

mRNAs containing extensive secondary structure in their 5' non-coding region translate efficiently in cells overexpressing initiation factor eIF-4E

Antonis E.Koromilas,
Anthoula Lazaris-Karatzas and
Nahum Sonenberg¹

Department of Biochemistry and McGill Cancer Centre, Faculty of Medicine, McGill University, Montreal, Canada H3G 1Y6

¹Corresponding author

Communicated by J.Schlessinger

Cellular eukaryotic mRNAs (except organellar) contain at the 5' terminus the structure m⁷(5')Gppp(5')N (where N is any nucleotide), termed cap. Cap recognition by eukaryotic initiation factor eIF-4F plays an important role in regulating the overall rate of translation. eIF-4F is believed to mediate the melting of mRNA 5' end secondary structure and facilitate 43S ribosome binding to capped mRNAs. eIF-4E, the cap-binding subunit of eIF-4F, plays an important role in cell growth; its overexpression results in malignant transformation of rodent cells, and its phosphorylation is implicated in signal transduction pathways of mitogens and growth factors. The molecular mechanism by which eIF-4E transforms cells is not known. Here, we report that overexpression of eIF-4E facilitates the translation of mRNAs containing excessive secondary structure in their 5' non-coding region. This effect may represent one mechanism by which eIF-4E regulates cell growth and transforms cells in culture.

Key words: helicase/transformation/translation control

Introduction

Translational control plays a significant role in regulation of gene expression in eukaryotes (Hershey, 1991). Most of this control is exerted at the level of translation initiation (Thach, 1992). Several features of the 5' non-coding region of eukaryotic mRNAs are important for the control of translation initiation. These include the 5' terminal cap structure [m⁷G(5')ppp(5')N; where N is any nucleotide] (Shatkin, 1976), and the primary and secondary structure of the 5' untranslated region (Hershey, 1991). The cap structure facilitates mRNA–ribosome binding, a step that requires the participation of three initiation factors: eIF-4A, eIF-4B, eIF-4F and ATP hydrolysis (Sonenberg, 1988). Cap function is mediated by the cap-binding complex, eIF-4F (Tahara *et al.*, 1981; Edery *et al.*, 1983; Grifo *et al.*, 1983), which is composed of three subunits: (i) a 24 kDa polypeptide, eIF-4E, which contains the cap-binding site (Sonenberg *et al.*, 1978), (ii) a 50 kDa polypeptide, eIF-4A, which exhibits RNA-helicase activity (Ray *et al.*, 1985; Rozen *et al.*, 1990), and (iii) a 220 kDa polypeptide, p220, whose function is unknown but its integrity is required for eIF-4F activity (Edery *et al.*, 1987). It is thought that eIF-4F

stimulates mRNA binding to the 40S ribosomal subunit by unwinding the secondary structure in the 5' untranslated region of the mRNA (Ray *et al.*, 1985; Rozen *et al.*, 1990). Consistent with this, several studies suggest that eIF-4E, as part of eIF-4F, acts as a discriminatory factor that relieves translational competition between mRNAs (Ray *et al.*, 1983; Edery *et al.*, 1984; Sarkar *et al.*, 1984).

eIF-4E, the smallest subunit of eIF-4F, is the least abundant of the translation initiation factors [0.05–0.25 molecules/ribosome as compared to 0.5–3.0 molecules/ribosome for the other initiation factors (Hiremath *et al.*, 1985; Duncan *et al.*, 1987)], consistent with a regulatory role for eIF-4E in translation. eIF-4E exists in both phosphorylated and non-phosphorylated forms (Hershey, 1989), and the major site of phosphorylation is ser-53 (Rychlik *et al.*, 1987). The phosphorylation state of eIF-4E correlates positively with the rate of translation. Phosphorylation of eIF-4E is increased in cells stimulated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (Morley and Traugh, 1989), insulin (Morley and Traugh, 1990), tumor necrosis factor (TNF) (Marino *et al.*, 1989), serum (Kaspar *et al.*, 1990), platelet-derived growth factor (PDGF) (Bu and Hagedorn, 1991; Frederickson *et al.*, 1991) as well as in *src*-transformed cells (Frederickson *et al.*, 1991), but is decreased during mitosis (Bonneau and Sonenberg, 1987; Huang and Schneider, 1991), in response to heat shock (Duncan *et al.*, 1987), and during the shut-off of host protein synthesis in adenovirus-infected cells (Huang and Schneider, 1991). Furthermore, eIF-4E phosphorylation is increased in PC12 cells in a *ras*-dependent manner in response to nerve growth factor (NGF) treatment (Frederickson *et al.*, 1992). The biological importance of the limiting status of eIF-4E is strikingly illustrated by the finding that overexpression of eIF-4E causes malignant transformation of rodent cells (Lazaris-Karatzas and Sonenberg, 1992; Lazaris-Karatzas *et al.*, 1990) and deregulation of HeLa cell growth (De Benedetti and Rhoads, 1990). Consistent with these findings, microinjection of recombinant murine eIF-4E or rabbit reticulocyte eIF-4F activates DNA synthesis in serum-starved NIH3T3 cells (Smith *et al.*, 1990). These findings demonstrate an important role for eIF-4E in cell growth and differentiation, most probably at the level of translation initiation.

Apart from the cap structure, the role of the 5' UTR in translation is not well understood. The 5' UTR has been shown to influence the efficiency of translation and the fidelity of initiation. mRNAs with excessive secondary structure in their 5' UTRs are discriminated against by the translational machinery and as a consequence inefficiently translated (Pelletier and Sonenberg, 1985; Kozak, 1986). However, little is known about which features of the 5' UTR of eukaryotic mRNAs are recognized by the translational machinery, since these regions are highly variable and do not possess common features. RNA molecules are capable of forming a variety of structures, including helices, loops,

bulges and pseudoknots, that may play important roles in the specific recognition and binding of proteins (Draper, 1989). In some mRNAs (e.g. ferritin) these structures regulate translation initiation (Klausner and Hartford, 1989).

