Translation driven by an eIF4G core domain *in vivo*

Ennio De Gregorio, Thomas Preiss and Matthias W.Hentze¹

Gene Expression Programme, European Molecular Biology Laboratory, Meyerhofstrasse 1, D-69117 Heidelberg, Germany

¹Corresponding author
e-mail: hentze@embl-heidelberg.de

Most eukaryotic mRNAs possess a 5' cap structure (m⁷GpppN) and a 3' poly(A) tail which promote translation initiation by binding the eukaryotic translation initiation factor (eIF)4E and the poly(A) binding protein (PABP), respectively. eIF4G can bridge between eIF4E and PABP, and—through eIF3—is thought to establish a link to the small ribosomal subunit. We fused the C-terminal region of human eIF4GI lacking both the eIF4E- and PABP-binding sites, to the IRE binding protein IRP-1. This chimeric protein suffices to direct the translation of the downstream cistron of bicistronic mRNAs bearing IREs in their intercistronic space *in vivo*. This function is preserved even when translation via the 5' end is inhibited. Deletion analysis defined the conserved central domain (amino acids 642–1091) of eIF4G as an autonomous 'ribosome recruitment core' and implicated eIF4A as a critical binding partner. Our data reveal the sufficiency of the conserved eIF4G ribosome recruitment core to drive productive mRNA translation in living cells. The C-terminal third of eIF4G is dispensable, and may serve as a regulatory domain.

Keywords: eIF4G/internal ribosome entry site/ribosome recruitment/translation initiation

Introduction

The recruitment of the small ribosomal subunit as part of the 43S translation pre-initiation complex normally represents the rate-limiting step in mRNA translation (Hershey, 1991; Sachs *et al.*, 1997). The 5' cap structure and the 3' poly(A) tail, with their respective binding proteins eIF4E and PABP/Pab1p, have been shown to play critical roles in translation initiation (Gallie, 1991; Iizuka *et al.*, 1994; Tarun and Sachs, 1995; Preiss and Hentze, 1998). The function of the protein with which both eIF4E and PABP/Pab1p interact, eIF4G, in ribosome recruitment is less clearly defined (Hentze, 1997). eIF4G is a subunit of the cap binding complex eIF4F which includes the cap recognition factor eIF4E and the RNA-dependent ATPase eIF4A (Figure 1A, upper scheme). Stimulated by eIF4B, eIF4A is thought to unwind secondary structure in the 5' UTR of the mRNA (Rozen *et al.*, 1990). eIF4G has a modular structure (see Figure 5A). It interacts with eIF4E (Mader *et al.*, 1995) and PABP/Pab1p (Tarun and Sachs, 1996; Imataka *et al.*, 1998) through

its N-terminal third, thus circularizing a capped and polyadenylated mRNA (Wells *et al.*, 1998). The central region of mammalian eIF4G bears a putative RNA recognition motif (RRM) (Goyer *et al.*, 1993; De Gregorio *et al.*, 1998) and binding sites for eIF4A (Imataka and Sonenberg, 1997) as well as eIF3, a multiprotein complex directly associated with small ribosomal subunits (Benne and Hershey, 1976; Lamphear *et al.*, 1995). The C-terminal third of eIF4G harbours a binding site for the eIF4E kinase Mnk1 (Pyrnnet *et al.*, 1999; Waskiewicz *et al.*, 1999), and a second binding site for eIF4A (Lamphear *et al.*, 1995; Imataka and Sonenberg, 1997). The multitude of interactions involving eIF4G suggests a central function for this factor in translation.

Not surprisingly, eIF4G and its binding partners are frequent targets for translational regulation. For example, eIF4G is cleaved by the proteases L or 2A during picornaviral infection to inhibit cellular protein synthesis and to favour the translation of viral RNAs (Jackson *et al.*, 1995; Gradi *et al.*, 1998). Furthermore, the eIF4E binding proteins, which competitively inhibit the binding of eIF4E to eIF4G, interfere with cap-dependent translation *in vivo* and *in vitro* (Haghighat *et al.*, 1995; Altmann *et al.*, 1997). Recently, it was also shown that the eIF4G–eIF3 interaction is controlled by the IRE/IRP system that regulates the translation of ferritin and other IRE-containing mRNAs: IRP-1 binding to an IRE located in the proximity of the cap structure blocks the productive assembly of translation initiation complexes beyond the step of eIF4F binding (Muckenthaler *et al.*, 1998).

The wealth of biochemical data and *in vitro* functional assays clearly suggest an important role for eIF4G in translation. However, little was known about the exact role of eIF4G in translation in the context of a living cell, where mRNAs compete for limiting translation initiation factors. To address the role of eIF4G *in vivo*, we adopted a tethered function approach in transfected HeLa cells: the N-terminal eIF4E- and PABP binding region of human eIF4GI was replaced by a specific RNA binding protein (RBP) and directed to a cognate binding site (B.S.) in the intercistronic space of a bicistronic reporter mRNA (Figure 1A, lower scheme). By assaying the translation of the second cistron, which is normally translated very poorly, this approach permits assessment of eIF4G function independent of eIF4E- and PABP binding and in the context of wild-type cells.

Here we show that a region of human eIF4GI (amino acids 642–1560) fused to the RNA binding protein IRP-1 (IRP-4G) suffices to direct translation of the downstream cistron of a bicistronic mRNA bearing an iron-responsive element (IRE) in the intercistronic space. The position and number of IREs modulate this effect. C-terminal deletions of eIF4GI demonstrate that the central region of eIF4GI (amino acids 642–1091) represents the critical core as fur-

Fig. 1. IRP-4G activates the expression of a downstream cistron in a binding-site-specific manner. (A) Schematic representation of cap-dependent recruitment of the small ribosomal subunit (upper panel) and of the experimental strategy adopted in this work (lower panel). (B) HeLa cells were transfected with combinations of three plasmids as follows: each reporter plasmid (MSC, IRE or 3×IRE; see Figure 4 and Table I for intercistronic structure) was tested with the effector plasmids pSGIRP (white bars), pSGIRP-4G (black bars), or the 'empty vector' pSG5 (set to 1 as a reference point, dashed line). In all cases, a plasmid expressing β -Gal (pCMV β) is co-transfected to correct for transfection efficiency. Each bar represents multiple repeat experiments (3–8) and gives CAT expression with standard deviation relative to the corresponding control transfection using pSG5. In addition, the specific translational activation of CAT by appending eIF4GI sequences to IRP-1 is given for each reporter construct as the ratio between the expression observed with pSGIRP-4G versus pSGIRP co-transfection (white numbers inside the bars). LUC expression data were treated in a completely analogous fashion and are given below the corresponding bars (translational activation in bold).

ther deletion into the eIF4A/eIF3 binding region on eIF4GI renders the fusion protein (amino acids 642–877) functionally inactive. Activation of translation of the downstream cistron is preserved when translation of the upstream cistron is inhibited by means of a stem-loop structure placed in the 5'UTR. The activity of the 4G/IRE-IRP module in driving translation in transfected HeLa cells ranges within an order of magnitude of the potency of natural viral internal ribosome entry sites (IRES).

