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Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E

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An important aspect of the regulation of gene expression is the modulation of translation rates in response to growth factors, hormones and mitogens. Most of this control is at the level of translation initiation. Recent studies have implicated the MAP kinase pathway in the regulation of translation by insulin and growth factors. MAP kinase phosphorylates a repressor of translation initiation [4E-binding protein (BP) 1] that binds to the mRNA 5′ cap binding protein eukaryotic initiation factor (eIF)-4E and inhibits cap-dependent translation. Phosphorylation of the repressor decreases its affinity for eIF-4E, and thus relieves translational inhibition. eIF-4E forms a complex with two other polypeptides, eIF-4A and p220, that promote 40S ribosome binding to mRNA. Here, we have studied the mechanism by which 4E-BP1 inhibits translation. We show that 4E-BP1 inhibits 48S pre-initiation complex formation. Furthermore, we demonstrate that 4E-BP1 competes with p220 for binding to eIF-4E. Mutants of 4E-BP1 that are deficient in their binding to eIF-4E do not inhibit the interaction between p220 and eIF-4E, and do not repress translation. Thus, translational control by growth factors, insulin and mitogens is affected by changes in the relative affinities of 4E-BP1 and p220 for eIF-4E.

Keywords: 4E-BP1/p220/translation initiation

Introduction

The expression of many eukaryotic genes is regulated at the level of translation initiation. This multistep event functions to position the ribosome at the AUG initiation codon. All eukaryotic cellular mRNAs (except organellar) contain a cap structure [m^{7}G(5′)ppp(5′)N; where N is any nucleotide] at their 5′ terminus (Shatkin, 1985). The cap structure is an important regulatory determinant of translational efficiency and functions to facilitate the attachment of the 40S ribosomal subunit to mRNA. Ribosome binding is a rate-limiting step in translation initiation and a frequent target for regulation. Ribosome binding to mRNA requires the participation of three eukaryotic initiation factors (eIF; eIF-4A, eIF-4B and eIF-4F) and ATP hydrolysis (Sonenberg, 1988). eIF-4F, which mediates cap function (Tahara *et al.*, 1981; Edery *et al.*, 1983; Grifo *et al.*, 1983), is composed of three subunits: (i) a 24 kDa cap binding polypeptide, eIF-4E (Sonenberg *et al.*, 1978); (ii) a 50 kDa polypeptide, eIF-4A, which exhibits RNA-dependent ATPase and bidirectional RNA unwinding activities (Ray *et al.*, 1985; Rozen *et al.*, 1990); and (iii) a 220 kDa polypeptide, p220, whose integrity is required for eIF-4F activity in cap-dependent translation, as its cleavage following poliovirus infection results in the shut-off of host protein synthesis (Sonenberg, 1987).

eIF-4E plays a key role in the regulation of translation (Hershey, 1991). It is present in limiting amounts in the cell (Hiremath *et al.*, 1985; Duncan *et al.*, 1987), consistent with a regulatory role in translation. There exists also a strong correlation between the phosphorylation state of eIF-4E and the rates of protein synthesis and cell growth. Increased eIF-4E phosphorylation occurs in response to growth factors, mitogens, hormones and cytokines (Morley and Traugh, 1989; Frederickson *et al.*, 1991). eIF-4E is hypophosphorylated during mitosis (Bonneau and Sonenberg, 1987a), following heat-shock (Duncan *et al.*, 1987) or infection with several viruses (Huang and Schneider, 1991; Feigenblum and Schneider, 1993), concomitant with a reduction in cap-dependent translation rates. The mechanism by which phosphorylation enhances translation is not well understood, but eIF-4E phosphorylation enhances its binding to the cap structure (Minich *et al.*, 1994).

The biological significance of the regulation of eIF-4E levels is highlighted by its ability, when overexpressed, to transform rodent cells (Lazaris-Karatzas *et al.*, 1990; Lazaris-Karatzas and Sonenberg, 1992) and to deregulate HeLa cell growth (De Benedetti and Rhoads, 1990). Consistent with its transforming activity, eIF-4E is mitogenic, as its microinjection into quiescent NIH3T3 cells activates DNA synthesis (Smith *et al.*, 1990). The microinjection of eIF-4E mRNA into early embryos of *Xenopus laevis* also leads to mesoderm induction (Klein and Melton, 1994). One plausible explanation for the transforming activity of eIF-4E is that its overexpression results in a more active eIF-4F complex, and hence increased unwinding activity and mitigation of the translational repression of the growth-promoting genes that are important for the control of cell growth. Indeed, the increased expression of cyclin D1 (Rosenwald *et al.*, 1993), ornithine decarboxylase (Shantz and Pegg, 1994) and c-*myc* (De Benedetti *et al.*, 1994) has been demonstrated in eIF-4E-overexpressing cells.

The activity of eIF-4E is modulated by two specific binding proteins (BP), termed 4E-BP1 and 4E-BP2 (Pause *et al.*, 1994b). These proteins exhibit high sequence homology (4E-BP1, 93% identity; 4E-BP2, 55% identity) to PHAS-I (Hu *et al.*, 1994). 4E-BP1 (PHAS-I) is a heat- and acid-stable protein which is phosphorylated by MAP kinase on serine 64 in response to insulin and growth

factors that signal through the MAP kinase pathway (Lin *et al.*, 1994). The association of 4E-BP1 with eIF-4E decreases exclusively the translation of capped but not uncapped mRNAs both *in vitro* and in cultured cells (Pause *et al.*, 1994b). However, this interaction is diminished dramatically upon the phosphorylation of 4E-BP1 in response to insulin, concomitant with the relief of the translational repression of capped mRNAs. These results explain previous reports on the enhancement of eIF-4F activity and the specific stimulation of cap-dependent translation following insulin treatment (Manzella *et al.*, 1991; Gallie and Traugh, 1994). Taken together, these findings indicate a key role for 4E-BPs in the regulation of protein synthesis and cellular growth and differentiation.

It has been suggested that the stimulatory action of insulin on translation results from the relief of 4E-BP1 inhibition of the interaction between eIF-4E and p220, hence leading to the formation of an active cap binding protein complex, eIF-4F, and subsequent ribosome binding (Pause *et al.*, 1994b). Here, we demonstrate that indeed 4E-BP1 blocks the interaction between eIF-4E and p220 *in vitro* using recombinant p220 from baculovirus. This inhibition is explained by the competition between p220 and 4E-BP1 for binding to eIF-4E through a similar binding site. In cultured cells, the eIF-4F complex precludes the association of 4E-BP1. Consistent with these results, 4E-BP1 inhibits the translation initiation of capped mRNAs by preventing the interaction between the 43S pre-initiation complex and the mRNA.

