

# Differential contribution of the m<sup>7</sup>G-cap to the 5' end-dependent translation initiation of mammalian mRNAs

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## ABSTRACT

Many mammalian mRNAs possess long 5' UTRs with numerous stem-loop structures. For some of them, the presence of Internal Ribosome Entry Sites (IRESes) was suggested to explain their significant activity, especially when cap-dependent translation is compromised. To test this hypothesis, we have compared the translation initiation efficiencies of some cellular 5' UTRs reported to have IRES-activity with those lacking IRES-elements in RNA-transfected cells and cell-free systems. Unlike viral IRESes, the tested 5' UTRs with so-called 'cellular IRESes' demonstrate only background activities when placed in the intercistronic position of dicistronic RNAs. In contrast, they are very active in the monocistronic context and the cap is indispensable for their activities. Surprisingly, in cultured cells or cytoplasmic extracts both the level of stimulation with the cap and the overall translation activity do not correlate with the cumulative energy of the secondary structure of the tested 5' UTRs. The cap positive effect is still observed under profound inhibition of translation with eIF4E-BP1 but its magnitude varies for individual 5' UTRs irrespective of the cumulative energy of their secondary structures. Thus, it is not mandatory to invoke the IRES hypothesis, at least for some mRNAs, to explain their preferential translation when eIF4E is partially inactivated.

## INTRODUCTION

Eukaryotic cells use two principal ways of translation initiation, a 5' end dependent (cap-dependent) mode and the mechanism of internal ribosome entry. The first mode can be employed by all eukaryotic cellular mRNAs, the second one was initially demonstrated for genomic RNAs from viruses with RNA positive genomes that replicate in the cytoplasm and have no m<sup>7</sup>G-cap at the 5' end (picornaviruses, some flaviviruses, pestiviruses) (1). The existence of IRES elements within the 5' UTRs of these viral RNAs was first inferred from experiments with dicistronic DNA constructs (2), and for some representatives of the picornavirus family, pestiviruses and flaviviruses was definitely proven by direct reconstitution of translation initiation complexes from purified components combined with site-specific mutagenesis (3–6). Moreover, the sites of binding of key canonical and auxiliary factors within the IRESes of these viral mRNAs were determined, thereby giving us some idea as to how these IRES elements work (4,7–9).

Later, the IRES elements were also proposed for 5' UTRs of many cellular mRNAs (see refs 10–13 and references cited therein) which are known to be all capped. They have usually been found within the 5' UTRs with complex secondary structure, and, as a rule, their corresponding mRNAs encode proteins with regulatory functions. The concept of cellular IRESes became very attractive because it helped to explain how ribosomes cope (10) with numerous stem-loop structures present within their 5' UTRs. The IRES concept is also appealing as an explanation of the relative resistance of some mRNA translation to special conditions of cell

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stress, mitosis or apoptosis when the cap-dependent translation is compromised (11).

However, unlike viral IRESes, for none of numerous cellular IRESes proposed to date has the molecular mechanism of their operation been dissected. To demonstrate the presence of an IRES in a 5' UTR, the dicistronic test has been employed as the 'gold standard'. According to this criterion, the sequence is declared as possessing IRES activity, if it only stimulates, *to any extent*, the translation of the second gene in the dicistronic construct, as compared to the 'empty' vector. Until recently, the most popular variant of the dicistronic test usually applied has been the transfection of cultured cells with a dicistronic DNA plasmid (for *in vivo* tests) and translation of the corresponding dicistronic transcript *in vitro* in nuclease treated rabbit reticulocyte lysate (RRL), the widely used mammalian cell-free system. However, both of these methods have been recently shown to produce a collection of artifacts (14–22). The RNA transfection of cultured cells has been suggested as an alternative approach. However, the poor activity of some cellular IRESes was revealed in these RNA dicistronic tests raising concern about its functional significance.

The classical IRES elements from viral RNAs can be quite distinct in secondary and tertiary structures (e.g. compare EMCV and HCV IRESes). However, all studied viral IRESes have one common feature: they all have highly specific sites for binding key translation initiation components (1). Consequently, even point mutations in these or other functionally important sites produce a killing effect on the IRES activity (4,7–9,23,24). To the best of our knowledge, such 'killing mutations' have not been reported for cellular IRESes. In most cases, attempts to localize a 'key sequence' or a 'critical structural element' within proposed cellular IRESes have been unsuccessful. In those few cases when such 'critical sequences' are found, the translation components that recognize them have not been identified (25,26). This situation prompted us to think about an alternative explanation for the differential effect of stress conditions on the translation initiation of individual mRNAs.

In this study, we have compared with each other the translation initiation potentials of several 5' UTRs (derived from  $\beta$ -actin,  $\beta$ -globin, LINE-1, Apaf-1, c-Myc, hsp70 and several viral mRNAs) in different constructs and under different conditions. We show that, unlike in monocistronic constructs, all the sequences tested, except viral IRESes, demonstrate an extremely low translation level when placed between two cistrons. Having proceeded to monocistronic constructs, we show that the selected capped 5' UTRs with putative IRES-elements are unexpectedly efficient in the cap-dependent translation, irrespective of their length and GC content, contradicting the generally accepted view (27).

The m<sup>7</sup>G-cap is still involved in the mechanism of translation initiation when the process is strongly inhibited by addition of the recombinant 4E-BP1 to the cell-free system, thereby mimicking stress conditions. However, the potential to direct translation initiation under these inhibitory conditions varies between different cap-dependent 5' UTRs, thereby providing an alternative

explanation for a relative resistance of translation of some mRNAs to stress conditions.

## MATERIALS AND METHODS

### Plasmid constructs

All dicistronic DNA constructs were prepared on the basis of pGL3R vector (28). The plasmids containing full-length LINE-1 5' UTR (29), hsp70-1A 5' UTR (the variant most widely represented in human EST sequences) (30), EMCV mut (30) and complete HCV IRES (nts 40–375) (29) were described previously. Rluc-HRV-Fluc plasmid (28) was a gift from A. Willis. For other constructs, RT-PCR fragments were obtained with total RNA from HEK293T cells (for  $\beta$ -actin, Apaf-1 and c-Myc) or RRL (for  $\beta$ -globin). For cDNA synthesis, dT<sub>16</sub> primer was used, followed by PCR with the pairs of gene-specific primers: CCGGCACTAGTGTAAATACGACTCACTATAGGACCGCCGAGACCGCGTCC and CGCGCCATGGTGAGCTGGCGGGCGGGTGTGGAC for  $\beta$ -actin, AAGAAGAGGTAGCGAGTGGACG and CGCGCCA TGGTCCCTCAGATCTTTCTCTCTCTG for Apaf-1, ACTTGCTTTTGACACAACCTG and CGCGCCATGG TCTGTCTGTTTTGGGGGATTGC for rabbit  $\beta$ -globin, CTGCGCGCAACTCGCTGTAGTAATTCC AGC and CGCGCCATGGAGATATCCTCGCTGGG CGC for c-Myc [which results in a fragment corresponding to the complete P2 promoter-directed 5' UTR plus the first 150 nt of coding region, see ref. (31)]. The resulting PCR products were inserted into pGL3R vector digested with PvuII and NcoI. The plasmid containing encephalomyocarditis virus (EMCV) IRES (nts 315–846) was obtained in a similar way using SmaI-NcoI fragment from the plasmid pTE1 (32). pRluc plasmid used for the monocistronic Rluc mRNA production was described in (29).

All mutants of the Apaf-1 5' UTR were prepared by PCR with corresponding primers; mut  $\Delta$ 1- $\Delta$ 4 correspond to deletions of nts 27–98, 106–345, 354–426 and 455–554, respectively. For the AUG1 mutant, nts 216–237 were replaced by aaaaccatggataaa (or by aaaacc taggataaa for UAG1). For AUG2 and UAG2 mutants, nts 434–443 were replaced by the same sequences as in the case of AUG1 and UAG1, respectively.