Results

IRP-4G mediates productive *in vivo* translation of the downstream cistron of a bicistronic mRNA in a binding-site-specific manner

To assay the effect of tethering eIF4G domains to a reporter mRNA *in vivo*, we constructed two classes of

plasmid vectors: the first, termed effector plasmids, express human iron regulatory protein-1 alone (pSGIRP), or fused to parts of human eIF4GI (depicted in Figure 5A); the second, termed reporter plasmids, produces bicistronic mRNAs coding for the reporter proteins luciferase (LUC, upstream) and chloramphenicol acetyltransferase (CAT, downstream). Binding sites for IRP-1 (iron-responsive elements, IREs) or for the bacteriophage MS2 coat protein as a negative control (MSC) were introduced into the intercistronic space (Figure 1A, intercistronic sequences are specified in Table I). These plasmids were co-transfected into HeLa cells together with a third plasmid expressing β -galactosidase (β -Gal), which served as a transfection efficiency control.

CAT and LUC activities were corrected for transfection efficiencies and the data expressed in relation to specificity controls in two ways: first, in relation to a transfection with the 'empty' effector plasmid pSG5 (termed 'relative CAT or LUC expression'; relative CAT expression: bar graphs, relative LUC expression: numbers below the histogram in Figure 1B); second, as a ratio between the expression of IRP-1 alone versus the IRP-4G fusion protein (CAT: white numbers inside the histograms, LUC: bold numbers below). Both methods of evaluation yielded consistent results. As expected, cotransfection with IRP-1 alone does not elicit CAT activity (white bars). By contrast, IRP-4G activates CAT expression ~3- to 5-fold when an IRE mediates its binding to the intercistronic space. Importantly, it fails to do so when the IRE is replaced by the MSC site (Figure 1B) which binds neither IRP-1 nor IRP-4G. A further activation in CAT expression is observed when the bicistronic mRNA contains three IREs instead of one (Figure 1B, see Table I and scheme in Figure 4). The effect of the IRP-4G fusion protein is specific for the downstream cistron, because any changes in LUC expression are only minor (1.2- to 1.6-fold) and independent of the binding sites in the intercistronic region (Figure 1B). Inhibition of LUC translation by the effector plasmids was never observed (data not shown).

Metabolic labelling followed by immunoprecipitation with antisera directed against IRP-1 (α IRP, Figure 2A) shows that the expression levels of IRP-1 and IRP-4G in cells transfected with the corresponding effector plasmids are similar (lanes 4 and 5). Gel mobility shift analysis (Figure 2B) demonstrates that overexpressed IRP-1 and IRP-4G both bind to an IRE probe (lanes 1–10) but not to an MSC probe (lanes 11–20). The lower mobility of complexes formed with IRP-4G (lanes 8–10) compared with IRP-1 (lanes 5–7) indicates that the IRP-4G fusion protein binds to the IRE probe. This was confirmed using specific antisera (Figure 2C). Incubation with α IRP disrupts the complexes formed with extracts containing either overexpressed IRP-1 (lane 7) or IRP-4G (lane 4), while antisera directed against human eIF4GI (α 4G) specifically 'supershift' the complex with overexpressed IRP-4G but not IRP alone (compare lanes 3 and 6). We also assessed the steady-state levels and integrity of the reporter mRNAs in the cotransfection experiments by Northern analysis (Figure 2D). Overexpression of IRP-1 (lane 3) or IRP-4G (lane 4) does not affect the level of the IRE reporter mRNAs. The same was found for the other reporter mRNAs used in this study (data not shown). A formal possibility was that monocistronic (uncapped)

Table 1. Sequences of the intercistronic regions of the reporter plasmids used in this study

IRE, +66, $\Delta 33$:	TAA..55nt. CTGGACGGTACCCGGgagatcctctcagatttaacctctccaacccaaggcctctcaggtcgaagtaacgatc GGATCCTGCTTCAAC AGTGCTTGGACGTGATCTTCTAGAGTCAGCTTCGACGAGATTTTCAGGAGCTAAGGAAGCTAAAAATG
MSC:	TAA..55nt. CTGGACGGTACCCGGGGATCCAAGAGACTAGACCATCAGGCTAGTCTCAATCTAGAGTCAGCTTCGACG AGATTTTCAGGAGCTAAGGAAGCTAAAAATG
3 $\times$ IRE:	TAA..55nt. CTGGAGATCCAAAAAATACGTCTGCTTCAACAGTGCTTGGACGACGTCAAAAAACTGCAGTGCTTCAACAGTGCTTGGACACTGCATAAAAAAGCTAGCTGCTTCAACAGTGCTTGGACGCTAGAGTCAGCTTCGACGAGAT TTTTCAGGAGCTAAGGAAGCTAAAAATG
$\Delta 3$, 1+ $\Delta 2$, $\Delta 2+1$:	TAA..55nt. CTGGACggtaccgggGATCCAAAAAATACGTCTGCTTCAACAGTGCTTGGACGACGTCAAAAAACTGCAGT GCTTCAACAGTGCTTGGACACTGCATAAAAAAGCTAGCTGCTTCAACAGTGCTTGGACGCTAGAGTtctagatCAG CTTCGACGAGATTTTCAGGAGCTAAGGAAGCTAAAAATG
BoxB:	TAA..55nt. CTGGACGGTACCCGGGGATCCGGGCCCTGAAGAAGGGCCCTTTCTTTCTAGAGTCAGCTTCGACGA GATTTTCAGGAGCTAAGGAAGCTAAAAATG

The luciferase stop codon and the CAT start codon are in bold and italics. The IREs, the boxB and the MSC sites are shown in bold letters. The sequence deleted in IRE- $\Delta 33$ is underlined. The sequence inserted in 66-IRE is represented in lower case letters. The particularities of constructing $\Delta 3$ result in minor additional sequences (in lower case letters) before and after the triple $\Delta 3 \times$ IRE which are not present in 3 $\times$ IRE, 1+ $\Delta 2$ or $\Delta 2+1$. The conserved cytosines which are deleted in the Δ constructs (see Figure 4B) are outlined.

cleavage products could contribute to CAT protein expression. Such shorter products of >1 kb were undetectable even on long autoradiographic exposures (Figure 2D), or when the analysis was extended to polysome-associated 3 $\times$ IRE mRNA (data not shown).

The data presented in Figures 1 and 2 collectively demonstrate that the IRP-4G fusion protein specifically activates the translation of the downstream CAT cistron in a binding-site-dependent manner. To exclude that our choice of the bulky IRP-1 (98 kDa) as an RNA binding domain could affect the binding of physiological interactors to the eIF4G moiety of the fusion protein and to establish the generality of these findings, we replaced IRP-1 by the short arginine-rich N-terminal domain (amino acids 1–22) of the bacteriophage λ transcriptional antiterminator protein N [λ N-(1–22)] (Tan and Frankel, 1995) (Figure 3A). The intercistronic IRE was replaced by the cognate hairpin, called boxB (Figure 3A). This complete exchange of components constituting the tether for eIF4G yielded site-specific activation of CAT translation of similar or even slightly higher potency (Figure 3B). Thus the effect of appropriate eIF4G fusion proteins on the downstream cistron translation reflects authentic functions of eIF4G. Furthermore, this experiment demonstrates the suitability of the λ N-(1–22)/ λ boxB couple for tethering approaches in heterologous systems.

