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STUDIES ON THE MECHANISMS OF mRNA BINDING TO RIBOSOMES IN EUKARYOTES

by

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A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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June 1997

Dedication

For my loving parents, Ali and Parvaneh, and my dear brother, Kamran,
for their unwavering love and support through the many years. Their
encouragement made possible an incredible expedition
of satisfying learning and living.
Thanks to them, I tasted the best of both.

"Lorsque tu veux vraiment une chose, tout l'univers conspire à te permettre de réaliser ton désir"

Paulo Coelho

SUMMARY

The binding of eukaryotic ribosomes to mRNA is a complex process that is considered to be rate-limiting in translation initiation, and consequently a key target for translational regulation. Ribosome binding to mRNA is facilitated by the 5' cap structure, m^7GpppN (where N is any nucleotide). The initiation factor eIF4F plays a key role in regulating translation rates. eIF4F is a three-subunit complex composed of eIF4E, the cap-binding protein; eIF4A, an RNA helicase; and eIF4G (p220), which bridges eIF4E and eIF4A, and enhances dramatically the interaction of eIF4E with the mRNA 5' cap structure. eIF4F in conjunction with another initiation factor, eIF4B, is thought to unwind the mRNA 5'-secondary structure to facilitate the binding of mRNA to ribosomes. The activity of eIF4F, and the regulation of mRNA binding to ribosomes is tightly correlated with the growth status of the cell. Recently, proteins that interact with eIF4E, termed 4E-BPs, have been identified; these proteins link translation initiation and growth promoting signal transduction pathways. Phosphorylation of 4E-BPs in response to insulin and mitogens decreases their affinity for eIF4E. 4E-BPs compete with eIF4G for binding to eIF4E through binding domains that share common sequence motifs. Consequently, 4E-BPs restrain eIF4E from forming an active cap-binding complex, eIF4F, and prevent subsequent binding of 40S ribosomal subunit to capped mRNAs. Under these conditions, the binding of eIF4E to the mRNA cap structure is extremely inefficient. As a result, cap- and eIF4E-dependent translation is downregulated. Modulation of eIF4F activity is also observed following infection by certain viruses. One of the most dramatic examples of this occurs upon picornaviral infection. As we report here, eIF4G alone is a relatively poor substrate for cleavage by the rhinovirus 2A proteinase ($2A^{pro}$). However, an eIF4G-eIF4E complex is cleaved efficiently by the $2A^{pro}$ suggesting that eIF4F is a preferred target for direct cleavage by rhinovirus $2A^{pro}$. Collectively, the studies presented in this thesis reveal novel mechanisms of regulating eIF4F function, and subsequent binding of mRNA to ribosomes in eukaryotes.

RÉSUMÉ

La liaison des ribosomes à l'ARN messager (ARNm) est l'étape limitante de l'initiation de la traduction chez les eukaryotes. La formation du complexe 48S est facilitée par la structure en coiffe (m^7GpppG, où N designe un nuléotide) en 5' de l'ARNm. Le facteur d'initiation eIF4F joue un rôle très important dans le contrôle de la traduction. eIF4F est composé de trois protéines: eIF4E, qui se lie directement à la structure en coiffe de l'ARNm; eIF4A, qui possède une activité de déroulement de structures secondaires; et eIF4G (p220) qui sert de lien entre eIF4E et eIF4A, et augmente la liason de eIF4E à la structure en coiffe de l'ARNm. Le déroulement de structures secondaires de l'ARNm par eIF4F et eIF4B permet l'accrochage de la sous-unité ribosomique 43S à l'ARNm. La modulation de l'activité du facteur d'initiation eIF4F est étroitement correlée avec l'activité de la croissance de la cellule. Deux protéines (4E-BP1 et 4E-BP2) interagissant avec eIF4E ont été identifiées; ces protéines relient les signaux extracellulaires à l'initiation de la synthèse protéique. 4E-BP1 est phosphorylé en réponse à l'insuline et aux mitogènes. Cette forme phosphorylée ne se lie plus à eIF4E. 4E-BP1 entre en compétition avec eIF4G pour un même site de liaison sur eIF4E. Par conséquent, 4E-BP1 séquestre eIF4E, et empêche ce dernier de former un complexe eIF4F fonctionnel. De cette manière, la traduction cap- et eIF4E-dépendante est diminuée. La modulation de l'activité de eIF4F est également observée après l'infection de cellules par certains virus. Un exemple de choix est apporté par l'infection cellulaire par les picornavirus. Comme rapporté ici, eIF4G en tant que tel est un substrat relativement peu effectif pour la protéinase 2A ($2A^{pro}$) de rhinovirus. Cependant, le complexe eIF4G•eIF4E est clivé efficacement par $2A^{pro}$; ceci suggère que eIF4F est une cible préferentielle pour un clivage direct par $2A^{pro}$. Collectivement, les resultats présentés dans ce travail de thèse révèlent un nouveau mécanisme de régulation de la fonction de eIF4F, et la liaison subséquente des ARN messagers aux ribosomes eukaryotes.

FOREWORD

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- While the inclusion of manuscripts co-authored by the candidate and others is acceptable, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent, and supervisors must attest to the accuracy of the claims at the PhD oral defence. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make the responsibilities of authors perfectly clear.

PREFACE

The results presented in chapters II, III and IV of this thesis have been published in the following journals:

Chapter II: A. Haghighat, S. Mader, A. Pause, and N. Sonenberg. (1995) *EMBO J.*, 14, 5701-5709.

Chapter III: A. Haghighat and N. Sonenberg. (1997) *J. Biological Chemistry*, 272, 21677-21680.

Chapter IV: A. Haghighat, Y. Svitkin, I. Novoa, E. Kuechler, T. Skern, and N. Sonenberg. (1996) *J. Virology*., 70, 8444-8450.

The technical aspects of the work presented in all chapters were performed by myself with the following contributions: For chapter II, S. Mader provided the cDNA for GST—4E-BP1 and GST—4E-BP1Δ, and A. Pause provided recombinant eIF4E. For Chapter IV, Y. Svitkin provided Krebs-2 ascites cell extracts, I. Novoa contributed some anti-p220 anitibody, and E. Kuechler and T. Skern supplied the preparation of HRV2 2A protease.

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ACKNOWLEDGMENTS

I am forever grateful to my director, Dr. Nahum Sonenberg, for the opportunity. My adventure in room 806 started when Nahum opened the door to his institute for me by giving me a little desk, some bench space, and a quasi-official project to go with it. Throughout the four years that followed, his constant and unusual strategy for drive provided for the most interesting and fulfilling learning experience. I thank him for believing in me, for his availability, and the years of fruitful interaction. As my teacher and mentor, he never bid me entry into his vast temple of wisdom, but taught me to persevere and reach the threshold of my own, what has proved ever so satisfying. This modesty remains one of Nahum's best hidden attributes. A great personality. I remain indebted to him for his support.

It is impossible to acknowledge properly all friends and colleagues who have helped to create and shape this adventure. So I apologize in advance for any shortcoming. The initial Han-Antony-Greg collection in 806, and the blasting music after 6:30 PM, made for an enjoyable working ambiance, which often stretched happily into the early hours of morning. But then Han left for the eleventh floor, Antony finished his time, Greg got married and became a father of two, and the radio broke down! Those early days were greatly missed in the later days. During these four years I have witnessed a great deal of reshuffling of members in the lab. Once there was a Greek malakas, Adonis, who made the lab meetings particularly very interesting. The Laura-and-Bo battles were amusing. Separately, however, I enjoyed their friendship and appreciated their input. During the last stretch of this journey, I also missed my Swiss neighbour, (Oliver) Donzé, for the fun and gossips. Nathalie must have had sore ears from listening to me model and remodel ideas that I ventured again and again; so I thank her. Since her departure, Yuri has become the next victim. I also thank him for sharing his secrets of the intricacies of translation assays and SDS-PAGE procedures.

I am very grateful to the past and present members of the Sonenberg group whose work set the stage for or aided mine, and the mentors who have directly or indirectly influenced my own work and thinking. Amim's helpful suggestions and Sylvie's contribution helped me experience my first true eureka effect. Contributions to my scientific endeavor also come from interesting discussions with 'consistent' friends Andrew and Greg among others. I therefore extend a special thanks to them. In the category of ex-Sonenbergians and influential mentors, I specially thank Jerry Pelletier for his interest. He remains an inspiration to most members.

Colin continues to provide invaluable assistance for all of us, and I thank him personally for his help. I just wish his time schedule would change because he puts most grad students to shame! Mina is another invaluable member of the force whose critical input is felt after long breaks in particular. Maureen, the mother of this department, has made my trip much smoother in the past, and I would like to acknowledge and thank her very much for being concerned. More people like her should be cloned.

Some extracurricular events were reasons for several of the highlights of my four year journey. The opportunities to teach undergraduate laboratory courses and to present a few lectures here and there helped discover some hidden skills. Thanks to Michel and Philippe, coordinators of 300D, for the initial appointments. I also acknowledge their interest and encouragement during the restructuring phase of the course that I had initiated. Of course, in this regard, the insight of my 300D students is also much appreciated. I would like to also thank members of the McGill Journal of Medicine for appointing me to their Editorial Board and allowing me to contribute to the development of the journal. The experience has been wonderful.

I thank all friends who provided the necessary distractions outside the work area that enabled me to continue in a sane manner. In particular, my great friend and roommate, Antoine, has been very supportive. Together, we created an apartment that became the ideal place to take refuge for writing this manuscript in a reasonable time.

Andrew, Yuri, and Greg were kind enough to critically review my thesis. I also appreciate Daniel's proof-reading of the French translation of the résumé to my thesis.

I would like to express my deepest gratitude to my companion, Roshanak, who has been there with me for the entire four years. Her continued presence, love and support has made these years that much more pleasant. I thank her for sharing these years with me. Moreover, I would like to extend my appreciation to her family (Ali, Nini, Rez, Chub, and Ruby the dog) who have treated me with extreme measures of love and kindness.

Last, but not least, a warm thanks to my twin brother, Kamran, who is of course my 'true' better half ! I thank him more than anyone for his support and motivation. He's been an inspiration. Despite the many recent years apart, and regardless of an inherent genetic link, he remains the closest person to me. Thanks to him though, I have become Bell Canada's most valuable student customer !

It has been fun!

CHAPTER I

General Introduction

1.1 PERSPECTIVE

The mechanisms of translational control in eukaryotes have received considerable attention in recent years and many aspects of this regulatory step have been analyzed in great detail. Eukaryotic protein synthesis is one of the most complex processes in a cell, and the elucidation of the underlying events has remained a challenging problem in molecular biology. More than 150 macromolecules comprise the eukaryotic protein synthesis machinery, and regulation of this multistep event is exerted at many levels. It has become evident that alterations in many steps of this process, particularly the initiation step, could lead to altered gene expression, cell growth and development, highlighting the fundamental importance of translational control.

For this reason, a brief overview of protein synthesis in eukaryotes is given, followed by a more detailed description of the steps and components of the translational apparatus that promote the binding of mRNA to the 40S ribosomal subunit. The focus will then be diverted onto the interaction of viruses with the cellular translational machinery, which includes an overview of the mechanism and prevalence of internal ribosome binding.

1.2 A BRIEF OVERVIEW OF EUKARYOTIC PROTEIN SYNTHESIS

Protein synthesis in eukaryotes is customarily divided into three phases: initiation, elongation, and termination. In each phase, the transient interactions between soluble protein factors, the ribosome and the mRNA ultimately lead to the decoding of the genetic imprint.

The *initiation* reaction of protein biosynthesis requires the assembly of a complex between a ribosome, an mRNA, and an aminoacyl-tRNA. The formation of this complex, which largely determines the rate of translation, is promoted by at least 11 initiation factors (eIFs), and energy in the form of ATP and GTP hydrolysis. A schematic for the pathway of translation initiation in eukaryotes is presented in Fig. 1. Initiation of translation starts with the generation of free 40S ribosomal subunits. Under physiological conditions 80S ribosomes predominate in active equilibrium with the dissociated subunits. Both subunits are targets for binding proteins which shift the equilibrium towards dissociation. eIF1A and eIF3 bind to the small 40S ribosomal subunit (Goumans *et al.*, 1980), and eIF6 binds exclusively to the larger 60S ribosomal subunit (Russell and Spremulli, 1979; Raychaudhuri *et al.*, 1984) and inhibit the formation of an 80S ribosome.

The next step is the formation of a ternary complex between the initiator methionyl-tRNA (Met-tRNA$_i$), eIF2, and GTP. The formation of this obligatory intermediate is stabilized by two ancillary factors, eIF2C and eIF3 (Gupta *et al.*, 1990). These factors also prevent the disruption of the complex in the presence of mRNA (Roy *et al.*, 1988). The ternary complex subsequently gains access to the P site of the 40S ribosomal subunit to form the 43S preinitiation complex (Safer *et al.*, 1976). This is followed by the binding of the 43S preinitiation complex to mRNA (48S preinitiation complex), an important regulatory step that requires energy derived from the hydrolysis of ATP, and the concerted action of several initiation fators including eIF4E, eIF4G, eIF4A, and eIF4B

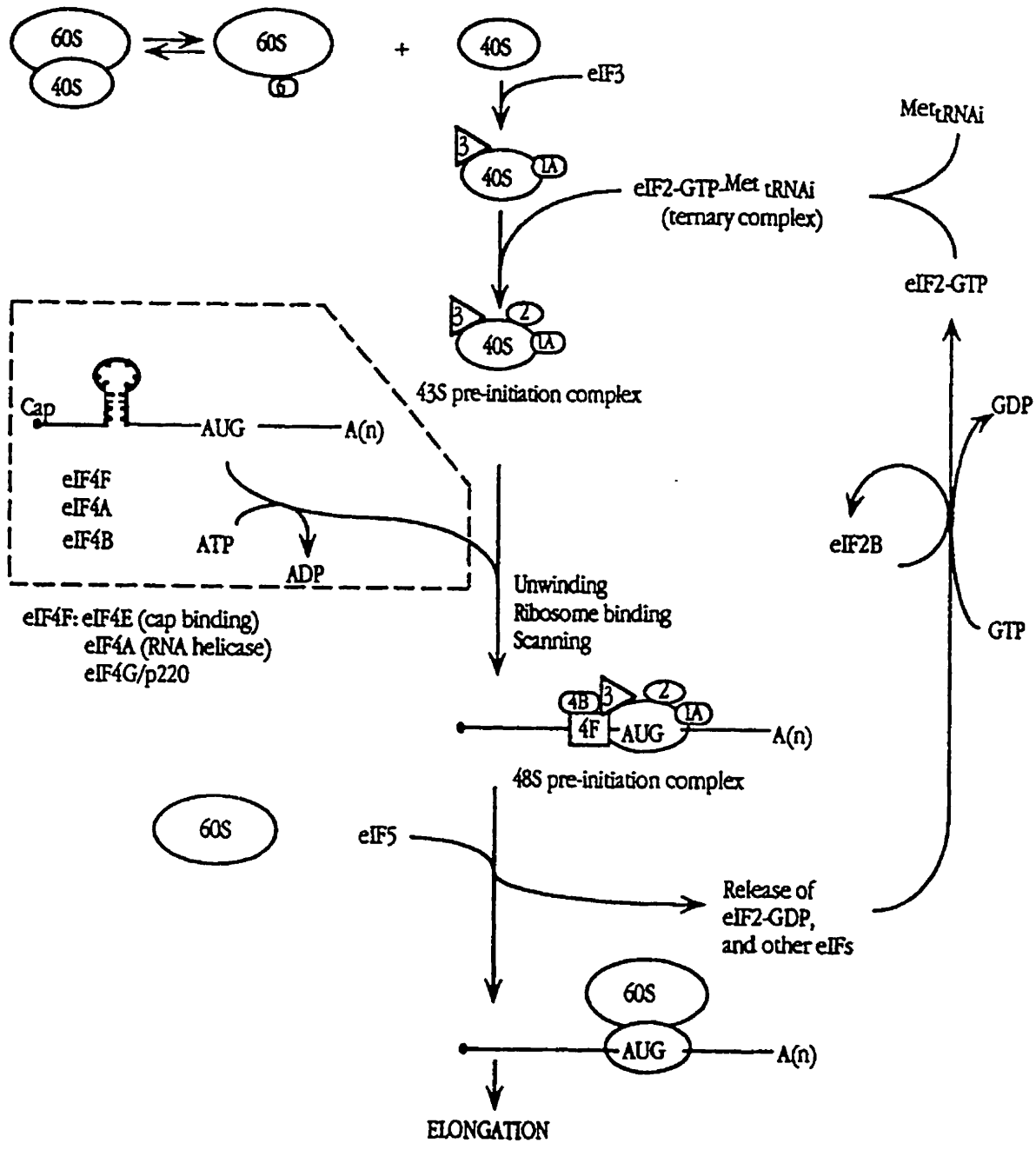


Figure 1.1: Pathway of Translation Initiation in Eukaryotes .

(Trachsel *et al.*, 1977; Benne and Hershey, 1978). One model posits that, following the binding of these initiation factors to the 5' cap structure [m^7G(5')ppp(5')N, where N is any nucleotide], unwinding of the secondary structures occurs in the vicinity of the cap which facilitates the binding of the ribosome to the mRNA (Sonenberg, 1988). This rate-limiting step is followed by the vectoral "scanning" of the mRNA by the 40S ribosomal subunit until it encounters the initiator AUG codon, as defined by its flanking sequences (Kozak, 1989). This specific alignment serves to decode the reading frame of the mRNA. Once a match is made between the anticodon of Met-tRNA$_i$ and the AUG initiator codon, eIF5 triggers the hydrolysis of the GTP carried by eIF2 (Trachsel *et al.*, 1977; Benne and Hershey, 1978). This event causes the release of initiation factors from the 48S preinitiation complex, and allows for the rapid joining of the 60S ribosomal subunit. The resulting 80S complex is then competent to enter the elongation phase of translation.

Elongation is perhaps the most complex step in the process of translation, and our knowledge of this event is primarily by analogy to mechanisms elucidated in prokaryotes (for a review, see (Moldave, 1985; Slobin, 1990)). The elongation step, which involves the repetitive cycle of codon-directed additions of aminoacyl-tRNA to the C-terminus of the growing peptide chain, begins with a peptidyl-tRNA in the P site and an empty A site in the 80S ribosome. Aminoacyl-tRNAs enter the A site as a ternary complex with eEF1A and bound GTP. Following the hydrolysis of GTP by eEF1A, and the release of this binary complex from the A site, the peptidyl-transferase center of the 60S ribosomal subunit catalyses the peptide-forming reaction between the aminoacyl-tRNA in the A site and the peptidyl-tRNA in the P site. Subsequently, an eEF2•GTP complex promotes the translocation of the ribosome along the mRNA by one codon, hence relocating the peptidyl-tRNA to the P site, and vacating the A site to accept the next ternary complex.

If either of three *termination* codons (UAA, UGA, UAG) are exposed in the A site, a release factor (eRF1•3) bound to GTP binds the stop codon and effects the hydrolysis of the aminoacyl-tRNA and the release of the peptide from the ribosome. Furthermore, it is

believed that eRF1 causes the dissociation of the mRNA and the ribosome so that these components may engage in another round of translation.

1.3 REGULATION OF TRANSLATION IN EUKARYOTES

Modulation of translation rates in eukaryotes is recognized to be of central importance in the control of cell proliferation. Protein synthesis is one of the early obligatory metabolic events that is required for entry into, and progression through the cell cycle (Brooks, 1977). The multistep process of translation is regulated at many levels, and integrates the activities of numerous elements, including a large group of initiation factors. The stability of eukaryotic mRNAs adds significance to a pathway for translational control. As such, a faster and more selective alteration in the translational efficiency of mRNAs may be attained in response to external stimuli, without invoking nuclear events of mRNA biogenesis and subsequent transport. It is generally assumed that initiation is the key regulatory step in protein synthesis, with the overwhelming preponderance of these intricate mechanisms being operative during this phase. Two particular steps of the initiation pathway which appear to be frequent targets for physiological control are the formation of ternary complexes, mediated by eIF2; and the binding of the ribosome to mRNA, mediated by eIF4E and associated initiation factors. As will be discussed below, the recurrent theme common to these check points is the reversible phosphorylation of key initiation components, such as eIF2 and eIF4E, in response to growth factor stimulation.

