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5′-Untranslated region of heat shock protein 70 mRNA drives translation under hypertonic conditions

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ABSTRACT

In mammalian cells, adaptation to hypertonic conditions leads to the activation of an array of early (cell shrinkage, regulatory volume increase) and late (accumulation of compatible osmolytes) responses and increased level of HSPs (heat shock proteins). Protein synthesis is strongly inhibited few minutes after the hypertonic challenge as demonstrated in whole cells and as reproduced under controlled conditions in cell-free systems. Different mechanisms known to mediate the accumulation of HSP70, such as mRNA transcription and stabilization, require fully active protein synthesis. We show that the 5′-untranslated region of HSP70 messenger drives a hypertonicity-resistant translation (up to 0.425 osmol/kg of water), whereas cap-dependent protein synthesis is almost totally blocked under the same conditions. The results, obtained in cell-free systems and in whole cells, might help to explain why HSP70 is accumulated in cells when total protein synthesis is impaired. We also observed that translation initiated by viral IRES (from Cricket paralysis virus) is highly efficient in cells exposed to hyperosmolarity, suggesting that the resistance to hypertonic conditions is a more general feature of cap-independent translation. The described mechanism may also play a role in the control of translation of other messengers encoding for proteins involved in the adaptation to hypertonicity.

1. Introduction

Mammalian cells exposed to hypertonic conditions face with the potential dangerous stress by activating sequential responses aimed at adaptation [1]. A few seconds after the challenge, they undergo shrinkage to balance the elevated extracellular hypertonicity with consequent large increase in cellular concentrations of Na^+ and K^+, although the intracellular content of these ions does not change. In a few minutes, the second response leads the cells to recover their volume by increasing the uptake of both the above mentioned ions and by the accompanying influx of water via $Na^+/K^+/Cl^-$ co-transport, Na^+/H^+ exchanger and Cl^-/HCO_3^- exchanger, until the net result is a rise in the intracellular content and concentration of K^+. However, both these responses cause specific impairment to important biochemical systems which control DNA homeostasis and protein synthesis. Indeed, the increase in ionic strength associated to instantaneous cell shrinkage induces DNA breaks during the earlier response, whereas protein synthesis is strongly impaired after 30 min exposure to hypertonicity due to the associated regulatory volume increase and prolonged enhancement of ionic strength. After a few days, cells counterbalance ionic strength by substituting compatible osmolytes (methylamines, polyols, neutral amino acids or amino acid derivatives) for inorganic ions. A compatible osmolyte is a small organic molecule that accumulates at high concentrations in cells accounting for intracellular osmolarity without affecting cell functions. Thus, cells recover the physiological ionic strength and the normal rate of protein synthesis is restored.

Direct evidence of what happens in the translation machinery of cells exposed to hypertonicity was obtained in cell-free protein synthesis systems, such as rabbit reticulocyte lysate translating endogenous globin mRNA [2]. A simple and well controlled cell-free system might allow the direct determination of the effect on protein synthesis of putative osmolytes and the construction of dose–response curves, avoiding problems arising from the use of cellular models (transport and biotransformation of the osmolytes). In this system both initiation and elongation are found to be inhibited by unusually high concentrations of inorganic ions, but not by the compatible osmolytes betaine or *myo*-inositol, nor

Abbreviations: CrPV, Cricket paralysis virus; FBS, fetal bovine serum; FLuc, *Photinus pyralis* luciferase; HSPs, heat shock proteins; HSP70, heat shock protein 70; IRES, internal ribosome entry site; RLuc, *Renilla reniformis* luciferase; SNAT-2, sodium-dependent neutral amino acid transporter-2; 5′-UTR, 5′-untranslated region.

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by neutral amino acids. Thus, increased osmolarity alone clearly did not affect protein synthesis, rather high concentrations of either betaine or *myo*-inositol actually stimulated translation. When both osmolarity and ionic strength were increased by the presence of relatively high concentrations of inorganic ions or charged amino acids, however, the rate of translation was greatly reduced. This pinpointed to the increased ionic strength as the factor responsible for the impairment of translation.

