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Distinctive Properties of the 5′-Untranslated Region of Human Hsp70 mRNA*

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A relaxed cap-dependence of translation of the mRNA-encoding mammalian heat shock protein Hsp70 may suggest that its 5′-untranslated region (UTR) possesses an internal ribosome entry site (IRES). In this study, this possibility has been tested in transfected cells using plasmids that express dicistronic mRNAs. Using a reporter gene construct, *Renilla* luciferase/*Photinus pyralis* luciferase, we show that the 216-nt long 5′-UTR of Hsp70 mRNA acts as an IRES that directs ribosomes to the downstream start codon by a cap-independent mechanism. The relative activity of this IRES (100-fold over the empty vector) is similar to that of the classical picornaviral IRESs. Additional controls indicate that this high expression of the downstream reporter is not due to readthrough from the upstream cistron, nor is it due to translation of cryptic monocistronic transcripts. The effect of small deletions within the 5′-UTR of Hsp70 mRNA on the IRES activity varies in dependence on their position within the 5′-UTR sequence. With the exception of deletion of nt 33–50, it is small for the 5′-terminal half of the 5′-UTR and rather strong for the 3′-terminal section. However, neither of these small deletions abolishes the IRES activity completely. Excision of larger sections (>50 nt) by truncation of the 5′-UTR from the 5′-end or by internal deleting results in a dramatic impairment of the IRES function. Taken together, these data suggest that the IRES activity of the 5′-UTR of Hsp70 mRNA requires integrity of almost the entire sequence of the 5′-UTR. The data are discussed in terms of a model that allows a three-dimensional rather than linear mode of selection of the initiation region surrounding the start codon of Hsp70 mRNA.

At least three mechanisms are thought to exist for eukaryotic translation initiation, namely 5′→3′ scanning, "shunting," and internal ribosomal entry. In the scanning model (1), the small ribosomal subunit binds to the 5′-end and is believed to subsequently travel in a 5′→3′ direction until it encounters an initiator AUG codon in a favorable context. The shunting mechanism (2, 3) is also 5′-terminus-dependent but differs from scanning in that the 40 S subunit bypasses part of the 5′-UTR by "jumping" to a region at or near the authentic initiation codon. Translation initiation by internal ribosome entry involves the binding of the 40 S ribosomal subunit to an internal ribosome entry site (IRES)[1] at or near the authentic AUG, thereby eliminating the requirement for scanning through the greater part of the 5′-UTR (for recent reviews see Refs. 4 and 5). Whether these three modes of translational initiation are mechanistically quite different or else they are just versions of the same mechanism of eukaryotic translational initiation is not clear.

IRESs were originally identified in the 5′-UTRs of picornaviral RNAs where these complex structural elements allow ribosomes to enter at a considerable distance from the 5′-end of the viral mRNA. Later, IRESs were also identified within the 5′-UTRs of cellular mRNAs. The list of cellular IRESs is constantly growing (5), giving an impression that every long and structured 5′-UTR of eukaryotic mRNAs may harbor an IRES or employ the shunting model, whereas a "purely" scanning mechanism may operate only for mRNAs with short and unstructured 5′-UTRs.

The cellular IRESs, as a rule, are also complex and highly organized structures. Of those known to date, the only cellular IRES that structurally stands by itself is that from the mRNA-encoding human immunoglobulin heavy chain binding protein (BiP) (6). The attempts to experimentally identify a specific structure within the relatively short 5′-UTR of BiP mRNA (210 nt) have been unsuccessful (7). Even more surprising is that the 5′-UTR of mammalian Hsp70 mRNA has been reported to be devoid of IRES properties (8, 9), despite the fact that its length, GC-content, and relaxed cap-dependence (10, 11) are similar to the 5′-UTR of BiP mRNA, and the corresponding mRNAs encode proteins with related functions (chaperones). The high G+C content of the 5′-UTR of mammalian Hsp70 mRNA seems more compatible with operation via an IRES than by means of the classical scanning mechanism.

The *Drosophila* homologue of the mammalian Hsp70 mRNA has been studied much more extensively. However, the results obtained for its 5′-UTR may be extended to the mammalian Hsp70 mRNA only with great caution. Indeed, the 5′-UTR of the former is strikingly enriched in adenylic residues (>50%), which should greatly facilitate the scanning process. Furthermore, a higher discrimination in translation between Hsp and normal mRNAs is observed in *Drosophila* as compared with mammalian cells (12).

Nevertheless, like the *Drosophil*a homolog, the mammalian

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[1] The abbreviations used are: IRES, internal ribosomal entry site; UTR, untranslated region; EMCV, encephalomyocarditis virus; HRV (hrv), human rhinovirus; nt, nucleotides; eIF, eukaryotic initiation factor; Rluc, *Renilla* luciferase; Fluc, firefly luciferase; PIPES, 1,4-piperazinediethanesulfonic acid.

Hsp70 mRNA demonstrates a reduced dependence on eIF4F, a principal initiation factor that recognizes the cap at the 5′-end of mRNA and initiates the search for the authentic initiation codon. Under heat shock conditions and soon after heat shock, the mammalian cap-binding initiation factor eIF4E is impaired (13–17) and the abundance of eIF4F complexes is reduced (14). In addition, 4E-BPs, repressors of eIF4E, become activated under these conditions due to their hypophosphorylation (18, 19). Thus, like mRNAs encoding other heat shock proteins, the translational activity of human Hsp70 mRNA is resistant to changes in activity of eIF4F.

