Contribution of internal initiation to translation of cellular mRNAs containing IRESs

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Abstract
A broad range of cellular stresses lead to the inhibition of translation. Despite this, some cellular mRNAs are selectively translated under these conditions. It is widely supposed that cap-independent internal initiation may maintain efficient translation of particular cellular mRNAs under a variety of stresses and other special conditions when cap-dependent protein synthesis is impaired. However, in spite of a large number of reports focused on the investigation of the regulation of IRES (internal ribosome entry site) activity in different tissues and under various stresses, only rarely is the real efficiency of IRES-driven translation in comparison with cap-dependent translation evaluated. When precisely measured, the efficiencies of candidate IRESs in most cases appeared to be very low and not sufficient to compensate for the reduction of cap-dependent initiation under stresses. The usually low efficiency of internal initiation of translation is inconsistent with postulated biological roles of IRESs.

IRESs (internal ribosome entry sites) in cellular mRNAs

Regulation at the level of translation of mRNA is one of the mechanisms of control of gene expression in eukaryotes. Translation of most eukaryotic mRNAs involves interaction of the mRNA 5′ m7GpppN cap with the eIF4E (eukaryotic initiation factor 4E) subunit of the eIF4F translation initiation complex [1–3]. The binding of eIF4F to mRNA further recruits other initiation factors as well as the 4S ribosomal subunit, and scanning for a favourable AUG initiation codon commences.

A broad range of cellular stresses lead to the inhibition of translation. This is accomplished by the phosphorylation of some initiation factors [4] or by proteolytic cleavage of several initiation factors [5].

An alternative mode of translation initiation that does not require eIF4E and the 5′-cap involves recruitment of the translation initiation complex by an IRES. Translation by an internal ribosome entry was first identified in picornaviruses, but a number of viral and cellular mRNAs have subsequently been found to contain an IRES [6]. Cellular IRES elements are found in a limited but growing number of mRNAs (reviewed in [7,8]). As a rule, such mRNAs contain IRES elements in their 5′-UTR (untranslated region). Interestingly, they are found preferentially in the mRNAs of genes involved in the control of cellular proliferation, survival and death (e.g. bFGF-2 (basic fibroblast growth factor-2) [9], VEGF (vascular endothelial growth factor) [10], the oncogene c-myc [11], BiP (immunoglobulin heavy-chain-binding protein) [12], NF-κB (nuclear factor κB)-repressing factor [13], PITSLRE protein kinase [also known as CDK11 (cyclin-dependent kinase 11)] [14], DAP5 (death-associated protein 5) [15], X chromosome-linked inhibitor of apoptosis protein XIAP (X-linked inhibitor of apoptosis) [16], Apaf-1 (apoptotic protease-activating factor 1) [17], anti-apoptotic protein Bcl-2 [18] and HIF-1α (hypoxia-inducible factor-1α) [19]). It is widely supposed that cap-independent internal initiation may maintain efficient translation of particular cellular mRNAs under a variety of stresses and other special conditions when cap-dependent protein synthesis is impaired (reviewed in [20]).

However, the debates over the existence and biological significance of cellular IRESs continue [21,22]. The standard assay for evaluation of IRES activity is the ‘bicistronic’ test. Bicistronic mRNA, which can be produced and translated either in vivo or in vitro, is designed to contain candidate IRESs between two reporter genes (also called cistrons). The first cistron measures cap-dependent initiation, whereas the second reflects the internal initiation of translation.

Some candidate IRESs were subsequently dismissed because the sequences were shown to harbour cryptic transcriptional promoters or splice sites (reviewed in [23]). This could lead to generation of monocistronic mRNA, containing only the second gene, that could be translated by the regular ribosome scanning mechanism.

Re-evaluation of the 5′-UTRs of PDGF (platelet-derived growth factor) [24], PIM-1 [25] and the human CDK inhibitor, p27Kip1 [26], has shown that they do not have IRESs as was initially thought [27–29], but the sequences are able to function as promoters. Cryptic promoter activity was detected in many other putative IRESs, including c-myc, VEGF and hepatitis C virus [30].

Key words: cap-independent initiation of translation, internal ribosome entry site (IRES), plant mRNA.

Abbreviations used: Apaf-1, apoptotic protease-activating factor 1; BiP, immunoglobulin heavy-chain-binding protein; CDK, cyclin-dependent kinase; crTMV, crucifer-infecting tobamovirus; DAP5, death-associated protein 5; eIF4E, eukaryotic initiation factor 4E; ECV, encephalomyocarditis virus; Fvuc, firefly luciferase; GFP, green fluorescent protein; HRIF-1α, hypoxia-inducible factor-1α; IRES, internal ribosome entry site; NF-κB, nuclear factor κB; RLuc, Renilla luciferase; RRL, rabbit reticulocyte lysate; TEV, tobacco etch virus; UTR, untranslated region; VEGF, vascular endothelial growth factor; XIAP, X-linked inhibitor of apoptosis.