In addition to the accumulation of compatible osmolytes in mammalian cells the later adaptive phase is characterized by increased production of HSPs (heat shock proteins) to combat stress-related molecular structure dysfunctions [3,4]. One of the most important family in this class is HSP70, composed of molecular chaperones which assist protein folding and protect against protein aggregation, hence reducing the detrimental effect of stressors, such as elevated ionic strength. The accumulation of inducible HSPs is due to different concurrent or alternative mechanisms depending on the cell type and on the nature of the stressor [1]: (i) activation of specific stress-dependent transcription factors, such as heat shock factor 1; (ii) post-transcriptional stabilization of HSP mRNAs; (iii) post-translational regulation increasing the stability of HSP70 protein. In mammalian cells a sudden hypertonic shock is not followed by the accumulation of HSP70 mRNA and protein [5,6], whereas the exposition for a few hours to a prolonged mild hypertonic condition causes an enhancement of HSP70 both at mRNA and at protein level [3,4,7]. Thus, in the latter case translation into proteins of HSP70 mRNA is required.

A peculiar characteristic of HSPs is that they might be synthesized and accumulated in cells when the global rate of cellular protein synthesis is strongly reduced. The initiation of eukaryotic translation is the rate-limiting step of protein synthesis during which capped mRNAs are recognized by several initiation factors that mediate their binding to 40S subunit. However, a subgroup of cellular mRNAs can also initiate translation by a cap-independent mechanism, when cap-dependent translation is down-regulated, as occurs under stress conditions [8,9]. One of the better characterized cap-independent translation initiation mechanisms requires the presence of long, structured, CG-rich 5′-UTR (5′-untranslated region) elements termed IRES (internal ribosome entry site) which are directly recognized by the small ribosomal subunit in the presence of few ancillary protein factors [10]. In the 5′-UTR of human HSP70 mRNA an IRES element capable of driving cap-independent translation initiation has been described [11].

In this paper, by using a novel application of the well-known rabbit reticulocyte cell-free protein synthesis system, we demonstrated that under hypertonic conditions the 5′-UTR of HSP70 messenger confers a comparative advantage in translation with respect to capped-messenger. The same hypertonic-dependent behavior was observed in cell transfected with constructs containing HSP70 5′-UTR or Cricket paralysis virus (CrPV) IRES.

2. Materials and methods

2.1. In vitro transcription

Two capped bicistronic mRNAs encoding for RLuc (*Renilla reniformis* luciferase) under the control of cap-dependent initiation and for FLuc (*Photinus pyralis* luciferase) under the control of either HSP70 5′-UTR or the CrPV–IRES were transcribed from HpaI linearized pR-HSP70-5′-UTR-F (a generous gift of Prof. I.N. Shatsky from Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, Moscow, Russian Federation) or from BglII linearized pR-CrPV–IRES-F (a generous gift of Dr. Ruggero from Helen Diller Family Comprehensive Cancer Center, University of California, San Francisco, CA USA), respectively, using the mMessage mMachine T7 kit (Ambion) according to manufacturer protocol.

2.2. Protein synthesis and luminescence assays

Rabbit reticulocyte lysate was prepared as in [12] and supplemented with 25 μM haemin starting from a stock 10 mM solution prepared as described in [13]. Translation *in vitro* was performed in reaction mixtures (22 μl) containing 30 mM Hepes/KOH, pH 7.5, 80 mM KCl, 1.8 mM magnesium acetate, 50 μM of each amino acid, 1 mM ATP, 0.25 mM GTP, 5 mM creatine phosphate, 0.18 mg/ml creatine phosphokinase, 0.5 mM dithiothreitol, 0.4 mM spermidine, 8.8 μl of lysate and the amounts of bicistronic mRNA reported in the figures. Where indicated, the osmolarity of the reaction mixture was increased by addition of KCl. The complete mixture was incubated at 30 °C for the times indicated in the figures, then the reaction was blocked by the addition of 5.5 μl of passive lysis buffer to provide stability and high performance of the reporter enzymes according to Dual-Luciferase® reporter assay system (Promega). To measure luciferase activity, the samples were treated following the manufacturer instructions: 100 μl of luciferase reagent II were pre-dispensed in multiwell plates, 20 μl of the sample was added, and after 5 s mixing by pipetting, the intensity of the FLuc-dependent luminescence was recorded with a Fluoroskan luminometer (2 s delay, 10 s integration time). The addition of 100 μl Stop & Glo® reagent (5 s vortex) allows quenching of the former reaction and initiation of the RLuc reaction whose luminescence was recorded as above. The osmolality under normal and hypertonic conditions was measured in parallel reaction mixtures with a vapour pressure osmometer (Wescor), the standard mixture being 0.358 osmol/kg of water.