Results

4E-BP1 inhibits the 43S pre-initiation complex binding to mRNA

To determine the step of translation that is inhibited by 4E-BP1, ribosome binding experiments were performed. We formed 80S initiation complexes with ^{32}P cap-labeled chloramphenicol acetyltransferase (CAT) RNA in a reticulocyte lysate in the presence of anisomycin, a peptide chain elongation inhibitor. The resulting complexes were resolved by centrifugation through linear 10–50% sucrose gradients. A large fraction (45%) of the input mRNA was bound to 80S ribosomes and disomes (Figure 1A). Disome formation occurred because the 5′ untranslated region (UTR) could accommodate the binding of two ribosomes (Pelletier and Sonenberg, 1985a). The addition of glutathione-S transferase (GST)–4E-BP1 inhibited mRNA binding to ribosomes (16% of mRNA bound, ~2.9-fold inhibition), causing displacement of the mRNA to the top of the gradient. To assess the cap specificity of the inhibition, we examined the effect of 4E-BP1 on ribosome binding to mRNAs which initiate translation via a cap-independent internal ribosome binding mechanism. A ^{32}P-labeled CAT mRNA containing the encephalomyocarditis (EMC) virus internal ribosome entry site (IRES) in its 5′ UTR was used. The IRES element serves as a direct ribosome landing pad, and hence translation of this RNA is mediated via a cap-independent pathway. In this experiment, ~30% of the mRNA input was bound to 80S and disomes (Figure 1B; we suspect disome formation, but we have not characterized the disome fractions). Strikingly, in contrast to the effect on CAT mRNA, GST–4E-BP1 had no significant effect on ribosome binding (Figure 1B, ~1.2-fold inhibition).

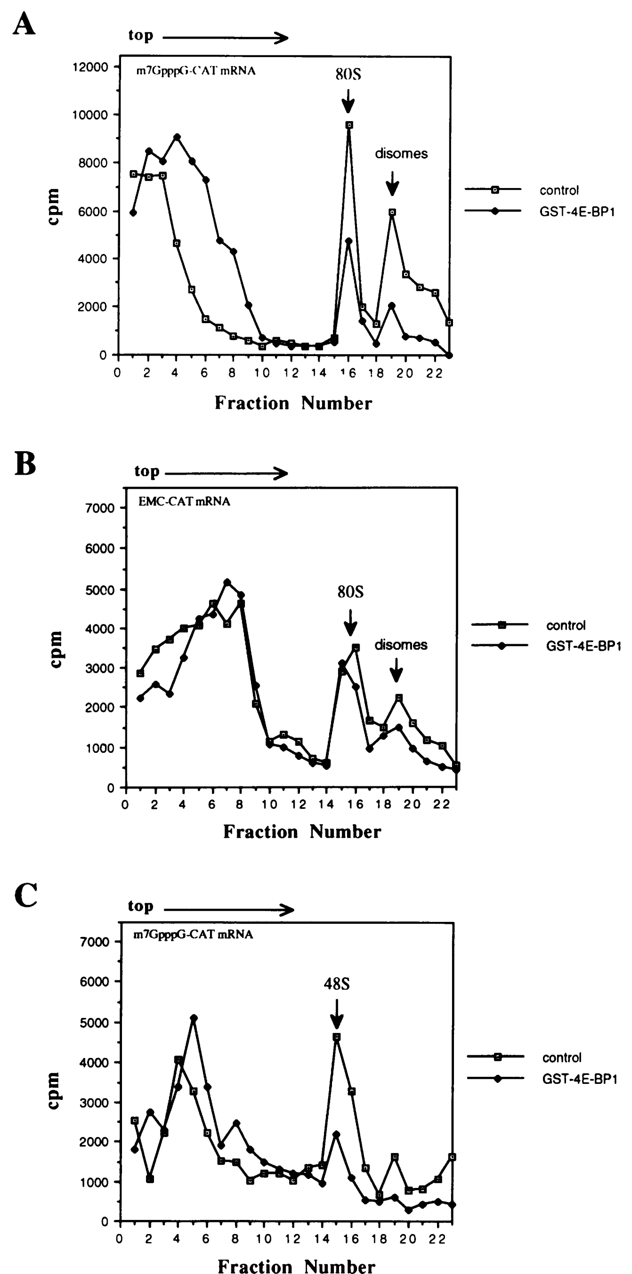


Fig. 1. 4E-BP1 inhibits mRNA–ribosome binding. Assays were performed in reticulocyte lysates with ~8×10^4 c.p.m. of ^{32}P-labeled capped CAT mRNA (A and C) or uncapped ^{32}P-labeled EMC–CAT mRNA (B). (**A**) Lysate (35 μl) was pre-incubated with buffer A (see Materials and methods) or 5 μg GST–4E-BP1 for 20 min at 30°C. Next, anisomycin and the other components were added and the reaction mixture was incubated for a further 20 min at 30°C. (**B**) As in (A), but ^{32}P-labeled EMC–CAT mRNA was used. (**C**) Lysate (35 μl) was pre-incubated with GST–4E-BP1 for 20 min at 30°C. Next, ^{32}P cap-labeled mRNA and the other components were added and the reaction mixture incubated for a further 20 min at 30°C in the presence of 10 mM GMP-PNP (Calbiochem). Initiation complexes were resolved on sucrose gradients as described in Materials and methods. Sedimentation was from left to right.

To examine the association of mRNA with 43S pre-initiation complexes, ribosome binding experiments were performed with capped CAT mRNA in the presence of guanylyl-imidodiphosphate (GMP-PNP). This non-hydro-

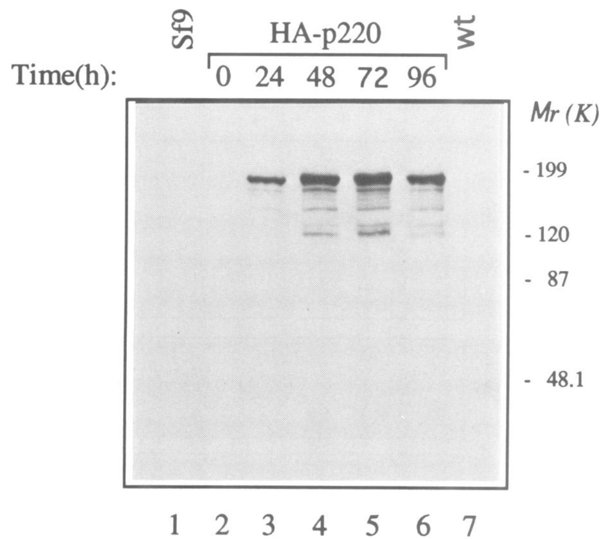


Fig. 2. Immunoblot analysis of p220 produced in *Sf9* cells. *Sf9* insect cells were infected with wild-type baculovirus or recombinant virus containing human p220 cDNA. Total protein was solubilized by cell lysis in buffer containing 50 mM Tris–HCl, pH 7.5, 100 mM KCl, 2 mM DTT, 1 mM EDTA, 1% Triton X-100, 1 mM $MgCl_2$, 10% glycerol, 1 mM PMSF, 20 μg/ml leupeptin, 50 μg/ml aprotinin and 1 mM benzamidine. Protein was resolved by SDS–8% PAGE and detected by Western blotting using an anti-HA monoclonal antibody (12CA5). Lane 1, uninfected *Sf9* cells; lanes 2–6, *Sf9* cells infected with recombinant p220 virus; lane 7, *Sf9* cells infected with wild-type virus. Molecular masses for protein standards (Bio-Rad) are indicated to the right of the panel.