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Relative efficiencies of internal initiation and cap-dependent translation

To prove the hypothesis that translation of a particular mRNA under stress conditions is due to IRES elements, it is necessary to evaluate the real contribution of IRES-driven initiation to the overall translation. However, in spite of a large number of reports focused on identification of new IRESs and investigation of the regulation of IRES activity in different tissues and/or under various stresses, only rarely is the real efficiency of IRES-driven translation in comparison with cap-dependent translation evaluated.

Tests for internal initiation often use the putative IRES from EMCV (encephalomyocarditis virus) [31] as a positive control. There were several attempts to determine the efficiency of EMCV IRES-dependent gene expression in comparison with that of cap-dependent expression. The results were highly variable depending on the experimental design. Mizuguchi et al. [32] compared IRES-dependent second gene expression with cap-dependent first gene expression of bicistronic RNA in several cultured cell lines and in mouse liver in vivo and found that the expression of the second gene ranged from 6 to 100% of that of the first gene, depending on the order of reporter genes and translation systems. However, the efficiency of internal initiation in principle should not depend on the choice of reporter genes. The control experiments confirming the integrity of mRNA were not reported in the above mentioned study. Bert et al. [33] reported 50% activity of EMCV IRESs in experiments on transfection of HeLa cells. Later, Wang et al. [25] transfected the in vitro-produced bicistronic mRNAs containing EMCV IRESs between Renilla and firefly luciferases (RLuc and FLuc respectively) into Cos-7 cells and found that the yield of FLuc from the 3’-cistron was only 3% of that of RLuc. These discrepancies may be explained by the recent finding of cryptic promoter in EMCV IRESs [34].

Nevertheless, EMCV is often used as a positive control in experiments on IRES investigation. Activity of Apaf-1, DAP5 and Bip IRESs was found to be less than 5% of EMCV IRES activity in HEK-293T [HEK-293 cells (human embryonic kidney cells) expressing the large T-antigen of SV40 (simian virus 40)], human cervical carcinoma (HeLa), human bladder carcinoma (T24), human glioblastoma (SF539), CHO (Chinese-hamster ovary) and mouse fibroblast (NIH 3T3) cell lines that were transfected with plasmids capable of producing bicistronic reporter RNAs (Table 1). However, etoposide-induced apoptosis enhanced Apaf-1 and DAP-5 IRES-dependent translation up to 3-fold in comparison with non-stress conditions in transfected 293T cells. It should be mentioned that the increase in IRES efficiency, in this case, leads to only 10% activity in comparison with EMCV IRESs. Thus Apaf-1 and DAP-5 IRESs show low activity, and elevation of IRES-dependent translation is not enough to compensate for the reduction of cap-dependent initiation [35].

In experiments on transfection of HeLa cells with in vitro-synthesized mRNA, it was demonstrated that bicistronic mRNA containing the HIF-1α, VEGF, c-myc and XIAP 5’-UTR consistently and reproducibly produced more downstream protein than the bicistronic control, which has only a linker sequence between the two cistrons, but the level of activity of these putative IRESs was only 1–2% of the activity of the EMCV IRES [33]. The level of expression of the reporter protein driven by these cellular IRESs was also less than 2% of that produced from capped monocistronic mRNAs containing these sequences in 5’-UTRs [33]. Together, these results suggest that the HIF-1α, VEGF, c-myc and XIAP IRESs support some degree of internal ribosome entry, but the IRES activities are very low in comparison with cap-dependent translation (Table 2).

IRES elements in plant viral and cellular RNAs

The above-mentioned IRESs were found in animal viruses or cells. The plant translational machinery can mediate cap-independent translation on IRESs from plant and mammalian viruses [36–38], but so far only a few examples of IRES elements in plant cellular mRNAs have been described.

Several examples of IRESs present in genomes of plant viruses are known. For example, IREScp148 precedes the CP (coat protein) gene of crTMV (crucifer-infecting tobacco virus). Comparison of the relative activities of the IREScp148

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**Table 1 | Activity of IRESs in comparison with EMCV IRES**

<table>
<thead>
<tr>
<th>IRES</th>
<th>Bicistronic vector</th>
<th>Expression system</th>
<th>Relative efficiency (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apaf-1</td>
<td>βgal-CAT</td>
<td>HeLa cells</td>
<td>2</td>
<td>[35]</td>
</tr>
<tr>
<td>DAP5</td>
<td>βgal-CAT</td>
<td>HeLa cells</td>
<td>2</td>
<td>[35]</td>
</tr>
<tr>
<td>Bip</td>
<td>βgal-CAT</td>
<td>HeLa cells</td>
<td>2.5</td>
<td>[35]</td>
</tr>
<tr>
<td>IREScp148</td>
<td>Cp-uda</td>
<td>Tobacco protoplasts</td>
<td>170</td>
<td>[40]</td>
</tr>
<tr>
<td></td>
<td>(gfp-uda)</td>
<td>HeLa cells</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Yeast cells</td>
<td>1000</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RRL</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>NIHFSF-1</td>
<td>gfp-uda</td>
<td>Tobacco protoplasts</td>
<td>120</td>
<td>[40]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>HeLa cells</td>
<td>270</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>RRL</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>NF-κb</td>
<td>RLuc-Fluc</td>
<td>C243 cells</td>
<td>1000</td>
<td>[46]</td>
</tr>
<tr>
<td>PITSRRE kinase</td>
<td>RLuc-Fluc</td>
<td>RRL</td>
<td>2</td>
<td>[14]</td>
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</tbody>
</table>
elements and the EMCV IRESs in plant, yeast and HeLa cells identified the 148-nt IREScp148 as the strongest element that also displayed IRES activity across all kingdoms. IREScp148 was 170% as active as EMCV in tobacco protoplasts, 300% in HeLa cells, 30% in RRL (rabbit reticulocyte lysate) and 1000% in yeast. However, it was shown later that the observed activity of IREScp148 in yeast cells is due to the presence of cryptic promoter in this sequence, which is active in yeasts [39]. EMCV does not provide internal initiation in wheatgerm extract at all, whereas IREScp148 provides effective translation of mRNA [40].