We wished to investigate the mechanism(s) by which eIF-4E regulates translation *in vivo*, which may explain how eIF-4E overexpression causes cell transformation. One possible scenario is that eIF-4E enhances translation of mRNAs encoding proteins that control cell growth. In this paper we show that eIF-4E overexpression facilitates the translation of mRNAs with extensive secondary structure in their 5' UTRs.

Results

eIF-4E overexpression facilitates the translation of mRNAs with 5' UTR extensive secondary structure

A model to explain why mRNAs with 5' terminus extensive secondary structure translate poorly posits that eIF-4F RNA-helicase activity is limiting in the cell, because of the low abundance of the eIF-4E subunit of the complex. This model predicts that eIF-4E overexpression should facilitate translation of such mRNAs. To test this prediction, we constructed a series of vectors bearing different amounts of secondary structure in the 5' UTR of the CAT gene. Secondary structure was introduced based on the protocol described by Pelletier and Sonenberg (1985), by insertion of one, three or five *Bam*HI linkers into the unique *Hind*III site 57 nucleotides downstream of the transcription initiation site in the pSV2CAT vector (herein referred to as HB0) (Figure 1). The theoretical standard free energies of the 5' non-coding regions of HB0, HB1, HB3 and HB5 are -17, -32.7, -57.7 and -81.7 kcal/mol respectively (Zucker and Stiegler, 1981).

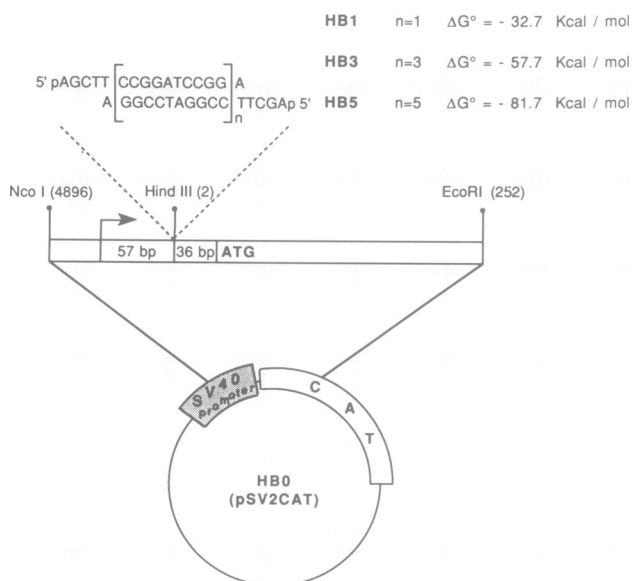


Fig. 1. Construction of insertion derivatives of chloramphenicol acetyltransferase (CAT) gene. Linkers were introduced in the unique *Hind*III site of pSV2CAT vector as described in Materials and methods. CAT transcription is directed by the early SV40 promoter and initiates 57 bp upstream of the *Hind*III site (black arrow). The predicted free energy values are given for the 5' non-coding region of CAT mRNA variants (1 cal=4.184 j). The numbers in parentheses represent the position of the indicated restriction endonuclease sites in HB0 (pSV2CAT) plasmid.

CAT insertion derivatives were transiently expressed in NIH3T3 and eIF-4E-overexpressing NIH3T3 [3T3 pMV7-4E(C1)] cells by calcium phosphate transfection. To control for possible differences in transfection efficiency between the two cell lines the amount of transfected plasmid DNA was determined. Low molecular weight plasmid DNA was isolated by the method of Hirt (Hirt, 1967) from transfected cells 48 h post-transfection. Linearized plasmids were analysed on agarose gels and quantitated by Southern blotting (Figure 2). The amount of plasmid DNA that adhered to cells (background control) was determined from an experiment in which cells were mixed with calcium phosphate-HB0 plasmid DNA co-precipitate (Figure 2a, lanes 1 and 6). The amount of transfected DNA of all