The relaxed cap-dependence of the 5′-UTR of human Hsp70 mRNA along with the current lack of evidence for an IRES within this 5′-UTR leaves open the possibility of translation initiation occurring via a shunting mechanism. This is usually revealed by a low sensitivity of translation initiation to insertion of a stable hairpin near the 3′-end of 5′-UTR. Indeed, insertion of a stable hairpin into the 3′-end of the 5′-UTR of human Hsp70 mRNA still allows the mRNA to direct translation of a reporter cistron. In addition, two short sequences complementary to a 3′-terminal hairpin of 18 S rRNA have been identified at positions 96–102 and 194–205 of this 5′-UTR. These sequences have been postulated to participate in a primary binding of the 40 S ribosomal subunit followed by its shunting to the authentic AUG codon (8).

However, insertion of the 3′-hairpin showed a large suppression (3-fold) of translation directed by the modified 5′-UTR, whereas, on the contrary, deletion of the sequences complementary to 18 S rRNA did not abolish completely the initiation activity of the 5′-UTR of Hsp70 mRNA (8). In addition, it is not immediately clear why a 5′-UTR that uses for ribosomal binding sequences complementary to 18 S rRNA is not capable of internal initiation.

Given incompleteness of current data on the translation initiation of mammalian Hsp70 mRNA, we have reinvestigated the issue using plasmids that express dicistronic mRNAs, a conventional approach to test eukaryotic 5′-UTRs for the presence of an IRES. Here we show that the 5′-UTR of human Hsp70 mRNA represents an IRES with the relative activity similar to that of the classical picornaviral IRESs. However, unlike many other IRES-elements, the activity of the Hsp70 mRNA IRES requires the integrity of almost the entire sequence of the 5′-UTR.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The initial dicistronic vector pGL3R containing reporter genes for *Renilla* luciferase (first cistron, Rluc) and *Photinus pyralis* firefly luciferase (second cistron, Fluc) was described (20). This vector contains an intercistronic linker with the sites for endonucleases 5′-*Xba*I, *Spe*I, *Pvu*II, and *Eco*RI-3′. To generate pGL3Rhsp70 that has the 5′-UTR of Hsp70 mRNA in the intercistronic position, a *Sal*I (blunt-ended with T4 DNA polymerase)-*NarI* fragment comprising the complete 5′-UTR of Hsp70 mRNA and 33 nt of the coding region of the Fluc gene, was inserted between the *Eco*RI (blunt-ended with T4 DNA polymerase) and *NarI* sites in the pGL3R. The *Sal*I-*NarI* fragment was excised from the dicistronic plasmid pGFP-hsp70-Fluc, kindly provided by Y. Rubtsov (Moscow State University). To shorten the remaining linker separating Rluc and the Hsp70 5′-UTR in pGL3Rhsp70, the *Xba*I site was converted to a *Spe*I site using the oligonucleotide 5′-CTCAAAAATGAACAATAATACTAGTGCTTATCGATACCG-3′ with the GeneEditor *in vitro* site-directed mutagenesis system (Promega). The resulting plasmid was digested with *Spe*I and re-ligated.

To obtain pGL3Rhsp70d33–50, a 315-bp fragment consisting of the last 280 nt of Rluc gene and the first 32 nt of the 5′-UTR of Hsp70 mRNA was amplified by PCR from pGL3Rhsp70 using the following primers: sense 5′-GGCCTCGTGAAATCCCG-3′ and antisense 5′-CTGTCGCAGCAGCTCCTC-3′. The PCR product was digested with *Spe*I, treated with T4 DNA polymerase, and inserted into pGL3Rhsp70d1–50 (see below), which was digested with *Spe*I and blunt-ended with T4 DNA polymerase. To create plasmids pGL3Rhsp70d1–23, d49–68, d72–95, d96–117, d118–140, d152–176, and d177–206, 1.5 kb fragments consisting of the Rluc gene and a part of the 5′-UTRof Hsp70 mRNA were amplified by PCR with Pfu Turbo DNA polymerase (Stratagene) from pGL3Rhsp70 using the reverse primer (Promega) and the following antisense primers: d1–23, 5′-TCTAGTATTATTGTTCATTTTTG-3′; d49–68, 5′-CGAAAAAGGTAGTGG-3′; d72–95, 5′-CTTGGGACAACGGG-3′; d96–117, 5′-CCGCACAGGTTCG-3′; d118–140, 5′-CTCGACGCGCCGG-3′; d152–176, 5′-GCGAGAAGAGCTCG-3′; and d177–206, 5′-GGGCTGGAAACGGAAC-3′. These products were digested with *Avr*II (fragments 1). Then 1 kb fragments consisting of a part of the Fluc gene and a part of the 5′-UTR of Hsp70 mRNA were amplified by PCR from pGL3Rhsp70 using the following primers: antisense 5′-GATCTCTGGCATGCGAGAATC-3′ and sense 5′-TCTGCGACA GTCCACTACC-3′, 5′-AAGGCTTCCCAGAG-3′, 5′-CTTGCAGGCACCGGC-3′, 5′-TTTCCGGCGTCCG-3′, 5′-GCTCTTCTCGCGG-3′, 5′-CCAATCTCAGAGCC-3′, and 5′-GGGAACCGGCATGGC-3′ for d1–23, d49–68, d72–95, d96–117, d118–140, d152–176, and d177–206, respectively. These products were digested with Bsp1407I (fragments 2). The corresponding fragments 1 and 2 were inserted between the *Avr*II and Bsp1407I sites in pGL3Rhsp70 in one step. The dicistronic inserts of the resulting constructs were sequenced. To generate plasmids pGL3Rhsp70d1–151 and pGL3Rhsp70d1–50, 63 and 170 bp fragments of the whole 5′-UTR of Hsp70 mRNA were amplified by PCR from pGL3Rhsp70 using the following primers: sense 5′-GGACTAGTGGATCCAGTGTTCCG-3′ for d1–151 and 5′-GACTAGTGACTCCCGTTG-3′ for d1–50, respectively, and antisense 5′-GGTGGCCATGCCGGTTC-3′. These products were digested with *Spe*I and *Bal*I and then inserted between the *Spe*I and *Bal*I sites in pGL3Rhsp70. To create pGL3Rhsp70d1–96, pGL3Rhsp70 was digested with *Spe*I and *Pst*I, treated with T4 DNA polymerase, and re-ligated. To construct pGL3Rhsp70d51–151, a 1.5-kb fragment covering the Rluc gene and first 50 nt of the 5′-UTR of Hsp70 mRNA was amplified by PCR from pGL3Rhsp70 using the following primers: sense 5′-CTAGCAAAATAGGCTGTCCC-3′ and antisense 5′-GGATCCCTCGAAAAAGGTAGTAGTGGAC-3′. This product was digested with *Eco*RV and *Bam*HI and inserted between the same sites in pGL3Rhsp70. To generate pGL3Rhsp70d97–151, pGL3Rhsp70 was cleaved with *Pst*I and *Bam*HI, blunt-ended with T4 DNA polymerase, and re-ligated.