lyzable GTP analog causes the accumulation of 48S pre-initiation complexes, as GTP hydrolysis is required prior to the joining of the 60S ribosomal subunit (Antony and Merrick, 1991). The sucrose concentration of the gradients was adjusted to 10–40% to improve the resolution between the 48S ribosome complex and the unbound mRNA. In this experiment, 42% of the mRNA input was associated with the 48S pre-initiation complex (Figure 1C). Pre-incubation of the reticulocyte lysate with GST–4E-BP1 resulted in the displacement of most of the mRNA to the top of the gradient, with ~17% of the mRNA input bound to the 48S complex (~2.5-fold inhibition). We conclude that 4E-BP1 inhibits translation initiation by preventing 40S ribosome attachment to the mRNA.

Purification of recombinant baculovirus and expression of p220 protein in Sf9 insect cells

We have hypothesized previously that 4E-BP1 competes with p220 for binding to eIF-4E, and thus inhibits eIF-4F complex formation and subsequent ribosome binding. To test this hypothesis, we expressed p220 as a fusion protein with the influenza hemagglutinin (HA) epitope tag in insect cells using a baculovirus expression system. Recombinant virus was purified using a series of screening techniques. Dot blot analysis revealed that most of the plaques contained the foreign gene (data not shown). High titer recombinant virus free from occlusion bodies was prepared and used to infect *Sf9* insect cells. The expression of p220 protein was examined by immunoblotting using an anti-HA monoclonal antibody (12CA5; Figure 2). p220 was first detected 24 h post-infection, and continued to accumulate up to 72 h. Uninfected cells (lane 1) and cells infected with wild-type virus (lane 7) showed no immunoreactivity. Lysates were prepared at ~72 h post-infection for all the experiments described below.

The major species of the p220 protein migrated as an ~190 kDa polypeptide. The fainter series of bands which migrated faster are most likely degradation products of p220. The cDNA of p220 encodes a 150 kDa polypeptide, but the protein migrates anomalously with an M_r of 220 kDa (Tahara *et al.*, 1981; Edery *et al.*, 1983; Yan *et al.*, 1992). The discrepancy in the migration of p220 expressed in *Sf9* insect cells with the authentic protein may be attributed to the extent to which this protein is processed in insect cells. An analysis of the amino acid sequence of p220 reveals potential sites for glycosylation, as well as phosphorylation by protein kinase C and protein tyrosine kinases (Yan *et al.*, 1992). p220 is highly phosphorylated *in vivo* (Morley and Traugh, 1989) and also in *Sf9* cells (data not shown). However, insect cells are known to be deficient in terminal glycosylases (Possee, 1986; Jarvis and Summers, 1989; Johnson *et al.*, 1989), which may explain the observed faster migration.

Analysis of the interaction between eIF-4E, p220 and 4E-BP1

Unlike the other subunits of eIF-4F (eIF-4E and eIF-4A), no specific biochemical activity has been documented for p220. p220 has been isolated from mammalian cells invariably as part of a complex with eIF-4E (Tahara *et al.*, 1981). Therefore, a functional p220 should form a complex with eIF-4E. To investigate this association, we studied the interaction of p220 with eIF-4E on an m^7GDP-coupled agarose resin to which eIF-4E binds specifically. Lysates of insect cells expressing HA–p220 were incubated with an m^7GDP-coupled agarose resin. HA–p220 alone did not bind to the resin, as determined by Western blotting (Figure 3A, lane 1). However, HA–p220 was retained by the resin in the presence of recombinant murine eIF-4E (lane 3). To test the hypothesis that 4E-BP1 and p220 compete for binding to eIF-4E, GST–4E-BP1 was pre-incubated with the m^7GDP-coupled resin containing bound eIF-4E. After extensive washing of the resin, the HA–p220-containing lysate of insect cells was added. As expected, GST–4E-BP1 bound to the resin only in the presence of eIF-4E (Figure 3A, compare lane 2 with lane 4). HA–p220 was no longer retained by the resin when the eIF-4E was complexed with GST–4E-BP1 prior to the addition of the p220-containing insect cell lysate (lane 4). These results strongly suggest that p220 and 4E-BP1 compete for binding to eIF-4E.

To substantiate this conclusion further, the reciprocal experiment was performed. An HA-p220-expressing *Sf9* cell lysate was pre-incubated with m^7GDP-bound eIF-4E prior to the addition of GST–4E-BP1. GST–4E-BP1 was retained by the resin only in the presence of eIF-4E (Figure 3B, compare lane 2 with lane 6). Similarly, HA–p220 bound to the resin only in the presence of eIF-4E (Figure 3B, compare lane 4 with lane 7). Pre-incubation of m^7GDP-bound eIF-4E with HA–p220-expressing *Sf9* lysates completely prevented the binding of GST–4E-BP1 to the cap column (lane 3). As a control, an uninfected *Sf9* cell lysate did not prevent the interaction between GST–4E-BP1 and eIF-4E (lane 5). Therefore, eIF-4E pre-bound to HA–p220 can no longer interact with GST–4E-