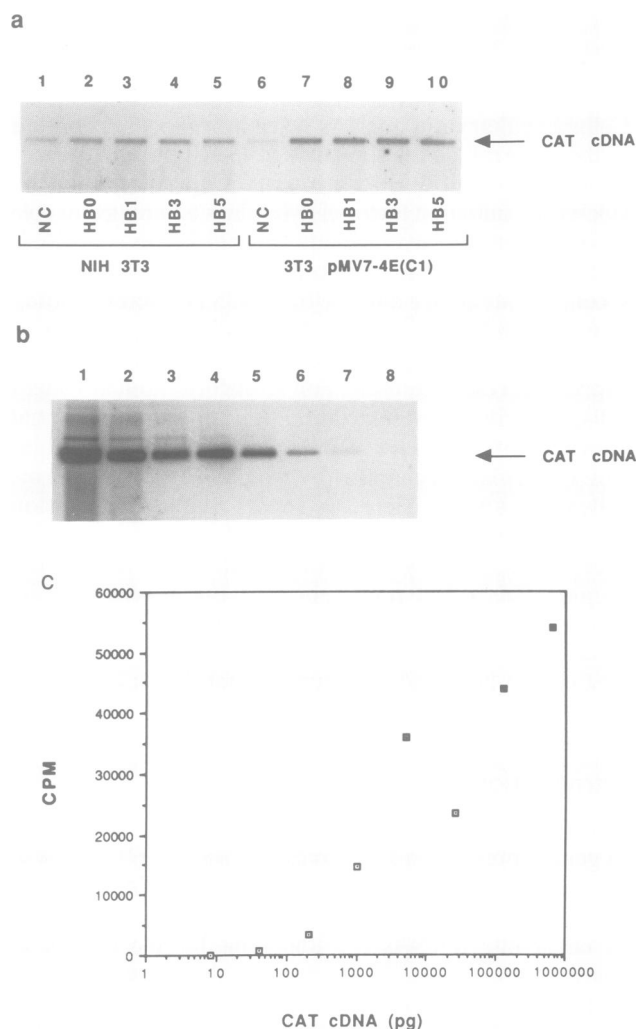


Fig. 2. Quantitation of plasmid DNA in NIH3T3 and 3T3 pMV7-4E(C1) transfected cells. (a) Plasmid DNA in NIH3T3 (lanes 2-5) and 3T3 pMV7-4E(C1) cells (lanes 7-10) was isolated by the Hirt method 48 h post-transfection. After *Hind*III digestion, DNA was electrophoresed on a 1% agarose gel, transferred to a nylon filter and hybridized with [³²P]CAT cDNA. To determine the background level NIH3T3 cells (lane 1) and 3T3 pMV7-4E(C1) cells (lane 5) were mixed with calcium phosphate-HB0 DNA coprecipitate. The cells were then washed in PBS and the DNA sticking to the cells was isolated and quantified. (b) Standard curve. Samples containing 5-fold serial dilutions of HB0 DNA were digested with *Hind*III, electrophoresed on 1% agarose gels, blotted and probed with [³²P]CAT cDNA. The radioactive bands were excised and counted in a liquid scintillator. (c) Plot of c.p.m. versus the amount of HB0 plasmid DNA.

insertion derivatives was 5-fold greater in 3T3 pMV7-4E(C1) cells (lanes 7–10) than in NIH3T3 cells (lanes 2–5). This difference was calculated based on a standard curve (Figure 2b and c). Similar results were obtained with another plasmid, pKCR.Tac-2A, which contains the interleukin-2 receptor (IL-2R) gene (α chain) under the control of SV40 promoter (Nikaido *et al.*, 1984). When pKCR.Tac-2A DNA was transfected into cells the number of 3T3 pMV7-4E(C1) cells expressing IL-2R was ~5-fold greater than NIH3T3 cells, as assessed by immunostaining and FACS analysis (data not shown). This difference could be explained by better transfection efficiency of eIF-4E-overexpressing cells.

Next, we measured CAT enzyme activity in transfected cells. Because of the differences in the levels of transfected DNA between the two cell lines, 5-fold more NIH3T3 than 3T3 pMV7-4E(C1) cell extract was used. In NIH3T3 cells, CAT expression from HB1, HB3 and HB5 vectors was markedly decreased, as the amount of secondary structure in 5' UTR was increased (Figure 3a, cf. lanes 1–4). However, strikingly, CAT expression did not significantly vary among the different insertion vectors when expressed

in 3T3 pMV7-4E(C1) cells (Figure 3a, lanes 5–8). The relative CAT activity (CAT activity of insertion derivative/CAT activity of HB0) for HB1, HB3 and HB5 was 2.5-, 5- and 13-fold higher respectively, in 3T3 pMV7-4E(C1) than in the parental NIH3T3 cells (Figure 3b). These results indicate that CAT insertion derivatives are better expressed in eIF-4E-overexpressing NIH3T3 cells than in the parental NIH3T3 cells.

To determine at what level the expression of CAT insertion derivatives is enhanced in 3T3 pMV7-4E(C1) cells, the amount of CAT mRNA was determined. Since CAT mRNA levels were barely detectable by the RNase protection assay, an accurate and highly reproducible polymerase chain reaction (PCR) method was employed (Robinson and Simon, 1991). Total RNA isolated from transfected cells was reverse transcribed and amplified by PCR. The amount of amplified CAT cDNA was quantified by Southern blotting (Figure 4). The exponential curve of the amplification reaction was determined on samples representing 5-fold serial dilutions of *in vitro* transcribed CAT RNA (Roy *et al.*, 1990) (Figure 4b). Equal amounts of CAT cDNA were detected after amplification for all the insertion derivatives in NIH3T3 cells (Figure 4a, cf. lanes 1–4). Likewise, similar amounts were detected in 3T3 pMV7-4E(C1) cells among all insertion derivatives (Figure 4a, cf. lanes 5–8). These levels were within the exponential phase of PCR amplification as determined by a standard curve (Figure 4b and c). Quantitation according to the standard curve (Figure 4c) shows that the amount of CAT mRNA for all insertion derivatives was ~5-fold higher in 3T3 pMV7-4E(C1) cells than in NIH3T3 cells. This difference corresponds exactly to the greater amount of plasmid DNA in 3T3 pMV7-4E(C1) cells. This result shows that transcription is not affected by insertion of secondary structure and the better expression of CAT insertion derivatives in eIF-4E-overexpressing cells is regulated at the translational level.