The construct containing the IRES element from human rhinovirus RNA in the intercistronic region of pGL3R as well as the construct with a low-energy stem preceding the Rluc cistron were a gift from A. Willis. The latter was used to obtain the construct with the 5′-UTR of Hsp70 mRNA in the intercistronic position (construct pGL3RH). To generate pGL3REMCVmut, a 400-bp fragment containing a truncated version of the EMCV IRES with a deletion of nt 701–763 was amplified by PCR from plasmid pTE10 (21) using the oligonucleotides: sense 5′-GCCGTCTTTTGGCCAATGTG-3′ and antisense 5′-GTCAATAACTCCTCTGG-3′. The product was digested with *Eco*RI and *Bal*I, blunt-ended with T4 DNA polymerase, and inserted into plasmid pGL3R treated with *Pvu*II and *Bal*I. The plasmid pGL3Rhsp70 lacking the SV40 promoter was prepared as suggested in Ref. 22.

Cellular RNA Purification—Total cellular RNA from 5×10^6 transfected cells was prepared by the NucleoSpin RNA II kit (Clontech) following the manufacturer's protocol.

RNase Protection Assay—A 626-bp DNA fragment from pGL3Rhsp70 was PCR amplified using primers 5′-GGCCTCGTGAAATCCCG-3′ and 5′-GCAATTGTTCCAGGAACC-3′. This product was blunt-end ligated into *Sma*I site of pSK$^+$ Bluescript (Stratagene). A [^{32}P]UTP-labeled riboprobe was then generated using T7 RNA polymerase with an *Xho*I-restricted DNA template. The transcripts were treated with DNase I and purified on a 4% polyacrylamide/7 M urea gel. The RNA probe was hybridized with total cellular RNA: 2 μg of RNA was dissolved in 30 μl of hybridization buffer (40 mM PIPES (pH 6.4), 400 mM NaCl, 1 mM EDTA, and 80% deionized formamide) containing the probe (3×10^5 cpm) and incubated at 45 °C for 16h. Then 500 μl of 10 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 300 mM NaCl containing 20 units of RNase ONE (Promega) were added, and the mixture was incubated at 37 °C for 30 min. After addition of 50 μg proteinase K and 10 μl of 20% SDS, the mixture was incubated at 37 °C for 15 min and then subjected to phenol extraction and ethanol precipitation with rRNA as a carrier. The RNA samples were then dissolved, denatured, and fractionated on a 4% polyacrylamide/7 M urea gel. The gel was dried and products were visualized by phosphorimaging analysis (Molecular Dynamics). Product sizes were determined using ^{32}P-labeled RNA size markers, which were produced by T7 transcription from a plasmid based on the vector pUC18 containing the HSP70 gene under control of the T7 promoter. Prior to T7 transcription, this construct was linearized with *Bal*I, *Nco*I, or *Pvu*II.

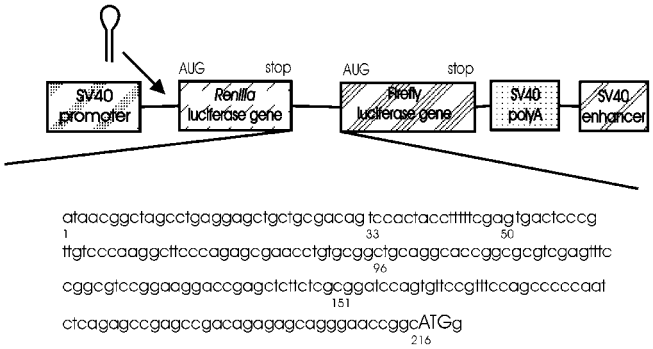

FIG. 1. **Schematic representation of the dicistronic construction in plasmid pGL3R.** The complete nucleotide sequence of the 5′-leader of human Hsp70 mRNA is shown as an insertion into the intercistronic region. Position of a low-energy hairpin in construct pGL3Rhsp70H is indicated by an *arrow*.