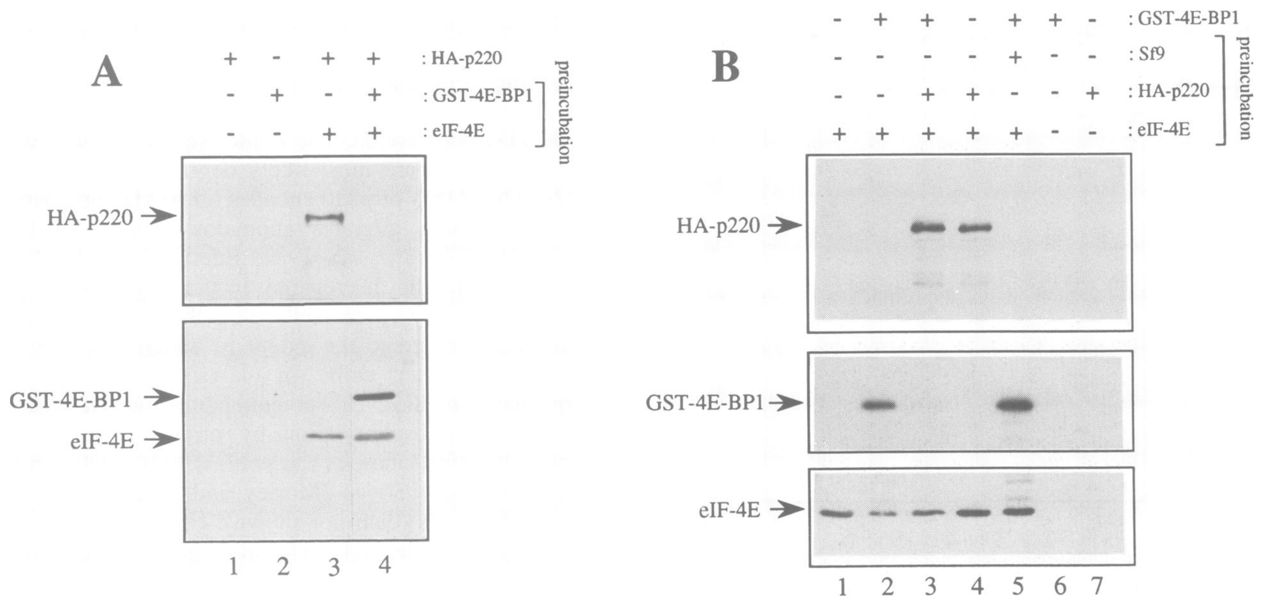


Fig. 3. 4E-BP1 and p220 compete for binding to eIF-4E. (**A**) m^7GDP-coupled agarose resin was incubated with buffer A (lanes 1 and 2) or 0.5 μg recombinant murine eIF-4E in buffer A. The resin was washed in buffer A (3×1 ml) and then incubated with either buffer A or 3 μg GST–4E-BP1 for 60 min at 4°C. The resin was washed in buffer A (3×1 ml) and incubated further with buffer A or 50 μl (~5×10^5 cells) of an HA–p220-expressing *Sf9* cell lysate for 60 min at 4°C. The resin was rinsed and bound proteins were eluted in SDS–sample buffer. Proteins were resolved on SDS–polyacrylamide gels and immunoblotting was performed as described in Materials and methods. (**B**) As in (A), but m^7GDP-bound eIF-4E was pre-incubated with 100 μl HA–p220-expressing *Sf9* cell lysate or 100 μl (~1×10^6 cells) of uninfected *Sf9* cell lysate before further incubation with 1 μg GST–4E-BP1. Incubations periods were as in (A). Minus signs indicate incubation with buffer A.

BP1. Taken together, these findings demonstrate that the binding of p220 and 4E-BP1 to eIF-4E is mutually exclusive.

Because the interaction of p220 and 4E-BP1 with eIF-4E was measured on a cap column, it is possible that the interaction of eIF-4E with the cap affected the outcome of the results. To circumvent this problem, we also used a glutathione column to bind GST–4E-BP1. In addition, this experiment was also designed to exclude the possibility that 4E-BP1 and p220 interact directly. GST–4E-BP1 bound to the glutathione–Sepharose column, as determined by Western blotting using an antibody to 4E-BP1 (Figure 4, lane 1). No signal was detected with an anti-HA antibody when a lysate of uninfected *Sf9* cells was incubated with either the resin alone (lane 2) or resin with bound GST–4E-BP1 (lane 3). Similarly, when an HA–p220-expressing *Sf9* cell lysate was incubated with either the resin alone (lane 4) or resin containing GST–4E-BP1 (lane 5), no HA–p220 was retained. This result demonstrates that p220 has no affinity for GST–4E-BP1. As anticipated, eIF-4E did not bind by itself to the glutathione column (lane 6) but interacted with GST–4E-BP1 (lane 7). A combination of the HA–p220-containing extract and eIF-4E also failed to bind the resin in the absence of GST–4E-BP1 (lane 8). Most importantly, when HA–p220 was pre-incubated with the resin containing eIF-4E already bound to GST–4E-BP1, no HA–p220 was retained on the resin (lane 9; this experiment was conducted at the same time as that shown in Figure 3 which contains a positive control for HA–p220). Taken together, these results and those shown in Figures 2 and 3 demonstrate that eIF-4E exists as a complex with 4E-BP1 or p220, but not with both.

The eIF-4F complex precludes the association of 4E-BP1

Notwithstanding the above results, it is possible that 4E-BP1 associates with eIF-4F in cells. In HeLa cells, eIF-4E exists in two forms: as a slowly sedimenting (<6S) form comprising only the 24 kDa CAP binding protein (CBP), and as part of a more rapidly sedimenting (~8–10S) complex, eIF-4F (Tahara *et al.*, 1981). In the light of the results described above, it is predicted that 4E-BP1 should not associate with the eIF-4F complex. To examine this, HeLa cells were lysed in high salt lysis buffer and subjected to velocity sedimentation on a 10–40% sucrose gradient. Catalase (11S), run in parallel on a separate gradient, sedimented at fractions 10 and 11 (Figure 5). eIF-2, which has a sedimentation coefficient of ~6S (Konieczny and Safer, 1983), was detected mainly in fractions 5 and 6, and serves as another sedimentation marker in this experiment. The immunoblot analysis of the fractions sedimenting slower than 11S revealed the two forms of eIF-4E (Tahara *et al.*, 1981): one centered in fraction 3, and the other at fractions 5–7. The higher molecular weight polypeptides p220 and eIF-4A cosedimented with eIF-4E, as expected if they were to be associated with eIF-4E to form the eIF-4F complex. eIF-4A sediments as a singular protein and as part of the eIF-4F complex (Nielsen and Trachsel, 1988). The trailing of this protein into lighter fractions represents the free form. In sharp contrast to the sedimentation of the different initiation factors, a Western blot analysis of 4E-BP1 revealed that the protein sedimented at the top of the gradient. No 4E-BP1 cosedimented with eIF-4F in fractions 5–7, indicating that 4E-BP1 is precluded from the eIF-4F complex. It is worth noting that some 4E-BP1