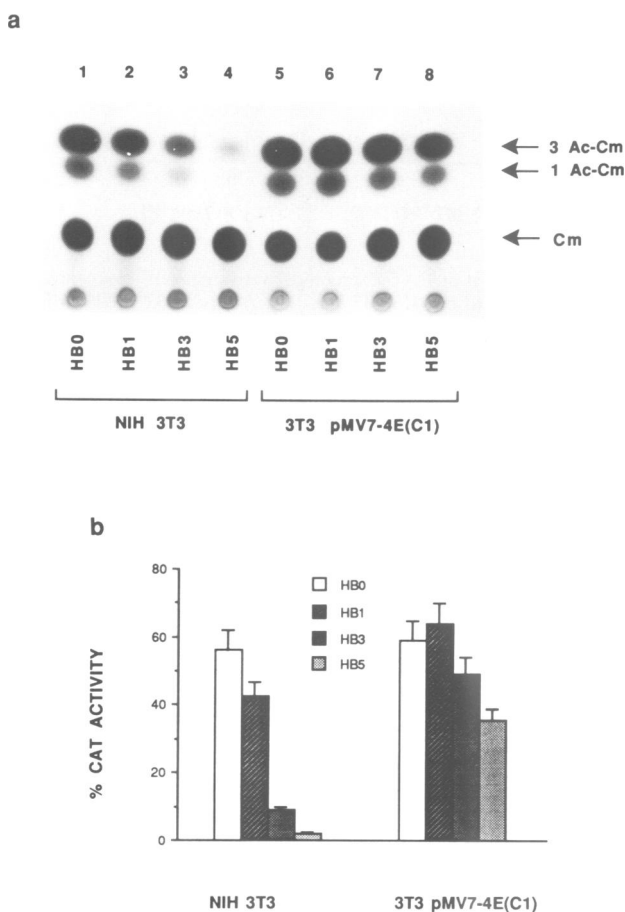


Fig. 3. The E2 DNA-binding domain is not required for replication. Lanes 1 and 2 contain DNA extracted from cells transfected with replicon DNA alone (lane 1), or replicon DNA and pCGEag-1235 (lane 2). Lanes 3–8 contain DNA extracted from cells co-transfected with the replicon, pCGEag-1235 and either wild-type C59 or mutated C59 DNA. Cells were transfected with C59 DNAs containing a TTL at aa 218 or aa 360 (lanes 5 and 7) or with C59 DNAs containing these TTLs and an additional deletion within the C-terminal domain, E2_{1–218/Δ377–410} and E2_{1–360/Δ377–410} (lanes 6 and 8). The replicated DNA was detected with a probe from the LCR region of BPV-1 (nt 6958–6936).

***eIF-4E* effect on translation is dependent on its phosphorylation at serine 53**

The major phosphorylation site of eIF-4E is ser-53. Substitution of ser-53 for alanine results in a non-functional eIF-4E protein, whose overexpression in NIH3T3 cells does not cause transformation (Lazaris-Karatzas *et al.*, 1990). To determine whether the translational activity of eIF-4E is dependent on its phosphorylation state, we examined the expression of DNA insertion derivatives in NIH3T3 cells overexpressing mutant eIF-4E ser⁵³-ala [3T3 pMV7-4E(Ala)]. Hirt extraction analysis of 3T3 pMV7-4E(Ala) and NIH3T3 cells showed equal amounts of plasmid DNA in both cell lines 48 h post-transfection (data not shown). The expression of insertion derivatives in 3T3 pMV7-4E(Ala) and 3T3 pMV7-4E(C1) cells 48 h post-transfection was compared. In 3T3 pMV7-4E(Ala) cells, CAT activity was inversely proportional to the amount of secondary structure in the 5' UTR of the expressed CAT genes (Figure 5, lanes 1–4), and the levels were similar to those in NIH3T3 cells (cf. Figures 5b and 3b). However, CAT activities for HB0, HB1, HB3 and HB5 were 1.5-, 6.5- and 13.0-fold higher respectively, in 3T3 pMV7-4E(C1) than in 3T3 pMV7-4E(Ala) cells (Figure 5b). This result suggests that eIF-4E phosphorylation is important for the expression of CAT mRNAs, and efficient translation of these mRNAs is dependent upon the amount of functional eIF-4E in the cell.

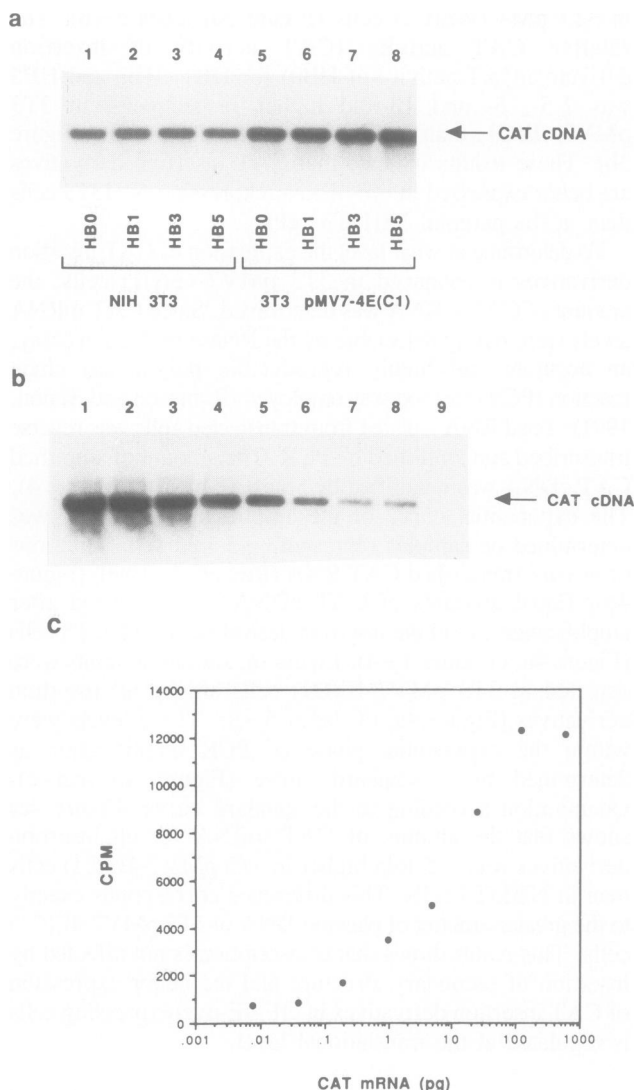


Fig. 4. RNA quantitation by the polymerase chain reaction (PCR). Total RNA (1 μ g) from transfected cells, and eight samples of 5-fold serial dilutions of T3 transcribed CAT RNA mixed with 1 μ g total RNA from NIH3T3 cells were reverse transcribed as described in Materials and methods. One-fifth of the reaction mixtures was used in PCR reactions for 25 cycles. Half of the PCR reactions were analysed on 2% agarose gels, transferred to a nylon filter and hybridized with [32 P]CAT cDNA. (a) Autoradiograph showing the levels of CAT cDNA after reverse transcription and PCR amplification of CAT mRNA from transfected NIH3T3 (lanes 1–4) and 3T3 pMV7-4E(C1) cells (lanes 5–8). (b) Standard curve. Autoradiograph showing the levels of CAT cDNA derived from samples containing 5-fold serial dilutions of *in vitro* transcribed CAT RNA after reverse transcription and PCR amplification (lanes 1–8). As a negative control, 1 μ g of total RNA from NIH3T3 cells was used (lane 9). The radioactive bands were excised and counted in a liquid scintillation counter. (c) Plot of c.p.m. versus the amount of CAT mRNA.