2.3. mRNA transfection

MCF-7 cells were obtained from ATCC. They were grown in RPMI 1640 (Euroclone) supplemented with 10% of FBS (fetal bovine serum, Euroclone), 2 mM L-glutamine (Euroclone), 100 U/mL penicillin and 100 μg/mL streptomycin (Euroclone) at 37 °C in humidified atmosphere containing 5% CO_2. The day before the transfection, 10^5 cells per well were seeded in a 6 well plate. The following day, cells were transfected with 0.4 μg bicitstronic mRNA/sample using Lipofectamine 2000 (Invitrogen) following the manufacturer instructions. After 4 h of transfection, medium was replaced with fresh complete medium in the presence and in the absence of 200 mM sucrose (Carlo Erba). After further 4 h, cells were harvested and analyzed with Dual-Luciferase® reporter assay system (Promega) as described above.

2.4. Statistics

A Student's *t* test was used to compare two groups. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Properties of the cell-free translating system

To measure in parallel the rate of translation of cap-containing and HSP70 5′-UTR-containing messengers under normal and hypertonic conditions we used the haemin-treated unfractionated rabbit reticulocyte lysate. This system is mainly constituted by polysomes actively engaged in the translation of endogenous globin messengers and, in the presence of haemin, the remarkable rate of protein synthesis is preserved for more than 60 min as the rate-limiting initiation step is fully efficient [13]. The lysate was

challenged with an *in vitro* transcribed bicistronic mRNA which drives both cap-dependent and HSP70 5′-UTR-dependent translation in competition with endogenous globin messengers. This avoids any perturbation of the system caused by the digestion of endogenous mRNA with nucleases. Moreover, supplementation of lysate with haemin was performed by adding small aliquots of a 10 mM concentrated stock solution to allow 1000-fold dilution of the osmotically active solvent ethylene glycol. Under these conditions, the osmolality of the complete protein synthesis assay is 0.358 osmol/kg of water and the concentrations of K^+ and Na^+ are 115 mM and 21 mM, respectively.

The structure of the *in vitro* transcribed mRNA is shown in Fig. 1A. The cap-dependent initiation drives the synthesis of the 36 kDa RLuc, the luciferase from *R.reniformis*, whereas direct recognition of HSP70 5′-UTR induces the translation of the 61 kDa firefly FLuc, the luciferase from *P.pyralis*. The two different luciferase activities are easily distinguished since the specific substrates and the reaction conditions of the two bioluminescence assays are different. This allows the measure of the rates of cap-dependent and HSP70 5′-UTR-dependent translation in the same sample at the end of the incubation.

Fig. 1B shows the efficiency of cap-dependent and cap-independent protein synthesis in the presence of different amounts of the bicistronic mRNA, after 2 h incubation at 30 °C. Although the absolute values of luminescence were quite different, both reactions reached a plateau in the presence of the maximum amount of bicistronic mRNA. HSP70 5′-UTR-dependent translation was linear up to 0.3 μg mRNA, while cap-dependent protein synthesis showed a steep increase in the presence of the lowest mRNA amount followed by a further non-linear increase with 0.3 μg mRNA. The latter amount of bicistronic mRNA was chosen in the following experiments. The time-course of protein synthesis (Fig. 1C) showed a lag time of approximately 30 min for HSP70 5′-UTR-dependent translation, conversely the cap-dependent engagement of mRNA is more rapid and efficient. In both cases translation rates progressively increased over 90 min, which is the time chosen for the following experiments. The lower absolute values obtained with cap-independent translation could reflect (i) the known lower efficiency (1–10%) of internal initiation [14] and (ii) the high specialization of reticulocytes in the cap-dependent synthesis of hemoglobin.

3.2. Comparative advantage of HSP70 5′-UTR-dependent translation under hypertonic conditions

Fig. 2 shows the effect of increased osmolarity and ionic strength on the translation system described above by addition of KCl (25–75 mM). This mimics the hypertonic conditions observed in whole eukaryotic cells before the accumulation of compatible osmolytes. In the representative experiment (Fig. 2A) the absolute values of protein synthesis are reported, whereas the means of translation percentage from 3 independent experiments are shown in Fig. 2B. According to previous results obtained in the same cell-free system with endogenous globin mRNA translation [2], under hypertonic conditions the rate of cap-dependent translation dramatically fell, with significant inhibitions observed at all the KCl concentrations studied. In contrast, HSP70 5′-UTR-dependent translation did not significantly changed up an osmolality