DNA Transfections and Reporter Gene Analysis—Approximately 2×10^5 293 or TE671 cells were grown on a 12-well dish in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen). The cells were transfected using the calcium phosphate DNA co-precipitation method as described previously (23), with 5 μg of pGL3R derivatives and 5 μg of β-galactosidase construct pCMV lacZ (Promega) as a transfection control. Cells were harvested after 48 h, luciferase expression was determined using the dual luciferase assay system (Promega), and β-galactosidase expression was estimated with the β-Galactosidase Enzyme Assay system (Promega). Luciferase activities were measured for 60 s following a 10 s delay on a Luminoscan TL (Labsystems) following the manufacturer's protocol. Variations in transfection efficiency were corrected by normalizing luciferase activity to β-galactosidase activity. All assays were performed in triplicate on three to five independent occasions.

RESULTS

Expression of a Downstream Reporter Gene Directed by the 5′-UTR of Hsp70 mRNA in a Dicistronic Vector—To investigate whether the 5′-UTR of Hsp70 mRNA could contain an internal ribosome entry segment, a dicistronic reporter assay was used. The whole 5′-UTR of Hsp70 mRNA was inserted between the two cistrons of the dicistronic vector pGL3R (20) (Fig. 1). The vector incorporates both the sea pansy (*Renilla reinformis*) and firefly (*P. pyralis*) luciferases as the first and second cistrons, respectively. As controls for the basal expression of the second cistron, the highly structured EMCV IRES with a deleted eIF4G-binding site (21, 24) was used along with the initial vector pGL3R. As a positive reference for the IRES activity, a construction with the human rhinovirus IRES element in the intercistronic position of the vector was employed. The activity of both luciferases was determined in transiently transfected human cells 48 h after transfection, and the activity of firefly luciferase (second cistron) was normalized to that of *Renilla* luciferase (first cistron) to generate values for the downstream cistron expression that were independent of transfection efficiency.

As seen in Fig. 2*A*, the translation efficiency of the downstream cistron in 293 cells is increased 100-fold over background after insertion of the 5′-UTR of human Hsp70 mRNA into the intercistronic position, and exceeds 3–4-fold the activity of the well documented rhinovirus IRES. As expected, the lowest IRES activity was found for the mutated EMCV IRES. The IRES properties of the Hsp70 5′-UTR are not affected by the origin of cultured cells. This is evident from comparison of the activities in 293 cells (kidney) (Fig. 2*A*) with that of human TE671 cells (muscles) (Fig. 2*B*). Although the overall IRES activity of the 5′-UTR of Hsp70 mRNA is somewhat lower in TE671 cells than in 293 cells, the relative activities of the Hsp70 IRES to the HRV IRES remained roughly the same.

IRES Properties of the 5′-UTR of Hsp70 mRNA Are Not due to Either Ribosomal Readthrough or the Presence of Cryptic Promoters or Splice Sites in the Dicistronic RNA—To ensure that the apparent internal ribosome entry was not observed due to either enhanced ribosomal readthrough or the presence of cryptic promoters or splice sites in the dicistronic RNA, three control assays were performed. In the first assay, vector pGL3RH (20) was used. This vector is analogous to dicistronic vectors pGL3R but contains a stem-loop structure with a free energy equivalent to -55 kcal mol^{-1} inserted approximately in the middle of the 5′-UTR of *Renilla* cistron. The effect of this insertion on translational efficiency was determined separately for the first and second cistrons (Fig. 2*C*). The *Renilla* and firefly luciferase activities were normalized to β-galactosidase synthesized from a co-transfected plasmid and expressed relative to those obtained from a similar construction but without the hairpin structure. It is evident that the hairpin decreases the translation efficiency of the first reporter gene, whereas translation of the second cistron remains unchanged.

In the second control experiment, to ensure that only intact dicistronic RNA was being transcribed from the dicistronic vector, RNase protection assay was carried out. A ^{32}P uniformly labeled antisense riboprobe (Fig. 3*A*) comprising the 3′-end of *Renilla* luciferase cistron (288 nt), the whole intercistronic region (216 nt), and the beginning of firefly luciferase reporter (122 nt) was annealed to mRNA isolated from cells that had been either mock transfected (Fig. 3*B*, *lane 4*) or transfected with the vector with the whole Hsp70 5′-UTR in the intercistronic position (Fig. 3*B*, *lane 3*) followed by RNase treatment. The data presented in Fig. 3*B*, *lane 3*, clearly show that the protected fragment corresponds to the expected size, 626 nt.

In the third control test, to ensure that there is no cryptic promoter either within the first reporter gene or within the intercistronic region, the SV40 promoter of plasmid pGL3Rhsp70 was destroyed as described in Ref. 22, and 293 cells were transfected with the resulting plasmid. No activity of *Renilla* or firefly luciferases was detected in such cells (data not shown). Taken together these results represent compelling evidence that the 5′-UTR of human Hsp70 mRNA does contain an IRES with a strong translation initiation activity.