1.3.1 TERNARY COMPLEX FORMATION

The formation of ternary complexes, and thus the binding of Met-tRNA$_i$ to 40S ribosomal subunits, is promoted by eIF2, and is one of the most important sites of translational regulation. Decrease in the activity of eIF2 in response to many physiological stimuli correlates with inhibition of translation and suppression of cell growth. eIF2

consists of three subunits with molecular masses of 36 (α), 38 (β), and 52 (γ) kDa, each exhibiting distinct activities. The β subunit is involved in RNA binding (Donahue *et al.*, 1988; Flynn *et al.*, 1994) and appears to play a role in AUG initiation codon selection (Donahue *et al.*, 1988). The γ subunit is the GTP-binding subunit (Gaspar *et al.*, 1994). The most significant feature of the α subunit of eIF2 is a conserved phosphorylation site at Ser51, which is the target for a family of protein kinases important in the regulation of protein synthesis.

Recognition of an initiator AUG codon, and the formation of 80S ribosomes result in the ejection of an eIF2•GDP complex. The GDP in this inactive complex is subsequently replaced by GTP in a reaction catalysed by a limiting factor called eIF2B (or GEF, Guanine nucleotide Exchange Factor). One physiological mechanism which regulates the recycling step involves the phosphorylation of the α subunit of eIF2. Three distinct kinases that specifically phosphorylate eIF2a are (i) the heme-controlled repressor (HCR), (ii) the double-stranded RNA-activated kinase PKR (also called DAI), and (iii) the *Saccharomyces cerevisiae* GCN2 [for a review see (Clemens, 1996)]. Classic examples of the mechanism of nucleotide exchange are through the activation of the heme-sensitive protein kinase, HCR; and PKR, a kinase activated by double-stranded RNA (eg. during viral infection). The phosphorylation of eIF2 α subunit leads to an increased affinity of eIF2 for eIF2B, thereby reducing the concentration of free eIF2B which could otherwise catalyze the recycling of the GDP on eIF2. The outcome is an overall reduction in protein synthesis (Hershey, 1991). The importance of these regulatory processes is underscored by the finding that overexpression of a dominant negative mutant of PKR (Koromilas *et al.*, 1992), or a nonphosphorylatable mutant of eIF2, causes transformation in NIH-3T3 cells (Donzé *et al.*, 1995).

1.3.2 mRNA BINDING TO RIBOSOMES

The recognition and binding of the mRNA by the 43S ribosomal preinitiation complex is usually the rate-limiting step in translation initiation. This step is energy dependent, requiring the hydrolysis of ATP, and the action of a number of initiation factors including eIF4E, eIF4A, eIF4G, and eIF4B. These factors are in turn regulated through their reversible phosphorylation in response to extracellular signals. Indeed, phosphorylation of these key regulatory factors positively correlates with enhanced protein synthesis and cellular growth.

Translational control at the level of mRNA binding to ribosomes is also realized through multiple mechanisms that specifically target structural features of the mRNA. In eukaryotes, several *cis*-acting elements distributed along the length of the mRNA modulate its intrinsic translational efficiency. Such structural features in the untranslated regions of mRNAs include the 5' cap structure, secondary structure, and the sequences flanking the initiator AUG codon.

1.3.2.1 *5' Cap Structure*

All eukaryotic cytoplasmic messenger RNAs are processed post-transcriptionally in the nucleus with the addition of a cap structure to the 5' end. The cap structure consists of a guanosine residue that is methylated at the N-7 position and linked to the penultimate nucleotide through an inverted 5'-5' triphosphate bond [reviewed in (Shatkin, 1976; Furuichi *et al.*, 1977)]. The cap structure plays a decisive role in pre-mRNA splicing (Konarska *et al.*, 1984; Edery and Sonenberg, 1985), and has been implicated as a positive signal for the nucleocytoplasmic export of mRNAs (Hamm and Mattaj, 1990; Dargemont and Kuhn, 1992), as well as 3' end processing (Hart *et al.*, 1985). Additionally, several studies have confirmed that the cap structure confers stability onto mRNAs through protection against 5' exonucleolytic activity. Indeed, the regulated process of mRNA

degradation in turn determines the level of expression of a gene [reviewed in (Sachs, 1993)].

The cap structure is an important regulatory determinant of translational efficiency, as capped messages are translated much more efficiently than their uncapped counterparts in several cell-free translation extracts (Both *et al.*, 1975; Muthikrishnan *et al.*, 1976). The facilitative effect of the cap structure on 40S ribosomal subunit binding to mRNA is in fact its best characterized attribute [see (Shatkin, 1976)]. The methyl group on the guanosine ring seems to be necessary for the translational activation *in vivo*. Importantly, *in vivo* experiments have provided the best evidence for a functional link between the 5' and 3' ends of the mRNA (Gallie, 1991). The cap structure has been shown to enhance poly(A)$^+$ mRNA better than poly(A)$^-$ mRNA. Furthermore, the poly(A) tail acts as an enhancer of translation of capped, and not uncapped, mRNAs. These findings suggest a synergy between the cap and the poly(A) tail function in translation, raising an intriguing possibility that the interacting mRNA termini could direct the terminating ribosomes back to the 5'UTR, and consequently promote reinitiation and enhance translation (Gallie *et al.*, 1988).

1.3.2.2 *The 5'UTR Secondary Structure*

The average length of the 5'UTR of vertebrate mRNAs varies between 20 to 100 nucleotides (Kozak, 1987). Several viral RNAs, as well as cellular mRNAs that code for products important for growth and development [e.g. *c-myc*, ornithine decarboxylase (ODC), and *c-sis*], however, contain remarkably long 5'UTRs in the range of 300-1000 nucleotides. Generally, long leaders have the potential to assume secondary structure conformations, which are inhibitory to translation initiation. This effect is position-dependent, with initial stages of mRNA binding to initiation factors and ribosomal subunits being most sensitive to impairment (Pelletier and Sonenberg, 1985a; Kozak, 1986). Stable secondary structures (ΔG=-50 to -60 kcal/mol) positioned 72 nucleotides downstream

from the cap allow for ribosome binding but strongly inhibit 40S ribosomal subunit scanning (Kozak, 1986). In contrast to stable hairpins, structures of moderate stability (ΔG=-30 kcal/mol) impede translation only when in close proximity (12 nucleotides) to the cap structure. In that position, weaker hairpin structures prevent access to the cap structure, and hence ribosome binding. Similar hairpin structures positioned further downstream of the cap can be readily melted by the initiation machinery (Pelletier and Sonenberg, 1985a). Accordingly, photochemical cross-linking assays have revealed that secondary structures close to the cap inhibit cross-linking of eIF4B to the cap structure (Pelletier and Sonenberg, 1985b). These results demonstrate that this feature of the mRNA determines its ability to compete for initiation factors, and hence influence the rate of initiation complex formation. Indeed, removal of naturally occurring secondary structures from the mRNA of *c-myc* and ODC increases their translation significantly (Parkin *et al.*, 1988; Manzella *et al.*, 1991).

In addition to the general role of the 5'UTR in the regulation of translation initiation, *cis*-acting elements in this region of specific mRNAs serve to selectively control ribosome binding. A well documented example, in which the binding of a repressor protein to the 5'UTR regulates translation, is that of the ferritin mRNA (Klausner *et al.*, 1993). The translation of this mRNA is regulated by the availability of iron. Upon iron deprivation, interaction of an iron regulatory factor (IRF) with conserved regulatory elements called iron-responsive elements (IRE) in the 5'UTR of ferritin mRNA prevents its translation. The inhibitory effect of the IRE is again position-dependent, and an IRF/IRE interaction interferes with the association of the 43S complex with the mRNA (Gray and Hentze, 1994). Furthermore, steric hindrance seems to be the operative mechanism of inhibition, as other RNA-binding proteins (e.g. U1A snRNP and the bacteriophage MS2 coat protein) can exert the same effect, provided their binding sites are inserted in the cap-proximal position (Stripecke and Hentze, 1992). Instances where repressors bind to

5'UTRs as means of regulating translation also apply to several reported autoregulatory feedback mechanisms. Thymidylate synthase (Chu *et al.*, 1991), dihydrofolate reductase (Chu *et al.*, 1993), and p53 (Mosner *et al.*, 1995) are examples of such cases.

1.3.2.3 *The Initiator AUG Codon*

Another dominant determinant of translational fidelity and efficiency in vertebrate mRNAs is the position of the initiation codon, AUG, and the contribution of surrounding sequences (Kozak, 1986). Greater than 95% of vertebrate mRNAs seem to initiate translation from the most 5'-proximal AUG. Furthermore, the AUG context **A/G**CCAUG**A/G** is optimal for initiation in mammalian cells, with the strongest contribution being from purines (preferably **A**) at position -3 and a **G** in position +4. More recent evidence has also implicated positions +5 and +6 in the initiation codon recognition process (Boeck and Kolakofsky, 1994). An optimal AUG context enhances translation by more than ten-fold *in vivo* and *in vitro*. The importance of this sequence to translation rates has been demonstrated with the rat preproinsulin mRNA, where single mutations at most positions had deleterious effects (Kozak, 1983). Although the importance of the appropriate sequence context within which the AUG codon is embedded has been well established, the molecular mechanism which governs the selection of the appropriate AUG codon is not well defined. Recent evidence has implicated trans-acting factors, such as the La autoantigen in the selection of the AUG start codon during eukaryotic translation initiation. Favourable AUG contexts have also been reported for *Drosophila melanogatser* and the yeast S. *cerevisiae*, although the requirement for specific nucleotides flanking the AUG codon appear less stringent than in higher eukaryotes (Cigan and Donahue, 1995; Cavener and Ray, 1991).

Departures from the first AUG rule have also been well documented. If the first AUG is followed by an in-frame termination codon, initiation from downstream start sites may occur. A prominent example of such a *'reinitiation'* process is that of the yeast

Saccharomyces cerevisiae GCN4 mRNA (Hinnebusch, 1996). Also, when the first AUG codon is in a suboptimal context, the 40S ribosomal subunit may bypass that site in a *'leaky scanning'* mechanism to initiate at a more favourable second AUG. Such a mechanism is operative in the production of the two proteins, LIP and LAP (transcriptional repressor and activator, respectively) from the same mRNA (Descombes and Schibler, 1991).

1.3.3 KEY INITIATION FACTORS AND THEIR ROLE IN mRNA RECRUITMENT TO RIBOSOMES

1.3.3.1 *eIF4E and Associated Proteins*

(a) eIF4E

eIF4E was initially identified by virtue of its ability to specifically interact with the mRNA cap structure (Sonenberg *et al.*, 1978). In HeLa cells, eIF4E exists in two forms: as a free 24 kDa protein, and as part of a high molecular weight complex, eIF4F (Tahara *et al.*, 1981). However, it is believed that the functional form of eIF4E is the complexed form. The observation that eIF4E localizes to the nucleus as well as the cytoplasm has raised the possibility that it may be implicated in the nucleocytoplasmic transport of mRNAs (Lejbkowicz *et al.*, 1992). An additional interesting feature of eIF4E from several species includes its high tryptophanyl content. Conveniently, the cap structure exhibits high affinity for tryptophans (Altmann *et al.*, 1988).

Of particular importance is the key role of eIF4E in the regulation of translation and cell growth. The limiting nature of eIF4E as compared to other initiation factors, and the positive correlation between its phosphorylation state and enhanced protein synthesis, renders eIF4E an excellent candidate as a key player in regulation of cell growth. The major phosphorylation site of eIF4E has been assigned to Ser-209 (Joshi *et al.*, 1995) Although there is evidence for and against the involvement of protein kinase C in the signalling pathway, it is conceivable that other kinases such as an insulin-stimulated

protamine kinase mediate the phosphorylation of eIF4E (Makkinje *et al.*, 1995). Increase in eIF4E phosphorylation is observed in response to a wide variety of growth-promoting signals such as mitogens, hormones, and cytokines (Rhoads, 1991). eIF4E is underphosphorylated during mitosis (Bonneau and Sonenberg, 1987a), following heat shock (Duncan *et al.*, 1987; Lamphear and Panniers, 1990), or infection with several viruses (Huang and Schneider, 1991; Feigenblum and Schneider, 1993), concomitent with a reduction in cap-dependent translation rates. The biological significance of eIF4E is underscored by the finding that its overexpression leads to transformation of rodent cells (Lazaris-Karatzas *et al.*, 1990; De Benedetti and Rhoads, 1990), and to the deregulation of HeLa cell growth (De Benedetti and Rhoads, 1990). Furthermore, microinjection of eIF4E into quiescent NIH 3T3 cells activates DNA synthesis (Smith *et al.*, 1990). The effects of eIF4E overexpression appears to be exerted via a ras-mediated pathway, as inhibition of Ras activity through overexpression of GAP (GTPase-activating factor) reversed the transformed phenotype (Lazaris-Karatzas *et al.*, 1992). One mechanism for the transforming activity of eIF4E posits that its increased levels lead to the formation of more active eIF4F, and hence increased unwinding activity and mitigation of translational repression of genes with secondary structure containing 5'UTRs (e.g. cyclin D1 and c-*myc*).

(b) eIF4E Binding Proteins

Recent studies have suggested that the availability of eIF4E is regulated by two specific binding proteins (BP), termed 4E-BP1 and 4E-BP2 (Pause *et al.*, 1994a; Lin *et al.*, 1994). The characterization of these two repressors of eIF4E activity has greatly enhanced our understanding of the mechanism which links growth-promoting signal transduction pathways and translation initiation. 4E-BP1 shares extensive sequence similarity with PHAS-I, a protein which is rapidly hyperphosphorylated in cells following treatment with insulin and growth factors (Hu *et al.*, 1994; Belsham and Denton, 1980). The major phosphorylation site of 4E-BP1 following activation is Ser-64, which regulates the

association of 4E-BP1 with eIF4E (Lin *et al.*, 1994; Haystead *et al.*, 1994). Indeed, *in vitro* phosphorylation of 4E-BP1 by the *m*itogen *a*ctivated *p*rotein (MAP) kinases ERK1 and ERK2 renders the protein incapable of interacting with eIF4E. Interaction of 4E-BP1 with eIF4E inhibits specifically translation of capped, and not uncapped, mRNA (Pause *et al.*, 1994a). The phosphorylation of 4E-BP1 is also mediated by the FRAP/ $p70^{s6k}$ pathway (Lin *et al.*, 1995; Beretta *et al.*, 1996). In support of this hypothesis, treatment of cells with the immunosuppressant drug rapamycin specifically inhibits $p70^{s6k}$ and blocks 4E-BP1 phosphorylation, without inhibiting the MAP kinase pathway (Von Manteuffel *et al.*, 1996). However, since $p70^{s6k}$ does not directly phosphorylate 4E-BP1 and 4E-BP2 *in vitro*, the kinase that phosphorylates these proteins *in vivo* remains to be identified. Additionally, the *in vitro* phosphorylation of 4E-BP1 can be mediated by casein kinase II (CKII) and PKC. It has been demonstrated that the interaction of 4E-BP1 with eIF4E can prevent the phosphorylation of eIF4E *in vitro*, suggesting a temporal relationship between eIF4E binding to 4E-BPs and eIF4E phosphorylation (Whalen *et al.*, 1996).

1.3.3.2 *eIF4A*

A 50 kDa polypeptide, eIF4A exhibits RNA-dependent ATPase and bidirectional RNA unwinding activities (Ray *et al.*, 1985; Rozen *et al.*, 1990). It is the most abundant initiation factor (3 molecules per ribosome) and appears to function both as a singular polypeptide and as a subunit of the eIF4F complex (Duncan and Hershey, 1983; Benne and Hershey, 1978; Grifo *et al.*, 1983). However, eIF4A as a subunit of eIF4F is 20-times more active as a helicase, than the singular form. Two separate, functional genes have been identified in mammalian cells that encode two proteins, eIF4AI and eIF4AII, which are 91% identical (Nielsen and Trachsel, 1989). While both forms incorporate into the eIF4F complex, a functional difference for these two proteins has not been demonstrated. Meanwhile, the two forms appear to be differentially expressed in various tissues. The physiological significance of this phenomenon is ill-understood. Disruption of the two

genes, *TIF1* and *TIF2*, in yeast causes complete abrogation of protein synthesis and lethality, demonstrating that eIF4A is essential for translation of all mRNAs (Blum *et al.*, 1989). Yeast and mouse eIF4A are 65% identical. Despite this high degree of conservation, mouse eIF4A fails to substitute for its yeast homologue. Reconstituted *in vitro* studies have revealed that eIF4A is essential for the recruitment of all mRNAs to the ribosome.

eIF4A is the prototype member of the DEAD box family that share nine conserved sequence elements (Linder *et al.*, 1989). Mutational analysis of recombinant eIF4A has served to assign functions to these conserved motifs (Pause and Sonenberg, 1992; Pause *et al.*, 1993). The ATPase A motif, near the amino terminus, is required for ATP binding, and the ATPase B motif (DEAD: asp-glu-ala-asp) participates in several processes such as ATP hydrolysis and RNA unwinding activities. It has been proposed that ATP binding results in a conformational change in eIF4A that allows it to bind single-stranded RNA through a C-terminal, arginine-rich motif (HRIGRXXR). Furthermore, mutants of eIF4A have been identified that function as dominant translational inhibitors of all mRNAs (Pause *et al.*, 1994b). The finding that these mutants exerted their inhibitory effect only on eIF4F-dependent helicase assays, and not the eIF4A-dependent assays, suggests that eIF4A functions by cycling through the eIF4F complex.

1.3.3.3 *eIF4B*

eIF4B is a phosphoprotein of 80 kDa that facilitates the binding of the mRNA to the 43S preinitiation complex (Abramson *et al.*, 1987; Benne and Hershey 1978). In addition, eIF4B is an RNA-binding protein that promotes the helicase activity of eIF4A (Abramson *et al.*, 1987; Rozen *et al.*, 1990). Consistently, disruption of the yeast eIF4B, *TIF3*, results in poor translation of mRNAs with extensive secondary structures in their 5'UTR. eIF4B contains a canonical RNA recognition motif (RRM) near its N-terminus, and an arginine-rich, non-specific RNA binding region in the C-terminus (Methot *et al.*, 1994; Naranda *et al.*, 1994). The non-specific RNA binding region mediates synergistic RNA

binding with eIF4A and RNA helicase activity. It has been suggested that the simultaneous association of eIF4B with 18S rRNA and a non-specific RNA serves as a bridge between the mRNA and the ribosome, and promotes unwinding.

Earlier studies indicated that the active form of eIF4B appears to be a homodimer (Abramson *et al.*, 1987). Recently the self association region of eIF4B was mapped to a portion of the molecule enriched in aspartic acid, arginine, tyrosine, and glycine (DRYG) (Methot *et al.*, 1996). Furthermore, the DRYG region mediates a direct interaction between eIF4B and the p170 subunit of eIF3 (Methot *et al.*, 1996). Despite these insights into the process of mRNA binding to ribosomes, an understanding of the mechanism by which eIF4B contributes to the helicase activity is lacking.