IRP-4G-mediated translational activation as a function of the position and number of binding sites in the intercistronic region

We next examined whether the exact position of the IRE to which the IRP-4G fusion protein binds affects the function of this protein. Increasing the distance of the IRE from the upstream cistron by 66 nucleotides (66-IRE) from 70 to 136 nucleotides yields a modest, reproducible improvement in CAT activation (Figure 4A). Reducing the distance between the IRE and the translation initiation codon of the downstream cistron from 46 to 13 nucleotides (IRE $\Delta 33$) has a negative effect. We did not assess whether translation initiation in IRE $\Delta 33$ was repositioned to AUG codons downstream of the authentic CAT start codon. The putative products of such initiation (N-terminally truncated or out of frame) are not expected to be detected by the

CAT-ELISA. Even if the optimal position of the binding site remains to be determined, these data show: (i) that specific activation of translation is observed from differently positioned sites, and (ii) that the positioning and context of the IRP-4G fusion protein within the intercistronic space exert quantitative effects on the translation of the downstream cistron.

In the light of these data and the finding that the 3 $\times$ IRE reporter plasmid yields more potent CAT activation (Figure 1B), we determined whether the latter is caused by the multiplicity of binding sites or an improved positioning of one of them. Derivatives of the 3 $\times$ IRE construct were generated (Figure 4B), which contain in each IRE position either a wild-type IRE or an IRE with a single nucleotide deletion (Δ) that reduces its affinity for IRP-1 (Goossen *et al.*, 1990). Mutations of the two upstream ($\Delta 2+1$) or the two downstream (1+ $\Delta 2$) IREs result in markedly weaker activation of CAT expression compared with the wild-type 3 $\times$ IRE construct. The CAT activities of the $\Delta 2+1$ and 1+ $\Delta 2$ constructs approximate those of the single-IRE constructs 66-IRE and IRE (compare Figure 4A and B), suggesting that increasing the distance between functional IRE and either the LUC (66-IRE/ $\Delta 2+1$ versus IRE) or the CAT (1+ $\Delta 2$ versus IRE) cistron only leads to marginal changes of CAT activation. Hence, the presence of multiple binding sites in 3 $\times$ IRE appears to explain better the more potent CAT activation seen with this construct compared with any of the intercistronic regions harbouring a single IRE in various different positions. As expected, mutation of all three IREs in construct $\Delta 3$ profoundly decreases, but does not completely abolish CAT activation. The small but reproducible effect mediated through the $\Delta 3$ sites most likely reflects the residual IRP-1 binding to the mutated IREs (Goossen *et al.*, 1990).

The central third of eIF4G functions as a 'ribosome recruitment core' in vivo

To map the functional core of eIF4G that directs downstream translation, C-terminal deletions (Figure 5A) were assayed. IRP-4G1091 lacks the C-terminal of the two eIF4A binding sites but retains the central eIF4A binding site, the putative RRM and the binding region for eIF3.

Fig. 2. Expression of the effector proteins and reporter mRNAs. (A) HeLa cells were transfected with pSG5 (lane 3), pSGIRP (lane 4) or pSGIRP-4G (lane 5) and incubated in the presence of [³⁵S]methionine. Equal amounts of labelled proteins were subjected to immunoprecipitation using polyclonal antisera against human IRP-1 (α IRP) and SDS-PAGE. Molecular mass markers (sizes in kDa) are shown in lane 1. The molecular masses of IRP-1 and IRP-4G are calculated to be 98 and 201 kDa, respectively. (B) RNA binding characteristics of IRP-1 and IRP-4G. HeLa cells were transiently transfected with the effector plasmids pSG5, pSGIRP or pSGIRP-4G and pCMV β . Similar transfection efficiencies were measured by β -Gal assay. Increasing amounts of cell lysates (7, 14 and 24 μ g cellular protein) were subjected to gel mobility shift analysis with ³²P-labelled IRE (lanes 2–10) or MSC (lanes 12–20) probes (P). Lanes 1 and 11 show the migration of free probes in the absence of cell extract (–). (C) IRP-4G fusion protein binding to the IRE. The IRE probe was incubated with extracts from cells transfected with pSGIRP-4G (lanes 2–4) or with pSGIRP (5–7) in the presence of 2 μ l of polyclonal antibodies directed against eIF4G (α 4G, lanes 3 and 6) or IRP-1 (α IRP, lanes 4 and 7). ‘Supershifted’ complexes were subsequently analysed by gel retardation assay. (D) Expression of IRP-1 or IRP-4G does not affect the levels and integrity of the bicistronic reporter mRNAs. Total RNA was extracted from untransfected cells (lane 1) or cells co-transfected with pSGIRE (lanes 2–4) and the effector plasmids shown on the left (lanes 2–4), and subjected to Northern analysis. Consecutive hybridizations were done with a probe covering both (LUC and CAT) coding regions of the reporter mRNA (upper panel) and with a probe for 18S rRNA (lower panel) as a loading control. The positions of RNA markers (sizes in kb) are shown on the left.

The fusion protein comprising IRP-1 and the central third of eIF4GI still activates CAT translation, albeit with lower (~60%) efficiency (Figure 5B). A further deletion of eIF4GI sequences (IRP-4G877), which preserves the putative RRM, results in a complete loss of activity (Figure 5B). This excludes the possibility that the activa-

Fig. 3. The fusion protein λ -4G activates the expression of a CAT cistron downstream of the boxB binding site. (A) Schematic representation of the λ -4G fusion protein and boxB reporter mRNA. The sequence of the λ N-(1–22) peptide is underlined. (B) Cells were co-transfected with the effector plasmid pSGλ-4G or with the empty vector pSG5 in combination with the reporter plasmids pSGboxB and pSGMSC. Relative CAT and LUC expression is given as in Figure 1B.

tion of translation by the core region of eIF4G is merely due to the presence of the RRM motif, a conclusion that is also supported by the stringent binding site specificity for the translation of the downstream cistron (Figures 1 and 4). As further controls, we confirmed that both IRP-4G1091 and IRP-4G877 fusion proteins bind an IRE probe (Figure 6A) but not the unrelated MSC control (data not shown). The level of complex formation roughly mirrors the steady-state levels of the overexpressed proteins as determined by α IRP-1 immunoblot (Figure 6B). This suggests that RNA binding by the IRP-1 moiety of the fusion is unaffected by adding or removing various eIF4GI sequences (Figures 2, 6A and B).