Mapping the Hsp70 IRES—To determine the position of the IRES within the 5′-UTR of Hsp70 mRNA, a series of dicistronic constructions was generated containing deletions of different size in the 5′-UTR of Hsp70 mRNA placed between the two cistrons (Fig. 4). The plasmids were transfected into 293 cells and the relative firefly luciferase activity was determined for each of these mutants. As seen from Fig. 5*A*, the IRES activity of the mutant constructs with relatively small deletions varies in dependence on their position within the 5′-UTR sequence. With the exception of deletion of nt 33–50 (construct hsp70d33–50), the effect is small for the 5′-terminal half of the 5′-UTR (deletion of the first 23 nucleotides did not affect the IRES activity) and much stronger for the 3′-terminal section. The most dramatic effect was found for construct d152–176. However, neither of these deleterious deletions abrogated completely the IRES activity of Hsp70 mRNA. This contrasts with the effect of small deletions or even point mutations on viral IRESs, at least those characterized to data (21, 25–32). Some of such mutations completely abolish their translation initiation activity (see, for example, the data for one of the EMCV IRES mutants in Fig. 2*A*).

IRES Activity of the 5′-UTR of Hsp70 mRNA Requires Integrity of Almost the Entire Sequence of the 5′-UTR—The data presented in the previous section may produce the impression that the IRES of Hsp70 mRNA is located mostly in the 3′-

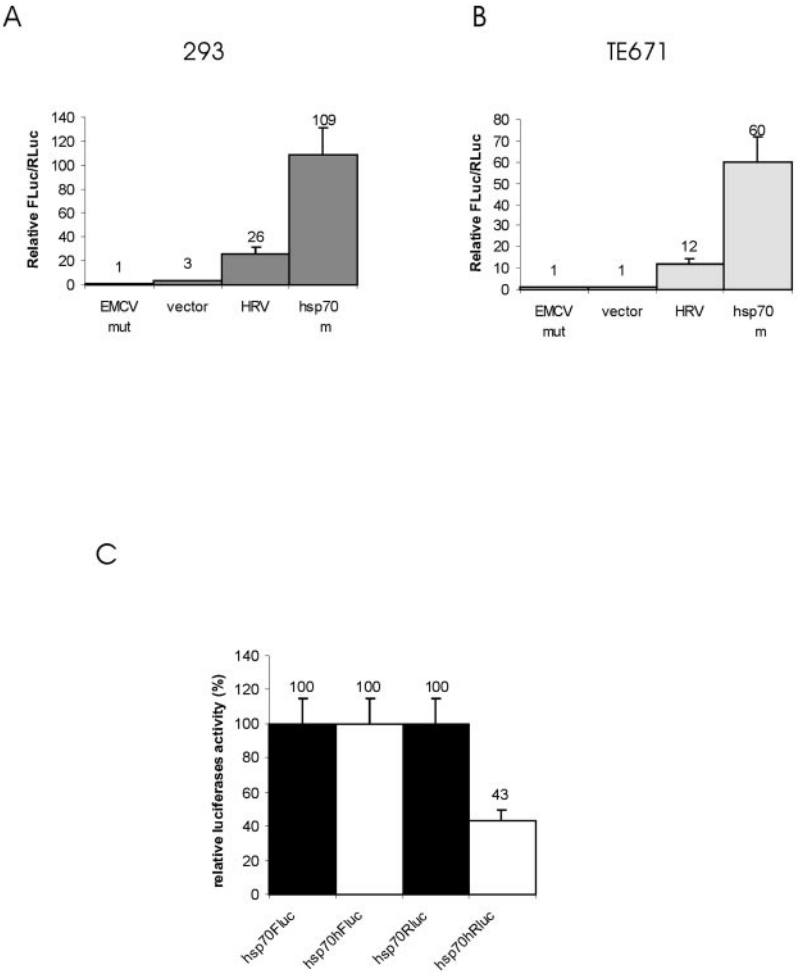

FIG. 2. This figure shows the effect of the 5′-UTR of HSP70 mRNA on expression of the downstream cistron in 293 (*A*) or TE671 (*B*) cells. Both luciferase activities were determined and normalized to β-galactosidase activity as described under "Experimental Procedures." Relative Fluc/Rluc activities were finally normalized to that for the inactivated EMCV IRES element. The ratio of *Renilla*/firefly luciferase activities for this EMCV IRES mutant was set at 1.0. The empty vector, pGL3R, was used as an additional negative control. As a positive control for the IRES activity, the HRV IRES element was employed. The data represent an average from 5 independent transfection experiments. *C*, effect of a low-energy stem-loop structure preceding the *Renilla* cistron on expression of the first and second cistrons in the construction with the 5′-UTR of Hsp70 mRNA in the intercistronic position. Vector pGL3Rhsp70H was transfected into 293 cells and luciferase activity measured as before. The luciferase activities for *Renilla* and *P. pyralis* were normalized to β-galactosidase and expressed relative to those obtained for vector pGL3Rhsp70. The results presented are an average of three independent transfection experiments.

terminal half of the 5′-UTR and the 5′-terminal half is less essential. If this is the case, removal of the 5′-terminal part of the 5′-UTR of Hsp70 mRNA should not abrogate the IRES activity. The results presented in Fig. 5*B* show that this is not the case. Deletion of just the first 50 nt from the 5′-UTR resulting in the intercistronic region, which constitutes 76% of the entire length of the 5′-UTR, leads to a dramatic reduction of the IRES activity. Even larger truncation of the 5′-UTR from the 5′-end further decreased the IRES efficiency (constructs hsp70d1–96) and in construct hsp70d1–151, which is lacking some important elements from the 3′-terminal half (see Fig. 4), it approached a background value (Fig. 5*B*). As expected, internal deletions larger than those used in the experiments shown in Fig. 5*A* almost abrogated the IRES activity. One may conclude that though the 3′-terminal half of the 5′-UTR of Hsp70 mRNA is more susceptible to mutations, almost the entire sequence of the 5′-UTR of Hsp70 mRNA is needed for the IRES activity. The data point to a specific configuration acquired by the 5′-leader of Hsp70 mRNA where many, if not all, parts of its sequence contribute (albeit to a different extent) to a maximal activity in the cap-independent mode of translation initiation.