### mRNA preparation

To prepare capped polyadenylated mRNAs, PCR products were first obtained from the corresponding plasmids. The same reverse primer T<sub>50</sub>AACTTGTTTAT TGCAGCTTATAATGG was used along with the T7 promoter containing 5' UTR-specific primers (CGCCGT AATACGACTCACTATAGGGACACTTGCTTTTGA CACAACCTGTG for  $\beta$ -globin, CCGGCACTAGTGTAA TACGACTCACTATAGG for  $\beta$ -actin, CCGCGTAAT ACGACTCACTATAGGGCGGAGGAGCCAAGATG GCCGAATAGG for LINE-1, CGCCGTAATACGACT CACTATAGGGAAGAAGAGGTAGCGAGTGGACG for Apaf-1, CGCCGTAATACGACTCACTATAGGG CTGCGCGCAACTCGCTGTAGTAATTCCAGC for c-Myc), or with universal primer CGCCGTAATACGA

CTCACTATAGGGAGCTTATCGATACCGTCG annealing to pGL3R region upstream of a 5' UTR (in the case of the viral IRESes). The PCR products were used then as templates for *in vitro* RNA transcription by T7 RiboMAX Large Scale RNA Production kit (Promega). For preparation of m<sup>7</sup>G- or A-capped transcripts the 3'-O-Me-m<sup>7</sup>GpppG or ApppG (NEB) was 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 PAGE.

### Cell culture and transfection procedures

HEK293T and HeLa cells were cultivated in DMEM supplemented with 10% FBS, as described (29). The day before transfection, exponentially growing cells were replated to 24-well plates at densities 1:2 or 1:3 (for RNA or DNA transfection, respectively). After 12–16 h of growth, when the cell density reached 60–80% (for RNA) or 50–60% (for DNA), transfection was performed using Lipofectamin 2000 (Invitrogen). DNA was transfected exactly as recommended by manufacturer. For a typical RNA transfection, 0.5 µg of reporter mRNA (or equimolar Fluc and Rluc encoding mRNA mix, 0.5 µg in total) was incubated with 1.3 µl of the transfection reagent in 50 µl DMEM for 20 min and then added into the growth medium. Two hours later, cells were harvested and luciferase activities were analyzed with the Dual Luciferase Assay kit (Promega). All the transfections were repeated several times in different cell passages. For the most important experiments, the procedure was repeated with an alternate transfecting reagent, Unifectin (RusBioLink) (29).

### Preparation of Krebs-2 cells S30 extract and *in vitro* translation

S30 extracts were prepared as described in (16). Briefly, Krebs-2 ascites cells were collected in a centrifuge tube with isotonic buffer (150 mM NaCl, 35 mM Tris-HCl pH 7.5), and four times washed with centrifugation (Beckman JA-20, 1200 rpm, 10 min) in equal volumes of the same buffer. After the fourth centrifugation, cells were re-suspended in 1.5 volumes of hypotonic buffer (10 mM Tris-HCl pH 7.5, 10 mM KCH<sub>3</sub>COO, 1.5 mM Mg(CH<sub>3</sub>COO)<sub>2</sub>, 2.5 mM DTT) and incubated on ice for 20 min. Then the cells were disrupted using a Dounce homogenizer (type B pestle, 15–20 strokes), and the lysate was centrifuged for 20 min at 15 000 rpm. The supernatant was aliquoted and stored at –75°C. Translation experiments were performed in a total volume of 10 µl, which contained 5 µl of the S30 extract, translation buffer (20 mM HEPES-KOH pH 7.6, 1 mM DTT, 0.5 mM spermidine-HCl, 0.6 mM Mg(CH<sub>3</sub>COO)<sub>2</sub>, 8 mM creatine phosphate, 1 mM ATP, 0.2 mM GTP, 120 mM KCH<sub>3</sub>COO and 25 µM of each amino acid), 2 units of Human Placental Ribonuclease Inhibitor (HPRI, Fermentas) and 0.25 pmol mRNA (or mRNA mix), for 1 h. The luciferase activities were measured using the Dual Luciferase Assay kit. Translation in nuclease treated RRL (Promega) was performed exactly as suggested by the manufacturer, with the addition

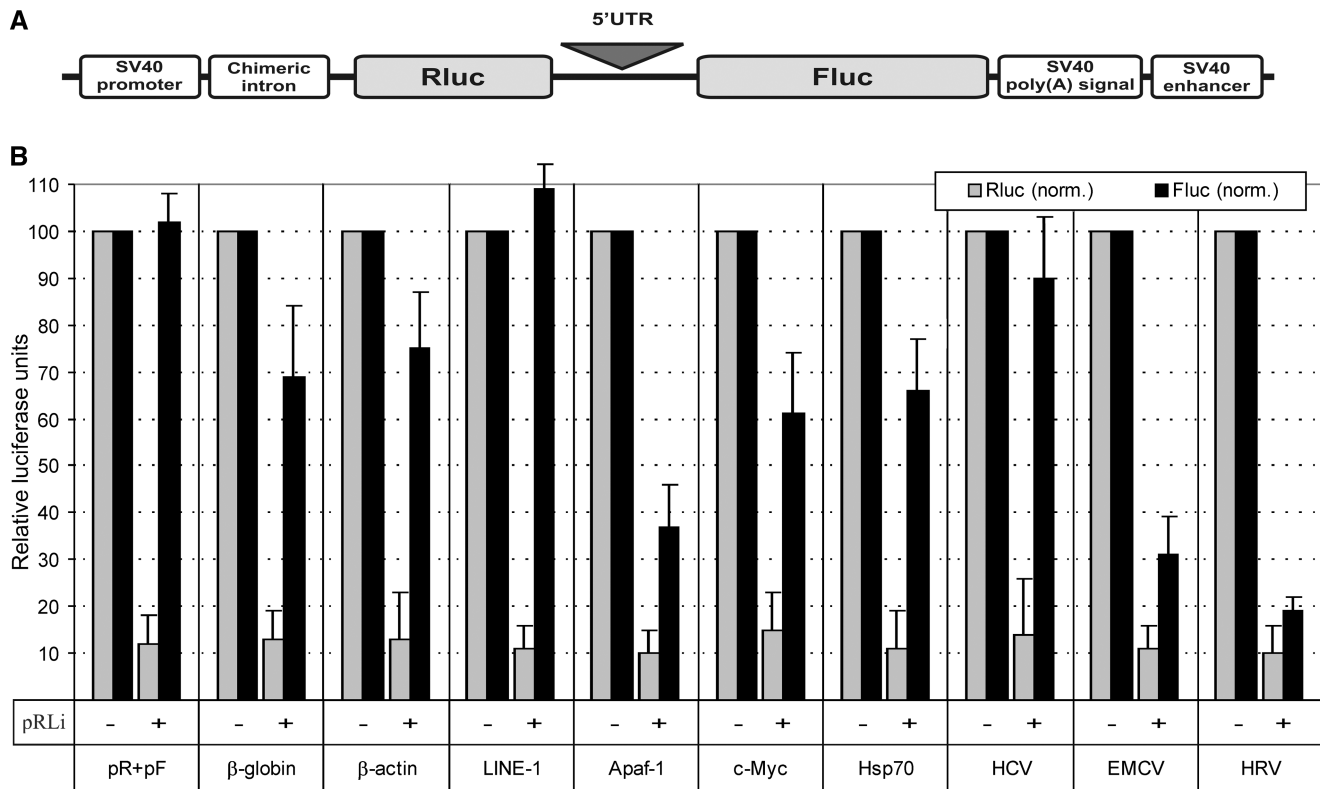
of 25 µM of L-methionine (instead of radiolabel [<sup>35</sup>S]methionine). When indicated, various amounts of m<sup>7</sup>GTP (equilibrated with respective amount of Mg(CH<sub>3</sub>COO)<sub>2</sub>) were added, and the translation mix was pre-incubated for 5 min before the addition of mRNA. For experiments with 4E-BP1, the recombinant protein was used. Plasmid pGEX-6p1-h4E-BP1 (a gift from Y. Svitkin and N. Sonenberg, McGill University, Montreal) encoding human 4E-BP1-GST fusion was expressed in *Escherichia coli*, and 4E-BP1 was purified using Glutathione-Sepharose 4B and PreScission Protease (Amersham) and dialyzed against buffer A100 (20 mM Tris-HCl pH 7.5, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 10% glycerol). Highly purified 4E-BP1 protein was added to the translation mix in a volume less than 1.5 µl and incubated for 5 min before the addition of reporter mRNAs. In the control, an equal amount of A100 buffer was added.