Discussion

A model has been proposed in which eIF-4F binds to mRNA via interaction with the cap structure. Subsequently, in combination with eIF-4B it unwinds mRNA 5' secondary structure in a process that requires ATP hydrolysis (Sonenberg, 1988). The unwinding step is believed to be rate limiting for ribosome binding. eIF-4E, the limiting component of eIF-4F, may regulate the amount of eIF-4F complex in the cell. Therefore, it is likely that in cells

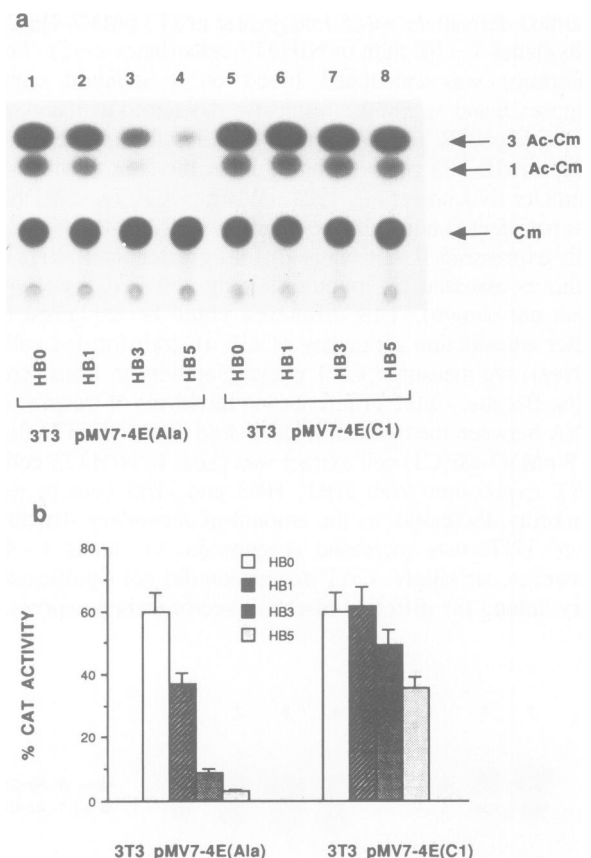


Fig. 5. Expression of CAT insertion derivatives in cells overexpressing ser⁵³-ala mutant of eIF-4E. 3T3 pMV7-4E(Ala) and 3T3 pMV7-4E(C1) cells were transfected with CAT insertion derivatives and 48 h later CAT enzyme activity was quantified in 15 and 3 μ g of cell extract respectively. (a) Autoradiograph showing the levels of CAT activity in transfected cells. 3Ac-Cm and 1Ac-Cm, monoacetylated products; Cm, [14 C]chloramphenicol substrate. (b) Quantitation of CAT activity. The CAT enzyme activities in 3T3 pMV7-4E(Ala) and 3T3 pMV7-4E(C1) cells were quantified as described in Figure 3. The results represent the means of three separate experiments. The standard deviation is 10%.

overexpressing eIF-4E more eIF-4A is recruited into eIF-4F resulting in efficient unwinding of RNA secondary structure and facilitated ribosome binding. Consistent with this model eIF-4F acts as a translational discriminatory factor (Sonenberg, 1988). Accordingly, mRNAs with long and structured 5' leaders are discriminated against by the translational machinery and are poorly translated.

Here, we used a model system to show that translation of mRNAs containing extensive secondary structure is enhanced by eIF-4E overexpression. We constructed a series of CAT gene derivatives whose 5' UTRs contain increasing amounts of secondary structure. We show that expression of these CAT gene derivatives is reduced in NIH3T3 cells at the translational level. This finding confirms previous results demonstrating the deleterious effect of secondary structure in the 5' UTR of mRNAs on translation (Pelletier and Sonenberg, 1985). However, translation of insertion derivative mRNAs is more efficient in cells overexpressing eIF-4E.

The effect of eIF-4E on translation may represent one of the mechanisms by which its overexpression causes cell transformation. Significantly, mRNAs of several proto-oncogenes, growth factors and growth-regulated genes, such

as PDGF-2 (Ratner *et al.*, 1987; Rao *et al.*, 1988), insulin-like growth factor I (IGF-I) receptor (Werner *et al.*, 1990), transforming growth factor $\beta 3$ (TGF- $\beta 3$; Arrick *et al.*, 1991) and ornithine decarboxylase (ODC; Brabant *et al.*, 1988; Manzella and Blackshear, 1990), possess long 5' UTRs with high amounts of secondary structure. Translation of these mRNAs is very inefficient in *in vitro* systems, but is dramatically enhanced by truncation of the long 5' UTR. Based on our data, it is possible that eIF-4E overexpression facilitates the expression of genes regulating cell growth at the level of translation initiation. This hypothesis is supported by the finding that conditioned media from eIF-4E-transformed cells is capable of stimulating DNA synthesis in serum-starved NIH3T3 cells (A. Lazaris-Karatzas and N. Sonnenberg, unpublished results).