DISCUSSION

This study focused on the molecular mechanism that allows Hsp70 mRNA to be translated without the cap even under normal conditions. Thus, the use of dicistronic mRNAs in this report, with the 5′-leader of Hsp70 mRNA in the intercistronic position, completely ensured cap-independence of translation of the reporter sequence directed by the 5′-UTR of Hsp70 mRNA under normal physiological conditions. An intriguing feature of the 5′-leader of Hsp70 mRNA that distinguishes it from Hsp70 mRNA from *Drosophila* is its elevated G/C content. This feature makes it poorly compatible with the requirements of the classical scanning model for efficient mRNAs and suggests employment of an alternative way of translation initiation. Here we present compelling evidence that the 5′-leader of Hsp70 mRNA possesses a strong IRES element comparable with that of translationally efficient picornaviral RNAs. However, unlike many other viral and cellular IRES-containing mRNAs studied to date where IRESs occupy an internal position within their long 5′-UTRs, the IRES of Hsp70 mRNA appears to be represented by almost the entire leader of this mRNA. No large section of the 5′-UTR can be excised without a dramatic deleterious effect on the expression of the second cistron. This conclusion nicely correlates with data of Vivinus *et al.* (9), who have recently shown the importance of the integrity of Hsp70 5′-UTR for its enhancing effect on translation of reporter genes in monocistronic constructions.

It should be noted that any data obtained by deletion experiments should be interpreted with great caution, in particular when the secondary structure of the corresponding RNA region is unknown and the resulting mutant RNA derivatives are not probed. Excision of a sequence may entail a substantial reorganization of the secondary structure of the corresponding RNA segment, and the resulting structure may have very little in common with the initial one, a fact that is neglected in many reports on identification and characterization of IRES-elements. This shortcoming is also inherent to the experimental design employed in this study. For instance, deletion of sequences 96–117 or 177–206 produces a remarkable negative

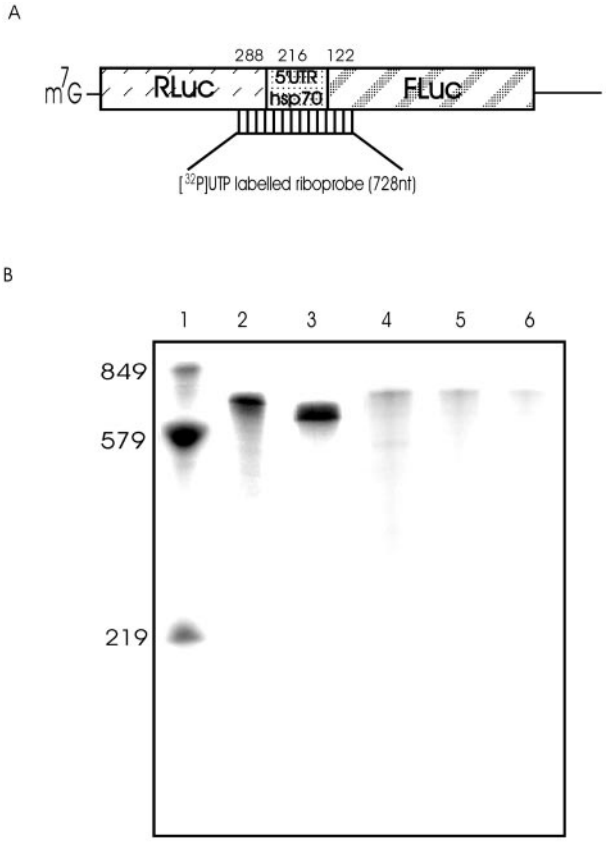

FIG. 3. **Test for the integrity of the dicistronic mRNA produced in 293 cells transfected with plasmid pGL3Rhsp70.** *A* shows the position of annealing of the ^{32}P-labeled riboprobe (728 nt) on the dicistronic mRNA transcribed from pGL3Rhsp70. *B* shows results of RNase protection assay of the riboprobe annealed with the total RNA isolated from cells transfected with pGL3Rhsp70. *1*, RNA size markers (the sizes are shown on the *left* of the gel); *2*, ^{32}P-labeled riboprobe before annealing and digestion with RNase ONE; *3*, fragment protected from nuclease digestion after annealing of the riboprobe with the total cellular RNA; *4*, products after RNase digestion of the riboprobe annealed with the total RNA from mock-transfected cells (control); *5*, products of RNase digestion after hybridization of the probe with the total calf liver tRNA; *6*, free riboprobe after treatment with RNase. For other details see "Experimental Procedures."