## RESULTS

### Transfection of cells with dicistronic DNAs produces artifacts even in the case of some viral IRESes

In our study we used 5' UTRs from nine different mammalian mRNAs (see Supplementary Table S1). 5' UTRs from Apaf-1, c-Myc and hsp70 mRNAs were reported to contain IRES-elements (28,30,31,33). In contrast, the relatively short β-globin (53 nt) and β-actin (84 nt) leaders represented classical cap-dependent 5' UTRs. The cap-dependent scanning mechanism was also exemplified by a very long leader (900 nt) from the LINE-1 mRNA (29). The well-characterized viral IRESes used as control were from encephalomyocarditis virus (EMCV), human rhinovirus (HRV) and hepatitis c virus (HCV) RNAs. They are known to be different in their structure and mechanisms of internal ribosome entry (34).

The standard Rluc-5' UTR-Fluc cDNA constructs under control of SV40 promoter (Figure 1A) were prepared with these 5' UTRs and co-transfected into human kidney cells (HEK293T) with or without plasmid pRLi. The pRLi test was proposed by R. Lloyd and co-workers (20). pRLi encodes an siRNA to the first cistron (RLuc). Its co-transfection with dicistronic cDNAs results in the degradation of RLuc sequences and a corresponding inhibition of RLuc protein synthesis in the cell. If Rluc and Fluc are translated exclusively from an intact dicistronic mRNAs, then both Rluc and Fluc activities should be proportionally reduced following the Rluc-targeted RNAi response. However, if an mRNA is monocistronic, encoding only Fluc, then the Fluc activity should be unaffected by the presence of RLuc siRNA. As seen from Figure 1B, only the dicistronic constructs with the HRV IRESes, and, to a lesser extent, with the 5' UTRs from the EMCV and Apaf-1 mRNAs passed this control test. The expression from the Fluc cistron when it was directed by other 5' UTRs was remarkably resistant to degradation of RLuc sequences. The HCV IRES does not stand the test, either, in agreement with data by Dumas *et al.* (18). It is important to note that similar conclusions were derived



**Figure 1.** Effect of RNA interference against Rluc in cells transfected by DNA dicistronic constructs on the activity of the second cistron (Fluc) directed by different 5' UTRs. (A) Schematic presentation of the DNA dicistronic constructs used in the experiments. (B) Results of the DNA transfections of HEK293T cells with and without pRLi plasmid. For each dicistronic construct the activities of Rluc and Fluc in the presence of pRLi were normalized to those in its absence, which were set to 100%. pR + pF is a control test where two monocistronic constructs pR and pF were cotransfected with or without pRLi.

for classical swine fever virus (CSFV), porcine Tescho virus-1 (PTV-1) and cricket paralysis virus (CrPV) IRES-elements (data not shown). This experiment is one more convincing illustration of why we cannot rely on the use of dicistronic cDNA transfections to test nucleotide sequences for IRES activity and should proceed to transfection of cells with RNAs.

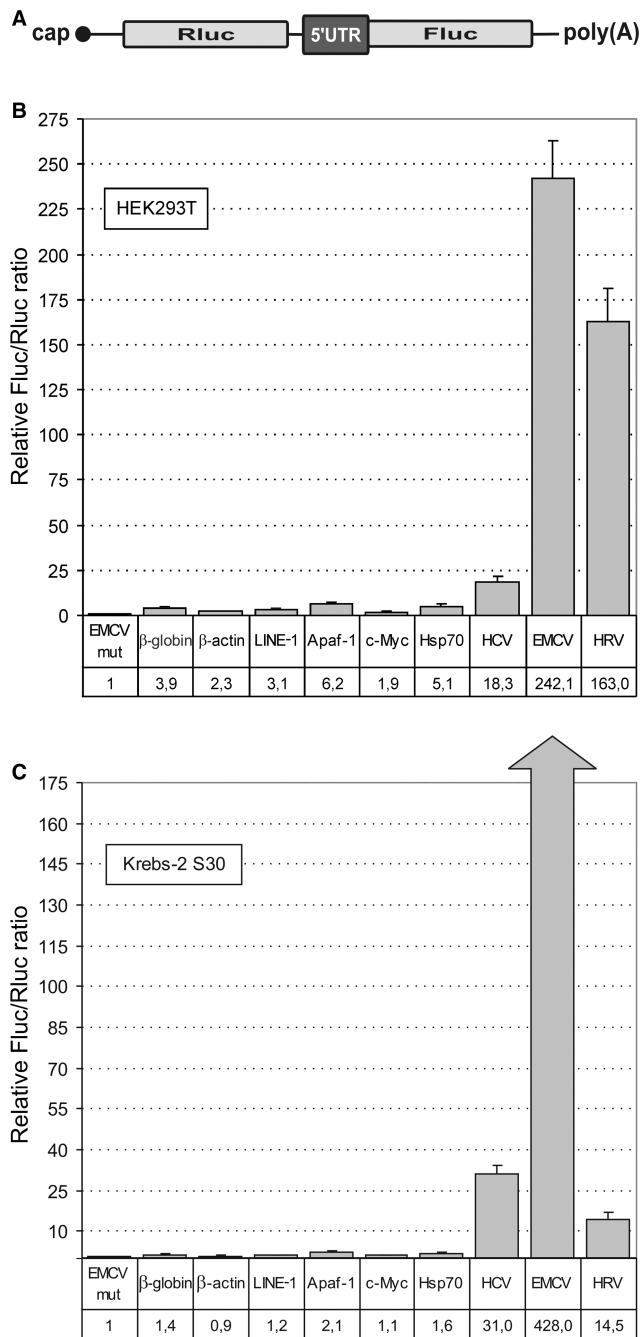
#### Identification of IRESes by RNA transfection: conventional criterion

As in the case of dicistronic DNAs, the conventional approach to identify IRES-elements from transfection experiments with dicistronic RNAs is to compare the second cistron expression (e.g. Fluc) driven by the test 5' UTR with its expression from the corresponding 'empty' dicistronic vector. Alternatively, one can use a construct where the second cistron is preceded by the sequence whose IRES activity is close to 0. In our work we used for this purpose the inactivated EMCV IRES (EMCVmut) (30).

$m^7G$ -capped and polyadenylated dicistronic mRNAs containing in the intercistronic position all cellular and viral 5' UTRs listed above (Figure 2A) were transfected into HEK293T cells. Their Fluc expression values normalized to Rluc are shown in Figure 2B. It can be seen that for all our constructs the Fluc activity was

higher than for the control EMCVmut construct. The stimulation of Fluc expression was from 2-fold ( $\beta$ -globin, c-Myc) to 6-fold (Apaf-1). Formally, it appears as if all the 5' UTRs of cellular mRNAs used in this study possess some IRES activity. Given that even the  $\beta$ -globin 5' UTR reveals a weak IRES activity, it is not clear what degree of stimulation should be observed to conclude that a particular sequence is an IRES: 4-fold, 10-fold or more? It was also clear that the IRES activity of all the tested cellular 5' UTRs was much lower than that for the classical viral IRESes, especially picornavirus IRESes (EMCV and HRV).