1.3.3.4 *eIF4G*

Formerly known as p220, eIF4G constitutes the largest subunit of eIF4F. The cDNA of eIF4G encodes a polypeptide of 154 kDa; yet the protein migrates aberrantly when examined on SDS-PAGE as a series of bands in the range of 210-220 kDa. This discrepancy is attributed to the amino-terminus of the protein (Ehrenfeld., 1996). Two yeast homologues (p130 and p150) of the mammalian eIF4G have also been identified which exhibit 53% identity to each other (Goyer *et al.*, 1993). While disruption of the gene that encodes p150 (tif4631) results in a slow growth phenotype, double gene disruption engenders lethality, demonstrating the importance of these genes for viability (Goyer *et al.*, 1993). eIF4G is phosphorylated at multiple sites. However, the specific effects of these posttranslational modifications on the activity of eIF4G are unknown.

eIF4G coordinates the activity of eIF4F by interacting specifically with eIF4E and eIF4A. The interaction sites for eIF4E and eIF4A have been mapped to the N-terminus and the central two-thirds of the protein, respectively (Mader *et al.*, 1995; Lamphear *et al.*, 1995). As eIF4G also copurifies with eIF3, it has been postulated that eIF4G may serve as a scaffold between the ribosome and the mRNA. In turn, cleavage of eIF4G by picornavirus

proteases separates the eIF4E and eIF3 binding regions (See section 1.5.1.2). As a result, translation of cellular mRNAs is compromised. It is also believed that eIF4G accounts for the avid interaction of eIF4F with RNA. This enhanced RNA binding may stabilize the interaction of eIF4A with its substrate RNA, and promote unwinding of secondary structures.

1.4 MECHANISMS OF CAP-DEPENDENT TRANSLATION

Currently, two working models outline the series of events that ultimately lead to the formation of an 80S complex. The first model posits that the first step of the initiation pathway involves an interaction between eIF4F and the mRNA cap structure (Sonenberg, 1988). eIF4F is composed of three subunits: eIF4E, eIF4G, and eIF4A. The specific interaction of eIF4F with the cap is mediated by the eIF4E subunit. The cap recognition step is one of several that determines the rate of initiation of mRNA translation, and is hindered by simple steric accessibility as governed by the mRNA secondary structure (see section 1.3.2.2). eIF4F, in combination with eIF4B, subsequently unwinds secondary structure in the 5'UTR of the mRNA, presumably creating a single-stranded region of RNA which serves as a binding site for the 43S pre-initiation complex. The joining of the 43S ribosomal subunit is thought to be mediated through an interaction of the eIF4G subunit and eIF3, the latter being part of the 43S subunit. It is believed that these components collectively scan the mRNA for an AUG codon.

An alternative model suggests that eIF4E alone binds the cap structure, which is then complexed with eIF4G that is already associated with the ribosome (Joshi *et al.*, 1994). Support for the first model is based on the observation that the affinity of eIF4F for capped mRNA is about 15-fold greater than that of eIF4E alone (Lawson *et al.*, 1988). Furthermore, eIF4E in extracts prepared from poliovirus-infected cells, where the eIF4G subunit is cleaved (see section 1.5.1.2 below), cross-links inefficiently to the cap structure

(Lee and Sonenberg, 1982). These results suggest that the binding of eIF4F to the mRNA is also partly determined by the RNA binding activity of its eIF4G subunit (H. Lee and N. Sonenberg, unpublished data). Indeed, eIF4F binds much more avidly to RNA than either eIF4E or eIF4A (Jaramillo *et al.*, 1991).

Regardless of the primary sequence of events depicted above, the models postulate that the scanning 40S ribosomal subunit unwinds any higher order structure that it encounters in the 5'UTR, before arriving at the initiator AUG codon. It is important to note, however, that these paradigms represent only rough approximations to reality, and more biochemical detail is required to fully understand the more subtle issues, such as how and when the eIF4F complex is assembled. The point in the pathway where ribosome binding occurs remains unknown. Furthermore, the models do not address how scanning is driven: if the eIF4F•eIF4B complex scans the mRNA while on the 43S ribosomal subunit, or whether they do so alone. Also, it is not clear whether the recycling of eIF4A through eIF4F (see section 1.3.3.2) takes place on the mRNA or in solution.

Exceptions to the general rule of linear scanning have also been documented, notably in the case of the 35S cauliflower mosaic virus (CaMV) RNA (Futterer *et al.*, 1993), as well as the late adenovirus mRNAs (Shneider, 1995). In the skipping or "ribosomal shunt" process, a portion of the scanning ribosomes bypasses a large segment of the 5'UTR, to land at the initiator AUG codon. In this instance, insertion of secondary structures upstream of the AUG codon does not affect the initiation rates. It is emphasized, however, that this mechanism differs from internal initiation of translation where ribosomes access the mRNA at specific regions referred to as internal ribosome entry sequences (See section 1.5.1.1, below).

1.5 THE INTERPLAY BETWEEN VIRUSES AND THE CELLULAR TRANSLATION APPARATUS

Viruses rely on their hosts to supply the energy and the machinery necessary for their replication. The dependence of these obligate intracellular parasites on their hosts is most apparent in protein synthesis, as none encode the elements of this intricate apparatus. As such, viruses have adopted regulatory mechanisms similar to their hosts, and at times even less orthodox strategies. In many instances, viruses dominate the host's protein synthesis machinery, imposing cunning and sophisticated approaches that would assure the preferential translation of their own mRNAs at the host's expense [For a review see (Mathews, 1996)]. The viral interference with the cellular macromolecule synthesis is referred to as "host-cell shutoff", a phenomenon which contributes to an accelerated monopolisation of biosynthetic pathways and, perhaps, an enhanced virus yield.

Despite the widespread occurrence of shutoff, the mechanisms which govern this phenomenon are diverse and at best controversial. In general, the virus tends to impose limitations upon the host which itself can bypass. Amongst the strategies are the destabilization of the host mRNA, inhibition of cytoplasmic transport of cellular mRNAs, and even the covalent modification of key initiation factors, thereby limiting their function. However, mRNA competition seems to be a common theme in many cases. For example, rRNA synthesis is prevented during herpesvirus infection and the host mRNA is destabilized. On the other hand, accumulation of overwhelming concentrations of viral mRNA in the case of vaccinia virus, vesicular stomatosis virus (VSV), and reovirus infections seem to be the major factor in the prevalence of these viruses (Lodish and Porter, 1981). In the case of adenovirus (Ad) infection, translational dominance is effected at multiple stages, attributed to the exertion of a block in the nucleocytoplasmic transport of host mRNAs, inhibition of rRNA processing, as well as the modification of initiation factors eIF4E and eIF2 (Shneider, 1996). Activation of PKR during Ad infection leads to the

phosphorylation of the a subunit of eIF2, hence limiting its function in translation initiation. Dephosphorylation of eIF4E correlates with the suppression of host protein synthesis following infection of cells with a number of viruses, including Adenovirus and Influenza virus. These viruses produce "strong" mRNAs with little secondary structure in their 5'UTR that become intrinsically efficient initiators. It transpires therefore that these tactics place cellular cap-dependent mRNAs at a disadvantage when competing with abundant, strong, and relatively eIF4E-independent viral RNAs.

A more radical departure from the more conventional mechanisms used by viruses has introduced the concept of cap-independent initiation of translation. Picornaviruses are the paradigm for this mode of translation which is effected by the direct association of ribosomes and a complement of cellular factors to internal ribosome entry sites (IRES) (formerly recognized as ribosome landing pads, RLP (Pelletier and Sonenberg, 1988)) present in their 5'UTR (Belsham and Sonenberg, 1996). Before indulging in the description of this unorthodox mechanism of translation, however, it would be more pertinent to present an overview of this family of viruses.

1.5.1 THE PICORNAVIRIDAE

Picornaviruses are small animal viruses that contain a single-stranded RNA genome of positive polarity, encapsidated in an icosahedral core. The family *Picornaviridae* is divided into five genera (Table 1.). Although the physical structure, genome size, and replication of all members are similar, they exhibit differences in host target specificity, pathogenicity, and various details of gene expression. The unique structural properties of the picornavirus RNA, and the unique translational features of the various members have made them ideal for studying the mechanisms of translational control in eukaryotes [for a review see (Rueckert, 1996)].

TABLE 1.1: Classification of Members of the Family Picornaviridae[1]

Picornaviridae Genus	Representative Species
Enteroviruses	**poliovirus, coxsackievirus, echovirus**
Rhinoviruses	**human Rhinoviruses (>100 serotypes)**
Aphthoviruses	**foot-and-mouth disease virus**
Cardioviruses	**EMCV, mengo virus, Theiler's virus**
Hepatoviruses	**hepatitis A**

The picornavirus genome extends approximately 7,500 nucleotides in length, and contains a long 5'UTR in the range of 650 to 1300 nucleotides, and a much shorter 3'UTR of approximately 70 nucleotides. They are polyadenylated at their 3'ends, but unlike most cellular and viral mRNAs the picornavirus genome RNA is devoid of a 5'cap structure. Instead, they contain a small protein termed VPg, covalently attached to their 5'ends, which, however, is rapidly cleaved off following the release of the genome into the host's cytoplasm. The 5'UTRs contain complex secondary structures with a common sequence motif which is a single-stranded polypyrimidine tract followed by an AUG codon. Furthermore, the 5'UTR of these viruses are burdened with mutliple AUG codons which are poorly conserved, even among different isolates of a serotype. The RNA genome encodes a single large polyprotein of 247 kDa, that undergoes processing by virus-encoded proteases to generate functional viral polypeptides.

1.5.1.1 *Internal Initiation of Translation*

The observation that the picornavirus RNA is naturally uncapped suggested that its translation must proceed in a cap-independent fashion. Consistently, earlier *in vitro*

[1] *Adapted from E. Ehrenfeld, (1996), In Translational Control, CSHL press*

experiments demonstrated that under conditions where the translation of capped mRNAs is abrogated (e.g. in poliovirus (PV)-infection, or in the presence of cap analogues), the translation of picornavirus mRNA proceeds with high efficiency. The possibility was therefore entertained that ribosomes bind directly to the 5'UTR of picornavirus mRNAs. Direct evidence for 'internal initiation' of translation was initially provided by Pelletier and Sonenberg (Pelletier and Sonenberg, 1988), who demonstrated that the insertion of the PV 5'UTR into the intercistronic spacer of a dicistronic reporter construct promoted efficient expression of the downstream cistron. Similar results were obtained with the EMCV 5'UTR (Hellen and Wimmer, 1985). Subsequent characterization of the 5'UTR of picornaviruses has confirmed the requirement for a minimal element within this region termed 'internal ribosome entry site' (IRES) for directing translational initiation independent of a cap structure. More recently, compelling evidence was also provided by Chen and Sarnow that the EMCV IRES can efficiently direct ribosomes onto a circular RNA, confirming that scanning from a free 5' terminus is not required for translation initiation of their RNA (Chen and Sarnow, 1995).

A growing number of vertebrate and invertebrate cellular mRNAs initiate translation by internal ribosome binding. These include the 5'UTR of BiP (immunoglobulin heavy-chain binding protein) mRNA (Macejak and Sarnow, 1991), fibroblast growth factor-2 mRNA (Vagner *et al.*, 1995), and the mRNAs for *Drosophila melanogaster* homeotic proteins Antennapedia and Ultrabithorax (Oh *et al.*, 1992). Recently, and rather intriguingly, a potential IRES has been identified in the 5'UTR of eukaryotic initiation factor 4G (Gan and Rhoads, 1996).

1.5.1.2 *Picornaviruses and Host Shutoff*

Infection of cells by picornaviruses belonging to several genera is marked by a dramatic inhibition of host cellular protein synthesis. As such, translation becomes confined to the exclusive production of viral proteins. The mechanisms that govern this precipitous

shutoff include both structural and functional modifications of the host's translational machinery. The ability of eukaryotic initiation factor 4F (eIF4F) to restore the translation of capped mRNAs in extracts prepared from poliovirus-infected cells initially implicated eIF4F as one target for such modifications (Tahara *et al.*, 1981). Indeed, the *Enterovirus*, *Rhinovirus*, and *Aphtovirus* genera of picornaviruses induce cleavage of the eIF4G subunit of eIF4F into an N-terminal fragment of about 50 kDa (which migrates as a set of two to three polypeptides of 110 to 130 kDa) and a carboxy terminal fragment of about 100 kDa. No cleavage is detected during infection of cells with members of the *Cardiovirus* and *Hepatovirus* genera (See below). As mentioned above, eIF4G serves as a scaffold: it interacts with both eIF4E and eIF4A, and its association with eIF3 is believed to promote the binding of the 43S preinitiation complex at the 5'end of mRNAs. Cleavage of eIF4G therefore results in the uncoupling of the cap recognition function of eIF4E from the helicase and ribosome binding activities of eIF4A and eIF3. Meanwhile, the C-terminus of eIF4G, together with eIF4A, can efficiently mediate internal entry of 43S preinitiation complexes (Pestova *et al.*, 1996).

After poliovirus infection, the cleavage of eIF4G correlates with the inhibition of cellular translation (Etchison *et al.*, 1982). However, proteolysis of eIF4G alone cannot account for the complete shutoff, as this correlation seems to hold only until ~70% inhibition (Bonneau and Sonenberg, 1987b). The supporting data indicate that the complete abrogation of cellular protein synthesis requires the contribution of other mechanisms. In poliovirus-infected cells eIF2α is phosphorylated (O'Neal and Racaniello, 1989), although this occurs as a late event and is most likely not significant. Another mechanism operative in PV infection, as well as in infection by cardioviruses (where no eIF4G cleavage is observed), is believed to be a virus-induced change in the ionic milieu of the cell so as to bias translational efficiency towards viral RNAs (Alonso and Carrasco, 1981).

Recently, shutoff in picornavirus-infected cells has been linked to the dephosphorylation of 4E-BP1 (Gingras *et al.*, 1996). This finding has been observed for

both EMCV and poliovirus. However, as dephosphorylation of 4E-BP1 occurs after the onset of inhibition of protein synthesis following poliovirus infection, it is apparent that the underlying mechanisms governing complete bias towards the translation of picornavirus RNA is not resolved.

1.5.1.3 *The Quest for the eIF4G Cleavage Activity*

Earlier experiments on poliovirus implicated the virus-encoded protease 2A ($2A^{pro}$) as the mediator of eIF4G cleavage. $2A^{pro}$ functions primarily to process the viral precursor polyprotein into functional polypeptides. A mutant virus containing a single-codon insertion in the $2A^{pro}$ gene displayed a small plaque phenotype, and was incapable of inhibiting host protein synthesis (Bernstein *et al.*, 1985). Most striking was the observation that the $2A^{pro}$ mutant virus could not effect the cleavage of eIF4G in infected cells. eIF4G cleavage was however detected when infection was performed in the presence of guanidine, an inhibitor of polovirus replication, ruling out the possibility that the lack of cleavage activity is attributed to reduced levels of viral proteins (Bonneau and Sonenberg, 1987a). Expression of $2A^{pro}$ alone in HeLa cells dramatically inhibited cellular protein synthesis, demonstrating that $2A^{pro}$ is the only viral protein required for eIF4G cleavage. Subsequent studies indicated however that the cleavage activity was separable from the bulk of poliovirus $2A^{pro}$ (Lloyd *et al.*, 1986). Furthermore, anti-$2A^{pro}$ antiserum did not inhibit the proteolysis of eIF4G, although it prevented $2A^{pro}$ -mediated processing of the virus polyprotein (Krausslich *et al.*, 1987; Wyckoff *et al.*, 1990). These results suggested an indirect mechanism whereby the cleavage of eIF4G is mediated by virus-activated cellular proteases. Experiments aimed at characterizing these cellular proteases identified eIF3 as an additional factor requisite to the cleavage of eIF4G in vitro (Wyckoff *et al.*, 1990). These conclusions form the premise for the work presented in Chapter 4.

Recently, the requirement for the rhinovirus $2A^{pro}$, as well as the unrelated FMDV leader (L) protease in the cleavage of eIF4G has been established (Kirchweger *et al.*,

1994; Lamphear *et al.*, 1993). The 2A protease is thought to be related to the class of small serine proteases, whereas the L protease appears to be part of a papain-like class of thiol proteases. The eIF4G cleavage site recognized by the L protease [Gly479 and Arg480; (Kirchweger *et al.*, 1994)] is seven amino acids upstream of the bond that is hydrolyzed by the $2A^{pro}$ [Arg 486 and Gly487; (Lamphear *et al.*, 1993)]. In contrast to the proteases of other family members, neither the L protease nor the 2A protein of cardioviruses contain any protease consensus motifs, or any demonstratable eIF4G cleavage activity. The mechanism of shutoff adopted by these viruses was discussed in section 1.5.1.2.

CHAPTER II

Repression of Cap Dependent Translation by 4EBP1: Competition with p220 for Binding to eIF4E.

2.1 SUMMARY

An important aspect of 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 implicated the MAP kinase pathway in the regulation of translation by insulin and growth factors. MAP kinase phosphorylates a repressor of translation initiation (4E-BP1) that binds to the mRNA 5' cap binding protein 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 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 compete with p220, and do not inhibit 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.

2.2 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 [$m^7G(5')ppp(5')N$; where N is any nucleotide] at their 5' terminus (Shatkin, 1976). 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 initiation factors: eIF-4A, eIF-4B, eIF-4F and ATP hydrolysis (Sonenberg, 1988). eIF-4F which mediates cap function (Edery *et al.*, 1983; Grifo *et al.*, 1983; Tahara *et al.*, 1981) 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 bi-directional 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 (Duncan *et al.*, 1987; Hiremath *et al.*, 1985), consistent with a regulatory role in translation. There also exists a strong correlation between the phosphorylation state of eIF-4E and the rate of protein synthesis and cell growth. Increased eIF-4E phosphorylation occurs in response to growth factors, mitogens, hormones and cytokines (Frederickson *et al.*, 1991; Morley and Traugh, 1989). eIF-4E is hypophosphorylated during mitosis (Bonneau and Sonenberg, 1987a), following heat-shock (Duncan *et al.*, 1987), or infection with several viruses (Feigenblum and Schneider, 1993; Huang and Schneider, 1991), 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). 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 translational repression of growth promoting genes that are important for the control of cell growth. Indeed, increased expression of cyclin D1 (Rosenwald *et al.*, 1993), ornithine decarboxylase (ODC; (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 termed 4E-BP1 and 4E-BP2 (4E-BPs for eIF-4E binding proteins; (Pause *et al.*, 1994a)). These proteins exhibit high sequence homology (93% 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 growth factors and insulin 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.*, 1994a). This interaction is dramatically diminished, however, upon phosphorylation of 4E-BP1 in response to insulin, concomitant with the relief of translational repression of capped mRNAs. These results explain previous reports on the enhancement of eIF-4F activity and specific stimulation of cap-dependent translation following insulin treatment

(Gallie and Traugh, 1994; Manzella *et al.*, 1991). 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.*, 1994a). 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 translation initiation of capped mRNAs by preventing the interaction between the 43S pre-initiation complex and the mRNA.