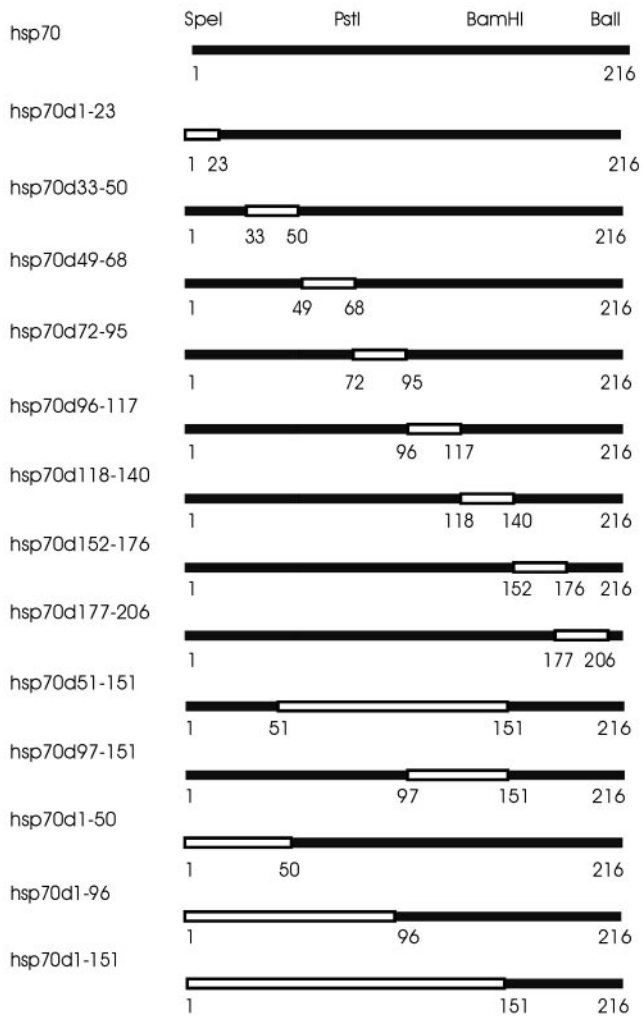

FIG. 4. **Position of different deletions (*open bars*) within the 5′-UTR of HSP70 mRNA inserted between the two reporter genes in the dicistronic vector.** For details see "Experimental Procedures."

effect on the IRES activity of Hsp70 mRNA. These deletions comprise nt 96–102 and 194–205, respectively, that have been recently postulated to be involved in complementary interactions with 18 S rRNA (8). In fact, our data only indicate that these sequences are important parts of a larger sequence forming the IRES element of the 5′-UTR of Hsp70 mRNA. The deleterious effect of their removal may be accounted for by both impairment of the interaction of the 40 S ribosomal subunit with this 5′-UTR and changing a specific secondary structure located in its 3′-half. Certainly, much finer structural analysis is needed to choose between these two possibilities.

Nevertheless, even taking into account these considerations, one may draw some conclusions as to how the IRES of Hsp70 mRNA is organized. The viral IRESs characterized to data all form autonomous and highly ordered structural domains (4). These domains include highly specific binding sites for canonical initiation factors, auxiliary mRNA-binding proteins, or the 40 S ribosomal subunit (4). That is why short deletions or even point mutations in many parts of these IRESs are able to completely destroy them (21, 25–32). This does not appear to be the case for the IRES of Hsp70 mRNA. With the exception of mutant d152–176, deletions within this IRES produce at most strong rather than dramatic effects. This suggests that it forms a relatively loose configuration, presumably with some redundancy of binding sites for mRNA recruiting translational components.

Assuming that the mode of cap-independent accommodation of the scanning machinery is similar for the 5′-terminal (monocistronic) and intercistronic positions of the Hsp70 mRNA 5′-UTR, we suggest a model for the cap-independent recruitment of the Hsp70 mRNA onto the 40 S ribosome (Fig. 6). In this model, the mRNA recruitment machinery binds to the sites of the Hsp70 5′-leader, which includes both its 5′-proximal and 3′-terminal segments as essential elements. The binding occurs in a way that a three-dimensional configuration of the 5′-UTR of Hsp70 mRNA ensures a close proximity of the sequence surrounding the start codon to the scanning apparatus. If so, any large deletion of the internal or terminal segments of the IRES would destroy either binding of the mRNA recruiting factors or the functional configuration of the IRES.

There is a well documented example of the three-dimensional recognition by eIF4A (as a part of eIF4F) of the sequence surrounding the initiation codon. This is the case for the EMCV IRES. Here the mRNA recruitment factors appear to have their fixed binding sites on the IRES (24, 33) and do not appear to move anywhere. The IRES seems to acquire a specific three-dimensional configuration that allows selection of the initiation triplet only within a narrow "starting window" (34, 35). It should be noted that the cap-dependent translation initiation