The 5′-leader of TEV (tobacco etch virus) genomic RNA directs efficient translation from the naturally uncapped viral mRNA. Two distinct regions within the TEV 143-nt leader confer cap-independent translation in vivo (carrot protoplasts) and in vitro (wheatgerm extract) when present in the intercistronic region of a bicistronic mRNA, indicating that the TEV leader contains an IRES [41].

The first IRES in plant cellular mRNA was found in the 5′-UTR of the maize HSP (heat-shock protein) gene HSP101 [42], the second example is the leader sequence of the Arabidopsis thaliana RPS18C gene, which is able to trigger the cap-independent translation in vitro [43]. The actual contribution of these IRES elements to the overall translation rate of the corresponding mRNA was not studied.

Adh1, the maize gene encoding ADH1 (alcohol dehydrogenase-1), mRNA is efficiently translated in O2-deprived roots of maize, whereas many normal cellular mRNAs are poorly translated. By using a reporter gene assay, we showed that adh1, the leader sequence of adh1 mRNA, provides efficient translation of the reporter GFP (green fluorescent protein) gene in Nicotiana benthamiana cells under oxygen shortage or heat shock [44]. The presence of this leader sequence in the 5′-UTR of mRNA did not change the level of expression under aerobic conditions, but under oxygen shortage or heat shock the levels of GFP expression were reduced 5–10-fold in the absence of the leader and remained unaffected if adh1 was present in 5′-UTR. Consequently, adh1 acts as a translational enhancer providing efficient translation of mRNA in plant cells under stress conditions [44].

Using bicistronic assays, we found [45] that adh contains IRESs active both in vivo, in N. benthamiana cells and in vitro, in the RRL translation system. Efficiency of adh-driven translation was comparable with that of IREScp148 [40] from crTMV.

Then, we quantitatively measured the contribution of cap-dependent ribosome scanning and internal initiation to translation of monocistronic mRNA with an adh leader in plant cells. Insertion of a 60-bp inverted repeat was expected to block cap-dependent translation, while deletion of adh removed internal initiation. Surprisingly, we found that the real contribution of adh-dependent internal initiation is only approx. 0.3% of the total translation level of mRNA in N. benthamiana cells under normal conditions and less than 1% under heat shock [45]. Consequently, IRES activity of adh is too weak to explain the activity of adh as a stress-specific translational enhancer.

**Concluding remarks**

Since IRES-mediated initiation avoids a requirement for eIF4E, it provides a logical mechanism for the preferable translation under stress conditions. However, although it was reported that the 5′-UTRs of c-myc, Apaf-1, Bcl-2, adh1, HIF-1α, VEGF, XIAP, DAP5, BiP and others do have greater IRES activity than a negative control RNA (only a linker sequence between the two cistrons), the amount of protein produced as a result of IRES activity is low compared with the amount that is produced from cap-dependent translation. It does not mean that these IRESs do not really exist, but it cannot be concluded that IRES elements are responsible for cell survival until it is shown that the level of IRES-dependent translation is sufficient to compensate for reduction of cap-initiated translation.

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### References


<table>
<thead>
<tr>
<th>IRES</th>
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<th>Expression system</th>
<th>Relative efficiency (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMCV</td>
<td>RLuc-Fluc</td>
<td>Cos-7 cells</td>
<td>3</td>
<td>[25]</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>RLuc-Fluc</td>
<td>HeLa cells</td>
<td>0.1</td>
<td>[33]</td>
</tr>
<tr>
<td>VEGF</td>
<td>RLuc-Fluc</td>
<td>HeLa cells</td>
<td>1</td>
<td>[33]</td>
</tr>
<tr>
<td>XIAP</td>
<td>RLuc-Fluc</td>
<td>HeLa cells</td>
<td>1</td>
<td>[33]</td>
</tr>
<tr>
<td>c-myc</td>
<td>RLuc-Fluc</td>
<td>HeLa cells</td>
<td>0.3</td>
<td>[33]</td>
</tr>
<tr>
<td>Cat-1</td>
<td>CAT-LUC</td>
<td>RRL</td>
<td>1</td>
<td>[47]</td>
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