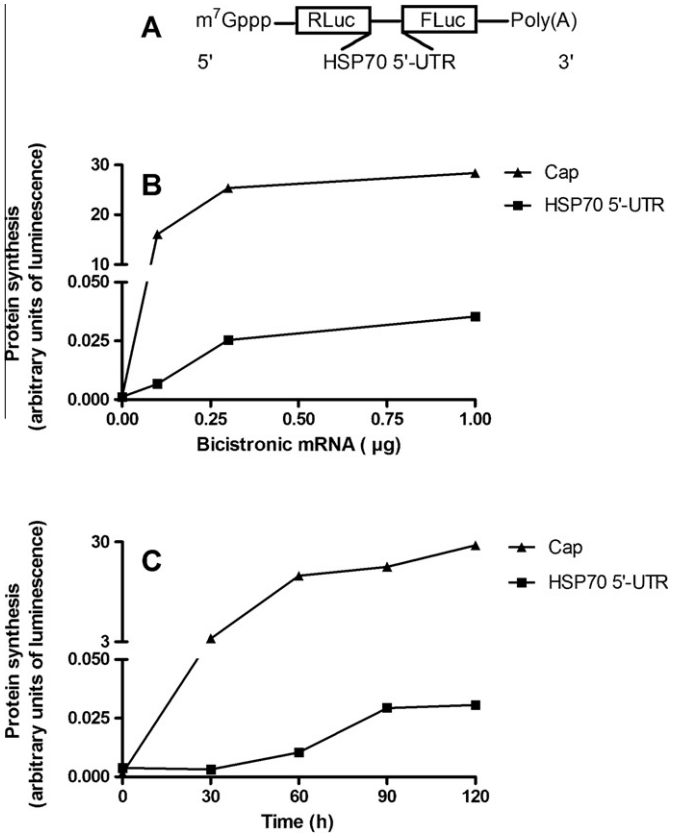

Fig. 1. Rabbit reticulocyte cell-free protein synthesis system translating a bicistronic mRNA. (A), Schematic structure of the bicistronic mRNA driving cap-dependent and HSP70 5′-UTR mediated protein synthesis. (B), Dose–response curve (2 h at 30 °C) in the presence of different amounts of bicistronic mRNA, and (C), Time course of cap-dependent and cap-independent translation in the presence of 0.3 μg of bicistronic mRNA.

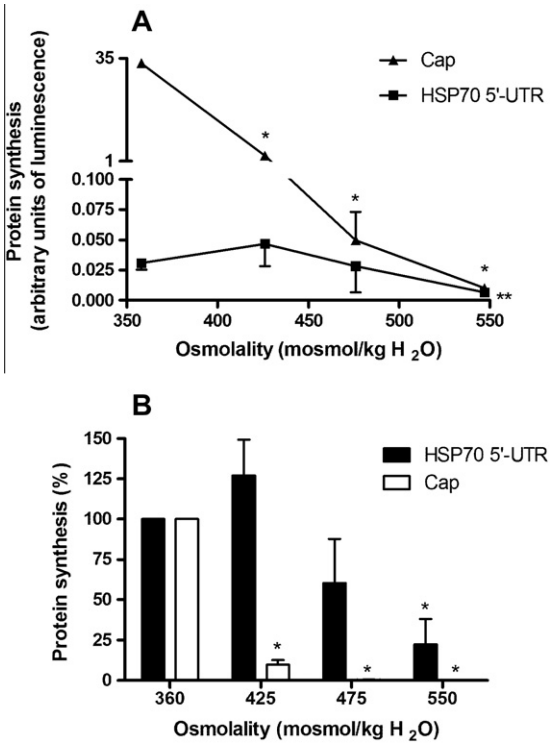

Fig. 2. Effect of increased osmolarity and ionic strength on cap-dependent and HSP70 5′-UTR mediated translation in rabbit reticulocyte cell-free system *in vitro*. (A), Representative experiment showing the opposite effects of increased KCl concentrations on cap-dependent or cap-independent translation. The conditions of the assay are described in materials and methods, incubation was 90 min at 30 °C in the presence of 0.3 μg of bicistronic mRNA. Values are the means ± SD of the experimental points performed in duplicate. (B), Histograms show the percentage values of protein synthesis (means ± SD) from three independent experiments. In the *x*-axis, categorized osmolality values are shown. $^{*}p \leqslant 0.01$; $^{**}p < 0.05$.

of approximately 0.475 osmol/kg of water, rather a non significant increasing trend was observed in mild hypertonic conditions (0.425 osmol/kg of water). Hence, when ionic strength moderately increases the presence HSP70 5′-UTR granted an advantage in mRNA translation with respect to capped messenger, which might explain the synthesis of these chaperones when global cap-dependent translation is strongly impaired.