Phosphorylation of eIF-4E is critical for eIF-4F activity, since CAT insertion mRNAs are poorly translated in cells overexpressing eIF-4E ser⁵³-ala mutant. In the eIF-4F complex, eIF-4E is phosphorylated to a greater extent than uncomplexed eIF-4E (Lamphear and Panniers, 1990, 1991). Also an eIF-4E ser⁵³-ala mutant fails to associate with 48S translation-initiation complexes resulting in decreased translation (Joshi-Barve *et al.*, 1990). These data suggest that eIF-4E phosphorylation is important for the function of eIF-4F in the formation of the 48S initiation complex. The correlation of eIF-4E phosphorylation and the relief of translational inhibition is also shown by the efficient translation of CAT insertion derivatives mRNAs in 3T3 pMV7-4E(C1) cells, since these cells contain higher levels of phosphorylated eIF-4E than parental NIH3T3 cells (Lazaris-Karatzas *et al.*, 1992). The effect of eIF-4E phosphorylation on translation has also been demonstrated by the stimulation of ODC synthesis in cells treated with insulin or phorbol esters (Manzella *et al.*, 1991). Similar mechanisms may explain how translation of highly structured mRNAs are regulated during early development in *Xenopus* (Fu *et al.*, 1991).

In summary, our results show that eIF-4E overexpression facilitates translation of mRNAs with high secondary structure in their 5' UTR. This effect may be responsible for eIF-4E transforming activity. Thus, cell transformation by eIF-4E overexpression is likely to be caused by translational relief of mRNAs encoding proteins that regulate cell growth. It would be of considerable interest to identify these mRNAs.

Materials and methods

Materials

Enzymes and reagents were purchased from the following sources: restriction endonucleases, calf intestine alkaline phosphatase, polynucleotide kinase, Klenow fragment of DNA polymerase I, and T4 DNA ligase from Boehringer Mannheim. Deoxynucleoside triphosphates were from Promega Biotec. RNase-free DNase I and Moloney murine leukaemia virus reverse transcriptase (Mo-MLV RT) from BRL. Taq DNA polymerase was from Bio/Can Scientific; T7 Sequencing TM kit from Pharmacia LKB. Taq polymerase sequencing kit (Taq Track TM) was from Promega Biotec; Biotyne B nylon membrane for Southern hybridizations was from Pall BioSupport Company. TLC sheets were from J.T. Baker Inc. and X-ray films from Kodak XAR-5. [¹⁴C]Chloramphenicol (dichloroacetyl; 54 mCi/mmol) was from ICN and [α -³²P]dATP (> 3000 Ci/mmol) from NEN. BamHI linker derivatives 5'-AGCTT(CCGGATCCGG)_nA-3', where $n = 1, 3$ and 5, and 5' CAT primer (5'-AACCAGACCGTTCAGTGG-ATATTACGGCC-3') and 3' CAT primer (5'-GCCACTCATCGCAG-TACTGTTGTAATTCAT-3') were synthesized by Sheldon Biotechnology Centre, Montreal.

General methods

Preparation of plasmid DNA, restriction enzyme digestion, agarose gel electrophoresis of DNA and bacterial transformation were carried out using standard methods (Sambrook *et al.*, 1989).

Plasmid constructions

Plasmid pSV2CAT contains the bacterial chloramphenicol acetyltransferase gene downstream from the SV40 early promoter (Gorman *et al.*, 1982). BamHI linkers were kinased with polynucleotide kinase and inserted, using T4 DNA ligase, into a unique HindIII site, 57 bp downstream of the transcription initiation site. Plasmids were amplified in *Escherichia coli* strain HB101, and colonies were analysed for their susceptibility to BamHI and NcoI digestion. NcoI cleaves 109 bp upstream and 551 bp downstream from the HindIII site. Plasmid DNA was labelled with [γ -³²P]ATP, and was analysed on a 7% polyacrylamide-8 M urea gel. Plasmids containing the correct size of NcoI fragments (660-710 bp) were sequenced from both strands by the dideoxy chain termination method (Sanger *et al.*, 1977) or Taq DNA polymerase system according to manufacturer's specifications. The three plasmid derivatives containing one, three and five BamHI linkers are termed HB1, HB3 and HB5 respectively.

Cell lines and DNA transfection

3T3 pMV7-4E(C1) and 3T3 pMV7-4E(Ala) cell lines overexpress wild-type and mutant ser⁵³-ala eIF-4E respectively, as previously described (Lazaris-Karatzas *et al.*, 1990). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated serum, 4 mM glutamine and 50 units/ml of penicillin and streptomycin.

For DNA transfection, plasmids were purified twice on caesium chloride gradients. Cells were incubated with Ca₃(PO₄)₂-DNA coprecipitates as described by Graham *et al.* (1980). Briefly, 1×10^5 cells were plated in 5 ml media in 60 mm Petri dishes. Twenty-four hours later, 0.8 ml of calcium phosphate coprecipitate containing 8 μ g of supercoiled DNA was added. After incubation at 37°C for 24 h, medium was removed, cells were washed with PBS (140 mM NaCl, 15 mM KH₂PO₄, pH 7.2 and 2.7 mM KCl) twice, and fresh medium was added. Cells were incubated for 48 h, harvested in PBS and were used for RNA extraction (Chomczynski and Sacchi, 1987) and quantitation of CAT enzymatic activity (Gorman *et al.*, 1982).