Similar data were obtained when the same constructs were translated *in vitro*, in nuclease untreated cytoplasmic extracts of Krebs-2 cells (Figure 2C). The significant difference was that, for some reason, the control HRV IRES was weaker in this system than in transfected HEK293T cells whereas in contrast, the other control, HCV IRES, worked much better. Otherwise, the results *in vivo* and *in vitro* correlated with each other. As it has been stressed earlier (16,35), nuclease untreated cytoplasmic extracts from cultured cells are the only available *in vitro* system which is adequate to study complex mechanisms of translation initiation and regulation (see also below). In this system the tested mRNAs are translated under conditions of competition with cellular mRNAs (35). In addition, in contrast to



**Figure 2.** Conventional test to identify IRES elements in the 5' UTRs of cellular and viral mRNAs using dicistronic RNA constructs. (A) Schematic presentation of the dicistronic RNA construct used to identify IRES elements within 5' UTRs of cellular mRNAs. The length of poly(A) tail was 50A residues. (B) Results of transfection of HEK293T cells with dicistronic mRNAs carrying in the intercistronic position various cellular 5' UTRs or viral IRESes. Fluc/Rluc ratio for the control EMCVmut construct was set to 1. Other Fluc/Rluc ratios were normalized to the Fluc/Rluc ratio of the EMCVmut and, therefore, the values in the bottom row reflect the extent of Fluc stimulation by different 5' UTRs in the intercistronic position over the EMCVmut control. (C) Translation of the same constructs in the nuclease untreated cytoplasmic extract of ascite carcinoma cells Krebs-2.

RRL, the cytoplasmic extract from cultured cells appears to contain the entire set of mRNA-binding proteins which can presumably modify the molecular mechanism and the efficiency of translation initiation (see 'Discussion' section).

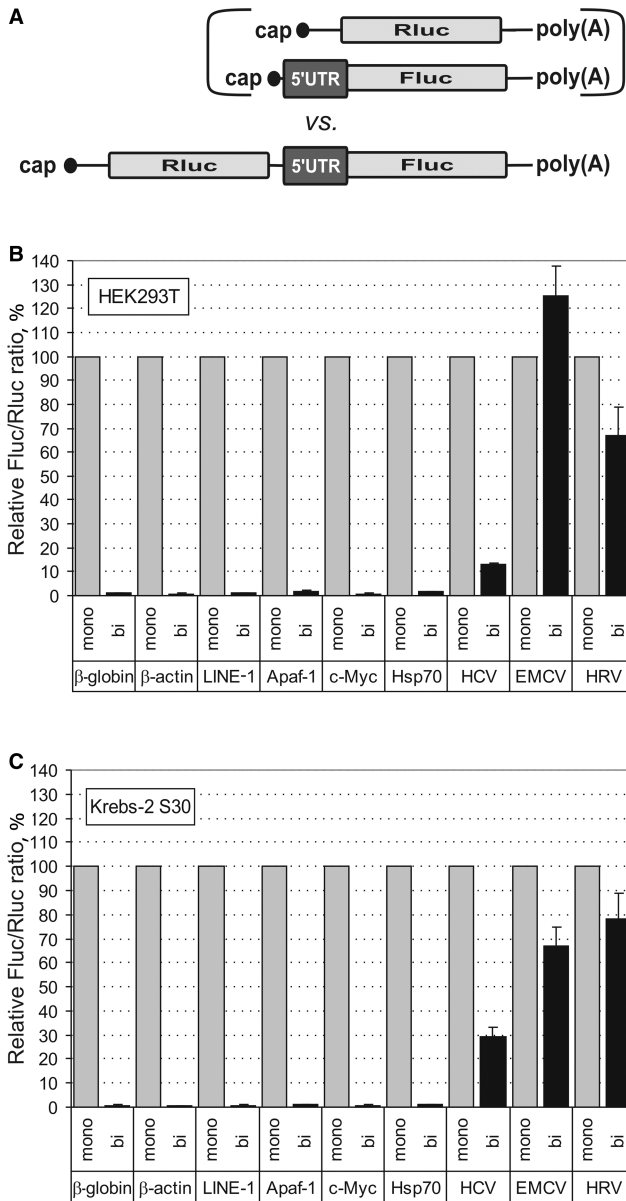
Thus, the Fluc/Rluc values obtained for cellular 5' UTRs in the intercistronic position in both systems produce the impression that they represent just background activities, inherent to any experimental system, and may be of no physiological significance. To clarify this point, it was important to use other approaches and alternative criteria. First of all, we wanted to know what was the actual translation initiation potential of selected 5' UTRs when they are in their natural situation, i.e. at the 5' end of m<sup>7</sup>G-capped monocistronic mRNAs.

### Comparison of the 5' UTR activities in mono- versus dicistronic capped mRNAs

All eukaryotic mRNAs are both monocistronic and m<sup>7</sup>G-capped, and dicistronic constructs therefore represent an artificial situation. To determine the actual translation initiation potential, it was important to compare translation initiation activities of tested 5' UTRs in their natural 5'-terminal position *vs.* the intercistronic one. To this end, m<sup>7</sup>G-capped polyadenylated mono- and dicistronic mRNAs (Figure 3A) were synthesized, transfected into two cell lines (HEK293T and HeLa) in the same molar amounts and the translational activity was determined for each of the constructs. Figure 3B shows the data for HEK293T cells, the level of Fluc/Rluc for monocistronic constructs being set to 100%. It is clear that the activities of cellular 5' UTRs in the dicistronic position are dramatically lower than in monocistronic mRNAs whereas the corresponding values do not differ significantly for picornaviral IRESes. Similar data were obtained for HeLa cells (see Supplementary Figure S1), and also *in vitro*, in the cell-free system prepared from ascite cells Krebs-2 (Figure 3C). (Interestingly, the HCV IRES also demonstrated a substantial difference in activity between the two constructs. The possible explanation of this phenomenon is given in the next section.) These data convincingly indicate that the putative cellular IRESes selected for this study do not meet by definition the critical criterion characteristic of true IRES-elements: they demonstrate only very low background activities when placed in the intercistronic position, especially in comparison with the corresponding monocistronic constructs. It is important to note that the all capped monocistronic mRNAs used in these experiments revealed very high absolute levels of luciferase activity (see below).

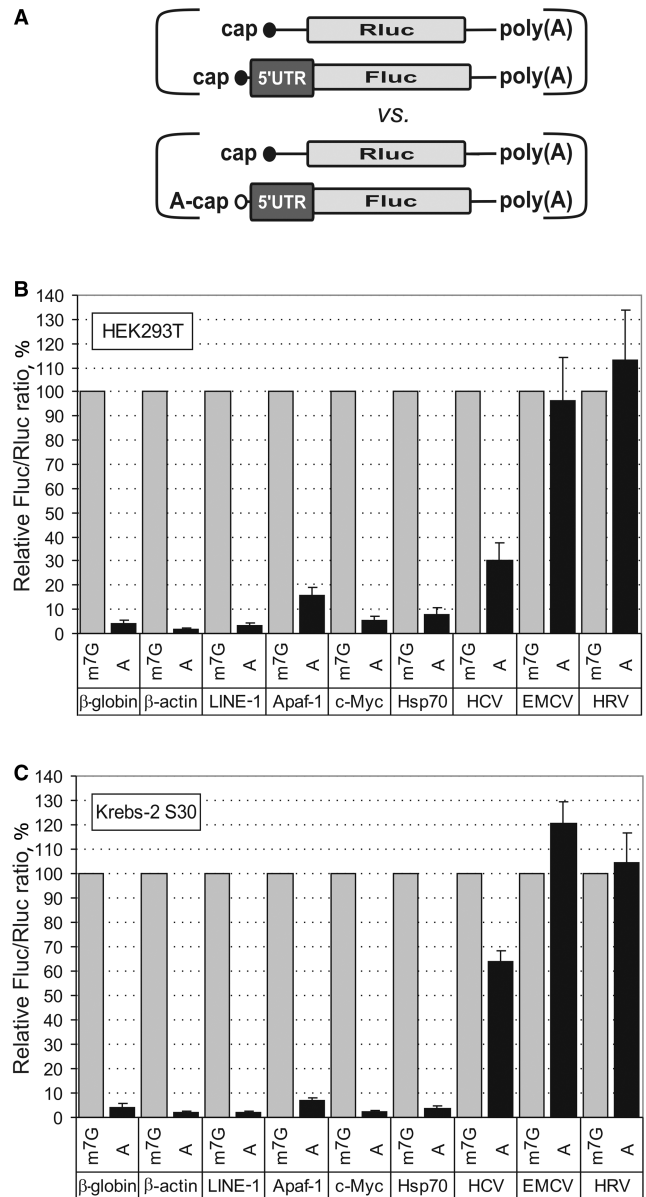
### Comparison of m<sup>7</sup>G-capped versus A-capped monocistronic mRNAs