It should be noted that the addition of 25 mM KCl (lower hypertonic point in Fig. 2) to controls at the end of protein synthesis did not affect the bioluminescence reaction catalyzed by FLuc (105.2 ± 7.4%; $p = 0.425$), thus the resistance of HSP70 5′-UTR-dependent translation to enhanced ionic strength cannot be explained by stimulating effect on the luciferase activity. Also the inhibition of cap-dependent translation is not related to the ionic *optima* of RLuc since the addition of 25 mM KCl rather stimulated the bioluminescence reaction (147.7 ± 15.1%; $p < 0.05$). Similar results were obtained with higher concentrations of added KCl (50 and 75 mM KCl) for both RLuc (stimulation) and FLuc (no influence) activities.

To extend the results obtained in the cell-free translating system in a whole cellular system, we tested the effect of increased osmolarity in a cultured cell line 4 h-transfected with the described *in vitro* transcribed bicistronic reporter mRNA (Fig. 3). MCF-7 cells were exposed to a medium containing 200 mM sucrose. This condition is known to induce an increase of the intracellular ionic strength. After further 4 h, the activities of the two reporter luciferases (RLuc and FLuc) were evaluated. Under hypertonic conditions the rate of cap-dependent translation was significantly reduced (Fig. 3A, left). In contrast, HSP70 5′-UTR-dependent translation was strongly increased (up to 2 times, Fig. 3A, middle). Also the ratio between FLuc and RLuc activities, indicating the normalized HSP70 5′-UTR-mediated translation initiation resulted highly increased (Fig. 3A, right). Taken together, these results confirm and extend the observation made in the cell-free system, strongly suggesting that in cells under hypertonic conditions HSP70 synthesis is driven by the cap-independent translation initiation mediated by 5′-UTR.

3.3. Hypertonicity-resistant translation driven by the IRES of Cricket paralysis virus

The inhibition of cap-dependent translation under various stress conditions and the escape of IRES–containing mRNAs from the inhibition has been well documented [10,15]. To assess whether hypertonicity-resistance is specific to HSP70 5′-UTR or instead a general feature of cap-independent translation, we evaluated the behavior of protein synthesis mediated by a well known viral IRES, i.e., CrPV–IRES, in the above described experimental system. A bicistronic reporter mRNA with CrPV–IRES instead of the HSP70 sequence was used to transfect MCF-7 cells then exposed to hypertonic conditions as described above. The results paralleled those obtained with HSP70 5′-UTR since cap-dependent translation was impaired by the same extent (Fig. 3B, left), whereas hypertonic conditions consistently induced an increase (about 4-fold) in CrPV–IRES-dependent translation (Fig. 3B, middle), also confirmed by the ratio between FLuc and RLuc activities (Fig. 3B, right). This suggests that the resistance to hypertonic conditions is a more general feature of cap-independent translation.

4. Discussion

Under adverse hypertonic conditions mammalian cells are capable of accumulating HSP70 protein to prevent and repair molecular dysfunctions imposed by the stress. Several mechanisms