Isolation of plasmid DNA by Hirt's method

Hirt extraction was done as previously described (Hirt, 1967) with a few modifications. Briefly, 48 h after DNA transfection, cells in 60 mm dishes were washed with PBS twice, and lysed with 200 μ l of 0.6% SDS-10 mM EDTA solution. The lysate was incubated at room temperature for 20 min and transferred to an Eppendorf tube. NaCl was added to a final concentration of 1 M. The samples were kept on ice for at least 5 h (usually overnight) and spun at 10 000 r.p.m. for 20 min. The supernatant was carefully removed and transferred to a new tube, phenol extracted and ethanol precipitated. The pellet was dissolved in 100 μ l of H₂O and treated with DNase-free RNase A (1 μ g/ml) for 15 min and proteinase K (400 μ g/ml) for 30 min. Following phenol-chloroform extraction and ethanol precipitation, the samples were digested with HindIII and electrophoresed on a 1% agarose gel.

RNA quantitation by the polymerase chain reaction (PCR)

For CAT mRNA quantitation, a previously described PCR protocol (Robinson and Simon, 1991) with slight modifications was used. Total RNA, isolated 48 h post-transfection, was treated with RNase-free DNase I (20 μ g/ml) and proteinase K (400 μ g/ml), followed by phenol extraction and ethanol precipitation. Each RNA sample (1 μ g) was mixed with 50 pmol of 3' CAT primer and 6 μ l of 5 $\times$ reverse transcription (RT) buffer to a final volume of 30 μ l. Samples were heated to 75°C for 3 min and cooled slowly at room temperature. Twenty microliters of a cocktail containing 1.25 μ l 20 mM dNTP, 5 μ l 0.1 M DTT, 4 μ l 5 $\times$ RT buffer and 200 units of Mo-MLV reverse transcriptase was added and the reaction allowed to proceed for 1 h at 37°C. Samples were boiled for 5 min and 10 μ l of the reaction were mixed together with 0.25 μ l 20 mM dNTP, 5 μ l 10 $\times$ Taq polymerase buffer, 50 pmol of 5' CAT primer, 40 pmol of 3' primer and 2 units of Taq polymerase in a volume of 50 μ l. PCR amplification was performed for 25 cycles as follows: one cycle at 94°C for 4 min, 65°C for 3 min, 72°C for 3 min and 24 cycles at 94°C for 1 min, 65°C for 1.5 min, 72°C for 2 min with a final extension at 72°C for 7 min. A 25 μ l aliquot from each PCR amplification reaction was electrophoresed on a 2% agarose gel and transferred to Biotyne B nylon filter according to the manufacturer's specifications.

Hybridization conditions of Southern blots

Filters were prehybridized first in 3 $\times$ SSC (15 mM NaCl, 1.5 mM sodium acetate) at room temperature for 30 min followed by 3 $\times$ SSC, 5 $\times$ Denhardt's solution for 1 h at 65°C. Filters were soaked in 10 ml of hybridization

solution (50 mM Tris-HCl pH 7.6, 400 mM NaCl, 10 mM EDTA, 1% SDS, 5× Denhardt's solution and 20 µg/ml denatured salmon sperm DNA) for 30 min at 65°C. Following hybridization with 1.5×10^6 c.p.m./ml (1×10^8 c.p.m./µg DNA) of random-primed-generated probe (Feinberg and Vogelstein, 1983) for 12–16 h at 65°C, the filters were washed once with 2× SSC at room temperature and four times with 0.1× SSC, 0.1% SDS for 20 min at 65°C. Filters were exposed to X-ray film for 4–8 h without intensifying screens, and autoradiographic signals were quantified by excising and counting the bands in a liquid scintillator.

Acknowledgements

We thank S.Roy for advice during the course of the work and J.Pelletier and F.Lejbkowitz for critical comments on the manuscript. This research was supported by grants from the Medical Research Council and the National Cancer Institute of Canada to N.S. A.E.K. is recipient of a long-term post-doctoral fellowship from the Human Frontier Science Programme Organization (HFSPO).