2.3 RESULTS

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

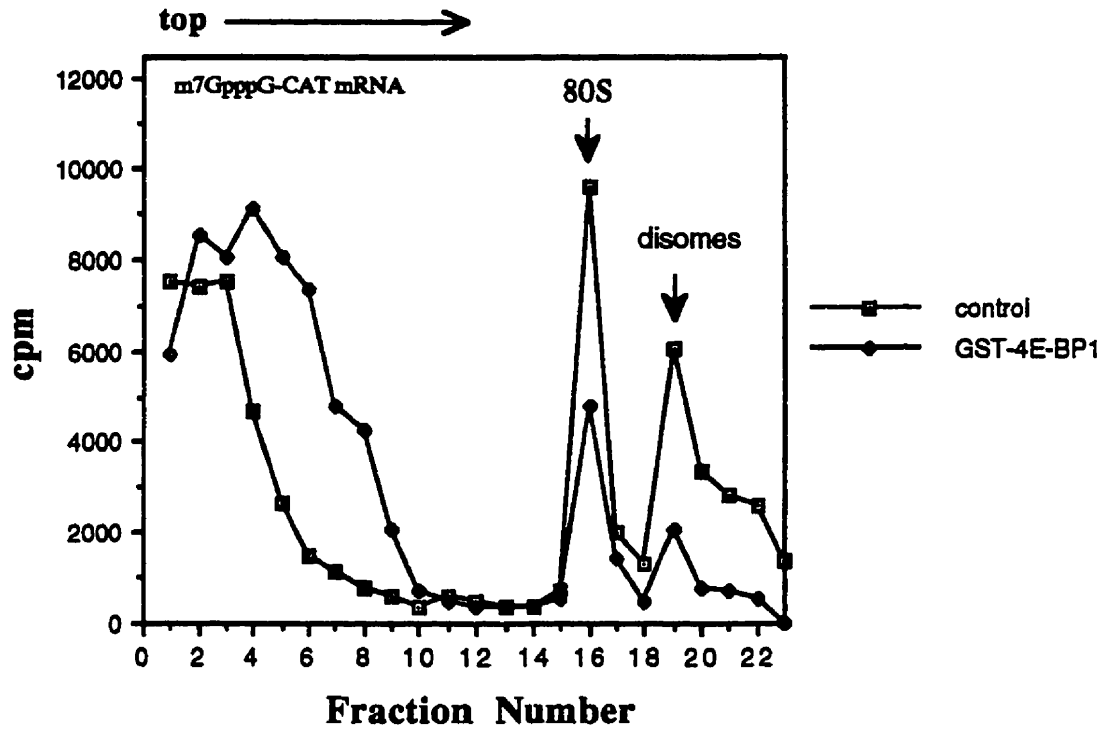
To determine the step of translation that is inhibited by 4E-BP1, ribosome binding experiments were performed. 80S initiation complexes were formed with ^{32}P-cap-labeled chloramphenicol acetyltransferase (CAT) RNA in a reticulocyte lysate in the presence of anisomycin, a peptide chain elongation inhibitor. The resulting complexes were resolved by centrifugation through linear 10-50% sucrose gradients. A large fraction (45%) of mRNA input was bound to 80S ribosomes and disomes (Figure 2.1A). Disome formation occurred because the 5'UTR could accommodate the binding of two ribosomes (Pelletier and Sonenberg, 1985a). Addition of glutathione-S transferase (GST)—4E-BP1 inhibited mRNA binding to ribosomes (16% of mRNA bound, ~2.9- fold inhibition), causing the 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 virus (EMCV) 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 2.1B; 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 2.1B, ~1.2-fold inhibition).

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-hydrolysable GTP analogue causes the accumulation of 48S pre-initiation complexes, as GTP hydrolysis is required prior to the

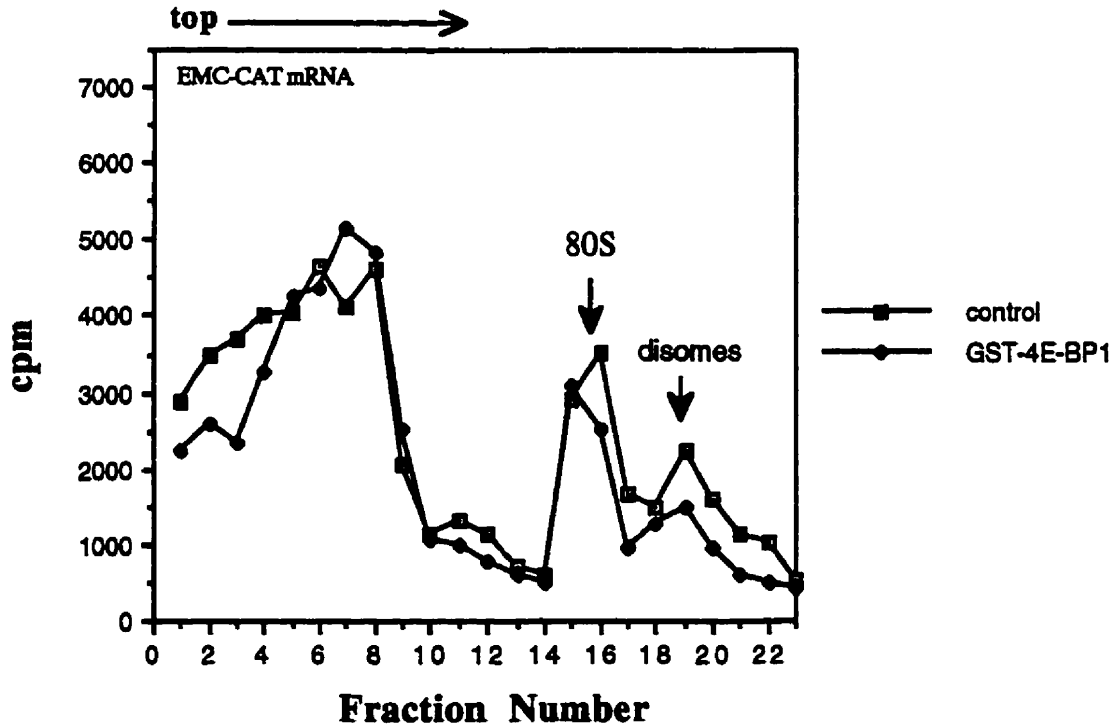
Figure 2.1. 4E-BP1 inhibits mRNA-ribosome binding.

Assays were performed in reticulocyte lysates with ~8 x 10^4 cpm 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^{o}C. Next, anisomycin and the other components were added and the reaction mixture was incubated for a further 20 min at 30^{o}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^{o}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^{o}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.

A


top
m7GpppG-CAT mRNA
80S
disomes
cpm
12000
10000
8000
6000
4000
2000
0
0 2 4 6 8 10 12 14 16 18 20 22
Fraction Number
control
GST-4E-BP1

B


top
EMC-CAT mRNA
80S
disomes
cpm
7000
6000
5000
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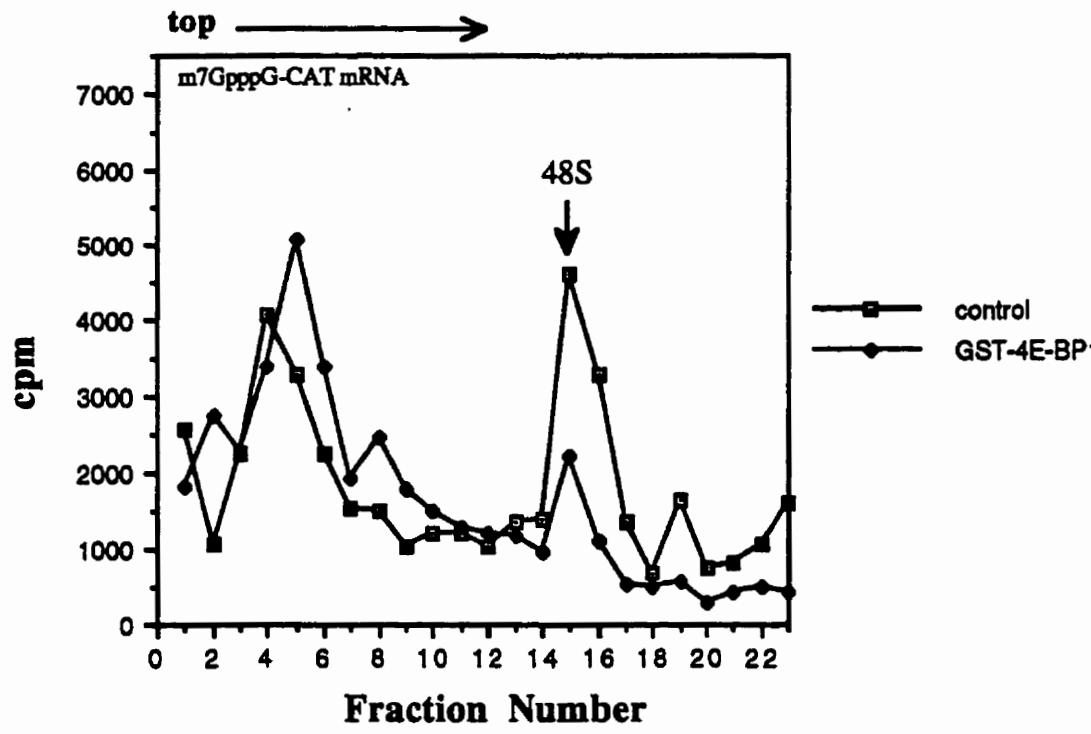
3000
2000
1000
0
0 2 4 6 8 10 12 14 16 18 20 22
Fraction Number
control
GST-4E-BP1

C


top
m7GpppG-CAT mRNA
48S
cpm
7000
6000
5000
4000
3000
2000
1000
0
0 2 4 6 8 10 12 14 16 18 20 22
Fraction Number
control
GST-4E-BP1

joining of the 60S ribosomal subunit (Anthony and Merrick, 1991). The sucrose concentration of the gradients was adjusted to 10-40% to improve the resolution between the 48S ribosome complex and unbound mRNA. In this experiment, 42% of the mRNA input was associated with the 48S pre-initiation complex (Figure 2.1C). Pre-incubation of the reticulocyte lysate with GST—4E-BP1 resulted in 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.

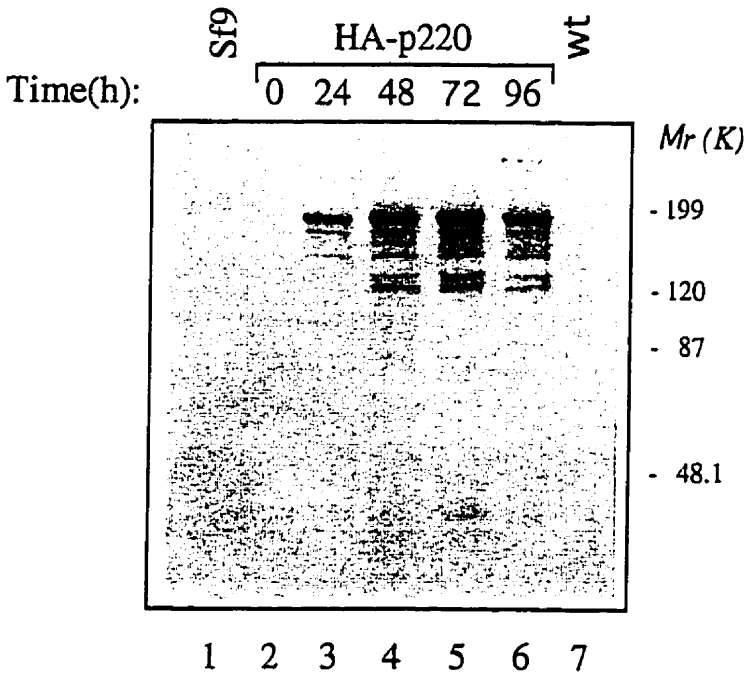
Purification of recombinant baculovirus and expression of p220 protein in Sf9 insect cells

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 haemagglutinin (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 2.2). p220 was first detected 24 h post-infection, and continued to accumulate up to 72 h. Uninfected cells (lanes 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

Figure 2.2. 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, 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


Sf9
HA-p220
wt
Time(h):
0 24 48 72 96
Mr (K)
- 199
- 120
- 87
- 48.1
1 2 3 4 5 6 7

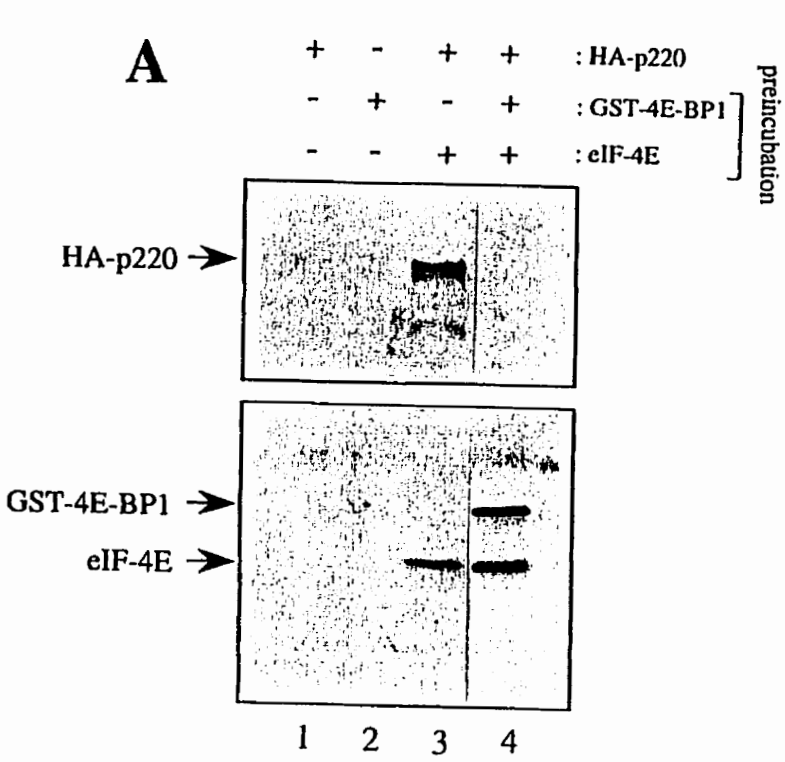
with an M_r of 220 kDa (Edery *et al.*, 1983; Tahara *et al.*, 1981; Yan *et al.*, 1992). The discrepancy in the migration of p220 expressed in Sf9 insect cells with the authentic protein may be attributed to the extent to which this protein is processed in insect cells. An analysis of the amino acid 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 the terminal glycosylases (Jarvis and Summers, 1989; Johnson *et al.*, 1989; Possee, 1986) which may explain the observed faster migration.

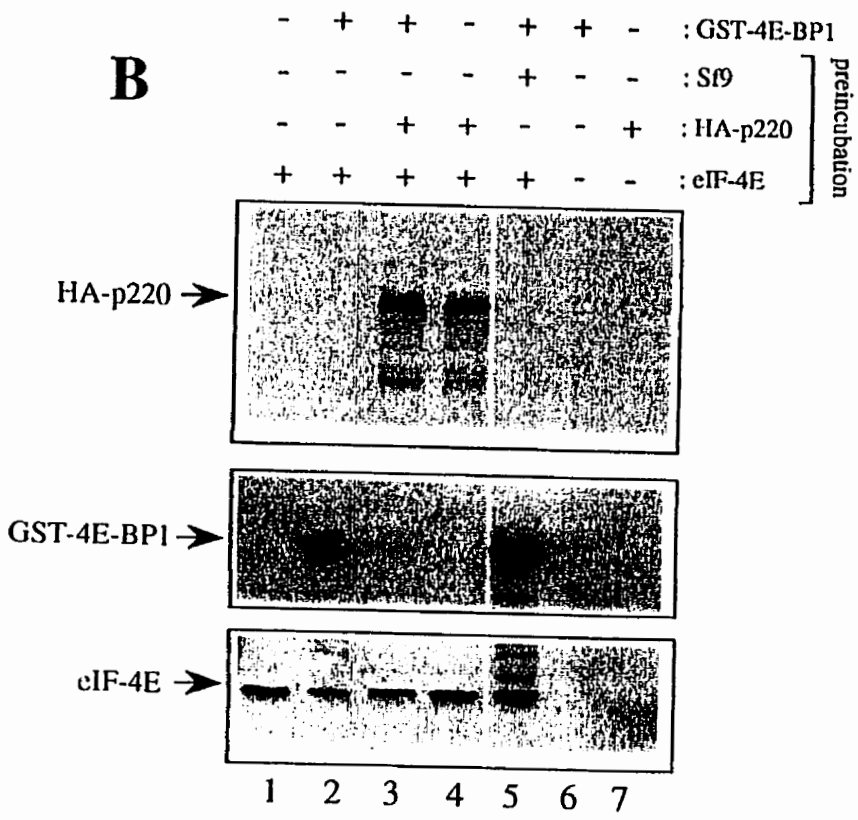
Analysis of the interaction between eIF-4E, p220, and 4E-BP1

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 2.3A, 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 2.3A, 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

Figure 2.3. 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 x 1 ml) and then incubated with either buffer A or 3 μg GST—4E-BP1 for 60 min at 4^{o}C. The resin was washed in buffer A (3 x 1 ml) and incubated further with buffer A or with 50 μl (~5 x 10^5 cells) of an HA-p220-expressing Sf9 cell lysate for 60 min at 4^{o}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 panel (A), but m^7GDP-bound eIF-4E was pre-incubated with 100 μl HA-p220-expressing Sf9 cell lysate or 100 μl (~1 x 10^6 cells) of uninfected Sf9 cell lysate before further incubation with 1 μg GST—4E-BP1. Incubation periods were as in (A). Minus signs indicate incubation with buffer A.


A
+ - + + : HA-p220
- + - + : GST-4E-BP1
- - + + : eIF-4E
preincubation
HA-p220
GST-4E-BP1
eIF-4E
1 2 3 4


B
- + + - + + - : GST-4E-BP1
- - - - + - - : Sf9
- - + + - - + : HA-p220
+ + + + + - - : eIF-4E
preincubation
HA-p220
GST-4E-BP1
eIF-4E
1 2 3 4 5 6 7

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 2.3B, 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-BP1. Taken together, these findings demonstrate that the binding of p220 and 4E-BP1 to eIF-4E is mutually exclusive.

Since 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. 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 (Fig. 2.4, lane 1). No signal was detected 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 it 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

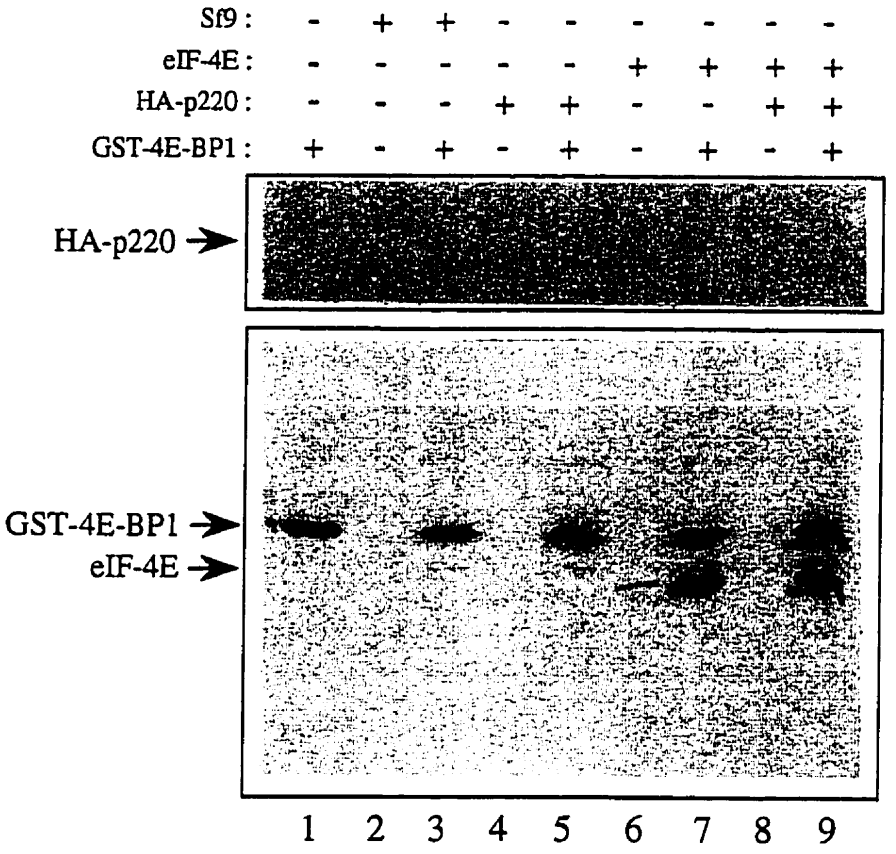
importantly, when HA-p220 was preincubated with the resin containing eIF-4E already bound to GST-4E-BP1, no HA-p220 was retained on the resin (lane 9; This experiment was conducted with that shown in Fig.2.3 which contains a positive control for HA-p220). Taken together, these results and those of Figs. 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 2.5). 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 co-sedimented 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 co-sedimented with eIF-4F in fractions 5-7, indicating that 4E-BP1 is precluded from the eIF-4F complex. It is

Figure 2.4. Affinity purification of complexes of GST—4E-BP1.