The immunoblot shown in Figure 5C demonstrates that the IRP-4G and IRP-4G1091 fusion proteins are expressed at comparable levels (lanes 3 and 4) when equal amounts of effector plasmids were transfected, whereas IRP-4G877 is less well expressed (lane 5). This prompted us to titrate the amount of effector plasmids used for transfection and to examine translational activation (Figure 6D) as a function of effector protein levels, measured by immunoblotting (Figure 6C). IRP-4G877 failed to stimulate CAT translation at any DNA concentration tested (Figure 6D), even when IRP-4G877 fusion protein levels approached

Fig. 4. Role of position and number of IRP-4G binding sites. Transfection experiments were performed as in Figure 1 using IRE-derived (A) or 3×IRE-derived (B) reporter constructs (upper panels: schematic representation of intercistronic regions, see Table I for sequences) in combination with pSGIRP-4G (black bars) or pSG5 (dashed line). (A) The position of the single IRE relative to both cistrons is changed by an upstream insertion of 66 nucleotides (66-IRE) or a downstream deletion of 33 nucleotides (IREΔ33). The results for IRE and MSC are shown as positive and negative controls. (B) The 3×IRE construct and variants carrying the ΔC mutation in some or all of the IREs (Goossen *et al.*, 1990) (indicated by a Δ in the scheme on top) were analysed as in (A). In 3×IRE, the first IRE is situated 72 nucleotides downstream from the LUC-ORF, while the distance between the third IRE and the CAT coding region is 41 nucleotides.

and exceeded those of IRP-4G (Figure 6C, compare transfection of 2 pmol of pSGIRP-4G877 with 0.5 pmol of pSGIRP-4G). Serendipitously, this experiment also revealed that transfection of 0.5–1.0 pmol pSGIRP-4G plasmid yields a further substantial increase in downstream cistron translation over the previously used 2.0 pmol (Figure 6D). Therefore, 1.0 pmol of pSGIRP-4G were used in the experiments shown in Figures 8 and 9.

The data shown in Figures 5 and 6 demonstrate that the central third of eIF4GI is sufficient to direct translation *in vivo* with an additional contribution by the C-terminus. Further deletion into this core region, as in IRP-4G877, yields a functionally inactive protein. To correlate these findings with the binding of the relevant interaction partners, we performed co-immunoprecipitation assays with immobilized αIRP-1 from lysates of cells transfected with all different variants of the effector plasmid (Figure 7). The material recovered from the affinity matrix was

Fig. 5. The central region of eIF4GI represents a 'ribosome recruitment core'. (A) Schematic representation of the functional domains of eIF4GI and of the IRP-4G fusion proteins used in this study. Boxed regions indicate interaction domains for PABP (Imataka *et al.*, 1998), eIF4E (Mader *et al.*, 1995), eIF4A and eIF3 (Imataka and Sonenberg, 1997; Lamphear *et al.*, 1995) as well as the putative RRM (Goyer *et al.*, 1993; De Gregorio *et al.*, 1998) (black box). Amino acids are numbered according to the revised eIF4GI sequence (Imataka *et al.*, 1998). [(As a reference, the viral protease 2A cleaves between amino acids 641 and 642 (amino acids 485 and 486 in the original cDNA) (Lamphear *et al.*, 1993).] (B) Effector plasmid pSGIRP-4G, pSGIRP-4G1091, pSGIRP-4G877, pSGIRP or pSG5 were co-transfected with the reporter constructs 3×IRE or MSC as in Figure 1. The graph shows the effects of the different fusion proteins on relative CAT expression. (C) Immunoblot performed with αIRP showing the expression of the plasmids described above. The molecular mass marker (sizes in kDa) is shown on the left. The position of IRP-1 is indicated on the right. The calculated molecular masses are: IRP-1, 98 kDa; IRP-4G, 201 kDa; IRP-4G1091, 149 kDa; IRP-4G877, 126 kDa. Due to the simplified cell lysis procedure for the CAT-ELISA, IRP-4G (lane 3) suffers substantial proteolysis. Note that such proteolysis is far less apparent in the immunoprecipitation experiment shown in Figure 2A.

subjected to SDS-PAGE and immunoblotting. Probing with αIRP-1 confirms that the antisera precipitate the endogenous IRP-1 protein as well as all overexpressed proteins by means of their IRP-1 moiety (Figure 7, upper panel). Reprobing with antibodies directed against human eIF4A (αeIF4A) (lower panel) demonstrates the strong and specific interaction of this factor with the long IRP-4G fusion protein. Deletion of the C-terminal eIF4A binding site in IRP-4G1091 strongly reduced the co-immunoprecipitation of eIF4A, while IRP-4G877 allows no detectable co-precipitation of eIF4A (lane 5). Using the polyclonal anti-eIF3 antisera available to us we failed to obtain interpretable results. Thus, the co-immunoprecipitation analysis revealed correlation between the occurrence and magnitude of translational activation by the

Fig. 6. (A and B) RNA binding properties of the fusion proteins IRP-4G1019 and IRP-4G877. (A) Gel retardation assay. Extract (24 μ g) from cells transfected with 2 pmol of effector plasmids pSG5 (lane 1), pSGIRP (lane 2), pSGIRP-4G1091 (lane 3) and pSGIRP-4G877 (lane 4) were incubated with the IRE probe in the presence of 2 μ l of α 4G. The positions of the IRE-IRP complex, of the α 4G-mediated supershift and of the unbound IRE probe are indicated. (B) The same cell extracts used for the gel retardation assay (A) were subjected to Western analysis performed with α IRP. Note that the strong IRP-1 signal in lane 2 results from endogenous plus transfected sources. (C and D) Dose-response relationships for IRP-4G and IRP-4G877 expression and its effects on CAT translation. Two pmol of empty vector pSG5 (0) or 0.5 to 2 pmol of effector plasmids were co-transfected with the reporter 3 $\times$ IRE. (A) IRP-4G and IRP-4G877 protein expression was determined by immunoblot performed with α IRP. The signals were quantified by densitometry. (B) CAT expression was measured in the samples used for (A).

IRP-4G fusion proteins and their ability and strength of eIF4A binding.

IRP-4G-mediated translational activation in comparison with IRES-mediated and cap-mediated translation

Internal ribosome entry sites that are present within some viral and cellular mRNAs are the only known RNA elements that can drive the translation of a downstream cistron of bicistronic mRNAs (Jang *et al.*, 1988; Pelletier and Sonenberg, 1988). To compare the relative strength of IRP-4G-mediated activation with the potency of a natural IRES, the full-length IRES from hepatitis C virus (Reynolds *et al.*, 1995) including the sequence downstream

Fig. 7. Interactions between the IRP-4G fusion proteins and eIF4A *in vivo*. Cells were transfected with 2 pmol of the indicated effector plasmids. Equal amounts of protein from the different extracts were immunoprecipitated with immobilized α IRP, separated by SDS-PAGE and subjected to immunoblotting. After transfer, the same membrane was incubated with α IRP (upper panel) or monoclonal antibodies against human eIF4A (α eIF4A, lower panel).

Fig. 8. IRP-4G-mediated translational activation in comparison with IRES-mediated translation. (A) Schematic representation of the bicistronic HCV construct (see Materials and Methods). (B) Expression of CAT in cells co-transfected with pSG3 $\times$ IRE and the empty vector pSG5 was set to 1; values for CAT expression are shown for cells co-transfected with pSG3 $\times$ IRE and 1 pmol of pSGIRP-4G or with pSGHCV.

from the translation start codon (see Materials and Methods), was cloned between the LUC and CAT cistrons of our reporter plasmid (HCV, Figure 8A). We chose this IRES, because it is highly active in transfection experiments using several different cell lines including HeLa cells, where it displays similar potency to the other intensively studied IRESes from polio and encephalomyocarditis virus (Borman *et al.*, 1997b). The IRP-4G/3 $\times$ IRE module is 10–15% as efficient as the HCV construct in directing CAT expression under the same conditions (Figure 8B), while luciferase activity was similar in both cases (after correction for transfection efficiency, data not shown).