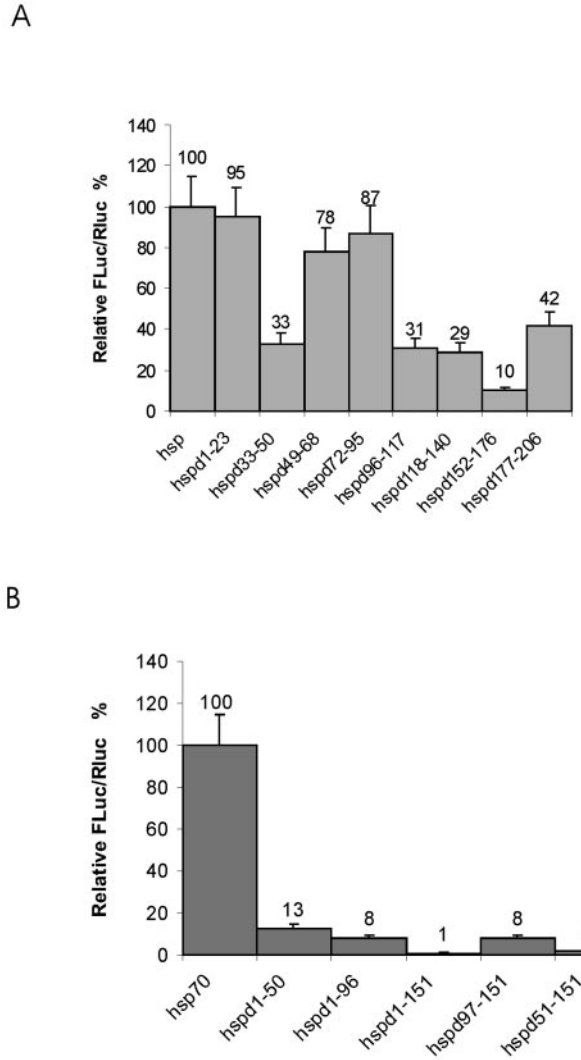

FIG. 5. **Effect of various deletions within the 5′-UTR of Hsp70 mRNA on its IRES activity.** The relative Fluc/Rluc activities were determined as before and expressed as a ratio of the activity for the intact Hsp70 IRES which was set at 100%. *A* represents the effect of smaller deletions, whereas *B* demonstrates the influence of larger deletions.

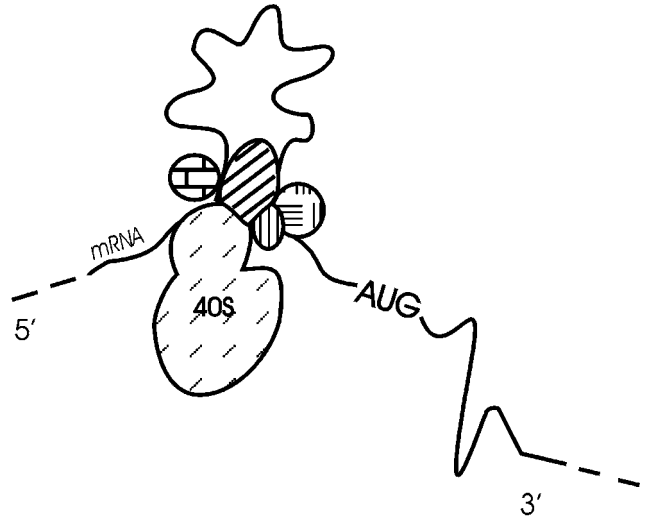

FIG. 6. **A scheme that illustrates how the start AUG codon of the 5′-UTR of Hsp70 mRNA may be selected in a cap-independent way.** The mRNA recruitment apparatus binds both to the 5′-proximal and 3′-proximal sites of the IRES. A temporal succession of these events is not known. A special three-dimensional configuration of the 5′-UTR of hsp70 mRNA ensures a contact of the scanning machinery with the region surrounding the initiation triplet and its proper orientation with respect to the mRNA-binding cleft of the 40 S ribosomal subunit.

does not exclude three-dimensional recognition of the initiation region, either. In that case, a presumably more flexible connection of eIF4F with an mRNA (through the cap) will only facilitate such a three-dimensional recognition.

This model substantially differs from the shunting mechanism of translation initiation of mammalian Hsp70 mRNA that has been recently proposed (8). It necessitates neither prior binding of the mRNA recruitment machinery at the cap nor a subsequent scanning of the 5′-proximal sequence of the 5′-leader of an mRNA followed by a mysterious jumping to the initiation codon. The model also explains why it is not amenable to testing by the conventional approach elaborated for identification of shunting mechanisms. This approach is known to be based on insertion of a low-energy hairpin structure between the 5′-end of an mRNA and its initiation codon. A small effect of such modifications on translational activity is regarded as evidence in favor of the ribosomal shunting. However, it is difficult to predict how such insertions would affect an overall configuration of the 5′-UTR and, hence, relative positions of the scanning machinery and the initiation codon. These considerations may help to explain why insertion of a stable hairpin in the distal position of the 5′-UTR of HSP70 mRNA results in a considerable loss (30% *versus* wt mRNA) of the translational activity (8).

The classical scanning model (1) is strictly linear. It should be stressed, however, that it was directly tested only for 5′-UTRs whose length did not exceed 100 nt. None of the existing reports excludes a three-dimensional way of selection of the target sequence near the initiation codon by initiation factors positioned on the 40 S ribosomal subunit. Thus, we speculate that the model described above is applicable not only to the case of the Hsp70 IRES but may have a more general application.

Certainly, a proximity of the scanning machinery apparatus to the initiation codon may not be the only principal parameter determining the rate and efficiency of translation initiation. A base-pairing and a nucleotide context of the sequence surrounding the initiation codon should greatly affect kinetics of the process. For standard capped mRNAs routinely used in laboratories, with their unstructured and relatively short 5′-UTRs, all three requirements (proximity, a low base-pairing, and a nucleotide context) are optimal, giving a strong preference for the cap-proximal initiation triplet.

We are aware that the model of translation initiation discussed in this paper is speculative. However, we hope that it may prove to be useful in understanding 1) why the overwhelming majority of the IRESs has been identified in long, GC-rich, and highly structured 5′-leaders of mammalian mRNAs, and 2) what the role of cellular IRESs is and how they may be regulated.

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