internal initiation from the IRES of enterovirus and rhinovirus is stimulated by elf4G cleavage (13-15), presumably through the generation of cleavage products, continued synthesis of elf4G may provide more cleavage products to aid in viral translation. Distinguishing between these possibilities will require additional experimentation.

Another physiological situation in which the predominant mode of translation can switch from cap-dependent to cap-independent is heat shock (reviewed in Ref. 33). In a variety of cell types and species, heat shock causes the synthesis of non-HSP proteins to decrease rapidly, but the synthesis of HSPs remains the same or increases. The change in translational specificity does not require transcription of HSP genes or the appearance of new HSPs in the cytosol. The defect produced by heat shock is preserved in cell lysates and can be corrected in both Ehrlich (34) and Drosophila (35) cell lysates by a complex of elf4E and elf4G. Cells with very low levels of both factors do not recover from heat shock (36). Notably, BIP, which is produced by internal initiation (9) and continues to be translated in poliovirus-infected cells (37), shares many properties with HSPs. It has partial sequence homology with HSP70 and becomes elevated during a variety of stress-inducing conditions. The finding that the 5′-UTR of elf4G mRNA directs internal initiation suggests that elf4G may also continue to be synthesized after heat shock and may accelerate recovery.

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