The cap-dependent translation of the upstream LUC cistron serves as another point of reference for IRP-4G-mediated translation of the downstream CAT cistron. Thus, we determined the absolute quantities of LUC and CAT

Table II. IRP-4G-mediated translational activation in comparison with cap-mediated translation

DNA (pmol)	IRP-4G				
	0	0.5	1	1.5	2
LUC (fmol)	490	830	1060	1307	1322
CAT (fmol)	3.6	46	48	34	30
CAT (%)	0.7	5.5	4.5	2.6	2.3

Reporter plasmid 3×IRE was co-transfected with 2 pmol of empty vector pSG5 (0) or with increasing amount (0.5–2 pmol) of IRP-4G effector plasmid (see Figure 6C and D). The amount of LUC and CAT protein present in equal amounts of cell extract is given in fmol. Percentage of CAT relative to LUC protein is stated below. All data were normalized for transfection efficiency.

Fig. 9. The IRE/IRP-4G module functions as an 'IRES by design'. Left panel: activation of CAT expression using 1 pmol of the effector plasmid pSGIRP-4G, pSGIRP or pSG5 in combination with the 3×IRE or G243 reporter plasmid. Right panel: schematic representation of the G243 mRNA showing the sequence. The calculated free energy of formation of the inhibitory stem-loop structure in the 5' UTR and its repressive effect on LUC translation are also given.

proteins in cell extracts from the experiment shown in Figure 6C and D using appropriate LUC and CAT protein calibration curves (see Table II and Materials and methods). From these measurements, we estimate that IRP-4G-mediated CAT translation is ~5% as efficient as the cap-dependent translation of luciferase (Table II, cotransfection of pSG3×IRE with 0.5 or 1 pmol of pSGIRP-4G).

We conclude from these quantitative comparisons that the translation of the downstream cistron driven by the IRP-4G is significant in comparison with a potent IRES and to cap-mediated translation in living cells.

IRP-4G-mediated translational activation is preserved even when translation of the 5' cistron is inhibited

IRES recruit ribosomes internally, independently of the translation of upstream cistrons (Chen and Sarnow, 1995). We finally wanted to address the mechanism of IRP-4G-directed ribosome recruitment. To block the translation of the upstream LUC cistron, a stable stem-loop structure ($\Delta G = -243$ kJ/mol; Kozak, 1986) was inserted into the 5' UTR of 3×IRE (Figure 9, right panel). While this insertion represses LUC expression very efficiently (96–

97%, Figure 9, right panel), it affects the translational activation of CAT by IRP-4G only by about one third (Figure 9, left panel). This result suggests that, while some of the ribosomes may be re-recruited from the upstream LUC reading frame, ribosomes translating the CAT coding region can be recruited from the free 43S complex pool.

Discussion

Biochemistry, yeast genetics and cell-free translation assays have helped to formulate models of the translation initiation pathway which suggest an important role of eIF4G in the recruitment of the small ribosomal subunit (e.g. Lamphear *et al.*, 1995; Pestova *et al.*, 1996; Tarun and Sachs, 1996; Tarun *et al.*, 1997; Imataka *et al.*, 1998). Information on the proteins that interact directly with eIF4G and their respective roles in translation has allowed the proposal that eIF4G serves as a scaffold or adapter protein between the cap structure and the poly(A) tail of mRNAs on the one hand and the 40S ribosomal subunit on the other (Hentze, 1997; Morley *et al.*, 1997; Sachs *et al.*, 1997). The inhibitory effect of the 4E binding proteins on translation by interference with the eIF4E–eIF4G interaction as well as the negative effect of picornaviral proteolytic cleavage of eIF4G on the translation of host cell mRNAs have underscored the central role of eIF4G in translation initiation (Haghighat *et al.*, 1995; Altmann *et al.*, 1997; Gradi *et al.*, 1998). Moreover, C-terminal fragments of human eIF4G have been shown to be able to stimulate the translation of uncapped mRNAs in a cell-free system by an unknown mechanism (Ohlmann *et al.*, 1996; Borman *et al.*, 1997a; De Gregorio *et al.*, 1998) and to suffice to mediate 40S subunit binding to the IRES of encephalomyocarditis virus (EMCV) (Pestova *et al.*, 1996). Similarly, internal initiation on bicistronic mRNAs lacking conventional IRES elements was stimulated by addition of purified eIF4F *in vitro* (Anthony and Merrick, 1991). All these activities possibly involve the known general RNA binding capacity of eIF4G, which may also serve to increase binding of eIF4E to the capped end of mRNAs (Haghighat and Sonenberg, 1997) thus contributing to efficient initiation of translation in a competitive cellular environment. In cell-free systems where mRNAs are limiting, the RNA binding capacity of eIF4G may function independently, while the EMCV-IRES may have evolved to engage this functionality of eIF4G with particular efficiency.

eIF4G, like several other initiation factors, appears to be required to mediate ribosome recruitment via the cap structure and the poly(A) tail both *in vivo* and *in vitro* (Tarun *et al.*, 1997; Gradi *et al.*, 1998). However, the information available did not permit the prediction of whether productive translation would ensue as a consequence of eIF4G binding to an mRNA, particularly in living cells. We asked whether eIF4G binding would suffice to recruit all necessary 'downstream' translation factors not only to elicit the binding of a functional 43S translation pre-initiation complex to an mRNA, but also to allow the positioning of this complex at a downstream translation initiation codon, and to support the subsequent joining of the 60S subunit for productive translation. To address this question, we devised the bicistronic mRNA

and tethered function approach depicted in Figure 1A. This strategy permits examination of the function of eIF4G (and subdomains thereof) in the context of a wild-type cell and without perturbing general translation.

Insights into translation initiation

Our results identify the central region of eIF4GI as an active 'ribosome recruitment core' which requires no more than a means to bind upstream of an open reading frame to recruit all additional factors necessary for at least basal translation *in vivo* (Figures 1, 3 and 5). Reconstitution experiments with pure translation initiation factors demonstrated that this core region of eIF4GI can bind directly to the EMCV-IRES (Pestova *et al.*, 1996; Kolupaeva *et al.*, 1998) and promote 40S ribosomal subunit binding *in vitro* (Pestova *et al.*, 1996). However, it was not determined whether this pre-initiation complex represented a functional initiation intermediate. Moreover, mutations in the EMCV-IRES have been described which disrupt IRES function (Witherell *et al.*, 1995) without affecting eIF4G binding *in vitro* (Kolupaeva *et al.*, 1998), indicating that eIF4G binding is not sufficient for translation from the EMCV IRES.