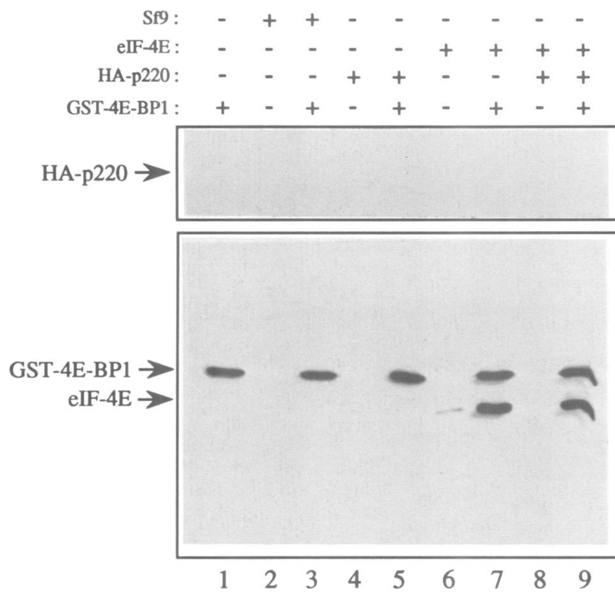


Fig. 4. Affinity purification of complexes of GST–4E-BP1. Glutathione-coupled Sepharose beads were incubated with either buffer A or ~0.4 µg GST–4E-BP1 for 30 min at 4°C. The resin was washed in buffer A before the addition of the following components: 0.5 µg recombinant murine eIF-4E and 50 µl of an uninfected or HA–p220-expressing *Sf9* cell lysate. Incubations were as in Figure 3. Minus signs indicate incubation with buffer A. Bound protein was eluted and analysed as described in the legend to Figure 3.

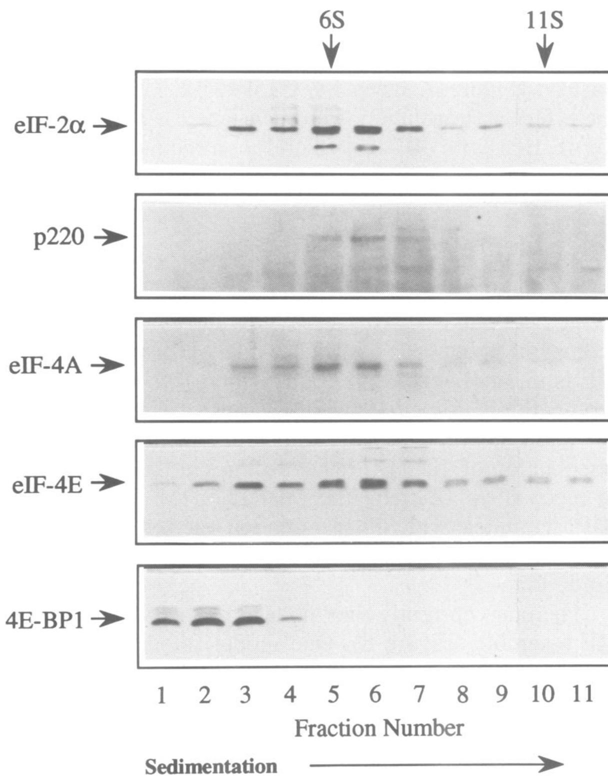


Fig. 5. Density gradient sedimentation of 4E-BP1 in HeLa cell extracts. HeLa cell extract was subjected to a density gradient sedimentation analysis on a 10–40% sucrose gradient (see Materials and methods). Equivalent samples of each fraction sedimenting slower than 11S were subjected to SDS–PAGE and analyzed by immunoblotting, as described in Materials and methods. Sedimentation is from left to right.

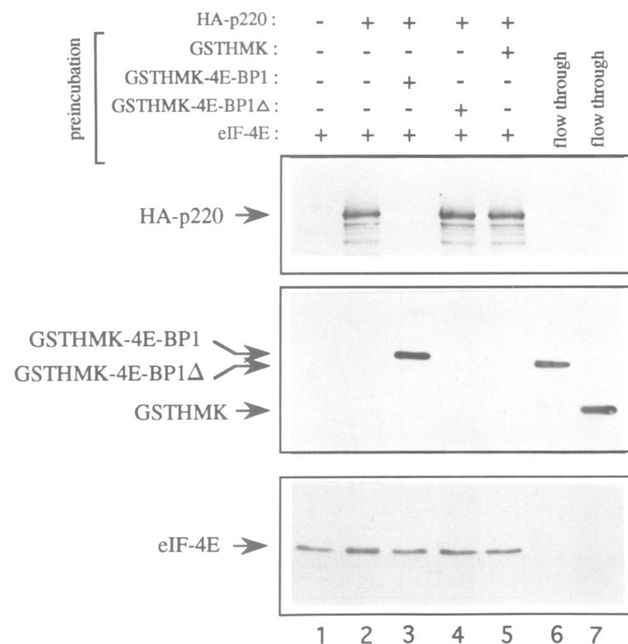
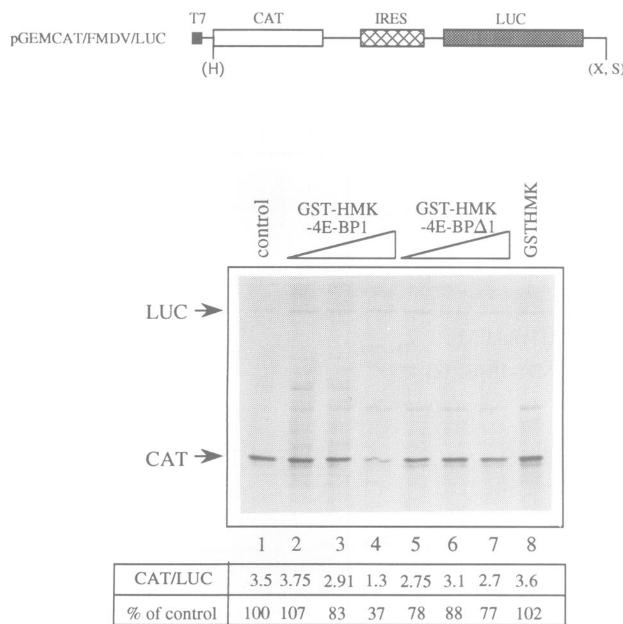


Fig. 6. A 4E-BP1 deletion mutant does not block the interaction between eIF-4E and p220. m^{7}GDP affinity purification of complexes was performed as described in the legend to Figure 3. eIF-4E (0.5 µg)-bound m^{7}GDP agarose resin was pre-incubated with GST fusion proteins of HMK–4E-BP1 (3 µg), HMK–4E-BP1Δ (3 µg) or HMK (3 µg). The resin was then washed (3×1 ml) in buffer A before the addition of 50 µl of an HA–p220-expressing *Sf9* cell lysate. Bound proteins were analysed as described in the legend to Figure 3. Lanes 6 and 7, flowthrough from pre-incubations indicated in lanes 4 and 5, respectively. GST fusion proteins were detected with a rabbit anti-GST polyclonal antibody (1:1000).