Glutothione-coupled Sepharose beads were incubated with either buffer A or with ~ 0.4 μg GST—4E-BP1 for 30 min at 4^{o}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 2.3. Minus signs indicate incubation with buffer A. Bound protein was eluted and analysed as described in the legend to Figure 2.3.


Sf9 : - + + - - - - - -
eIF-4E : - - - - - + + + +
HA-p220 : - - - + + - - + +
GST-4E-BP1 : + - + - + - + - +
HA-p220
GST-4E-BP1
eIF-4E
1 2 3 4 5 6 7 8 9

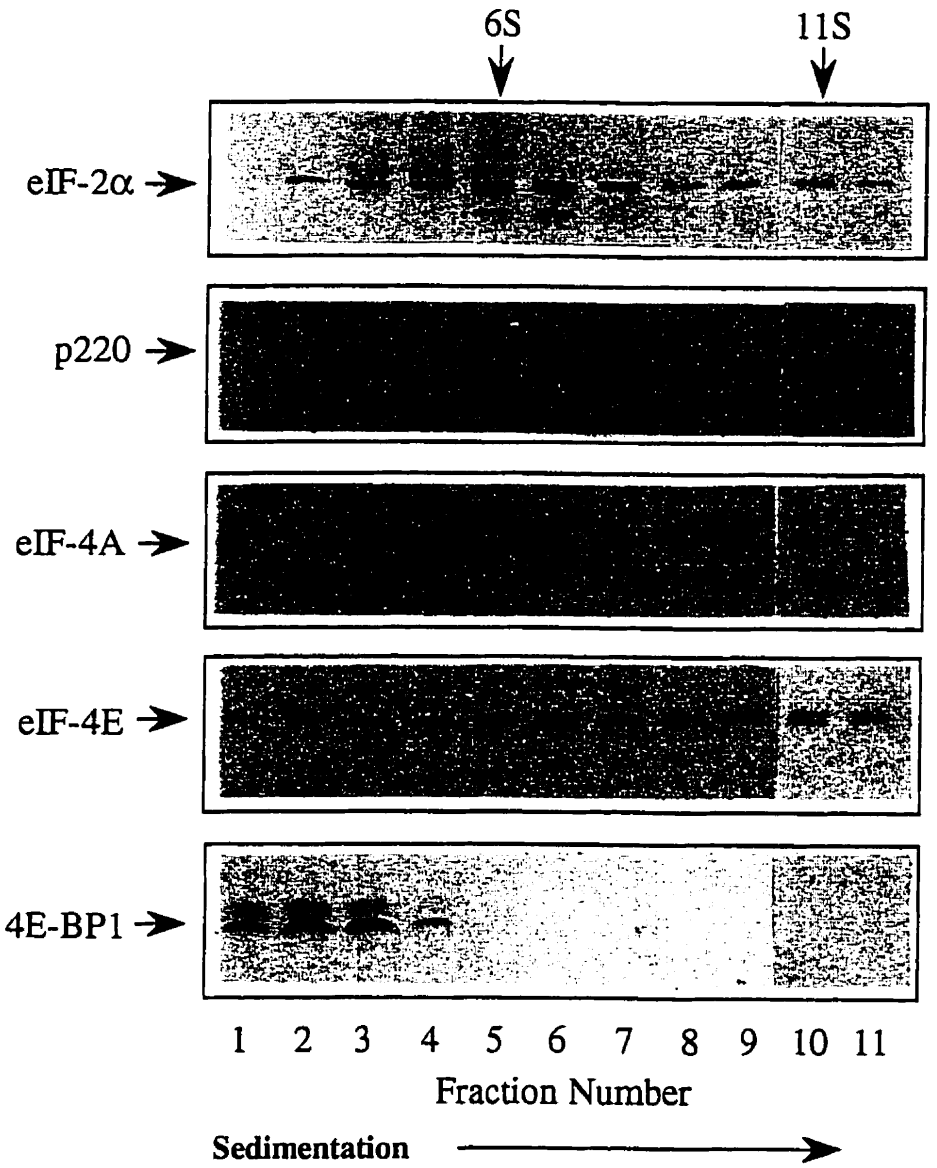
worth noting that some 4E-BP1 cosedimented with eIF-4E close to the top of the gradient (fractions 2 and 3), presumably in a complex form. These results further confirm the conclusion that eIF-4E in a complex with p220 cannot interact with 4E-BP1 in the cell.

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

p220 shares a region of homology with 4E-BP1 which is required for binding 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 E**KKRYDREFL**LGF and T**RII****YDRKFL**MEC (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*. Incubation of an eIF-4E-bound m^7GDP-coupled agarose resin with HA-p220-expressing Sf9 extract resulted in the interaction between the two polypeptides (Figure 2.6, 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 in the presence of eIF-4E (Figure 2.6, lanes 4 and 5, respectively), but instead were present in the flowthrough fractions (Figure 2.6, 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.

Figure 2.5. 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.


6S
11S
eIF-2α
p220
eIF-4A
eIF-4E
4E-BP1
1 2 3 4 5 6 7 8 9 10 11
Fraction Number
Sedimentation

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 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 2.7). 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 2.7, 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 2.7, 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 2.7, lanes 5-7). Likewise, GST—HMK had no effect on translation (Figure 2.7, lane 8). The interaction of 4E-BP1 with eIF-4E is therefore a requisite for the 4E-BP1 inhibitory effect on cap-dependent translation.

Figure 2.6. A 4E-BP1 deletion mutant does not block the interaction between eIF-4E and p220.

m^7GDP affinity purification of complexes was performed as described in the legend to Figure 3. eIF-4E (0.5 μg)-bound m^7GDP 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 x 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 2.3. 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).

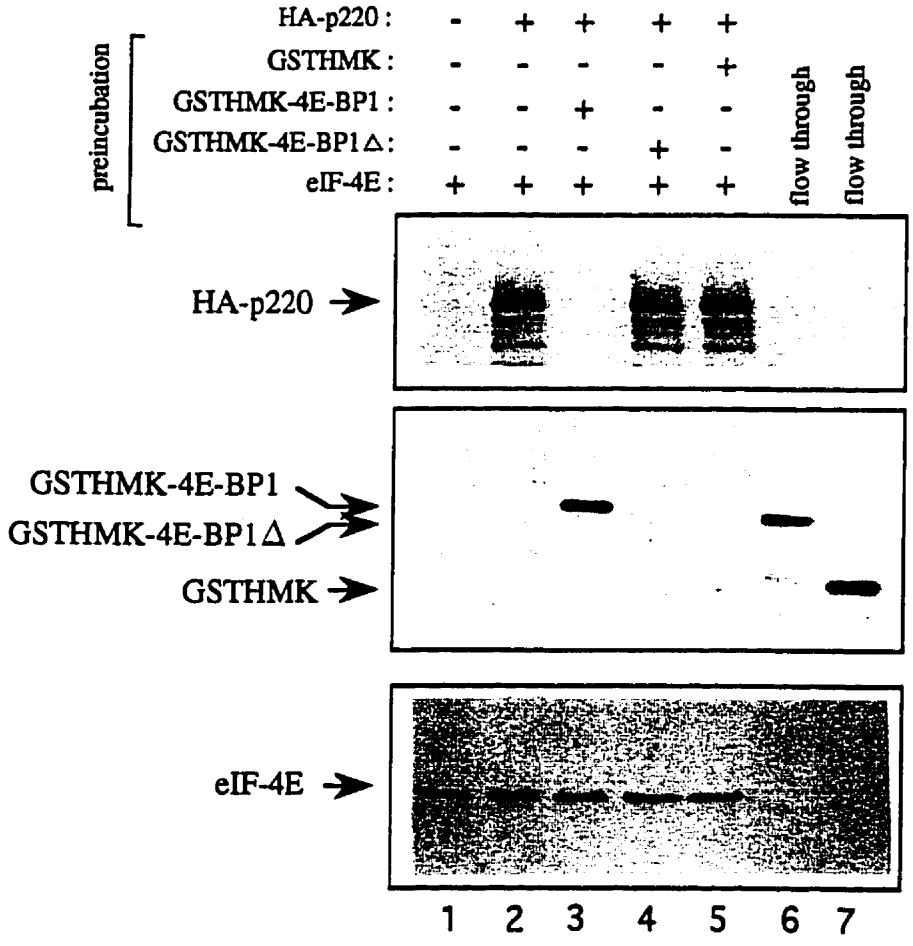
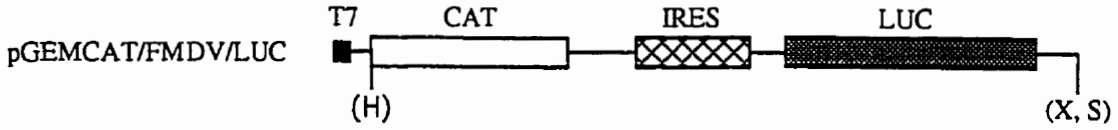
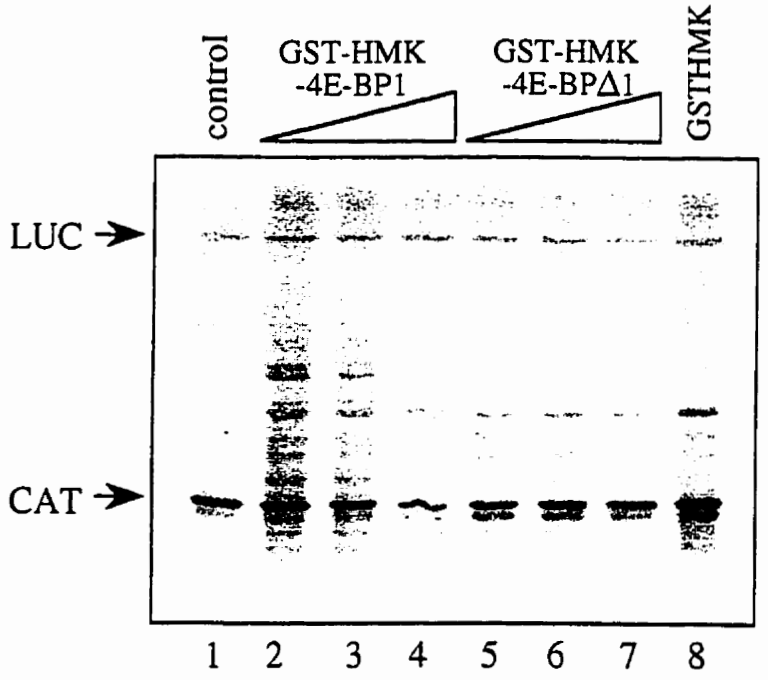

HA-p220 : - + + + +
GSTHMK : - - - - +
GSTHMK-4E-BP1 : - - + - -
GSTHMK-4E-BP1Δ : - - - + -
eIF-4E : + + + + +
preincubation
flow through
flow through
HA-p220
GSTHMK-4E-BP1
GSTHMK-4E-BP1Δ
GSTHMK
eIF-4E
1 2 3 4 5 6 7

Figure 2.7. 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.





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

2.4 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 mutally 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 2.8). 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 cross-linking experiments (Lee *et al.*, 1985). Furthermore, following poliovirus infection, which leads to the cleavage of p220, cross-linking 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, due to 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 2.8, 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 favour 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 this and earlier studies (Pause *et al.*, 1994a) that 4E-BPs do not affect 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). Translation of viral RNA requires eIF-4F function (Pause *et al.*, 1994b). 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 complete inhibition of host protein synthesis after poliovirus infection (Bonneau and Sonenberg, 1987b). Thus, it is 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.*, 1994a). 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 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 an 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.

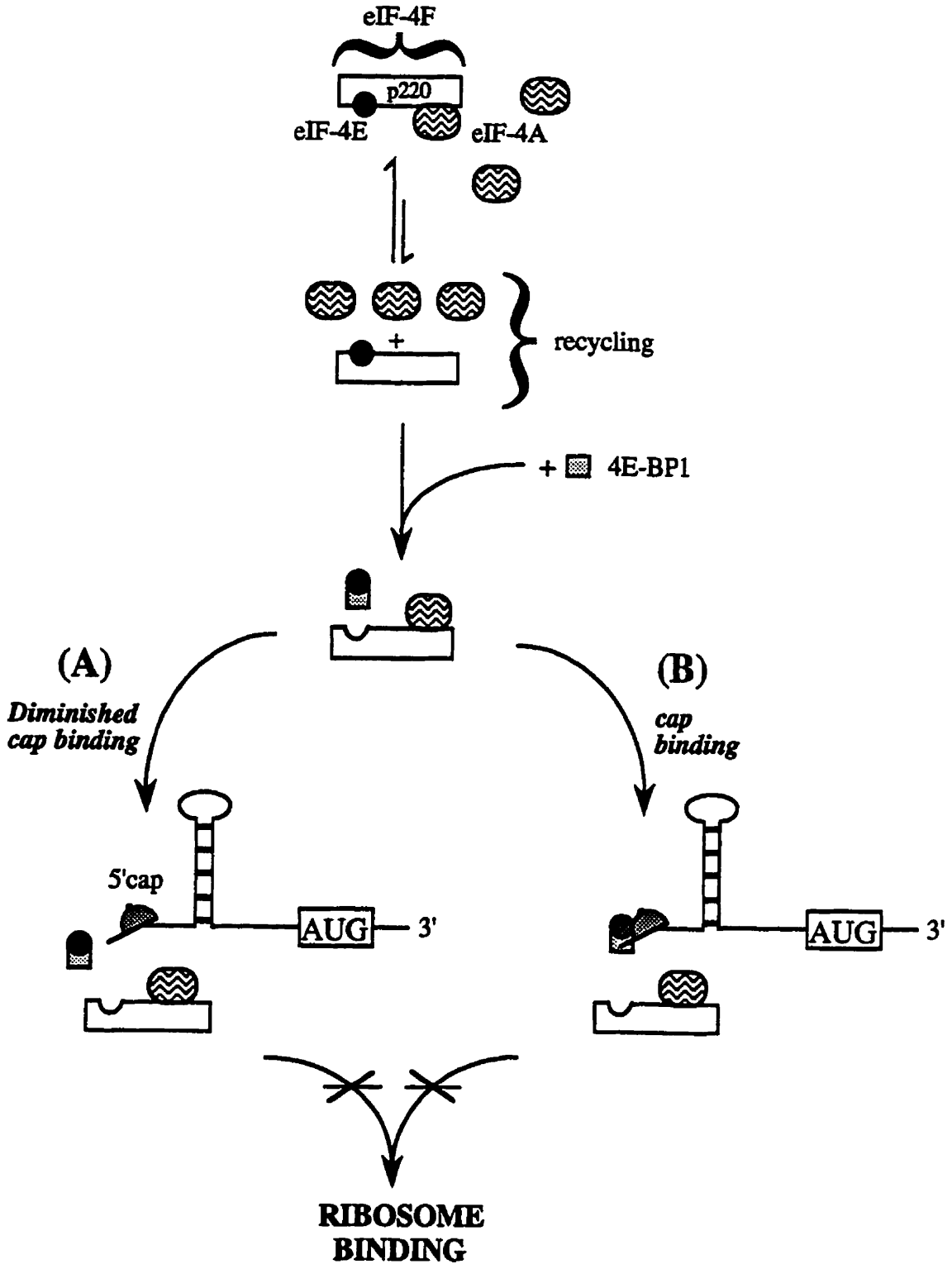


Figure.2.8. Models for the inhibition of cap-dependent translation by 4E-BP1(See text for details).

2.5 MATERIALS AND METHODS

Cell culture and protein factors

Spodoptera frugiperda (Sf9) 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.*, 1994a). 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 three 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, 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 tranfer 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 *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-BPΔ1, 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 analysed by SDS-PAGE. Gels were fixed, treated with EN^{3}Hance and processed for autoradiography. The intensity of the bands corresponding to CAT and LUC was quantified using a Fuji BAS2000 phosphorImager.

m^{7}GDP column chromatography

m^{7}GDP-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 x 1 ml) in the same buffer, and then pre-incubated for 60 min with either 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 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 units 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 with GST—4E-BP1 for 20 min at 30^{o}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^{o}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^{o}C for 4 h. Fractions (0.4 ml) were collected and counted directly in 5 ml EcoLite scintillation fluid.

2.6 ACKNOWLEDGMENTS

We thank members of the Sonenberg laboratory for their helpful comments during the course of this study. Special thanks to Hiroaki Imataka, Kyoko Kohara, and Olivier Donzé for critical reading of the manuscript. We also gratefully acknowledge A-C. Gingras, L.M. O'Brien and M. Tremblay for providing antibodies to 4E-BP1, eIF-2α and HA-TAG, respectively. We thank Dr. R.E. Rhoads for the human p220 cDNA. This work was supported by grants from the Medical Research Council and The National Cancer Institute of Canada to N.S.

CHAPTER III

eIF4G Dramatically Enhances the Binding of eIF4E to the mRNA 5' Cap Structure.

3.1 SUMMARY

The cap structure, m^7GpppN, is present at the 5' end of all eukaryotic cellular (except organellar) mRNAs. Initiation of translation is mediated by the multi-subunit initiation factor eIF4F, which binds the cap structure via its eIF4E subunit and facilitates the binding of mRNA to ribosomes. Here, we used recombinant proteins to reconstitute the cap recognition activity of eIF4F *in vitro*. We demonstrate that the interaction of eIF4E with the mRNA 5' cap structure is dramatically enhanced by eIF4G, as determined by a UV-induced cross-linking assay. Furthermore, assembly of the eIF4F complex at the cap structure, as well as ATP hydrolysis, is shown to be a requisite for the cross-linking of another initiation factor, eIF4B, to the cap structure. In addition, the stimulatory effect of eIF4G on the cap recognition of eIF4E is inhibited by the translational repressor, 4E-BP1. These results suggest that eIF4E initially interacts with the mRNA cap structure as part of the eIF4F complex.

3.2 INTRODUCTION

Cap-dependent binding of ribosomes to mRNA is mediated by several initiation factors: eIF4F, eIF4A, and eIF4B, and requires energy derived from ATP hydrolysis (Sonenberg, 1996). eIF4F is a three-subunit complex composed of (i) eIF4E, (ii) eIF4A, and (iii) eIF4G. eIF4E is a 24 kDa polypeptide that specifically interacts with the 5' cap structure (m^7GpppN; where N is any nucleotide) (Sonenberg *et al.*, 1978). eIF4A is a 50 kDa protein that exhibits RNA-dependent ATPase activity and, in conjunction with eIF4B, RNA helicase activity (Ray *et al.*, 1985; Rozen *et al.*, 1990). eIF4G is a 154 kDa polypeptide that binds to both eIF4E and eIF4A (Lamphear *et al.*, 1995; Mader *et al.*, 1995). eIF4G also exhibits sequence-nonspecific RNA-binding activity that is most probably responsible for the RNA binding activity of eIF4F (Jaramillo *et al.*, 1991; H. Lee, unpublished data).

eIF4E activity is regulated by two proteins, termed 4E-BP1 and 4E-BP2 (Lin *et al.*, 1994; Pause *et al.*, 1994). Interaction of 4E-BP1 with eIF4E inhibits specifically cap-dependent translation (Pause *et al.*, 1994). 4E-BPs are rapidly hyperphosphorylated in cells following treatment with insulin and growth factors (Belsham and Denton, 1980; Haystead *et al.*, 1994). The phosphorylation of 4E-BPs decreases the association of 4E-BP1 with eIF4E (Pause *et al.*, 1994). Consequently, phosphorylation of 4E-BPs leads to stimulation of translation. 4E-BP1 competes with eIF4G for binding to eIF4E through similar sequence motifs (Haghighat *et al.*, 1995). Furthermore, the association of 4E-BP1 with eIF4E prevents the *in vitro* phosphorylation of eIF4E by PKC, raising the possibility of a temporal relationship between eIF4E binding to 4E-BPs and eIF4E phosphorylation (Whalen *et al.*, 1996).