The ribosome recruitment core region of eIF4GI defined in this study retains a putative RRM (Goyer *et al.*, 1993; De Gregorio *et al.*, 1998) and interaction sites for eIF4A (Imataka and Sonenberg, 1997) and eIF3 (Lamphear *et al.*, 1995). Both eIF4A and eIF3 have been assigned central roles in current models of translation initiation (see introduction) and likely represent essential components of a minimal productive initiation complex for cellular mRNAs. In support of this, we show here that the ability of the IRP-4G fusion proteins to bind eIF4A correlates with translational activation. Technical limitations precluded us from ascertaining whether the same is true for eIF3; however, the inactive IRP-4G877 lacks part of a region previously found to be required for both eIF3 and eIF4A binding *in vivo* (Imataka and Sonenberg, 1997), allowing the prediction that IRP-4G877 does not bind eIF3 either. Refined mutational studies will be required to address possible individual contributions of eIF3- and eIF4A binding to the translational activity of the eIF4G core region. The C-terminal third of eIF4G harbouring the second eIF4A binding site is able to stimulate the function of the core region, and might thus function as an enhancer or regulatory domain.

IRP-4G-mediated translation of the downstream cistron is preserved even when translation via the 5' end is inhibited (Figure 9). This suggests that ribosomes can be recruited from the free 43S pool by the IRE/IRP-4G module, which acts as an 'IRES by design'. How potent is this module in mediating translation in living cells? We have addressed this issue directly in comparison with cap-mediated (Table II) and IRES-mediated (Figure 8) translation on the one hand, and 'background' termination-reinitiation on the other (Table II). As expected, the background translation of the downstream cistron is low in the absence of a functional IRE/IRP-4G module (0.7% of the translation of the upstream cistron; Table II). By contrast, IRE/IRP-4G-mediated translation is 10–15% as efficient as the HCV-IRES, and still ~5% as efficient as cap-dependent translation. We consider these values to be highly significant. However, it is also important to ask

which aspects of our experimental conditions currently limit the expression of the CAT cistron to these values. Future experiments with the IRE/IRP-4G module will attempt to define the optimal spatial positioning of the 'ribosome recruitment core' in relation to the translation initiation site and to elucidate whether the 'natural' non-covalent binding of eIF4E and PABP to eIF4G is superior to the covalent attachment within the IRP-4G fusion protein. Such experiments may further improve the activity of eIF4G fusion protein-driven translation of the downstream cistron and, importantly, shed light on the role of 'scanning'. It will also be interesting to introduce binding sites for eIF4E and PABP into the IRE/IRP-4G module to assay for potential conformational effects of eIF4E binding to eIF4G or a contribution of the poly(A) tail to the translation of the downstream cistron. Following refinement, we envisage practical utility of our findings in the design of expression vectors that allow control of gene expression at the translational level. Moreover, the sufficiency of eIF4G binding for productive translation of a downstream cistron can be utilized to examine RNA-protein and protein-protein interactions.

Common themes in translation and transcription

The role of the 'ribosome recruitment core' in translation is reminiscent of the function of transcription activation domains, which—when fused to DNA binding proteins—can drive transcription from promoters bearing (multiple) suitable binding sites. This highlights an analogy between these elementary steps in gene expression (Sachs and Buratowski, 1997).

In translation, as well as transcription, cells exploit several strategies to form productive initiation complexes on the nucleic acid templates. The cap structure and the poly(A) tail of a typical cellular mRNA could be viewed as fulfilling a similar function to the IRE in our experiments, while eIF4E and PABP would physiologically play the role adopted by the IRP portion of the IRP-4G fusion protein: to cooperate in the recruitment of eIF4G to the mRNA (Imataka *et al.*, 1998; Preiss and Hentze, 1998; Wells *et al.*, 1998). During translation of rotavirus mRNAs, which are capped but not polyadenylated, the function of PABP appears to be replaced by the viral protein NSP3, which binds to a specific site in the 3' UTR and interacts with eIF4G (Piron *et al.*, 1998). Thus, the various strategies that have evolved to recruit eIF4G to the mRNA could all serve similar purposes.

In summary, eIF4G has long been known to be involved in translation initiation. The most significant advance of the work reported here is the demonstration—in living cells—that eIF4G binding suffices to initiate all downstream events for productive mRNA translation. This finding itself as well as the technical approach used here should pave the way for a better understanding of the translation initiation pathway in mammalian cells, complementing *in vitro* and genetic approaches.

Materials and methods

Plasmid constructs

Effector plasmids: human eIF4GI cDNA sequences were excised from p220Bam (De Gregorio *et al.*, 1998) with *Bam*HI–*Hind*III and inserted in-frame behind the IRP-1 coding region, into the *Msc*I–*Hind*III sites of

pSGIRP (Kollmus *et al.*, 1996) to create plasmid pSGIRP-4G. A *Bam*HI–*Eco*RI fragment from pGEX4G935 (De Gregorio *et al.*, 1998) or a *Bam*HI–*Nsi*I fragment from p220*Bam* were inserted into the *Msc*I site of pSGIRP to create plasmids pSGIRP-4G1091 and pSGIRP-4G877, respectively. A synthetic DNA fragment coding for the λ -N-(1–22) peptide (Tan and Frankel, 1995) was cloned in the *Sac*I–*Bam*HI sites of p220*Bam* to generate p λ -4G. A *Sac*I–*Xho*I fragment from p λ -4G was inserted into the *Sac*I–*Xho*I sites of pSG5 to generate pSG λ -4G. Reporter plasmids: p Δ 4 $\times$ IREGH, p Δ 3+1 $\times$ IREGH and p2+ Δ 2IREGH (Goossen *et al.*, 1990) were amplified by PCR using the primers: 3 $\times$ 5: GACGGAT-CCAAAAAATAC and 3 $\times$ 3: GGTCTAGAACTCTAGCGTCCAA-GCAC; the amplification products were digested with *Bam*HI–*Xba*I and cloned into the *Bam*HI–*Xba*I sites of pIRE.CAT (Preiss *et al.*, 1998) creating p Δ 3CAT, p Δ 2+1CAT and p1+ Δ 2CAT. *Sac*I–*Hind*III fragments from pIRE.CAT, pIREscap.CAT (Preiss *et al.*, 1998), pIRE100 (Paraskeva *et al.*, 1999), pMSC-CAT (Stripecke and Hentze, 1992), p Δ 3CAT, p Δ 2+1CAT p1+ Δ 2CAT or a *Bam*HI–*Hind*III fragment from p4 $\times$ IRE-CAT were ligated into the *Xho*I sites of pSGluc.trs3 (Pantopoulos and Hentze, 1995) to generate the reporter plasmids: pSGIRE, pSGIRE Δ 33, pSG66-IRE, pSGMSC, pSG Δ 3, pSG Δ 2+1, pSG1+ Δ 2 and pSG3 $\times$ IRE (see Table I for sequences of all intercistronic regions). A synthetic DNA fragment corresponding to the boxB site was inserted into the *Bam*HI–*Xba*I sites of pIRE.CAT to generate pB-CAT. A *Sac*I–*Hind*III fragment from pB-CAT was cloned into the *Xho*I sites of pSGluc.trs3 to generate pSGboxB (see Table I for the sequence of the intercistronic region). pSGG243 was created by inserting a synthetic fragment of dsDNA into the *Avr*II site of pSG3 $\times$ IRE (Figure 9). The HCV-IRES element (nucleotides 40–373, including the 5'UTR plus 29 nucleotides of the coding sequence) was amplified by PCR and cloned into the *Bam*HI–*Xba*I sites of pIRE.CAT creating pHCV-CAT. A *Kpn*I–*Hind*III fragment of pHCV-CAT was inserted into the *Xho*I sites of pSGluc.trs3 to generate pSGHCV (see Figure 8A). All plasmid manipulations were verified by sequencing.