cosedimented with eIF-4E close to the top of the gradient (fractions 2 and 3), presumably in a complex form. These results further confirm the conclusion that eIF-4E in a complex with p220 cannot interact with 4E-BP1 in the cell.

p220 and 4E-BP1 compete for eIF-4E through binding domains that share common sequence motifs

p220 and 4E-BP1 share a region of homology which is required for the binding of either protein to eIF-4E, as deletions or conserved point mutations in this region abrogate the interaction. The respective amino acid sequences of the common motifs in p220 and 4E-BP1 are EKKRYDREFLLGF and TRIIYDRKFLMEC (identical amino acids are in bold), representing amino acids 412–424 and 50–62, respectively (Mader *et al.*, 1995). To examine the possibility that 4E-BP1 and p220 compete for eIF-4E through this binding domain, a GST–4E-BP1 mutant containing a deletion of amino acids 54–62 was used. This sequence bears a high homology to the eIF-4E binding site in human p220 (Mader *et al.*, 1995). GST fusion proteins of heart muscle kinase (HMK)–4E-BP1, HMK–4E-BP1Δ (deletion mutant) and HMK were prepared in *Escherichia coli*. The incubation of an eIF-4E-bound m^{7}GDP-coupled agarose resin with HA–p220-expressing *Sf9* extract resulted in the interaction between the two polypeptides (Figure 6, lane 2). This interaction was abrogated by pre-incubation with HMK–4E-BP1, as expected (lane 3). However, neither the deletion mutant, HMK–4E-BP1Δ, nor HMK were retained on the resin



	1	2	3	4	5	6	7	8
CAT/LUC	3.5	3.75	2.91	1.3	2.75	3.1	2.7	3.6
% of control	100	107	83	37	78	88	77	102

Fig. 7. Effect of a 4E-BP1 deletion mutant protein on translation in Krebs-2 ascites cell extracts. Extracts were pre-incubated with buffer A (see Materials and methods; control lane) or increasing amounts of the GST fusion protein HMK–4E-BP1 (0.1, 0.3, 0.8 µg), HMK–4E-BP1Δ (0.1, 0.3, 0.8 µg) or HMK (0.9 µg) as indicated. Translation was performed as described in Materials and methods. The bicistronic construct is shown at the top of the figure. The IRES element is derived from the 5′ UTR of FMDV. Indicated restriction enzyme sites are as follows: H, *Hin*dIII; X, *Xho*I; S, *Sal*I. Translation of CAT and LUC was quantified using a PhosphorImager. The ratio of CAT to LUC is indicated in the bottom of the figure.

in the presence of eIF-4E (Figure 6, lanes 4 and 5, respectively), but instead were present in the flowthrough fractions (Figure 6, lanes 6 and 7, respectively). Therefore, failure of the mutant 4E-BP1 to interact with eIF-4E allowed for the association and retention of p220 by eIF-4E. These results confirm that 4E-BP1 and p220 compete for binding to eIF-4E through a common sequence motif.

4E-BP1 deletion mutant does not inhibit translation

To show that the interaction of 4E-BP1 with eIF-4E is responsible for the inhibitory effect of 4E-BP1 on translation, the effect of the 4E-BP1 deletion mutant was studied in an *in vitro* translation assay. A bicistronic mRNA containing the IRES from foot-and-mouth disease virus (FMDV) was used to assess the cap specificity of the inhibition (Figure 7). The translation of CAT is cap-dependent, whereas the translation of luciferase (LUC) directed by the FMDV IRES proceeds by a cap-independent mechanism. The translation of this RNA in a Krebs-2 ascites cell extract produced both CAT and LUC, although the expression of LUC was less efficient than that of CAT (Figure 7, lane 1). Pre-incubation of the lysate for 10 min with the GST–HMK–4E-BP1 prior to the addition of RNA resulted in a specific inhibition (~3-fold; at the maximum amount of GST–4E-BP1) of the cap-dependent translation of CAT, with no significant effect (~1.1-fold) on the IRES-directed translation of luciferase (Figure 7, lanes 2–4). Pre-incubation of the lysate with the deletion mutant GST–HMK–4E-BP1Δ exhibited no significant effect on the translation of either cistron (Figure 7, lanes 5–7). Likewise, GST–HMK had no effect on translation (Figure 7, lane 8). The interaction of 4E-BP1 with eIF-4E is therefore a requisite for the 4E-BP1 inhibitory effect on cap-dependent translation.

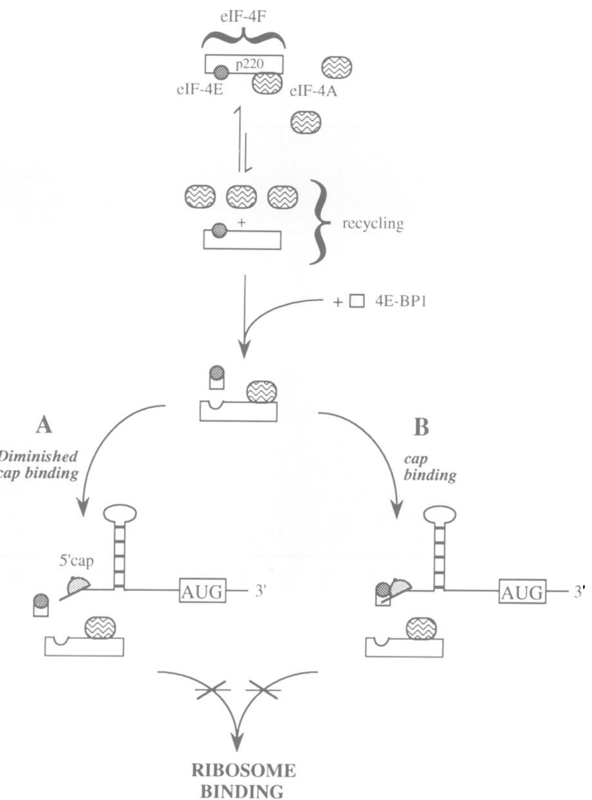


Fig. 8. Models for the inhibition of cap-dependent translation by 4E-BP1 (see text for details).

Discussion

Here we have characterized the interaction of eIF-4E with p220 and 4E-BP1. We provide evidence that the competition between 4E-BP1 and p220 for binding to eIF-4E is mutually exclusive. Furthermore, we show that this competition is mediated via a common sequence motif. In addition, the eIF-4F complex in cells precludes the association of 4E-BP1, hence ruling out the possibility of a mechanism whereby the interaction of an eIF-4F–4E-BP1 complex with other initiation factors is abrogated, leading to an inhibition of cap-dependent translation initiation.