The strikingly poor initiation activities of cellular 5' UTRs in the intercistronic position may be accounted for by the inhibitory effect of upstream nucleotide sequences, by the absence of the m<sup>7</sup>G-cap or both. The negative influence of upstream sequences is difficult to analyze as



**Figure 3.** Comparison of activities of various 5' UTRs in monocistronic versus dicistronic contexts. (A) Schematic representation of mono- and dicistronic mRNAs used in the assay. (B) HEK293T cells were transfected with either dicistronic mRNA derivatives indicated or equimolar mix of the corresponding monocistronic reporter (coding for Fluc) and the normalizing mRNA (Rluc). Fluc/Rluc values for monocistronic mRNAs was taken as 100% for each individual construct. (C) Translation of the same constructs, as in (B), *in vitro*, in the cytoplasmic S30 extract from cells Krebs-2.

we do not know the mechanism of translation initiation on intercistronic sequences. However, it is easy and important to assess the contribution of the m<sup>7</sup>G-cap to the translation initiation of different cellular 5' UTRs, those which use a classical cap-dependent mechanism and those which are claimed to possess IRES activity. To this end, both m<sup>7</sup>G-capped and uncapped monocistronic constructs were synthesized. As above, all transcripts were polyadenylated (50 A-residues). In so-called 'uncapped' transcripts the functional m<sup>7</sup>G-cap



**Figure 4.** Cap-dependence of Fluc mRNAs with various cellular and viral 5' UTRs. (A) Schematic representation of pairs of monocistronic mRNAs used in the assay. Note: the same reference mRNA, cap-Rluc-poly(A), was used to normalize activities of capped and uncapped (A-capped) Fluc constructs containing the tested 5' UTRs. To avoid an interference between capped and uncapped transcripts, the cap-Rluc-poly(A) was taken in a very low amount (1 ng) in these experiments. (B) Comparison of indicated capped *vs.* uncapped monocistronic transcripts in the RNA transfection assay for HEK293T cells. Fluc/Rluc values for capped mRNAs were taken as 100% for each individual construct. (C) Translation of the same pairs of constructs as in (B) and (C) in the S30 extract from cells Krebs-2.

was substituted by a non-functional A-residue to protect the 5'-end of mRNA from the 5'-exonuclease degradation. Hereafter, the corresponding transcripts are referred to as m<sup>7</sup>G-capped and A-capped mRNAs, respectively (Figure 4A). The results of RNA transfection experiments with these transcripts are shown for cells HEK293T in Figure 4B. It is clear that the omission of the m<sup>7</sup>G-cap from the 5' end of the mRNAs dramatically inhibited the

translation directed by all tested cellular 5' UTRs but had almost no effect on the picornaviral IRESes. Similar conclusions can be drawn from experiments with transfected HeLa cells (Supplementary Figure S2) and the cytoplasmic extract of Krebs-2 cells (Figure 4C). It is interesting that capping of the HCV IRES strikingly stimulates translation of the reporter gene, especially in living cells. Thus, m<sup>7</sup>G capping of the HCV IRES switched its mode of translation initiation from IRES-dependent to cap-dependent, the latter being more efficient. [The same observation was made with a transcript containing the CrPV IRES (data not shown)]. This conclusion is in agreement with the data of Wiklund *et al.* (36) and the recent report from Belsham group (37).

However, quantitatively, the m<sup>7</sup>G-cap omission did not exert the same effect on different cellular 5' UTRs. The most dramatic negative effect was noted for the 5' UTR of  $\beta$ -actin mRNA, the lowest—for the Apaf-1 5' UTR, especially in HEK293T cells. The data obtained for the same mRNA pairs *in vitro* (see Figure 4C) nicely correlated with those obtained in experiments with transfected cells.

It was very important to know whether degradation of mRNA affected values obtained, in particular when we determined the contribution of the m<sup>7</sup>G-cap to the translational initiation of individual mRNAs with different 5' UTRs. It is logical to think that the A-capped mRNAs are more sensitive to degradation since they have no initiation factors protecting the 5' end and are presumably less loaded with translating ribosomes. If a preferential degradation of A-capped mRNAs occurs and its extent is different for different mRNAs used in the study, then all our ratios m<sup>7</sup>G-capped mRNA/A-capped mRNA won't reflect the real contribution of the m<sup>7</sup>G-cap to the translational activity.

A conventional way to determine the integrity of a RNA is to extract it from cells and perform Northern assay or real-time PCR. However, in the case of RNA delivery by lipofection when the translation activity is measured soon after transfection, these methods are not correct to apply (38). Instead, we analyzed the time course of cap-dependence for various 5' UTRs used in the study. To this end, ratios of expression levels of m<sup>7</sup>G capped mRNAs *vs.* those for A-capped mRNAs were determined. We found that these ratios are nearly constant until 3 h post-transfection (see Supplementary Figure S3). Thus, the detected translational activities and their ratios are not significantly affected by degradation of A-capped mRNAs within the indicated period since all our results on the m<sup>7</sup>G-cap contribution to the translational potentials of various 5' UTRs shown on Figure 4A were obtained at 2 h post-transfection.

#### **m<sup>7</sup>GTP or eIF4E-BP differentially inhibit translation of m<sup>7</sup>G-capped mRNAs with different cellular 5' UTRs**

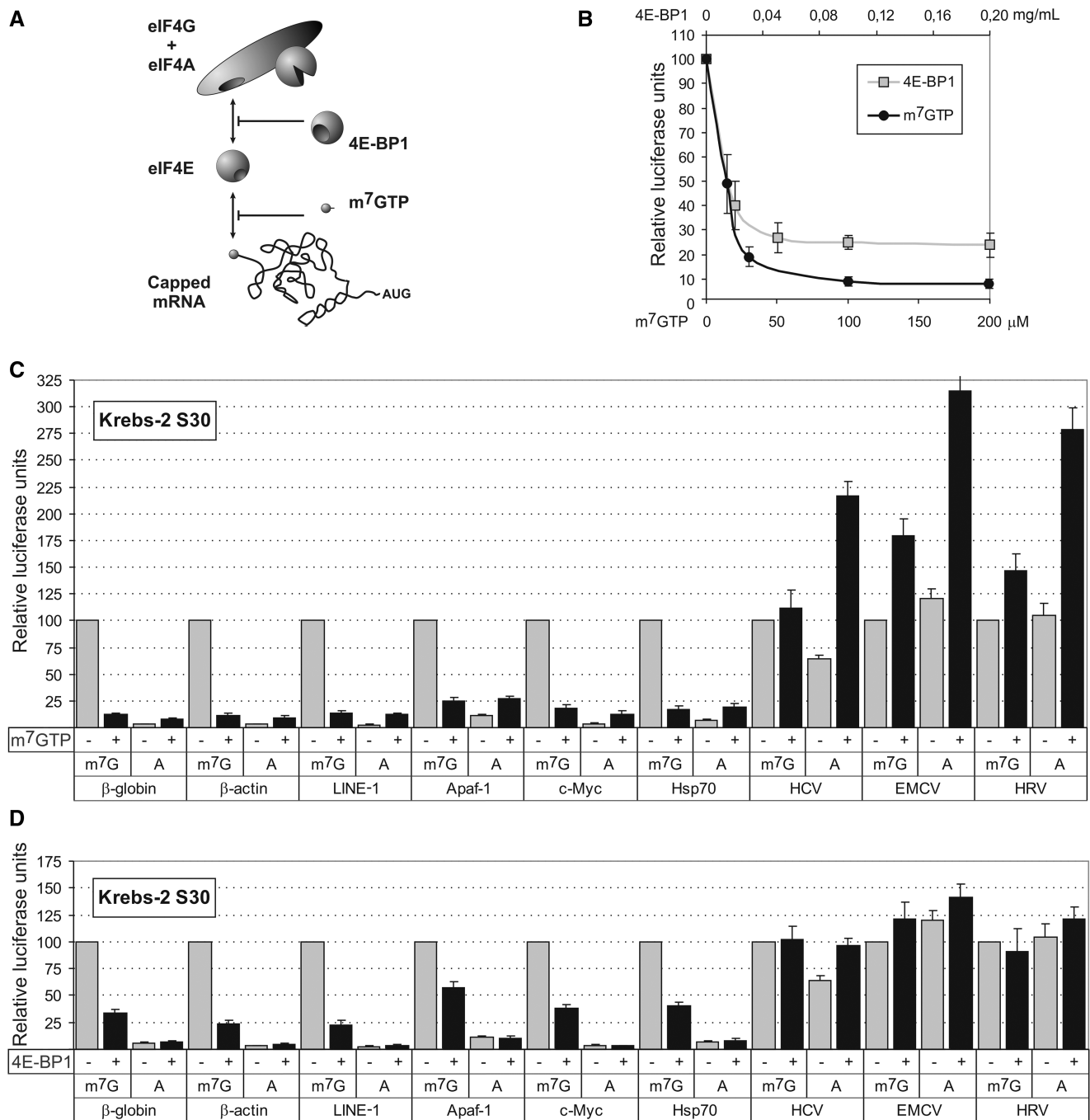
The different influence of the m<sup>7</sup>G-cap omission on the translation directed by individual 5' UTRs prompted us to compare the effect of known inhibitors of the cap-dependent initiation, m<sup>7</sup>GTP and eIF4E-BP (Figure 5A),