Cell transfection and protein analysis

HeLa cells were transiently transfected by the calcium phosphate method using 2 pmol of effector plasmids (unless indicated otherwise), 2 pmol of bicistronic vector and 1 pmol of pCMV β expressing β -Gal. CAT assays were performed using a CAT-ELISA kit (Boehringer Mannheim) following the manufacturer's instructions. Luciferase and β -Gal expression were measured by enzymic activity. CAT and LUC calibration curves were obtained using CAT (Boehringer Mannheim) and luciferase (Promega) standards. Cell lysis for mobility shift analysis was carried out as previously described (Pantopoulos and Hentze, 1995). Binding reactions were performed in the presence of 4 mg/ml heparin at room temperature and samples were resolved as described (Pantopoulos and Hentze, 1995).

Proteins were analysed by metabolic labelling followed by immunoprecipitation as described, using polyclonal antisera against recombinant human IRP-1 (Pantopoulos and Hentze, 1995).

For co-immunoprecipitation analysis, cells were lysed by incubation in 800 μ l of TMGK buffer (20 mM Tris pH 8, 20 mM MgCl₂, 110 mM KCl, 1% Triton X-100, 1 mM PMSF) for 30 min at 4°C. Cellular debris was pelleted by centrifugation at top speed in a microfuge. Supernatants were incubated 1 h at 4°C with antisera against human IRP-1 immobilized on protein A–Sepharose beads with 20 mM dimethylpimelidate as described (Harlow and Lane, 1988). After incubation the beads were washed three times with TMGK buffer and processed for SDS–PAGE as described (Pantopoulos and Hentze, 1995).

For Western blotting proteins were transferred to a reinforced cellulose nitrate membrane. After blocking for 1 h in TTBS (20 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween 20) containing 5% dried milk, membranes were incubated with the appropriate dilution of α IRP (1/500) or α eIF4A (1/50) in TTBS for 1 h at room temperature. Then they were washed four times in TTBS and incubated with a 1/5000 dilution of anti-rabbit or anti-mouse IgG coupled to horseradish peroxidase (Amersham) and subjected to ECL detection (Amersham). Membranes were stripped of the antibodies by incubation in 2% SDS, 68 mM Tris pH 6.7, 100 mM 2-mercaptoethanol for 30 min at 55°C.

Acknowledgements

We thank Martina Muckenthaler, Antje Ostareck-Lederer and Katharina Weber for discussions and experimental help. We thank H.Trachsel for α eIF4A. This work was supported by the European Commission Biotechnology Programme (BIO4-CT95-0045).

References

- Altmann,M., Schmitz,N., Berset,C. and Trachsel,H. (1997) A novel inhibitor of cap-dependent translation initiation in yeast: p20 competes with eIF4G for binding to eIF4E. *EMBO J.*, **16**, 1114–1121.
- Anthony,D.A. and Merrick,W.C. (1991) Eukaryotic initiation factor (eIF)-4F. Implications for a role in internal initiation of translation. *J. Biol. Chem.*, **266**, 10218–10226.
- Benne,R. and Hershey,J.W.B. (1976) Purification and characterization of initiation factor IF-E3 from rabbit reticulocytes. *Proc. Natl Acad. Sci. USA*, **73**, 3005–3009.
- Borman,A.M., Kirchweiger,R., Ziegler,E., Rhoads,R.E., Skern,T. and Kean,K.M. (1997a) eIF4G and its proteolytic cleavage products: effect on initiation of protein synthesis from capped, uncapped and IRES-containing mRNAs. *RNA*, **3**, 186–196.
- Borman,A.M., Le Mercier,P., Girard,M. and Kean,K.M. (1997b) Comparison of picornaviral IRES-driven internal initiation of translation in cultured cells of different origins. *Nucleic Acids Res.*, **25**, 925–932.
- Chen,C. and Sarnow,P. (1995) Initiation of protein synthesis by the eukaryotic translational apparatus on circular RNAs. *Science*, **268**, 415–417.
- De Gregorio,E., Preiss,T. and Hentze,M.W. (1998) Translational activation of uncapped mRNAs by the central part of human eIF4G is 5' end-dependent. *RNA*, **4**, 828–836.
- Gallie,D. (1991) The cap and poly(A) tail function synergistically to regulate mRNA translational efficiency. *Genes Dev.*, **5**, 2108–2116.
- Goossen,B., Caughman,S.W., Harford,J.B., Klausner,R.D. and Hentze,M.W. (1990) Translational repression by a complex between the iron-responsive element of ferritin mRNA and its specific cytoplasmic binding protein is position-dependent in vivo. *EMBO J.*, **9**, 4127–4133.
- Goyer,C., Altmann,M., Lee,H.S., Blanc,A., Deshmukh,M., Woolford,J.J.L., Trachsel,H. and Sonenberg,N. (1993) TIF4631 and TIF4632: two yeast genes encoding the high-molecular-weight subunits of the cap-binding protein complex (eukaryotic initiation factor 4F) contain an RNA recognition motif-like sequence and carry out an essential function. *Mol. Cell. Biol.*, **13**, 4860–4874.
- Gradi,A., Svitkin,Y.V., Imataka,H. and Sonenberg,N. (1998) Proteolysis of human eukaryotic initiation factor 4GII, but not eIF4GI, coincides with the shutoff of host protein synthesis after poliovirus infection. *Proc. Natl Acad. Sci. USA*, **95**, 11089–11094.
- Haghighat,A., Mader,S., Pause,A. and Sonenberg,N. (1995) Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor 4E. *EMBO J.*, **14**, 5701–5709.
- Haghighat,A. and Sonenberg,N. (1997) eIF4G dramatically enhances the binding of eIF4E to the mRNA 5'-cap structure. *J. Biol. Chem.*, **272**, 21677–21680.
- Harlow,E. and Lane,D. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Hentze,M.W. (1997) eIF4G: a multipurpose ribosome adapter? *Science*, **275**, 500–501.
- Hershey,J.W.B. (1991) Translational control in mammalian cells. *Annu. Rev. Biochem.*, **60**, 717–755.
- Iizuka,N., Najita,L., Franzusoff,A. and Sarnow,P. (1994) Cap-dependent and cap-independent translation by internal initiation of mRNAs in cell extracts prepared from *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **14**, 7322–7330.
- Imataka,H. and Sonenberg,N. (1997) Human eukaryotic translation initiation factor 4G (eIF4G) possesses two independent binding sites for eIF4A. *Mol. Cell. Biol.*, **17**, 6940–6947.
- Imataka,H., Gradi,A. and Sonenberg,N. (1998) A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation. *EMBO J.*, **17**, 7480–7489.
- Jackson,R.J., Hunt,S.L., Reynolds,J.E. and Kaminski,A. (1995) Cap-dependent and cap-independent translation: operational distinctions and mechanistic interpretations. *Curr. Top. Microbiol. Immunol.*, **203**, 1–29.
- Jang,S.K., Kräusslich,H.-G., Nicklin,M.J.H., Duke,G.M., Palmenberg,A.C. and Wimmer,E. (1988) A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. *J. Virol.*, **62**, 2636–2643.
- Kollmus,H., Hentze,M.W. and Hauser,H. (1996) Regulated ribosomal frameshifting by an RNA-protein interaction. *RNA*, **2**, 316–323.