There are currently two models for the pathway of eIF-4F assembly (Figure 8). One model (model A) suggests that the eIF-4F complex is pre-assembled prior to binding to the 5′ cap structure and subsequent association with ribosomes. According to this model, 4E-BP1 prevents the assembly of eIF-4F and consequently binding to the cap structure. Although eIF-4E alone binds efficiently to a cap affinity column (Bonneau and Sonenberg, 1987a), its binding to the mRNA cap structure is much more efficient as a subunit of the eIF-4F complex, as determined by crosslinking experiments (Lee *et al.*, 1985). Furthermore, following poliovirus infection, which leads to the cleavage of p220, crosslinking of eIF-4E to the mRNA cap structure

is reduced dramatically (Lee and Sonenberg, 1982; Pelletier and Sonenberg, 1985b; Rozen and Sonenberg, 1987). An eIF-4E complexed to one of the cleavage products of p220 can be isolated by the cap affinity column (Lee *et al.*, 1985). It is likely that eIF-4F, because of its strong RNA binding activity (Jaramillo *et al.*, 1991), interacts efficiently with the RNA in the vicinity of the cap structure to stabilize the interaction of eIF-4E with the cap, and that the RNA binding moiety of p220 is separated from the eIF-4E binding domain following cleavage of p220.

An alternative model (Figure 8, model B) for eIF-4F complex formation suggests that the complex assembles on the mRNA (Joshi *et al.*, 1994). According to this model, eIF-4E binds alone to the mRNA cap structure, whereas p220 binds separately to the 40S ribosomal subunit, presumably through its interaction with another initiation factor, eIF-3. mRNA is thus bound to ribosomes following an interaction between eIF-4E and p220. Therefore 4E-BP1 would inhibit 40S–mRNA association, but not eIF-4E binding to the cap structure. We favor the first model based on the results of the experiments performed previously and cited above (Lee and Sonenberg, 1982; Pelletier and Sonenberg, 1985b; Rozen and Sonenberg, 1987). Regardless of the mechanism of eIF-4F assembly, 4E-BP1 is expected to inhibit 40S association with the mRNA, and this was observed here using ribosome binding assays.

It is clear from our and earlier studies (Pause *et al.*, 1994b) that 4E-BPs do not affect the internal initiation of translation which is cap-independent. Some picornavirus infections result in the cleavage of the p220 subunit of eIF-4F and the subsequent shut-off of host protein synthesis (Sonenberg, 1987). The translation of viral RNA requires eIF-4F function (Pause *et al.*, 1994a). One can envisage that the cleavage of p220 might result in the loss of the eIF-4E subunit, where the modified eIF-4F remains functional for internal initiation but cap-dependent translation is blocked. However, cleavage of p220 alone is not sufficient for the complete inhibition of host protein synthesis after poliovirus infection (Bonneau and Sonenberg, 1987b). It is thus possible that dephosphorylation of 4E-BPs, and subsequent increased affinity for eIF-4E, also contribute to the shut-off of host protein synthesis. Such a mechanism could even play a more substantial role in the abrogation of host protein synthesis following EMC virus infection inasmuch as no p220 cleavage occurs in this case.

Another important issue is the relative contribution of the two identified and studied repressors of eIF-4E function, 4E-BP1 and 4E-BP2, to the regulation of eIF-4E activity. Both proteins appear to bind with similar affinities to eIF-4E and inhibit translation both *in vivo* and *in vitro* (Pause *et al.*, 1994b). They both contain the motif that is homologous to the eIF-4E binding site on p220 (Mader *et al.*, 1995). However, the two proteins exhibit a differential tissue distribution, and it is possible that they modulate the translation of specific mRNAs in a tissue-dependent manner (A.-C.Gingras and N.Sonenberg, unpublished results). It is also worth noting that 4E-BP2 contains a potential phosphorylation site for protein kinase A which is absent in 4E-BP1, suggesting an additional level of regulation of translation by diverse extracellular signals (A.-C.Gingras and N.Sonenberg, unpublished results).

Further studies should address the cellular parameters that affect the competition between p220 and 4E-BPs for binding to eIF-4E. For example, the phosphorylation of eIF-4E and p220 is enhanced following the stimulation of cells with growth factors and insulin (Morley and Traugh, 1990). In addition, phosphorylated eIF-4E forms a more stable complex with p220 (Bu *et al.*, 1993). Likewise, phosphorylated p220 could have a higher affinity for eIF-4E. As a result, under conditions of optimal cell growth, where 4E-BPs are phosphorylated and incapable of binding to eIF-4E, the affinities of eIF-4E and p220 for each other increase. Thus, the integration of several phosphorylation pathways might be required for efficient cap recognition and translation initiation.

Materials and methods

Cell culture and protein factors

Spodoptera frugiperda (*Sf*9) insect cells were obtained from Rosanne Tom, Biotechnology Research Institute, Montreal, Canada. Cells were cultured in Grace medium (Gibco-BRL) supplemented with 10% fetal calf serum (FCS), TC Yeastolate, lactalbumin hydrolysate, 50 μg/ml gentamicin sulfate and 2.5 μg/ml amphotericin B (Fungizone) in either T flasks or spinner flasks at 27°C, as described previously (Summers and Smith, 1987). HeLa R19 cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL) supplemented with 10% FCS. GST fusion proteins of 4E-BP1, HMK–4E-BP1 and HMK–4E-BP1Δ were expressed in *E.coli* BL21 and purified as described previously (Pause *et al.*, 1994b). Murine eIF-4E protein was expressed in *E.coli* K38 and purified as described previously (Edery *et al.*, 1988).

Generation of recombinant baculovirus

Recombinant baculovirus was generated by cationic liposome cotransfection of the p10HAp220 construct with Invitrogen linearized genomic AcMNPV DNA according to the manufacturer's instructions (Invitrogen). Recombinant virus containing the HAp220 cDNA was isolated by plaque assays and amplified as described previously (Summers and Smith, 1987). Infected cells were overlain with 1% Seaplaque agarose diluted with Grace medium (10 ml per 100 mm culture plate). At 3 days postinfection, culture plates were overlain with 1% agarose in Grace medium containing 150 μg/ml X-Gal (3 ml per plate; Biosynth AG). Blue plaques were picked with a sterile Pasteur pipette and the virus was eluted in Grace medium overnight at room temperature before being subjected to another round of plaque assay. Usually, five to six rounds of plaque assays were necessary to generate pure recombinant virus.