on the cellular and viral 5' UTRs selected for this study. Whereas m<sup>7</sup>GTP is not a naturally occurring inhibitor, the translational repressor 4E-BP1 appears to be involved in many control mechanisms switched on under various stress conditions (39). All experiments were performed in an *in vitro* system where we could control precisely the concentrations of inhibitors. As before, the nuclease untreated S30 extract from ascite cells Krebs-2 was employed.

First, we performed titration of the system with different concentrations of m<sup>7</sup>GTP and 4E-BP1 to find concentrations of each that produced a maximum inhibition effect on the standard cap-dependent  $\beta$ -actin 5' UTR (Figure 5B). It is important to note that the maximum inhibition we could achieve with 4E-BP1 was only 4-fold. However, this result is in a good agreement with the known effect of 4E-BP1 overexpressed in cells [2.5-fold inhibition of cap-dependent translation by non-phosphorylatable 4E-BP1 mutant—see, e.g. ref. (40)]. This suggests that under conditions of profound inhibition of translation by this translational repressor, the eIF4E–eIF4G interaction is not completely excluded from the initiation process even for classical cap-dependent mRNAs. As expected (see Figure 5C and D), the m<sup>7</sup>GTP caused a much more dramatic inhibitory effect on the translation of mRNAs directed by cellular 5' UTRs than 4E-BP1. In fact, the initiation activity for all cellular m<sup>7</sup>G-capped mRNAs at the concentration of m<sup>7</sup>GTP as high as 100  $\mu$ M was reduced to the level observed for the corresponding A-capped constructs, also in the presence of the m<sup>7</sup>GTP (A-capped mRNAs were stimulated by addition of the analog, presumably because of relieving the competition with endogenous capped mRNAs). However, even this background activity was not the same for all the cellular 5' UTRs selected for this study. The differential response of individual cellular 5' UTRs to the inhibition of initiation at the level of the eIF4E was more pronounced in the experiments with 4E-BP, an inhibitor with another mechanism of action (see Figure 5A). Some of the 5' UTRs ( $\beta$ -globin,  $\beta$ -actin and LINE-1 5' UTRs) responded stronger, whereas Apaf-1, c-Myc and hsp70 were more resistant to inhibition (Figure 5D). It is of interest that under the selected concentration (0,1 mg/ml) the 4E-BP1 inhibited the Apaf-1 5' UTR less than twice (see columns Apaf-1 in Figure 5D). As expected, the activity of viral IRES-elements was not suppressed.

#### **mRNAs with long and highly structured 5' UTRs reveal efficient cap-dependent translation initiation**

We have recently reported that the 900 nt long and highly structured 5' UTR of LINE-1 mRNA is capable of efficient cap-dependent translation initiation and that this mRNA uses an efficient scanning rather than shunting mechanism to locate its initiation codon (29). It was not clear, however, whether this mRNA represented a unique (exotic) case and hence all these features were accounted for by a special organization of its 5' UTR. However, when performing the experiments described in the previous sections, we noted that the

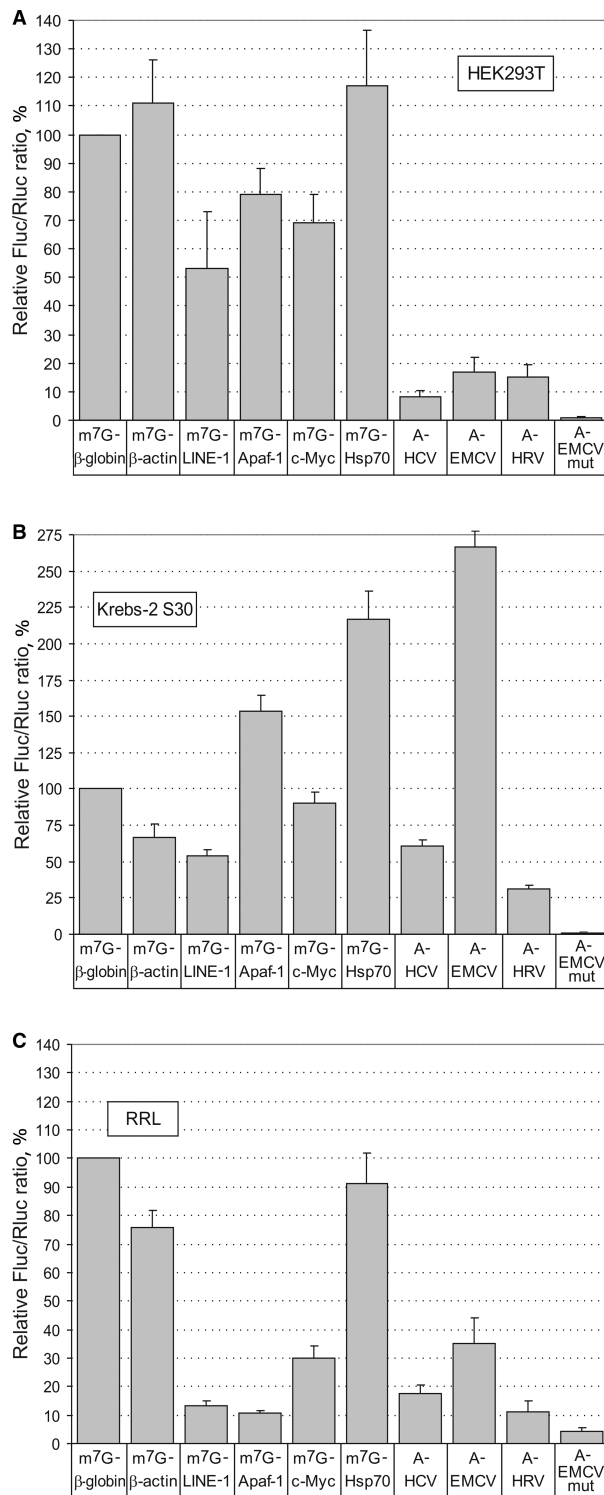


**Figure 5.** Differential inhibition of translation of capped mRNAs containing different cellular 5' UTRs by m<sup>7</sup>GTP or 4E-BP1 in the cytoplasmic extract of cells Krebs-2. (A) Interpretative drawing showing distinct mechanisms of inhibition of the cap-dependent translation by m<sup>7</sup>GTP or eIF4E-BP1. (B) Titration curves to determine the concentrations of m<sup>7</sup>GTP or 4E-BP1 which exert maximum inhibitory effects on the translation of the standard cap-dependent mRNA (β-actin 5' UTR directed Fluc mRNA). (C) and (D) Effects of m<sup>7</sup>GTP (100 μM) and 4E-BP1 (0.1 mg/ml) on the translation of m<sup>7</sup>G-capped and uncapped (A-capped) monocistronic mRNAs containing various cellular 5' UTRs and viral IRESes in the cytoplasmic extract of cells Krebs-2. As before, the extract was not treated with micrococcal nuclease to ensure competitive conditions for translation of exogenous mRNAs.