- Kolupaeva,V.G., Pestova,T.V., Hellen,C.U. and Shatsky,I.N. (1998) Translation eukaryotic initiation factor 4G recognizes a specific structural element within the internal ribosome entry site of encephalomyocarditis virus RNA. *J. Biol. Chem.*, **273**, 18599–18604.
- Kozak,M. (1986) Influences of mRNA secondary structure on initiation by eukaryotic ribosomes. *Proc. Natl Acad. Sci. USA*, **83**, 2850–2854.
- Lamphear,B.J., Yan,R., Yang,F., Waters,D., Liebig,H.D., Klump,H., Kuechler,E., Skern,T. and Rhoads,R.E. (1993) Mapping the cleavage site in protein synthesis initiation factor eIF-4 γ of the 2A proteases from human coxsackievirus and rhinovirus. *J. Biol. Chem.*, **268**, 19200–19203.
- Lamphear,B.J., Kirchweiger,R., Skern,T. and Rhoads,R.E. (1995) Mapping of functional domains in eukaryotic protein synthesis initiation factor 4G (eIF4G) with picornaviral proteases. *J. Biol. Chem.*, **270**, 21975–21983.
- Mader,S., Lee,H., Pause,A. and Sonenberg,N. (1995) The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 γ and the translational repressors 4E-binding proteins. *Mol. Cell. Biol.*, **15**, 4990–4997.
- Morley,S.J., Curtis,P.S. and Pain,V.M. (1997) eIF4G: translation's mystery factor begins to yield its secrets. *RNA*, **10**, 1085–1104.
- Muckenthaler,M., Gray,N.K. and Hentze,M.W. (1998) IRP-1 binding to ferritin mRNA prevents the recruitment of the small ribosomal subunit by the cap-binding complex eIF4F. *Mol. Cell*, **2**, 383–388.
- Ohlmann,T., Rau,M., Pain,V.M. and Morley,S.J. (1996) The C-terminal domain of eukaryotic protein synthesis initiation factor (eIF) 4G is sufficient to support cap-independent translation in the absence of eIF4E. *EMBO J.*, **15**, 1371–1382.
- Pantopoulos,K. and Hentze,M.W. (1995) Nitric oxide signaling to iron-regulatory protein (IRP): Direct control of ferritin mRNA translation and transferrin receptor mRNA stability in transfected fibroblasts. *Proc. Natl Acad. Sci. USA*, **92**, 1267–1271.
- Paraskeva,E., Gray,N.K., Schläger,B., Wehr,K. and Hentze,M.W. (1999) Ribosomal pausing and scanning arrest as mechanisms of translational regulation from cap-distal iron-responsive elements. *Mol. Cell. Biol.*, **19**, 807–816.
- Pelletier,J. and Sonenberg,N. (1988) Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature*, **334**, 320–325.
- Pestova,T.V., Shatsky,I.N. and Hellen,C.U.T. (1996) Functional dissection of eukaryotic initiation factor 4F: the 4A subunit and the central domain of 4G subunit are sufficient to mediate internal entry of 43S preinitiation complexes. *Mol. Cell. Biol.*, **16**, 6870–6878.
- Piron,M., Vende,P., Cohen,J. and Poncet,D. (1998) Rotavirus RNA-binding protein NSP3 interacts with eIF4G and evicts the poly(A) binding protein from eIF4F. *EMBO J.*, **17**, 5811–5821.
- Preiss,T. and Hentze,M.W. (1998) Dual function of the messenger RNA cap structure in poly(A)-tail-promoted translation in yeast. *Nature*, **392**, 516–520.
- Preiss,T., Muckenthaler,M. and Hentze,M.W. (1998) Poly(A)-tail-promoted translation in yeast: implications for translational control. *RNA*, **4**, 1321–1331.
- Pyronnet,S., Imataka,H., Gingras,A.-C., Fukunaga,R., Hunter,T. and Sonenberg,N. (1999) Human eukaryotic translation initiation factor 4G (eIF4G) recruits Mnk1 to phosphorylate eIF4E. *EMBO J.*, **18**, 270–279.
- Reynolds,J.E., Kaminski,A., Kettinen,H.J., Grace,K., Clarke,B.E., Carroll,A.R., Rowlands,D.J. and Jackson,R.J. (1995) Unique features of internal initiation of hepatitis C virus RNA translation. *EMBO J.*, **14**, 6010–6020.
- Rozen,F., Edery,I., Meerovitch,K., Dever,T.E., Merrick,W.C. and Sonenberg,N. (1990) Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F. *Mol. Cell. Biol.*, **10**, 1134–1144.
- Sachs,A.B. and Buratowski,S. (1997) Common themes in translational and transcriptional regulation. *Trends Biochem. Sci.*, **22**, 189–192.
- Sachs,A.B., Sarnow,P. and Hentze,M.W. (1997) Starting at the beginning, middle, and end: translation initiation in eukaryotes. *Cell*, **89**, 831–838.
- Striebeck,R. and Hentze,M.W. (1992) Bacteriophage and spliceosomal proteins function as position-dependent *cis/trans* repressors of mRNA translation *in vitro*. *Nucleic Acids Res.*, **20**, 5555–5564.
- Tan,R. and Frankel,A.D. (1995) Structural variety of arginine-rich RNA-binding peptides. *Proc. Natl Acad. Sci. USA*, **92**, 5282–5286.
- Tarun,S.Z., Jr and Sachs,A.B. (1995) A common function for mRNA 5' and 3' ends in translation initiation in yeast. *Genes Dev.*, **9**, 2997–3007.
- Tarun,S.Z., Jr and Sachs,A.B. (1996) Association of the yeast poly(A) tail binding protein with translation initiation factor eIF-4G. *EMBO J.*, **15**, 7168–7177.
- Tarun,S.Z., Jr, Wells,S.E., Deardorff,J.A. and Sachs,A.B. (1997) Translation initiation factor eIF4G mediates *in vitro* poly(A) tail-dependent translation. *Proc. Natl Acad. Sci. USA*, **94**, 9046–9051.
- Waskiewicz,A.J., Johnson,J.C., Penn,B., Mahalingam,M., Kimball,S.R. and Cooper,J.A. (1999) Phosphorylation of the cap-binding protein eukaryotic translation initiation factor 4E by protein kinase Mnk1 *in vivo*. *Mol. Cell. Biol.*, **19**, 1871–1880.
- Wells,S.E., Hillner,P.E., Vale,R.D. and Sachs,A.B. (1998) Circularization of mRNA by eukaryotic translation initiation factors. *Mol. Cell*, **2**, 135–140.
- Witherell,G.W., Schultz-Witherell,C.S. and Wimmer,E. (1995) Cis-acting elements of the encephalomyocarditis virus internal ribosome entry site. *Virology*, **214**, 660–663.

Received May 14, 1999; revised and accepted July 12, 1999