Two models were proposed for the pathway of eIF4F assembly and subsequent ribosome binding. One model posits that the first step of ribosome binding is the interaction between eIF4F and the mRNA cap structure (Sonenberg, 1996). According to this model, eIF4F in combination with eIF4B and eIF4A, unwinds secondary structure in the 5'UTR of the mRNA, to create a single-stranded region of RNA, which serves as a

binding site for the 43S pre-initiation complex. eIF4B and eIF4A were shown to cross-link to the cap structure only in the presence of eIF4F in a process that requires ATP hydrolysis (Sonenberg, 1981; Grifo *et al.*, 1983; Edery *et al.*, 1983). Joining of the 43S ribosomal complex is thought to be mediated through an interaction of the eIF4G subunit and eIF3, the latter being part of the 43S pre-initiation complex. An alternative model for cap recognition postulates that eIF4E alone binds first the cap structure, which is then complexed with eIF4G that is already associated with the ribosome (Joshi *et al.*, 1994). This model is based on the finding that *in vitro* translated eIF4G is bound to the 43S pre-initiation complex (Joshi *et al.*, 1994). Support for the first model stems from the observation that the affinity of eIF4F for capped mRNA is about 15-fold greater than that of eIF4E alone (Lawson *et al.*, 1988). Lee et al. showed that eIF4F cross-linked much more efficiently to the cap-structure than did eIF4E alone (Lee *et al.*, 1985). Furthermore, eIF4E in extracts prepared from poliovirus-infected cells, where the eIF4G subunit is cleaved and as a result eIF4E is associated with the N-terminal fragment of eIF4G, cross-links extremely inefficiently to the cap structure (Lee and Sonenberg, 1982; Pelletier and Sonenberg, 1985b). These results suggest an important function played by eIF4G in the cap recognition process.

In this report we reconstituted the eIF4F cap recognition activity *in vitro* using recombinant components. In a photochemical cross-linking assay, we demonstrate directly that eIF4G increases the affinity of eIF4E for the cap structure. Binding of the eIF4F complex to the cap structure, as well as ATP hydrolysis, is shown to be a prerequisite for the cross-linking of eIF4B to the cap structure. In addition, 4E-BP1 is shown to inhibit cap-binding activity of the eIF4E-eIF4G complex.

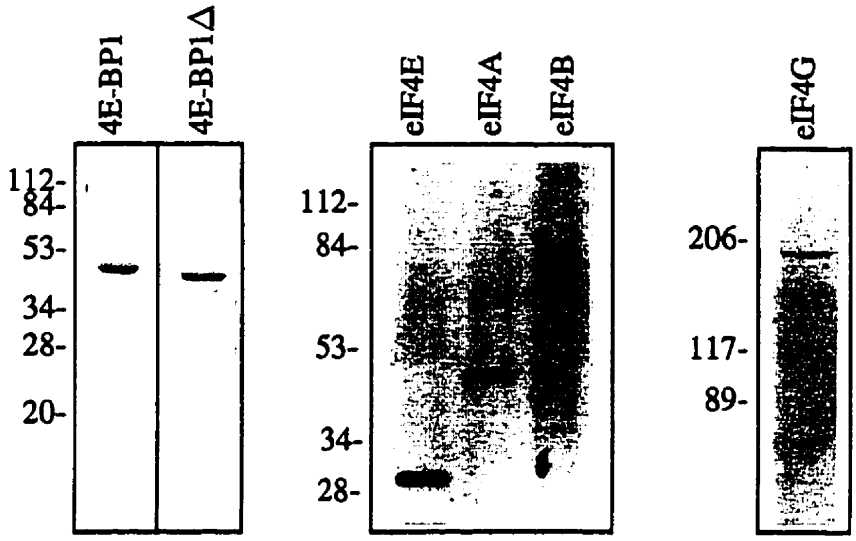
3.3 RESULTS AND DISCUSSION

Analysis of the interaction of cap binding proteins with mRNA.

To study the requirements for the interaction of eIF4E with the mRNA cap structure, purified recombinant initiation factors (Fig. 3.1) were used in a photochemical cross-linking assay (Pelletier and Sonenberg, 1985b). mRNA labeled with ^{32}P in the cap structure was incubated with protein factors, irradiated with UV light and RNase digested. Labeled proteins were then analyzed by SDS-PAGE followed by autoradiography. In the photochemical cross-linking experiments, RNA is the limiting component in the reaction mixtures. No detectable signal was observed in the absence of protein (Fig. 3.2A, lane 1). Similarly, no cross-linking of eIF4E to the cap-labeled mRNA was observed with 10 and 50 ng of purified eIF4E (lanes 2 and 3, respectively). Cross-linking was observed with 100 ng of eIF4E, albeit very inefficiently (lane 4), consistent with previous data (Lee *et al.*, 1985; Sonenberg *et al.*, 1979; Pelletier and Sonenberg, 1985b). We next examined the effect of eIF4G on the cross-linking of eIF4E to the cap structure. No cross-linking of eIF4G to the cap structure was observed (lane 5). Cross-linking of eIF4E to the cap structure was dramatically enhanced in the presence of eIF4G (lanes 6-8). As little as 10 ng of eIF4E was efficiently cross-linked to the cap structure in the presence of flag-eIF4G. Comparison of lanes 4 and 8 reveals an ~7 fold increase in the cross-linking of eIF4E to the cap structure in the presence of eIF4G. The cross-linking of eIF4E was cap-specific as the interaction was inhibited with 0.6 mM m^7GDP (lane 9). To determine the stoichiometry between eIF4E and eIF4G required for efficient cap binding, 10 ng of eIF4E was preincubated with increasing amounts of flag-eIF4G before the addition of the other components (Fig. 3.2B). While eIF4E alone did not cross-link to the cap structure (lane 1), addition of increasing amounts of flag-eIF4G enhanced eIF4E cross-linking to the cap structure in a dose dependent fashion (lanes 2-6). Under these conditions, ~10 ng of flag-eIF4G enhanced significantly the cross-linking of eIF4E to the cap (lane 2), with optimum binding occurring

Figure 3.1. Overview of purified recombinant factors.

Protein (2 μg) was analyzed by electrophoresis on polyacrylamide gels and Coomassie blue staining. 4E-BP1 and 4E-BP1Δ were expressed as GST fusion proteins. eIF4G was expressed as a fusion protein with a flag epitope tag. The positions of molecular weight markers are indicated to the left of each panel


4E-BP1
4E-BP1Δ
112-
84-
53-
34-
28-
20-
eIF4E
eIF4A
eIF4B
112-
84-
53-
34-
28-
eIF4G
206-
117-
89-

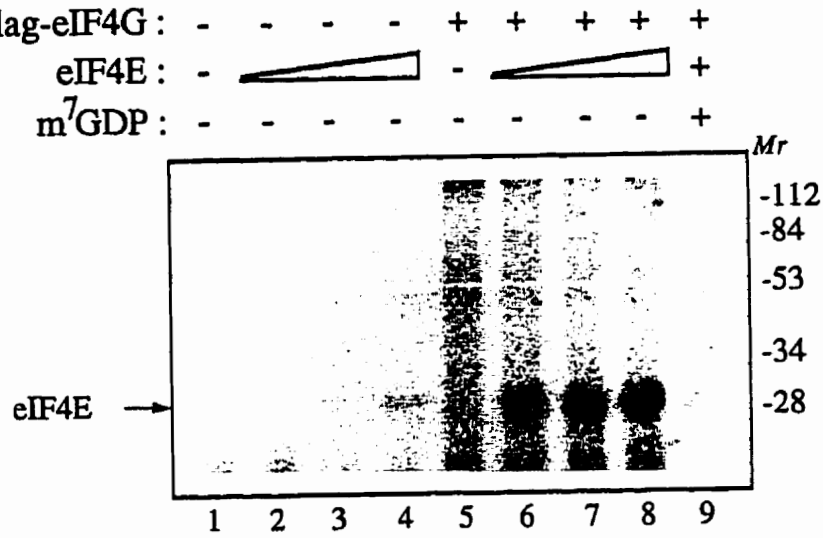
at a stiochiometry of 1:1 (lane 4). Cross-linking was inhibited by m^7GDP (lane 7), but not by GDP (lane 8).

We next analyzed the effect of other initiation factors on the cross-linking of eIF4E to the cap structure (Fig. 3.3). eIF4E alone did not cross-link to the cap structure (Fig. 3, Lane 1), as observed above. The interaction of eIF4E with the cap structure was not affected by the presence of eIF4A (lane 5), eIF4B (lane 6), or a combination of eIF4B and eIF4A (lane 7). Cross-linking of eIF4A alone was not observed either (lane 3). Cross-linking of eIF4A to the cap structure can be detected only when using the chemical cross-linking assay, where periodate oxidized mRNA is used (Sonenberg, 1981; Edery *et al.*, 1983; Grifo *et al.*, 1983; Pelletier and Sonenberg, 1985b). Similarly, eIF4B failed to cross-link to the cap structure under these conditions (lanes 2, 6 and 7). As expected, cross-linking of eIF4E to the cap structure was dramatically enhanced in the presence of flag-eIF4G (lane 8). Flag-eIF4G did not promote, however, the cross-linking of either eIF4A or eIF4B when present alone (lanes 9 and 10, respectively), or in combination (lane 11), to the mRNA cap structure. Furthermore, a combination of flag-eIF4G and eIF4E failed to promote cross-linking of eIF4B (lane 12) or eIF4A (lane 13) to the cap structure. Cross-linking of eIF4B was observed only in the presence of all the subunits of the eIF4F complex (eIF4A, eIF4E, and flag-eIF4G; lane 14), as shown earlier (Lee *et al.*, 1985). As expected, the specific interaction of eIF4E and eIF4B with the cap structure was insensitive to 0.6 mM GDP (lane 15), and was inhibited by 0.6 mM m^7GDP (lane 16). This confirms earlier findings that cross-linking of eIF4B to the cap structure is dependent on eIF4F and ATP hydrolysis (Lee and Sonenberg, 1982; Pelletier and Sonenberg, 1985b; Edery *et al.*, 1983). Taken together, these results provide direct evidence for the stimulatory effect of eIF4G on the interaction of eIF4E with the cap structure.

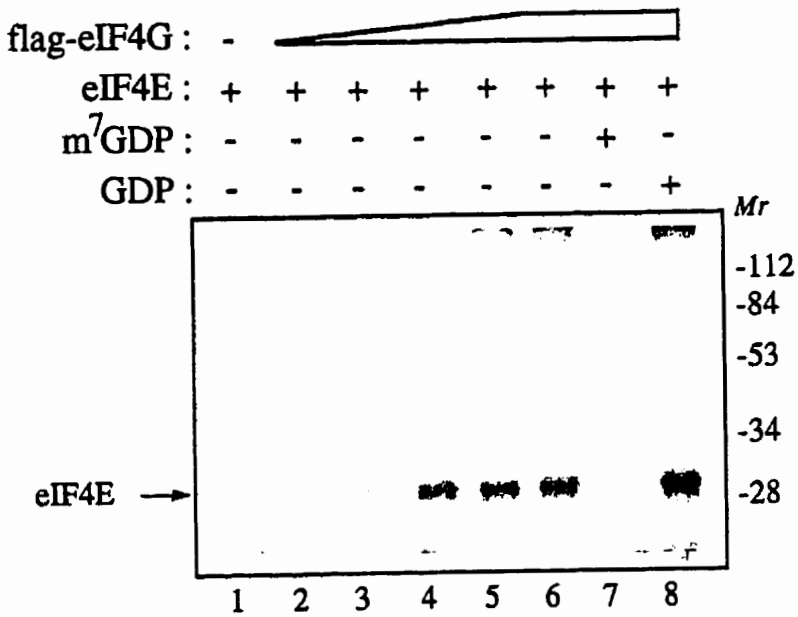
Figure 3.2. eIF4G enhances the cross-linking of eIF4E to mRNA cap structure.

eIF4E was cross-linked to ^{32}P cap-labeled RNA (~2 × 10^4 cpm) in the presence or absence of flag-eIF4G. **(A)** Reactions contained the following amounts of eIF4E: lanes 2 and 6, 10 ng; lanes 3 and 7, 50 ng; lanes 4 and 8, 100 ng. Where indicated flag-eIF4G (200 ng) was preincubated with eIF4E. m^7GDP (0.6 mM) was included where indicated. **(B)** eIF4E (10 ng) was preincubated with the following amounts of flag-eIF4G : lane 2, 10 ng; lane 3, 20 ng; lane 4, 60 ng; lane 5, 120 ng; lane 6, 240 ng. GDP or m^7GDP (0.6 mM each) was included where indicated.

A


flag-eIF4G : - - - - + + + + +
eIF4E : - - +
m7GDP : - - - - - - - - +
Mr
-112
-84
-53
-34
-28
eIF4E
1 2 3 4 5 6 7 8 9

B


flag-eIF4G : - +
eIF4E : + + + + + + + +
m7GDP : - - - - - - + -
GDP : - - - - - - - +
Mr
-112
-84
-53
-34
-28
eIF4E
1 2 3 4 5 6 7 8

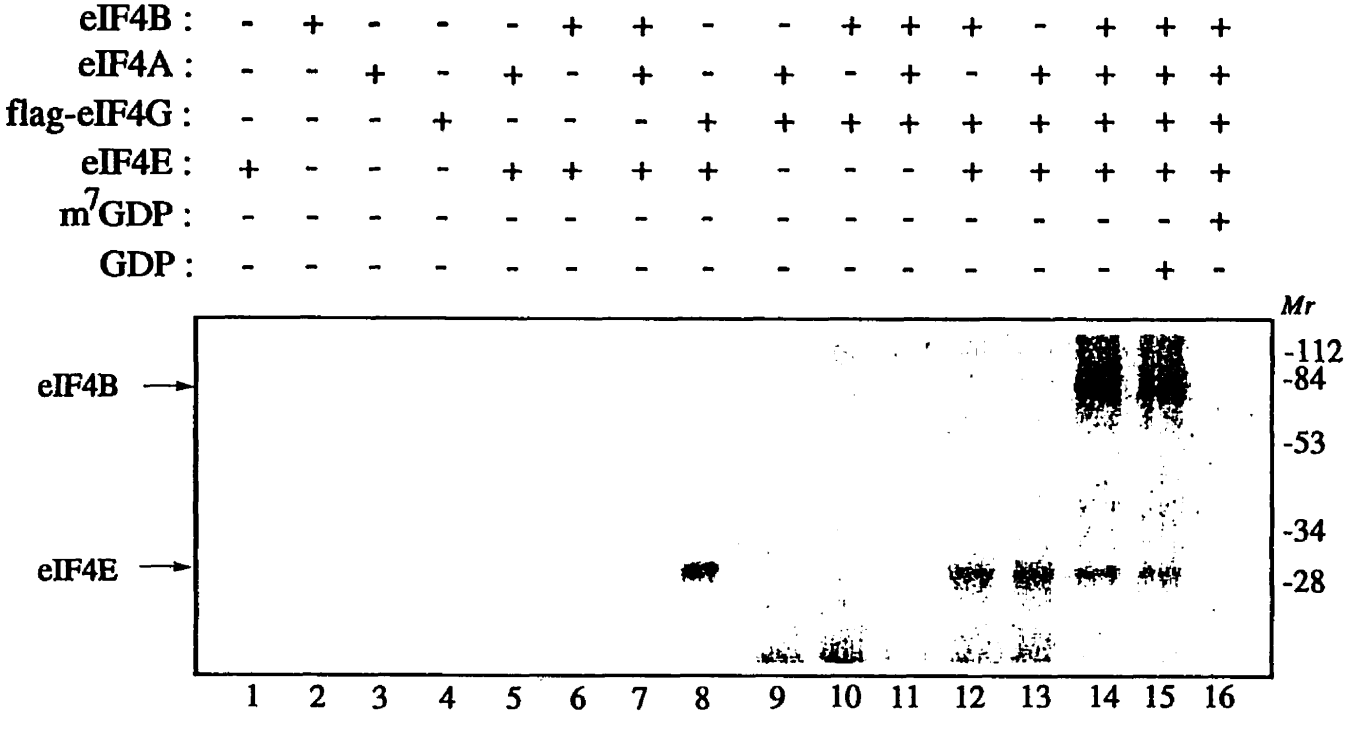
4E-BP1 prevents the stimulatory effect of eIF4G on the cap binding activity of eIF4E.

The activity of eIF4E is inhibited by 4E-BP1, which binds to eIF4E and prevents its interaction with eIF4G to form the eIF4F cap binding protein complex (Lin *et al.*, 1994; Pause *et al.*, 1994; Haghighat *et al.*, 1995). Earlier reports showed that binding of 4E-BP1 to eIF4E did not prevent the interaction of eIF4E with a cap-bound matrix (Pause *et al.*, 1994; Haghighat *et al.*, 1995). However, the effect of 4E-BP1 on eIF4E binding to the cap structure as part of the mRNA has not been determined. As we have shown above, the interaction of eIF4E with the mRNA cap structure is dramatically enhanced when it is bound to eIF4G. This is consistent with the finding that following poliovirus infection, which leads to the cleavage of eIF4G, the cross-linking of eIF4E to the mRNA cap structure is drastically reduced (Lee and Sonenberg, 1982; Pelletier and Sonenberg, 1985b; Lee *et al.*, 1985). Under these conditions, eIF4E complexed to the amino terminal cleavage product of eIF4G binds a cap-bound matrix (Lee *et al.*, 1985). Similarly, eIF4E complexed to 4E-BP1 can efficiently bind the cap affinity column (Pause *et al.*, 1994).

Based on the above observations, it is predicted that the cross-linking of eIF4E in an extract (where it binds tightly as part of eIF4F) should be diminished in the presence of 4E-BP1 (Haghighat *et al.*, 1995). To examine this, photochemical cross-linking to the mRNA cap structure was performed in a rabbit reticulocyte lysate. Cross-linking was done in the presence of ATP-Mg^{2+} to detect also eIF4B binding. UV irradiation induced cross-linking of polypeptides of 24, 65, and 80 kDa (Fig. 3.4A, lane 1), as previously shown (Pelletier and Sonenberg, 1985b). The 24 and 80 kDa polypeptides correspond to eIF4E and eIF4B, respectively, while the identity of the 65 kDa polypeptide is not known (Pelletier and Sonenberg, 1985b). The cross-linking of eIF4E and eIF4B was insensitive to 0.6 mM GDP (lane 2), but was inhibited by the same concentration of m^7GDP (lane 3). In contrast, the cross-linking of the 65 kDa polypeptide was not affected by either nucleotide. Strikingly, pre-incubation of the reticulocyte lysate with GST-4E-BP1 drastically reduced the cross-linking of eIF4E and eIF4B to the cap structure (lane 4).