cap-dependent translation efficiency of Apaf-1 and c-Myc 5' UTRs was rather high, in spite of the fact that they had a highly developed secondary structure. Some sequences making up these 5' UTRs form not only standard stem-loop structures but reveal also long distance interactions (41). Therefore, we decided to directly compare the

translation initiation potentials of all 5' UTRs used in this study in transfected HEK293T cells (Figure 6A). Similar pattern was observed at different time points (from 0.5 to 12 h, see Supplementary Figure S4) suggesting that degradation of mRNAs, if any, does not affect the levels observed. Analogous ratios of the





**Figure 6.** Demonstration of efficient cap-dependent translation initiation of mRNAs with long and highly structured 5' UTRs both in transfected cells and the nuclease untreated cytoplasmic extract from ascite carcinoma cells Krebs-2. (A) Translation in HEK293T. Exactly the same molar amounts of m<sup>7</sup>G-capped and polyadenylated mRNAs carrying different 5' UTRs were used in transfection assays. Viral IRESes were not capped (A-capped) to assess their *actual* translation initiation potentials in these cell lines. (B) and (C) The same mRNAs but translated in the extract from cells Krebs-2 and RRL, respectively. In all experiments Fluc activities of these constructs were normalized to that for the Fluc mRNA directed by β-globin 5' UTR.

translational levels were obtained for another cell line (HeLa, see Supplementary Figure S5).

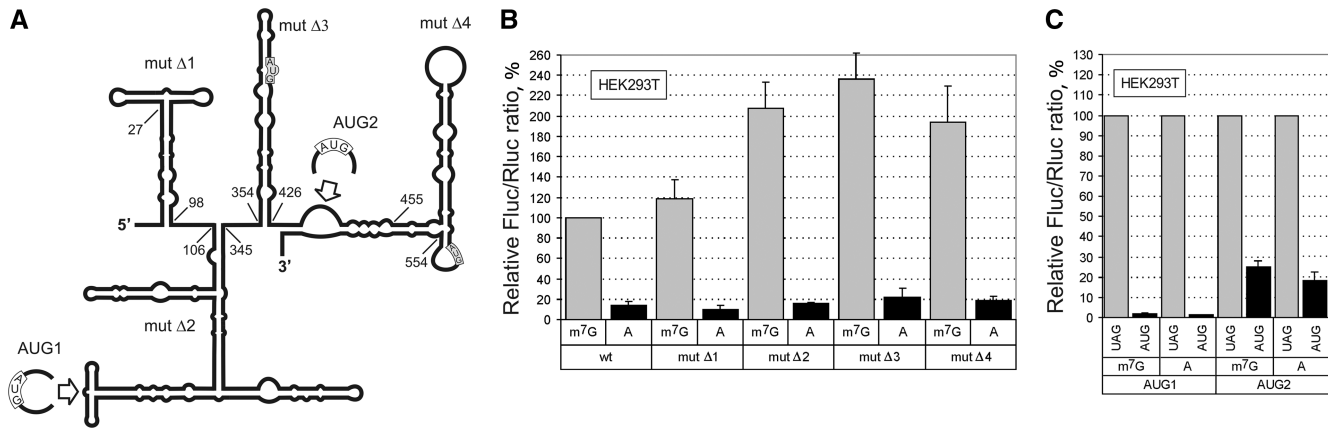
One can see that the translation initiation efficiency of Apaf-1 and c-Myc 5' UTRs is only half that for the 5' UTRs from β-globin and β-actin mRNAs in HeLa cells. The difference between these leaders is even less significant in HEK293T cells. One should recall that the short 5' UTRs of β-globin and β-actin mRNAs are regarded as standard efficient natural mRNAs that use the classical cap-dependent scanning mechanism. Among cellular 5' UTRs, the lowest activity was found for the LINE-1 5' UTR. However, as was shown in our recent report (29), this is accounted for by the existence of a short uORF at the very 5' end of this 5' UTR rather than by its length or highly developed secondary structure. Notably, all tested viral IRESes were significantly less active than the cap-dependent mRNAs, at least in HEK293T and HeLa cell lines.

The conclusion that the highly structured Apaf-1 and c-Myc 5' UTRs are efficient in the m<sup>7</sup>G-cap-assisted translation initiation was reinforced by experiments in the S30 extract (Figure 6B). In this case, the translation initiation activity of Apaf-1 mRNA even exceeded that of the 5' UTRs of β-globin or β-actin mRNAs.

The finding that the 5' UTRs of Apaf-1, c-Myc and LINE-1 mRNAs do not yield significantly to the 5' UTRs of β-globin or β-actin mRNAs in translation activity is not a trivial observation since it looks as contradicting the scanning rules (27). This observation is not without precedent (see 'Discussion' section). Nevertheless, this fact had not been given due consideration presumably because researchers normally used the translation in the standard nuclease treated RRL. The advantages of nuclease untreated cytoplasmic extracts of cultured cells have not yet become widely recognized. Thus, we decided to compare the translation initiation activities of different 5' UTRs in RRL with those in the extract from cells Krebs-2. These data are shown in Figure 6B and C. They demonstrate a striking difference in the relative translation efficiencies of 5' UTRs in these two cell-free systems. As expected from numerous previous reports, the classical cap-dependent 5' UTRs of β-globin or β-actin mRNAs proved to be very active in RRL whereas long and structured 5' UTRs are not. However, in the S30 extract they all have substantial activity. As the relative translation efficiencies in the nuclease untreated S30 extract reflect much better the situation observed in transfected cells, we conclude that RRL is not an adequate system to study the translation initiation mechanisms for mRNAs with complex 5' UTRs, a conclusion that had been already made in our recent reports (16,17).

#### The sequence of the Apaf-1 5' UTR is efficiently scanned by the ribosome to locate the start codon

What are the mechanisms of cap-dependent and cap-independent translation initiation on highly structured 5' UTRs? Certainly, every particular 5' UTR should be analyzed separately. We decided to focus our attention on the Apaf-1 5' UTR since this highly structured leader is the least cap-dependent among those studied in this



**Figure 7.** Mutation analysis of the 5' UTR of Apaf-1 mRNA to reveal the mechanism by which ribosomes select the start codon. (A) Schematic presentation of the secondary structure of the 5' UTR of Apaf-1 mRNA [adapted from ref. (40)]. The deletions of principal structural domains (mut  $\Delta$ 1 through mut  $\Delta$ 4) are denoted by numbers. Positions of additional upstream start sites (AUG1 and AUG2) are indicated with arrows; natural uAUG triplets are also shown. (B) Fluc expression from transcripts with additional uAUG1 and uAUG2 codons in transfected HEK293T cells. Similar transcripts with uUAGs in analogous positions were used as control; (C) Effect of the deletions shown in (A) on the translation efficiency of the corresponding transcripts, both m<sup>7</sup>G and A-capped, in HEK293T cells.

article and was therefore more likely to harbor some 'IRES-like' sequences. We made deletions of principal structural domains (42) within this 5' UTR (see Figure 7A) and analyzed the corresponding capped and uncapped transcripts in transfected HEK293T cells. As seen from Figure 7B, for the m<sup>7</sup>G-capped mRNAs these deletions resulted in some stimulation of translation though the maximum effect did not exceed 2-fold. More important results were obtained with A-capped transcripts: none of the deletions produced a strong effect on translation, thereby, once again, arguing against a true IRES in this 5' UTR. Moreover, insertion into the Apaf-1 5' UTR of additional AUG triplets (but not termination UAG codons) in a good Kozak context dramatically inhibited translation of both m<sup>7</sup>G- and A-capped transcripts (Figure 7C). We conclude that the 40S ribosome 'sees' AUG codons within the entire sequence of this highly structured 5' UTR and hence is able to efficiently scan it regardless of whether the Apaf-1 5' UTR is capped or not.