Plasmids and vector constructions

Plasmids pSP64–CAT and pEMC–CAT (containing the EMC virus 5′ UTR fused to the CAT sequence) were constructed as described previously (Jang *et al.*, 1988; Pelletier and Sonenberg, 1988). pGEMCAT/FMDV/LUC (G.Belsham, unpublished data) contains the FMDV 5′ UTR between the CAT and the LUC coding regions. For the construction of a baculovirus transfer vector, the human p220 cDNA was excised from the plasmid pSK(–)HFC1 (a kind gift from R.E.Rhoads; Yan *et al.*, 1992) with *Eco*RV and subcloned blunt into the *Eco*RI site of KS. The triple HA tag (*Hin*DIII–*Xba*I) encoding the influenza hemagglutinin antigen from the plasmid PACTAG-2 (a gift from A.Charest) was ligated blunt into the *Sma*I site of KS in front of the p220 sequence to generate KSHAp220. The *Xba*I (cohesive)–*Eco*RI (blunt) fragment from the latter construct was subsequently subcloned into the *Nhe*I (cohesive)–*Bam*HI (blunt) sites of the p10 transfer vector (Vialard *et al.*, 1990).

In vitro transcription and translation

The plasmid pGEMCAT/FMDV/LUC was linearized with *Xho*I. pSP64–CAT and pEMC–CAT were linearized with *Bam*HI. Transcription was performed by either T7 (pGEMCAT/FMDV/LUC) or SP6 (pSP64–CAT and pEMC–CAT) RNA polymerase, as described previously (Pelletier and Sonenberg, 1985a). Capped transcripts were obtained in a reaction mixture containing 50 μM GTP and 500 μM m^7GpppG. Labeled RNA was generated by including [α-^{32}P]GTP (100 μCi; 3000 Ci/mmol) in

the transcription reactions. The integrity of RNAs was analysed on a formaldehyde–agarose gel, and amounts were quantitated by spectrophotometry or liquid scintillation. Translations were performed in Krebs-2 ascites cell extracts, as described previously (Svitkin and Agol, 1978), in a final volume of 16 μl. Where indicated, extracts were pre-incubated with GST fusion proteins of HMK–4E-BP1, HMK–4E-BP1Δ or HMK for 10 min at 30°C prior to the addition of the mRNA (300 ng) and translation ingredients. Translation reactions were incubated at 30°C for 90 min and subsequently analyzed by SDS–PAGE. Gels were fixed, treated with EN^3Hance and processed for autoradiography. The intensity of the bands corresponding to CAT and LUC was quantified using a Fuji BAS2000 PhosphorImager.

m^7GDP column chromatography

m^7GDP-coupled agarose resin (Edery et al., 1988) was bound to eIF-4E in buffer A [20 mM Tris–HCl, pH 7.5, 100 mM KCl, 2 mM dithiothreitol (DTT), 2 mM EDTA]. The resin was washed (3×1 ml) in the same buffer, and then pre-incubated for 60 min with GST–4E-BP1, HA–p220-expressing *Sf9* cell lysates or uninfected *Sf9* cell lysates, as indicated in the figure legends. The resin was washed in buffer A and incubated further with either HA–p220-expressing *Sf9* cell lysates or GST–4E-BP1 for 60 min, as indicated in the figure legends. The resin was rinsed and bound proteins were eluted in SDS–sample buffer. Proteins were analysed by Western blotting on either an SDS–8% polyacrylamide gel (for HA–p220) or an SDS–15% polyacrylamide gel (for eIF-4E and GST–4E-BP1).

Sucrose density gradient analysis

HeLa cells were rinsed three times with ice-cold PBS and resuspended in 200 μl of lysis buffer containing 10 mM HEPES, pH 7.5, 3 mM $MgCl_2$, 50 mM KCl, 5% glycerol, 1 mM DTT, 0.5% NP-40 and 1 mM phenylmethylsulfonyl fluoride. The suspension was adjusted to 400 mM KCl and incubated at 4°C for 30 min with slow agitation. Cell debris was pelleted and the supernatant was sedimented through a 10–40% sucrose gradient in buffer B (20 mM Tris–HCl, pH 7.5, 3 mM $MgCl_2$, 85 mM KCl, 1 mM DTT) in a Beckman SW40 rotor at 38 000 r.p.m. at 4°C for 12 h. Catalase (11S) was run on a separate gradient as a sedimentation marker. Gradients were displaced by heavy sucrose [60% (w/v)] using an ISCO model 640 gradient fractionator equipped with a spectrophotometer flow cell for the direct recording of absorbance at 254 nm. Protein in each fraction was precipitated with five volumes of 80% acetone at –20°C, solubilized in SDS–sample buffer and resolved on SDS–polyacrylamide gels. Proteins were electroblotted onto a nitrocellulose membrane for immunoblot analysis.

Western blot analysis

Nitrocellulose membranes were incubated for 60 min in Tris-buffered saline containing 0.2% Tween-20 (TBST) and 5% dry milk at room temperature. Membranes were incubated further with the appropriate primary antibody for 120 min. The primary antibodies included a mouse anti-HA monoclonal antibody (1:1000; 12CA5; generous gift from M.Tremblay), a rabbit anti-p220 polyclonal antibody (1:1000; Lee et al., 1985), an anti-eIF-4A monoclonal antibody (1:10; a kind gift from H.Trachsel), a mouse monoclonal antibody to eIF-2α (1:2000; a generous gift from L.M.O'Brien), a rabbit polyclonal antibody to eIF-4E (1:1000; Frederickson et al., 1991), a rabbit anti-4E-BP1 polyclonal antibody (1:1000; kindly supplied by A.-C.Gingras) and an anti-GST polyclonal antibody (1:1000; A.Pause, unpublished results). After washing with TBST, membranes were treated with peroxidase-linked anti-mouse or anti-rabbit IgG in combination with the ECL system (Amersham), and exposed to X-ray film (Du Pont).

Ribosome binding assays

Uncapped RNA encoding CAT was capped and methylated with 6 U of vaccinia virus guanylyltransferase (Gibco-BRL) in the presence of 0.4 mM *S*-adenosyl-L-methionine and [α-^{32}P]GTP (100 μCi). Ribosome binding assays were performed using rabbit reticulocyte lysates (Promega) and labeled RNA in a total volume of 50 μl. Briefly, 35 μl of lysate were pre-incubated with buffer A or GST–4E-BP1 for 20 min at 30°C, followed by the addition of a mixture of RNAsin, amino acids, ^{32}P-labeled RNA and anisomycin (250 μg/ml; Fluka) or GMP-PNP (10 mM; Calbiochem). After a further 30 min of incubation at 30°C, reaction mixtures were chilled on ice and layered on sucrose gradients in buffer B (20 mM Tris–HCl, pH 7.5, 3 mM $MgCl_2$, 85 mM KCl, 1 mM DTT). Centrifugation was in a Beckman SW40 rotor at 38 000 r.p.m. at 4°C for 4 h. Fractions (0.4 ml) were collected and counted directly in 5 ml EcoLite scintillation fluid.

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