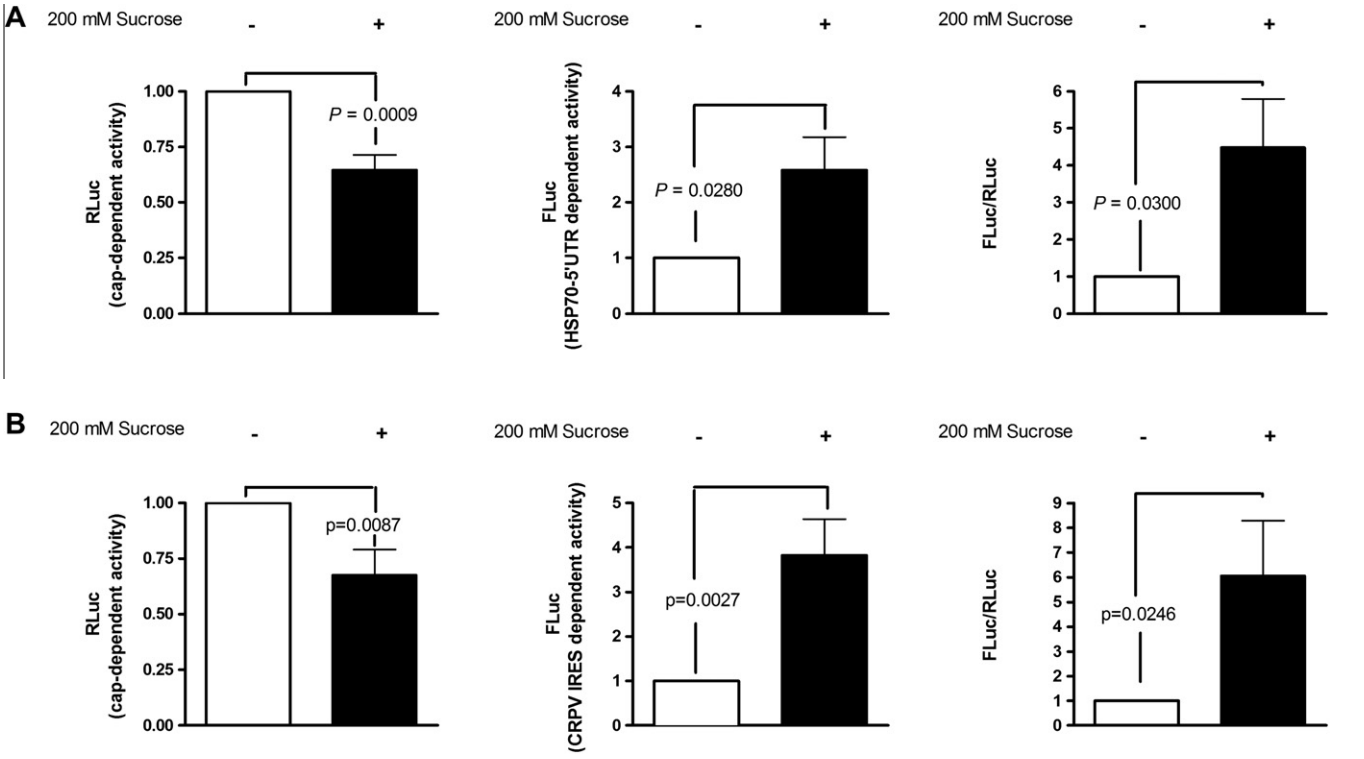

Fig. 3. Effect of increased osmolarity and ionic strength on cap-dependent and HSP70 5′-UTR or CrPV–IRES mediated translation in MCF-7 cells. Cap-dependent translation assessed by measuring the RLuc activity in MCF-7 cells transfected with bicistronic mRNAs containing HSP70 5′-UTR (A, left) or CrPV–IRES (B, left) and treated for 4 h with 200 mM sucrose in complete medium. HSP70 5′-UTR-dependent (A, middle) or CrPV–IRES-dependent translation (B, middle) assessed by measuring the FLuc activity in MCF-7 cells transfected with the bicistronic mRNA and treated for 4 h with 200 mM sucrose in complete medium. Normalized HSP70 5′-UTR-mediated (A, right) or CrPV–IRES-mediated (B, right) translation initiation assessed by using the ratio between firefly and *Renilla* luciferase activities. Histograms represent means ± SD from three independent experiments.

leading to HSP70 accumulation have been described such as transcription activation, mRNA and protein stabilization. While the latter mechanism is independent by *de novo* protein synthesis, the former require the translation into protein of the synthesized messengers. Our results obtained in cell-free systems and in whole cells show that protein synthesis driven by the 5′-UTR of HSP70 mRNA is maintained under hypertonic conditions which strongly impair cap-dependent translation. This explains why HSP70 is synthesized under conditions that dampen the translation of most of the other cellular messengers. In particular this mechanism may be activated in cells challenged with mild hypertonic conditions in which the accumulation of HSP70 requires *de novo* protein synthesis. Accordingly, our experiments in cell-free systems show a fully efficient or even stimulated 5′-UTR-driven translation under mild hypertonic conditions (up to 425 osmol/kg of water), when cap-dependent protein synthesis is more than 90% inhibited. This phenomenon was also observed in whole cells transfected with mRNAs containing HSP70 5′-UTR or a structurally different viral IRES (CrPV–IRES) suggesting that a similar advantage afforded by sequences present in 5′-UTR may be envisaged also for other proteins synthesized by cap-independent mechanisms. Several compatible osmolytes are rapidly accumulated in adapted cells and their transport systems are induced within few hours [1]. Interestingly, the messenger encoding for neutral amino acid transporter SNAT-2 possesses an IRES sequence in the 5′-UTR [16]. Thus, the comparative advantage in translation observed in this paper for HSP70 5′-UTR and for CrPV–IRES might be operative also for other messengers which are actively translated during the adaptive response to hypertonicity.

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