Figure 3.3. Effect of initiation factors on the cross-linking of eIF4E to the cap structure.

eIF4E was cross-linked to ^{32}P cap-labeled RNA (~2 × 10^4 cpm) in the presence or absence of the following initiation factors: eIF4A (0.2 μg), eIF4B (50 ng), flag-eIF4G (60 ng). GDP or m^7GDP (0.6 mM each) was included where indicated. Following UV irradiation, labeled polypeptides were resolved on an 12.5% polyacrylamide-SDS gel which was processed for autoradiography. The positions of eIF4E and eIF4B are denoted by arrows to the left of the figure.


eIF4B : - + - - - + + - - + + + - + + +
eIF4A : - - + - + - + - + - + - + + + +
flag-eIF4G : - - - + - - - + + + + + + + + +
eIF4E : + - - - + + + + - - - + + + + +
m7GDP : - - - - - - - - - - - - - - - +
GDP : - - - - - - - - - - - - - - + -
Mr
-112
-84
-53
-34
-28
eIF4B
eIF4E
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

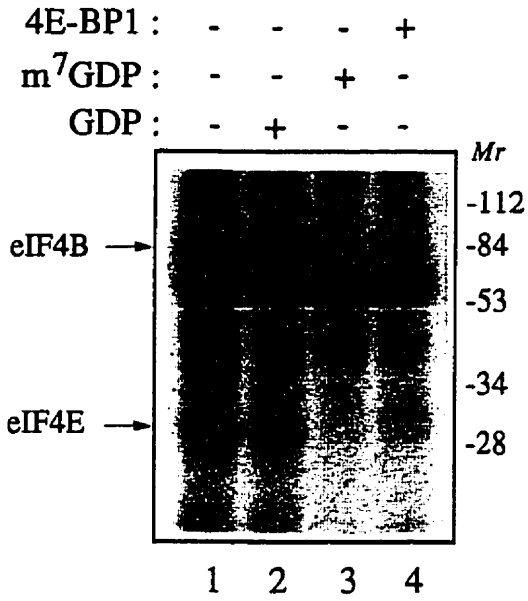
Since eIF4B cross-linking is dependent on eIF4F, it is inhibited in the presence of 4E-BP1, which prevents eIF4F complex formation. These results indicate that eIF4E as a complex with 4E-BP1 interacts weakly with the cap structure, as compared to eIF4E as a subunit of eIF4F.

To further substantiate these conclusions, the UV-induced cross-linking assay was performed using purified components as in Figs. 2 and 3. eIF4E alone did not cross-link to the cap structure (Fig. 3.4B, lane 1). To examine the effect of 4E-BP1 on the cross-linking of eIF4E to the mRNA cap structure in this reconstituted system, 4E-BP1 was pre-incubated with eIF4E before the addition of the other components. 4E-BP1 prevented the efficient eIF4E cross-linking that occurs in the presence of flag-eIF4G (lane 2). To verify that the inhibitory effect was the result of a direct interaction between GST-4E-BP1 and eIF4E, a mutant of 4E-BP1 (GST-4E-BP1Δ) was used. GST-4E-BP1Δ contains a deletion of the 4E binding domain, and does not repress translation (Haghighat *et al.*, 1995) (Mader *et al.*, 1995). Preincubation of eIF4E with GST-4E-BP1Δ had no effect on the stimulatory effect of eIF4G on the cross-linking of eIF4E to the cap structure (lane 3). The cross-linking of eIF4E to the cap structure in the presence of flag-eIF4G was sensitive to inhibition by 0.6 mM m^7GDP (lanes 5). Taken together, these results demonstrate that 4E-BP1 prevents the facilitative effect of eIF4G on the interaction of eIF4E with the mRNA cap structure.

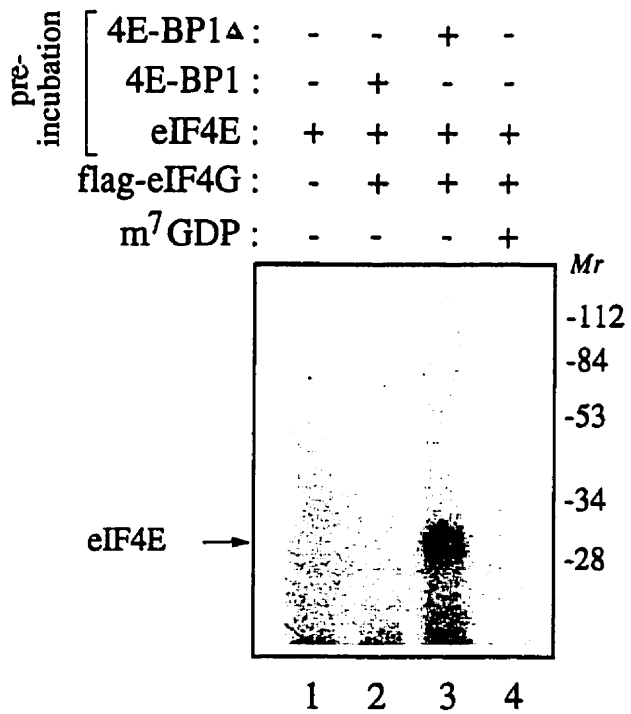
Figure 3.4. Effect of 4E-BP1 on the interaction of eIF4E with the cap structure.

(A) UV cross-linking of cap-labeled mRNA to proteins in reticulocyte lysate. Cross-linking was carried out as described in Materials and Methods, in the absence (-) or presence (+) of GST-4E-BP1 (0.7 μg), GDP (0.6 mM), m^7GDP (0.6 mM), as indicated above the Figure. Labeled proteins were analyzed by SDS-PAGE and autoradiography. **(B)** 4E-BP1 inhibits the facilitative effect of eIF4G on the association of eIF4E with the cap structure. Reactions were performed as in Fig. 3. Where indicated, eIF4E (10 ng) was pre-incubated with GST-4E-BP1 (40 ng) or GST-4E-BP1Δ (40 ng) before the addition of the other components. m^7GDP (0.6 mM) was included where indicated.

A


4E-BP1 : - - - +
m7GDP : - - + -
GDP : - + - -
Mr
-112
-84
-53
-34
-28
eIF4B
eIF4E
1 2 3 4

B


pre-incubation
4E-BP1Δ : - - + -
4E-BP1 : - + - -
eIF4E : + + + +
flag-eIF4G : - + + +
m7GDP : - - - +
Mr
-112
-84
-53
-34
-28
eIF4E
1 2 3 4

In summary, we have reconstituted the cap recognition step of eukaryotic translation initiation *in vitro* using purified components. We have demonstrated that eIF4G significantly enhances the cap recognition activity of eIF4E, suggesting that eIF4G plays an important role in the mechanism of mRNA cap recognition during eukaryotic translation initiation. Furthermore, 4E-BP1 inhibited the stimulatory effect of eIF4G on the cap binding activity of eIF4E. However, a 4E-BP1•eIF4E complex can be isolated by a cap affinity column (Pause *et al.*, 1994; Haghighat *et al.*, 1995). It is conceivable that eIF4G, because of its RNA binding activity (Pestova *et al.*, 1996; H. Lee and C. Goyer, unpublished data), interacts with the RNA in the vicinity of the cap structure and facilitates a stable association between eIF4E and the mRNA 5' cap structure. Indeed, eIF4F binds much more avidly to RNA than either eIF4E or eIF4A (Jaramillo *et al.*, 1991). Our results also indicate that eIF4B can gain access to the cap structure only in the presence of an intact eIF4F complex and ATP hydrolysis. In support of this conclusion, disruption of eIF4F complex by 4E-BP1 interfered with the efficient cross-linking of eIF4B to the cap structure. Taken together, our results indicate that eIF4E initially interacts with the cap structure as a subunit of eIF4F.

Several other observations support the hypothesis that eIF4F complex assembly occurs prior to the cap recognition step of translational initiation (Lee *et al.*, 1985; Lee and Sonenberg, 1982; Pelletier and Sonenberg, 1985b). The equilibrium constant (k_{eq}) of m^7GpppG-eIF4E (mammalian) complex formation has been determined by spectroscopic studies to be 4.8×10^{-5} M (Carberry *et al.*, 1989; Ueda *et al.*, 1991). This indicates a weak interaction between eIF4E and the cap structure that is unlikely to be favoured *in vivo*. eIF4E as a subunit of eIF4F cross-links 20-fold better to the cap structure than eIF4E alone (Edery *et al.*, 1987). Furthermore, Pelletier and Sonenberg showed that insertion of secondary structures 38 nucleotides downstream from the cap structure had no effect on UV cross-linking of initiation factors to the cap structure, whereas binding of ribosomes to mRNA was impaired, suggesting that eIF4F interaction with the cap structure precedes ribosome binding to mRNA (Pelletier and Sonenberg, 1985a; Pelletier and Sonenberg,

1985b). Based on our results and the studies cited above (Pelletier and Sonenberg, 1985a; Pelletier and Sonenberg, 1985b; Lee and Sonenberg, 1982), we favour the model where some localized unwinding of the 5'UTR of mRNA precedes ribosome attachment.

Other important parameters have also been implicated in effecting the interaction between eIF4E and the cap structure. Phosphorylation of both eIF4E and eIF4G is enhanced following treatment of cells with growth factors and insulin (Morley and Traugh, 1990). eIF4E is more phosphorylated as a subunit of eIF4F (Lamphear and Panniers, 1990; Tuazon *et al.*, 1990), and phosphorylated eIF4E forms a more stable complex with eIF4G (Bu *et al.*, 1993). Furthermore, phosphorylated eIF4E was reported to bind better to the cap structure relative to its unphosphorylated form (Minich *et al.*, 1994), and only the phosphorylated form of eIF4E is present in the 48S pre-initiation complex (Joshi-Barve *et al.*, 1990). In addition, the association of 4E-BP1 with eIF4E in vitro prevents the phosphorylation of eIF4E (Whalen *et al.*, 1996). Taken together these findings lend support to a model where prior assembly of eIF4F complex is a requisite for the cap recognition and subsequent ribosome binding steps of translation initiation in eukaryotes.

3.4 MATERIALS AND METHODS

Protein factors

Murine eIF4E protein was expressed in E. coli K38 and purified as described previously (Edery *et al.*, 1988). Recombinant flag-eIF4G was expressed in Sf9 insect cells and purified as described previously (Haghighat *et al.*, 1996). Glutathione-S-transferase (GST) fusion proteins of HMK-4E-BP1 and HMK-4E-BP1Δ were expressed in E. coli BL21 and purified as described previously (Mader *et al.*, 1995). Recombinant eIF4B was expressed in Sf9 insect cells as follows: for the construction of the baculovirus transfer vector, eIF4B cDNA was excised from the plasmid pGEM3-eIF4B (Methot *et al.*, 1994) with BamHI, and subcloned blunt into the NheI site of the p10 transfer vector (Vialard *et al.*, 1990). Recombinant baculovirus was generated by cationic liposome cotransfection of p10eIF4B construct with the linearized genomic AcMNPV DNA according to the manufacturer's instructions (Invitrogen). Recombinant virus was isolated (Summers and Smith, 1987), and eIF4B was purified as described previously (Pause and Sonenberg, 1992).

UV-induced cross linking assay

Uncapped RNA encoding chloramphenicol acetyl transferase (CAT) was capped and methylated with 6 units of vaccinia virus guanylyltransferase (Gibco-BRL) in the presence of 0.4 mM S-adenosyl-L-methionine and [α-^{32}P] GTP (100 μCi). UV-induced cross linking was performed in the presence of 1 mM ATP as described previously (Pelletier and Sonenberg, 1985b). Briefly, 2×10^4 cpm of ^{32}P-mRNA was incubated with initiation factors in a total volume of 20 μl in 20 mM HEPES (pH 7.5), 0.5 mM magnesium acetate, 2 mM dithiothreitol, 3% glycerol, 100 mM potassium acetate at 30°C for 10 min. Reaction mixtures were irradiated at 4°C at a distance of 4 cm with a G15T8 germicidal lamp for 45

min. The RNA was next digested for 30 min at 37°C with 20 μg of RNase A. Samples were analyzed on acrylamide gels followed by autoradiography. Quantitations were performed using a Fuji BAS2000 phosphorImager.

3.5 ACKNOWLEDGMENTS

We thank Greg Cosentino, Andrew Craig, and Kianoush Khaleghpour for critical reading of the manuscript. We gratefully acknowledge Arnim Pause for providing recombinant eIF4A, and appreciate the technical assistance of Nathalie Methot in the purification of eIF4B. This work was supported by a grant from the Medical Research Council of Canada to N. S. N.S. is the recipient of an MRC Distinguished Scientist Award from the Medical Research Council of Canada, and is an Howard Hughes International Scholar.

As mentioned previously, modulation of eIF4F activity is also observed following infection by certain viruses. A dramatic example of this occurs upon picornaviral infection. The study presented in chapter IV describes one sophisticated mechanism whereby certain members of the family picornaviridae assure the preferential translation of their own mRNAs at the host's expense.

CHAPTER IV

The eIF4G-eIF4E complex is the Target for Direct Cleavage by the Rhinovirus 2A Proteinase.

4.1 SUMMARY

The 2A proteinases ($2A^{pro}$) of certain picornaviruses induce the cleavage of the eIF4G subunit of the cap binding protein complex, eIF4F. Several reports have demonstrated that $2A^{pro}$ of rhinovirus and coxsackievirus B4 cleave eIF4G directly. However, it was suggested that in poliovirus infection, the $2A^{pro}$ induces the activation of a cellular proteinase which in turn cleaves eIF4G. Furthermore, it is not clear whether eIF4G is cleaved as part of the eIF4F complex or as an individual polypeptide. To address these issues, recombinant eIF4G was purified from Sf9 insect cells and tested for cleavage by purified rhinovirus $2A^{pro}$. Here we report that eIF4G alone is a relatively poor substrate for cleavage by the rhinovirus $2A^{pro}$. However, an eIF4G-eIF4E complex is cleaved efficiently by the $2A^{pro}$, suggesting that eIF4F is a preferred substrate for cleavage by rhinovirus $2A^{pro}$. Furthermore, $2A^{pro}$ drastically reduced the translation of a capped mRNA. An eIF4G-eIF4E complex, but not eIF4G alone, was required to restore translation.

4.2 INTRODUCTION

Infection of cells by picornaviruses belonging to several genera results in a precipitous and dramatic inhibition of host cellular mRNA translation (Ehrenfeld., 1996). In the case of poliovirus, this inhibition precedes any substantial synthesis of viral proteins (Belsham and Sonenberg, 1996). In contrast to cellular mRNA translation, viral RNA translation proceeds with high efficiency during the infection. The differential translation of viral mRNAs can be explained by the unique translational features of picornaviruses. Picornavirus RNAs, in contrast to cellular mRNAs, do not contain a 5' cap structure (Hewlett, 1976; Nomoto *et al.*, 1976), and their translation is mediated by ribosome binding to an internal ribosome entry site (IRES) that is present in the 5' untranslated region (Pelletier and Sonenberg, 1988).

Picornaviruses induce both structural and functional modifications of the translational machinery. The ability of eukaryotic initiation factor 4F (eIF4F) to restore the translation of capped mRNAs in extracts prepared from poliovirus-infected cells initially implicated eIF4F as one target for such modifications (Tahara *et al.*, 1981). eIF4F is a cap-binding multisubunit complex which facilitates mRNA unwinding, and subsequent ribosome binding to mRNA (Sonenberg, 1996). It is composed of three polypeptides: eIF4E, eIF4A, and eIF4G. eIF4E, a 24-kDa polypeptide, mediates the cap binding function of the complex (Sonenberg *et al.*, 1978) and plays a critical role in the control of translation rates (Sonenberg, 1996). eIF4A, a 50-kDa polypeptide, exhibits RNA-dependent ATPase activity and, in association with eIF4B, bidirectional RNA helicase activity (Ray *et al.*, 1985; Rozen *et al.*, 1990). Recent evidence suggests that eIF4G may serve as a scaffold: it interacts with both eIF4E and eIF4A, and its association with eIF3 is suggested to promote ribosome binding at the 5' end of mRNAs (Lamphear *et al.*, 1995; Mader *et al.*, 1995).

The *Enterovirus*, *Rhinovirus*, and *Aphthovirus* genera of picornaviruses cause cleavage of eIF4G into an N-terminal fragment of about 50 kDa (which migrates as a set of two or three polypeptides of 110 to 130 kDa), and a carboxy-terminal fragment of about 100 kDa. The 2A proteinase ($2A^{pro}$) of poliovirus, coxsackievirus, and rhinovirus is required for the cleavage of eIF4G, and mutants of poliovirus $2A^{pro}$ are defective in eIF4G cleavage (Bernstein *et al.*, 1985). Similarly, the L proteinase of foot-and-mouth disease virus cleaves eIF4G (Devaney *et al.*, 1988; Kirchweger *et al.*, 1994; Lloyd *et al.*, 1988). However, there is conflicting evidence as to whether these proteinases exert their effects directly or indirectly through the activation of cellular proteinases. Wyckoff *et al.* (1990) had reported that the activity which cleaves eIF4G does not copurify with poliovirus $2A^{pro}$, suggesting an indirect mechanism whereby poliovirus-activated cellular proteases mediate proteolytic cleavage. In addition, anti-$2A^{pro}$ serum capable of inhibiting poliovirus polyprotein processing does not inhibit eIF4G cleavage (Krausslich *et al.*, 1987; Lloyd *et al.*, 1986; Wyckoff *et al.*, 1990). More recently, a role for eIF3 in the cleavage of eIF4G was reported. In these experiments eIF4G was not cleaved by an *Ecsherichia coli* extract expressing poliovirus $2A^{pro}$, but cleavage occurred when purified eIF3 was added (Wyckoff *et al.*, 1992). In contrast, experiments with recombinant $2A^{pro}$ of human rhinovirus 2 or of coxsackievirus B4 and the L proteinase of foot-and-mouth disease virus demonstrated direct cleavage of the eIF4G subunit in the eIF4F complex (Kirchweger *et al.*, 1994; Lamphear *et al.*, 1993; Liebig *et al.*, 1993). Such an activity was not examined with purified poliovirus $2A^{pro}$. It is important to note that the eIF4G substrates used in the various studies were different. Wyckoff *et al.* (1992) used eIF4G that was partially purified in a form dissociated from the other eIF4F polypeptides. On the other hand, the eIF4G substrate used in experiments with $2A^{pro}$ of rhinovirus and coxsackievirus (Kirchweger *et al.*, 1994; Lamphear *et al.*, 1993; Liebig *et al.*, 1993) was purified as part of the intact eIF4F complex. While it is highly unlikely that the mechanism of action of poliovirus $2A^{pro}$ is different from those of coxsackievirus and rhinovirus, it is possible that eIF4G, in a

complex with the other eIF4F subunits, assumes a conformation which renders it a substrate for $2A^{pro}$.

To address these questions and to determine the substrate for $2A^{pro}$ (eIF4G or eIF4F), we examined directly whether recombinant eIF4G is a substrate for HRV2 $2A^{pro}$. Here, we demonstrate that $2A^{pro}$ from HRV2 cleaves purified recombinant eIF4G directly *in vitro*, although relatively poorly. In contrast, a complex of eIF4G with eIF4E is a preferable substrate for HRV2 $2A^{pro}$. We therefore propose that eIF4F, and not the eIF4G subunit alone, is the primary target for cleavage by HRV2 $2A^{pro}$. Consistent with these results, we show that restoration of cap-dependent translation in $2A^{pro}$-treated extracts requires both the eIF4E and eIF4G subunits of the eIF4F complex.