## DISCUSSION

The m<sup>7</sup>G-cap structure is present at 5'-termini of all nuclear encoded eukaryotic mRNAs and plays a central role in the scanning mechanism of AUG selection in eukaryotes. According to the scanning model proposed by Kozak (27), the secondary structure of 5' UTRs of eukaryotic mRNAs causes an inhibitory effect on translation initiation, since it should interfere with movement of the 40S ribosomal subunit along the polynucleotide chain (43). Thus, existence of efficiently translated mRNAs with long and structured 5' UTRs is poorly compatible with the prevailing idea on the scanning mechanism. This accounts for a widely accepted opinion that such mRNAs should be weak cap-dependent mRNAs.

In this article we show that this view can be challenged. It has most probably been implanted in the mind of many

researchers owing to the results of translation assays performed in RRL with various 5' UTRs including those used in this study. To the best of our knowledge, no one has systematically compared the absolute translation initiation potentials of different 5' UTRs in systems other than RRL, especially in living cells. We have decided to fill this gap, at least partially, with this study.

We show [(29) and this work] that three long and highly structured 5' UTRs from cellular mRNAs (Apaf-1, c-Myc and LINE-1) which were arbitrarily selected for our studies, reveal a high translation initiation potential, comparable with that for standard cap-dependent mRNAs ( $\beta$ -globin and  $\beta$ -actin). Our observations are not without a precedent. Indeed, (i) Hensold *et al.* (44) showed that the 5' UTR of Spi-1 (PU.1) mRNA was highly structured. It decreased translation of Spi-1 transcripts in RRL 8- to 10-fold. However, the effect of the 5' UTR on translation *in vivo* was negligible; (ii) Stonely *et al.* (45) showed that the m<sup>7</sup>G-capped c-Myc 5' UTR was even more efficient in Balb/c 3T3 and MCF-7 cells than the short leader of the standard Luc mRNA transcribed from the pGL3 vector; (iii) van der Velden *et al.* (46) reported that the 5' UTR (leader 1) of insulin-like growth factor II (592 nt) was highly structured, did not contain an IRES and did not use shunting to initiate translation. The leader 1 driven reporter expression was low in RRL but was very efficient in cells; (iv) Bert *et al.* (15) noted that the monocistronic firefly luciferase mRNAs containing the HIF-1 $\alpha$  or c-Myc 5' UTRs gave rise to luciferase levels that are 62%–77% of what was produced from the control monocistronic luciferase mRNA which had only a short, relatively unstructured 5' UTR. The authors concluded that the GC-rich HIF-1 $\alpha$  and c-Myc UTRs were not particularly inhibitory to translation but did not discuss this point further.

Thus, in disagreement with the currently prevailing view, the efficiency of mRNA translation in cultured cells and crude S30 cell-free system is little affected by the 5' UTR length and GC-richness. The m<sup>7</sup>G-cap plays

a crucial role in this high initiation activity. Again, we did not find any correlation of the m<sup>7</sup>G-cap contribution with the length, complexity or initiation efficiency of 5' UTRs. For instance, the highly structured Apaf-1 5' UTR (42) is less cap-dependent than the simple 5' leader of beta-actin mRNA.

What is the mechanism that is used by highly structured 5' UTRs to efficiently initiate translation in a 5'-end-dependent mode? Certainly, every individual mRNA should be investigated separately. Using as an example the Apaf-1 5' UTR, we show that the ribosome is able to efficiently scan the entire Apaf-1 leader in spite of its well developed secondary structure. Indeed, we present compelling evidence [see analogous data for the LINE 5' UTR (29)] that the 40S ribosome recognizes additional AUGs inserted into its sequence. In contrast, none of the deletions within uncapped Apaf-1 5' UTR produces a significant deleterious effect on the translation initiation thereby arguing against the existence of an IRES within this leader. These data taken together with those obtained recently for the IRES-less 900 nt long 5' UTR from the LINE mRNA strongly suggest that in cultivated cells or corresponding cytoplasmic extracts 40S ribosomes hardly 'notice' natural stem-loop structures in the course of initiation. We speculate that various mRNA-binding proteins present in the system mostly facilitate rather than inhibit the process of the start codon location. This may explain why the inhibition is observed in RRL: this system is known for its highly reduced content of many mRNA-binding proteins. The fact that the translation initiation efficiencies of 200 and 900 nt long 5' UTRs are comparable (29) strongly suggests that the unwinding of stem-loops occurs sequentially. Therefore, in our opinion, the current practice to calculate cumulative energies of 5' UTRs to get some idea about their translation efficiency makes no sense.

We think that the set of novel criteria used in this study allows us to determine the *actual* rather than *possible* modes of translation initiation used by these 5' UTRs. These criteria include: (i) comparison of translation initiation potentials for tested 5' UTRs in capped dicistronic versus monocistronic mRNAs; (ii) comparison of capped (m<sup>7</sup>G-capped) versus uncapped (A-capped) monocistronic mRNAs; (iii) evaluation of the effect of m<sup>7</sup>GTP or eIF4E-BP on the translation initiation directed by 5' UTRs of natural mRNAs; (iv) effect of deletions within 5' UTRs on the initiation activity of uncapped monocistronic mRNAs: for true IRESes, the deletions should cause dramatic reduction of the initiation activity. Using these criteria we come to conclusion that, at least under normal conditions, the 5' UTRs from Apaf-1, c-Myc and hsp70 mRNAs and also LINE-1 mRNA (29) reveal only background activities when placed in the intercistronic positions, in comparison with their natural (capped 5'-terminal) context. Very poor activities in the intercistronic position of dicistronic transcripts have been reported for several other putative cellular IRESes (15,20,22).

The artifacts of the cDNA dicistronic method appear to be responsible for hot debates over the concept of cellular IRESes (20,47–50). Without coming to grips with them,

and without questioning the existence of cellular IRESes in principle (especially, as only three of them have been investigated in this article), we simply propose a view which could explain a change in the relative expression of individual mRNAs observed under stress conditions but is alternative to the concept of cellular IRESes. We suggest that individual 5' UTRs have a different m<sup>7</sup>G-cap contribution and are resistant to inhibition of eIF4E activity to a different extent. This view is supported by the data presented in Figure 5 of this article. This idea is not new and has been proposed by N. Sonenberg and co-workers many years ago (51,52). However, we add here a substantial modification to this widely shared opinion: we show that this different cap-dependence is not directly related to the overall stability of the secondary structure, number of stem-loops or length of the 5' UTR. It should be emphasized here that we dealt with real 5' UTRs rather than artificial 5' leaders into which extremely stable stem-loop structures (e.g. several BamHI linkers) were artificially inserted. It is difficult to find such perfect structures in natural cellular mRNAs.

What might be the mechanism for the differential resistance of mammalian 5' UTRs to the inhibition of the cap-assisted initiation? Our working hypothesis is that some elements within 5' UTRs and some mRNA-binding proteins may be involved in recruiting key initiation factors (e.g. eIF4G, eIF3, eIF2, etc.) or/and 40S ribosomal subunits to mRNAs. These elements may be rudiments of the true IRESes found in viral mRNAs. However, unlike the case of classical viral IRESes this recruitment occurs with a low specificity and affinity. It simply augments the local concentration of translation components near the 5' end of an mRNA. We believe that the 5' terminus of an mRNA [whether it is capped or uncapped, see ref. (53)] is the only available site on eukaryotic mRNAs that can accommodate the translation initiation machinery and start going the scanning process when IRESes are absent. It should be noted that we are far from thinking that variations of cap-dependence and resistance of some mRNAs to stress conditions are determined exclusively by their 5' UTRs. Other parts of the mRNAs may be also involved. The solution of this and other problems requires investigation of each individual 5' UTR with sophisticated approaches and, hence, is beyond the scope of this study. Work in this direction is one of the priorities in our laboratory.

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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