References

- Arrick, B.A., Lee, A.L., Grendell, R.L. and Derynck, R. (1991) *Mol. Cell. Biol.*, **11**, 4306–4313.
- Bonneau, A.M. and Sonenberg, N. (1987) *J. Biol. Chem.*, **262**, 11134–11139.
- Brabant, M., McConlogue, L., van Daalen Wetters, T. and Coffino, P. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 2200–2204.
- Bu, X. and Hagedorn, C.H. (1991) *FEBS Lett.*, **283**, 219–222.
- Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.*, **162**, 156–159.
- De Benedetti, A. and Rhoads, R.E. (1990) *Proc. Natl. Acad. Sci. USA*, **87**, 8212–8216.
- Draper, D.E. (1989) *Trends Biochem. Sci.*, **14**, 335–338.
- Duncan, R., Milburn, S.C. and Hershey, J.W.B. (1987) *J. Biol. Chem.*, **262**, 380–388.
- Edery, I., Humbelin, M., Darveau, A., Lee, K.A.W., Milburn, S., Hershey, J.W.B., Trachsel, H. and Sonenberg, N. (1983) *J. Biol. Chem.*, **258**, 11398–11403.
- Edery, I., Lee, K.A.W. and Sonenberg, N. (1984) *Biochemistry*, **23**, 2456–2462.
- Edery, I., Pelletier, J. and Sonenberg, N. (1987) In Ilan, J. (ed.), *Translational Regulation of Gene Expression*. Plenum Publishing Corp., New York, pp. 335–366.
- Feinberg, A.P. and Vogelstein, B. (1983) *Anal. Biochem.*, **132**, 6–13.
- Frederickson, R.M., Montine, K.S. and Sonenberg, N. (1991) *Mol. Cell. Biol.*, **11**, 2896–2900.
- Frederickson, R.M., Mushynski, W.E. and Sonenberg, N. (1992) *Mol. Cell. Biol.*, **12**, 1239–1247.
- Fu, L., Ye, R., Browder, L.W. and Johnston, R.N. (1991) *Science*, **251**, 807–810.
- Gorman, L.C., Moffat, L.F. and Howard, B.H. (1982) *Mol. Cell. Biol.*, **2**, 1044–1051.
- Grifo, J.A., Tahara, S.M., Morgan, M.A., Shatkin, A.J. and Merrick, W.C. (1983) *J. Biol. Chem.*, **258**, 5804–5810.
- Graham, F.L., Bacchetti, S., McKinnon, R., Stanners, C., Cordell, B. and Goodman, H. (1980) In Baserga, R., Croce, C. and Rovera, G. (eds), *Introduction and Macromolecules into Viable Mammalian Cells*. Alan R. Liss, New York, pp. 3–25.
- Hershey, J.W.B. (1989) *J. Biol. Chem.*, **264**, 20823–20826.
- Hershey, J.W.B. (1991) *Annu. Rev. Biochem.*, **60**, 717–755.
- Hiremath, L.S., Webb, N.R. and Rhoads, R.E. (1985) *J. Biol. Chem.*, **260**, 7843–7849.
- Hirt, B. (1967) *J. Mol. Biol.*, **26**, 365–369.
- Huang, J. and Schneider, R.L. (1991) *Cell*, **65**, 271–280.
- Joshi-Barve, S., Rychlik, W. and Rhoads, R.E. (1990) *J. Biol. Chem.*, **265**, 2979–2983.
- Kaspar, R.L., Rychlik, W., White, M.W., Rhoads, R.E. and Morris, D.R. (1990) *J. Biol. Chem.*, **265**, 3619–3622.
- Klausner, R.D. and Harford, J.B. (1989) *Science*, **246**, 870–872.
- Kozak, M. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 2850–2854.
- Lamphear, B.J. and Panniers, R. (1990) *J. Biol. Chem.*, **265**, 5333–5336.
- Lamphear, B.J. and Panniers, R. (1991) *J. Biol. Chem.*, **266**, 2789–2794.
- Lazaris-Karatzas, A. and Sonenberg, N. (1992) *Mol. Cell. Biol.*, **12**, 1234–1238.
- Lazaris-Karatzas, A., Montine, K.S. and Sonenberg, N. (1990) *Nature*, **345**, 544–547.
- Lazaris-Karatzas, A., Smith, M.R., Frederickson, R.M., Jaramillo, M.L., Liu, Y.-L., Kung, H.-F. and Sonenberg, N. (1992) *Genes Dev.*, in press.
- Manzella, J.M. and Blackshear, P.J. (1990) *J. Biol. Chem.*, **265**, 11817–11822.
- Manzella, J.M., Rychlik, W., Rhoads, R.E., Hershey, J.W.B. and Blackshear, P.J. (1991) *J. Biol. Chem.*, **266**, 2383–2389.
- Marino, M.W., Pfeffer, L.M., Guidon, P.T. and Donner, D.B. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 8417–8421.
- Morley, S.J. and Traugh, J.A. (1989) *J. Biol. Chem.*, **264**, 2401–2404.
- Morley, S.J. and Traugh, J.A. (1990) *J. Biol. Chem.*, **265**, 10611–10616.
- Nikaido, T., Shimizu, A., Ishida, N., Sabe, H., Teshigawara, K., Maeda, M., Uchiyama, T., Yodoi, J. and Honjo, T. (1984) *Nature*, **311**, 631–635.
- Pelletier, J. and Sonenberg, N. (1985) *Cell*, **40**, 515–526.
- Rao, C.D., Pech, M., Robbins, K.C. and Aaronson, S.A. (1988) *Mol. Cell. Biol.*, **8**, 284–294.
- Ratner, L., Thielan, B. and Collins, T. (1987) *Nucleic Acids Res.*, **15**, 6017–6036.
- Ray, B.K. et al. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 663–667.
- Ray, B.K., Lawson, T.G., Kramer, J.C., Cladaras, M.H., Grifo, J.A., Abramson, R.D., Merrick, W.C. and Thach, R.E. (1985) *J. Biol. Chem.*, **260**, 7651–7658.
- Robinson, M.O. and Simon, M.I. (1991) *Nucleic Acids Res.*, **19**, 1557–1562.
- Roy, S., Parkin, N., Rosen, C., Itovitch, J. and Sonenberg, N. (1990) *J. Virol.*, **64**, 1402–1406.
- Rozen, F., Edery, I., Meerovitch, K., Dever, T.E., Merrick, W.C. and Sonenberg, N. (1990) *Mol. Cell. Biol.*, **10**, 11343–1144.
- Rychlik, W., Russ, M.A. and Rhoads, R.E. (1987) *J. Biol. Chem.*, **262**, 10434–10437.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Sanger, F., Nicklen, S. and Coulson, A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
- Sarkar, G., Edery, I., Gallo, R. and Sonenberg, N. (1984) *Biochim. Biophys. Acta*, **783**, 122–129.
- Shatkin, A.J. (1976) *Cell*, **9**, 645–653.
- Smith, M.R., Jaramillo, M., Liu, Y.-I., Dever, T.E., Merrick, W.C., Kung, H.F. and Sonenberg, N. (1990) *New Biol.*, **2**, 648–654.
- Sonenberg, N. (1988) *Prog. Nucleic Acids Res. Mol. Biol.*, **35**, 173–207.
- Sonenberg, N., Morgan, M.A., Merrick, W.C. and Shatkin, A.J. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 4843–4847.
- Tahara, S.M., Morgan, M.A. and Shatkin, A.J. (1981) *J. Biol. Chem.*, **256**, 7691–7694.
- Thach, R.E. (1992) *Cell*, **68**, 177–180.
- Werner, H., Stannard, B., Bach, M.A., LeRoith, D. and Roberts, C.T. (1990) *Biochem. Biophys. Res. Commun.*, **169**, 1021–1027.
- Zucker, M. and Stiegler, P. (1981) *Nucleic Acids Res.*, **9**, 133–148.

Received on July 8, 1992