4.3 RESULTS

Expression and purification of recombinant eIF4G from Sf9 insect cells

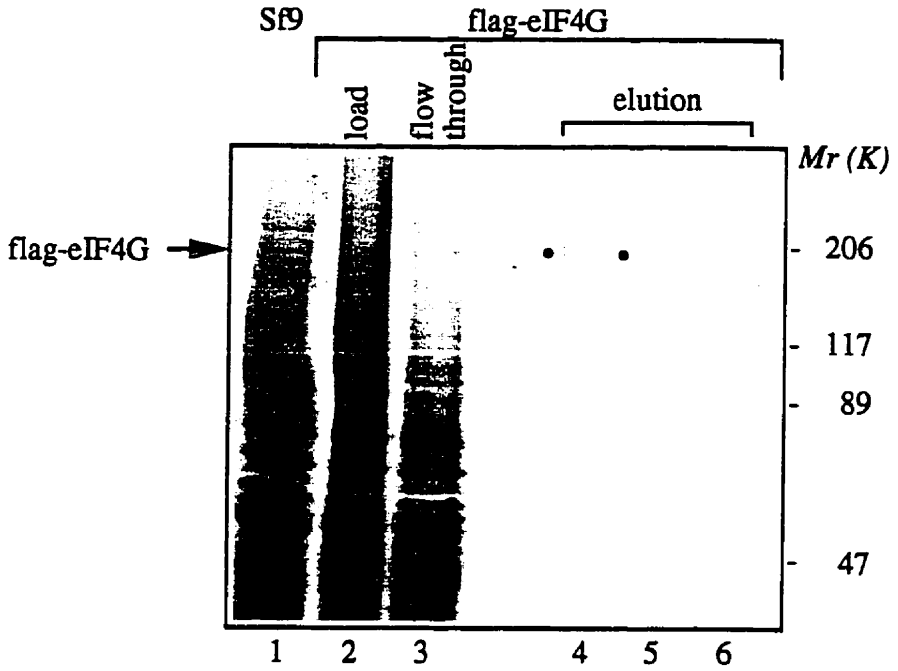
Human eIF4G was expressed as a fusion protein with flag-HMK epitope tag in insect cells by using a baculovirus expression system. High-titer virus (~2 x 10^8 PFU/ml) was generated and used to infect Sf9 insect cells. Cytoplasmic cell lysates were prepared at 72 h postinfection and eIF4G was immunopurified on an anti-flag column (Fig. 4.1A). The eluate contained a major polypeptide of about 200 kDa (lanes 4 to 6). The identity of the eluted band was determined by immunoblotting with an anti-eIF4G polyclonal antibody [the antibody detects both the amino- and carboxy-terminal cleavage products of eIF4G (Aldabe *et al.*, 1996)]. Uninfected cells showed no immunoreactive material (Fig. 4.1B, lane 1). Flag-eIF4G was detected in the load (lane 2), flowthrough (lane 3), and eluate fractions (lanes 4 to 6). In HeLa S10 extracts the antibody recognized eIF4G, which migrates at about 220 kDa (lane 7). Previously, we reported that HA-eIF4G expressed in Sf9 insect cells migrated at about 190 kDa (Haghighat *et al.*, 1995). The slower migration reported here is most probably due to the flag epitope.

To examine whether insect eIF4E copurifies with the recombinant eIF4G, the ability of eIF4G to be retained on an m^7GDP-coupled agarose resin was determined. Recombinant flag-eIF4G alone did not bind to the resin, as determined by Western blotting (data not shown). Flag-eIF4G was retained on the m^7GDP-coupled agarose resin only in the presence of exogenous recombinant murine eIF4E (data not shown) (Haghighat *et al.*, 1995). This finding also demonstrates that the recombinant eIF4G expressed in insect cells exhibits eIF4E binding activity.

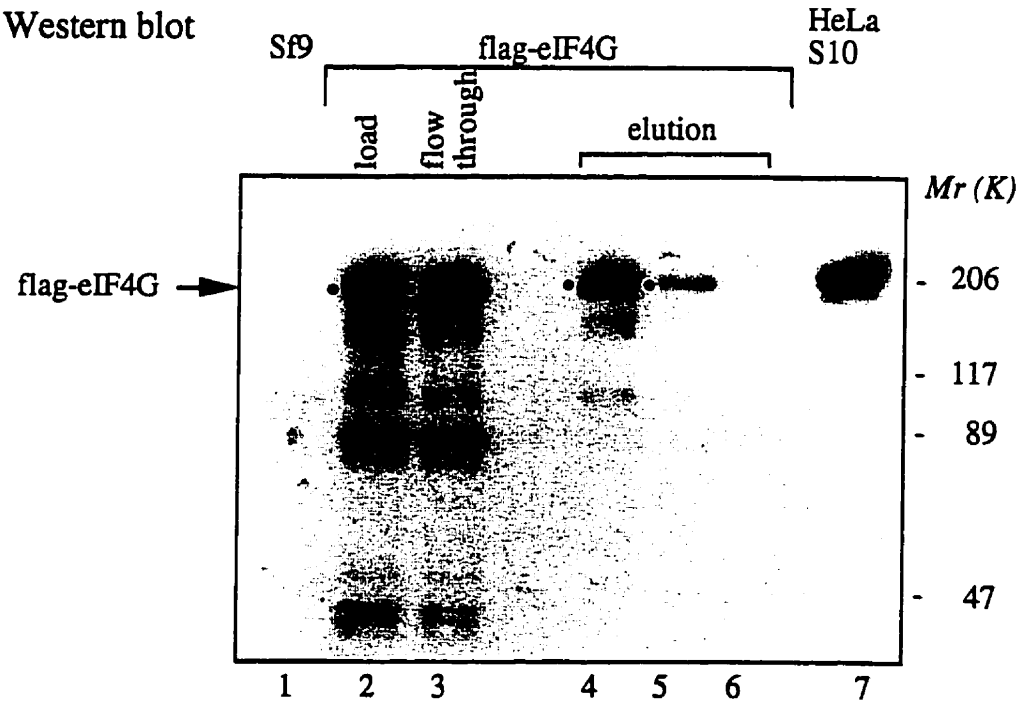
Figure 4.1. Expression of recombinant flag-eIF4G in Sf9 insect cells and purification on an anti-flag column.

Samples were resolved on an SDS-8% polyacrylamide gel and analyzed by Coomassie blue staining **(A)** and Western blotting **(B)**. The following samples were loaded on the gel: lane 1, ~70 μg of uninfected Sf9 insect cell lysate; lane 2, ~70 μg of infected Sf9 insect cell lysate (load); lane 3, ~70 μg of flowthrough; lanes 4 to 6, 10 μl (from a total of 1 ml for each fraction) of eluate. The Western blots of duplicate samples contain one-fifth of the material used in the Coomassie blue stain. Lane 7, ~12 μg of a HeLa S10 extract. Molecular masses of protein standards (Bio-Rad) are indicated on the right. The position of flag-eIF4G is indicated by an arrow and black dots.

A : Coomassie stain



B : Western blot



Inefficient cleavage of pure eIF4G by HRV2 2A^{pro} in vitro

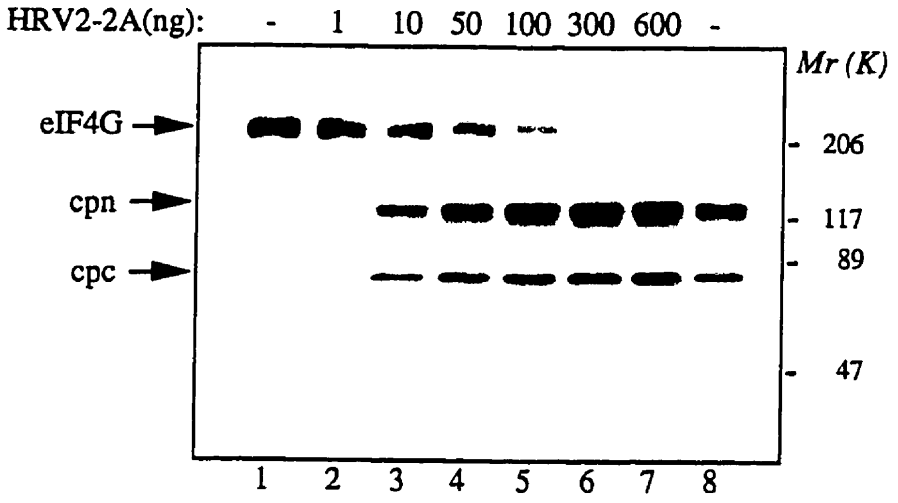
To test whether recombinant eIF4G is cleaved by purified HRV2 2A^{pro}, HeLa S10 extract or purified flag-eIF4G was incubated with increasing amounts of the 2A^{pro}. The cleavage of eIF4G was monitored by immunoblotting with an anti-eIF4G polyclonal antibody (Aldabe *et al.*, 1996). eIF4G in the HeLa S10 extract was stable when incubated with buffer alone (Fig. 4.2A, lane 1) but was efficiently cleaved into its characteristic cleavage products when incubated with increasing amounts of HRV2 2A^{pro} (lanes 2 to 7), consistent with the reports that 2A^{pro} is the only virally encoded protein required for the induction of eIF4G cleavage (Krausslich *et al.*, 1987; Liebig *et al.*, 1993). Under these conditions, 10 ng of HRV2 2A^{pro} cleaved approximately 50% of the eIF4G, as reported by Klump et al. (Klump *et al.*, 1996). The migration of the cleavage products resembled the pattern observed in extracts from poliovirus-infected HeLa S3 cells (lane 8). The cleavage products derived from the amino and carboxy termini were designated cpn (for cleavage product N terminus) and cpc (for cleavage product C terminus).

In contrast to the efficient cleavage of eIF4G in the HeLa S10 extract, recombinant flag-eIF4G was a relatively poor substrate for cleavage by the HRV2 2A^{pro} (Fig. 2B). While buffer alone had no effect on the stability of flag-eIF4G (lane 1), 600 ng of HRV2 2A^{pro} was required to cleave about 60% of the flag-eIF4G (lanes 2 to 7 [note that the antibody recognizes the flag-eIF4G cleavage products less efficiently than it recognizes the intact protein; the reason for this is not known—see also Fig. 4.3 and 4.4]). The cleavage product derived from the carboxy terminus of flag-eIF4G comigrated with the corresponding fragment in the control lane, whereas the amino-terminal product displayed a higher mobility than its counterpart in the control lane (compare lanes 7 and 8). In addition, the cpn derived from flag-eIF4G migrated as a single band at about 110 kDa whereas three or four bands are observed following cleavage of the authentic protein. Neither the

Figure 4.2. Cleavage of eIF4G by the HRV2 $2A^{pro}$ *in vitro*.

(A) HeLa S10 extract (~12 μg) was incubated at 30°C for 30 min with the indicated amounts of HRV2 $2A^{pro}$. Samples were analyzed as described in Materials and Methods. Lanes: 1, buffer B; 2 to 7, HRV2 $2A^{pro}$; 8, ~12 μg of poliovirus-infected HeLa S3 extract. **(B)** A ~80 ng portion of flag-eIF4G, equivalent to the eIF4G content of ~12 μg of HeLa S10 extract, as determined by Western blotting, was treated as in panel A. Lanes: 1, buffer B; 2 to 7, HRV2 $2A^{pro}$; 8, ~12 μg of poliovirus-infected HeLa S3 extract. The position of intact eIF4G and the N-terminal (cpn) and C-terminal (cpc) cleavage products are indicated by arrows.

A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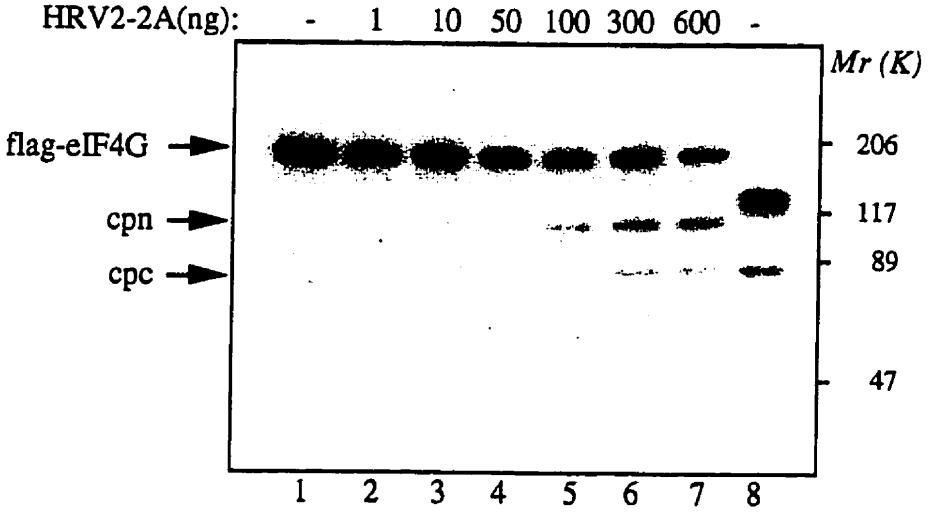
eIF4G
cpn
cpc
206
117
89
47
1 2 3 4 5 6 7 8

B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Mr (K)
flag-eIF4G
cpn
cpc
206
117
89
47
1 2 3 4 5 6 7 8

heterogeneity observed with the authentic protein (Ehrenfeld, 1996) nor the aberrant mobility exhibited by the amino-terminal third of flag-eIF4G is understood. A conformational change or the absence of a posttranslational modification in insect cells at the amino terminus may account for the altered mobility.

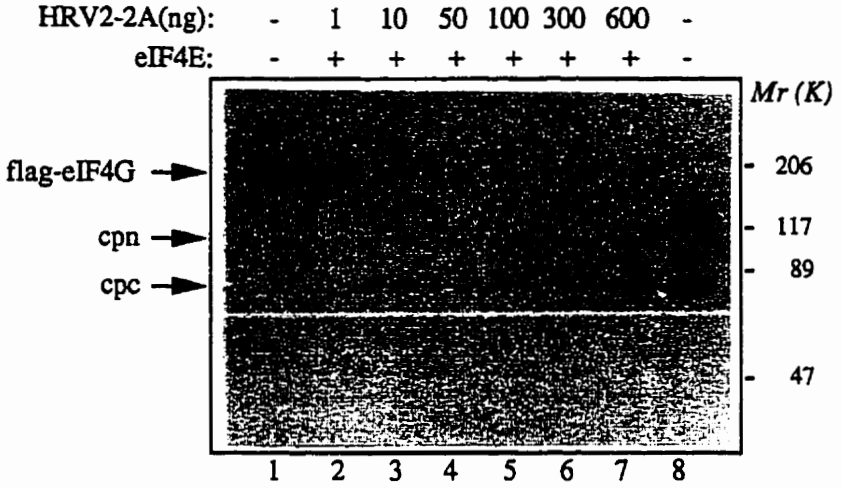
eIF4E enhances the cleavage of eIF4G by HRV2 2A^{pro}

To determine whether eIF4E enhances eIF4G cleavage, recombinant eIF4G was preincubated with eIF4E to allow for complex formation, and then increasing amounts of HRV2 2A^{pro} were added. Incubation in the presence of buffer alone did not induce any cleavage (Fig. 4.3A, lane 1). While no significant cleavage of eIF4G in the presence of eIF4E occurred with 1 ng of HRV2 2A^{pro} (lane 2), 10 ng of HRV2 2A^{pro} cleaved more than 75% of the input flag-eIF4G (lane 3). Approximately 9% of input eIF4G was resistant to cleavage by 2A^{pro} (lanes 4 to 7). Increasing amounts or eIF4E did not enhance cleavage of the resistant material (data not shown), which might be misfolded and unable to interact with eIF4E. To address this possibility, flag-eIF4G was preincubated with excess eIF4E and subsequently purified as a complex with eIF4E by chromatography on an m^{7}GDP-coupled agarose resin. This procedure is expected to eliminate the misfolded eIF4G that cannot interact with eIF4E. Incubation with buffer alone did not result in cleavage of eIF4G (Fig. 4.3B, lane 1), ruling out the possibility that eIF4E induces the cleavage of eIF4G. However, about 20% and in excess of 80% of the eIF4G was cleaved with 1 and 10 ng of the HRV2 2A^{pro}, respectively (lanes 2 and 3), and cleavage was complete with increasing amounts of enzyme (lanes 4 to 7). Addition of eIF4A or eIF3 did not change the rate of appearance or the mobility of the cleavage products (data not shown). Taken together, these results indicate that eIF4G in a complex with eIF4E is more susceptible to cleavage by HRV2 2A^{pro} than is eIF4G alone.

Figure 4.3. Cleavage of eIF4G by the HRV2 $2A^{pro}$ is enhanced by eIF4E.

(A and C) Similar amounts of flag-eIF4G **(A)** or HeLa S10 extract **(C)** to those in Fig. 4.2 were preincubated with buffer A (lane 1) or 50 ng of murine eIF4E for 5 min at $30^{o}C$. The mixture was subsequently treated as the experiment in Fig. 4.2. **(B)** Purified flag-eIF4G was incubated with purified murine eIF4E, and the mixture was applied to an $m^{7}GDP$-coupled agarose resin. The eluted eIF4G-eIF4E complex in buffer A, containing the same amount of eIF4G as in panel A, was treated with either buffer B (lane 1) or HRV2 $2A^{pro}$, as in the experiment in Fig. 2. **D)** Quantitative analysis of the results in panel C. Symbols: ■, HeLa S10; □, HeLa S10 plus eIF4E. **(E)** Quantitative analysis of the results in panels A and B. Symbols: ■, eIF4G; □, eIF4G plus eIF4E; □, eIF4F. The amount of intact eIF4G present in each lane was quantitated with a Bas 2000 phosphorimager and is presented as a percentage of input eIF4G.

A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cpn
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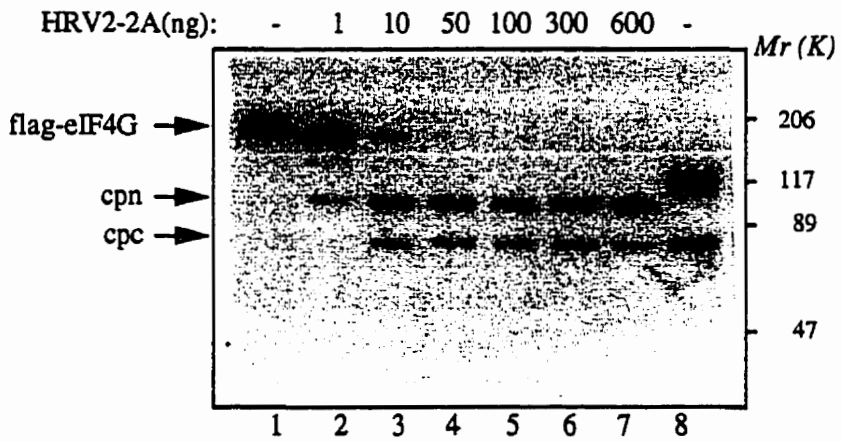
B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flag-eIF4G
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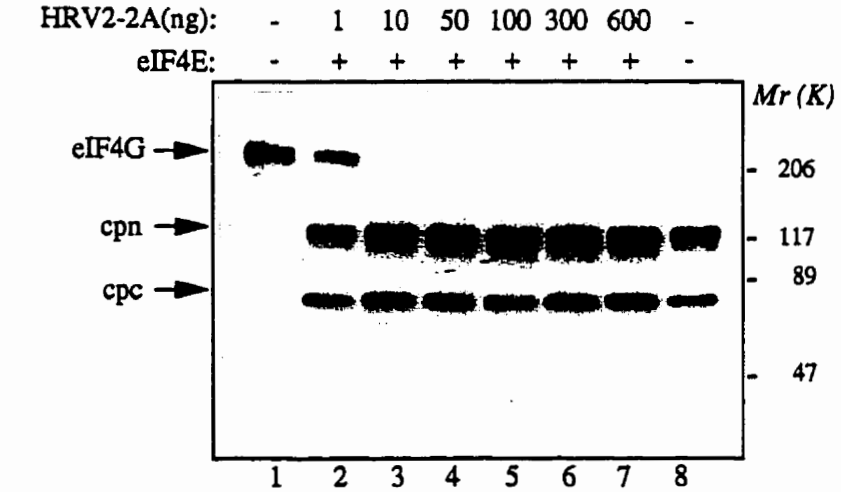
C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Mr (K)
eIF4G
cpn
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206
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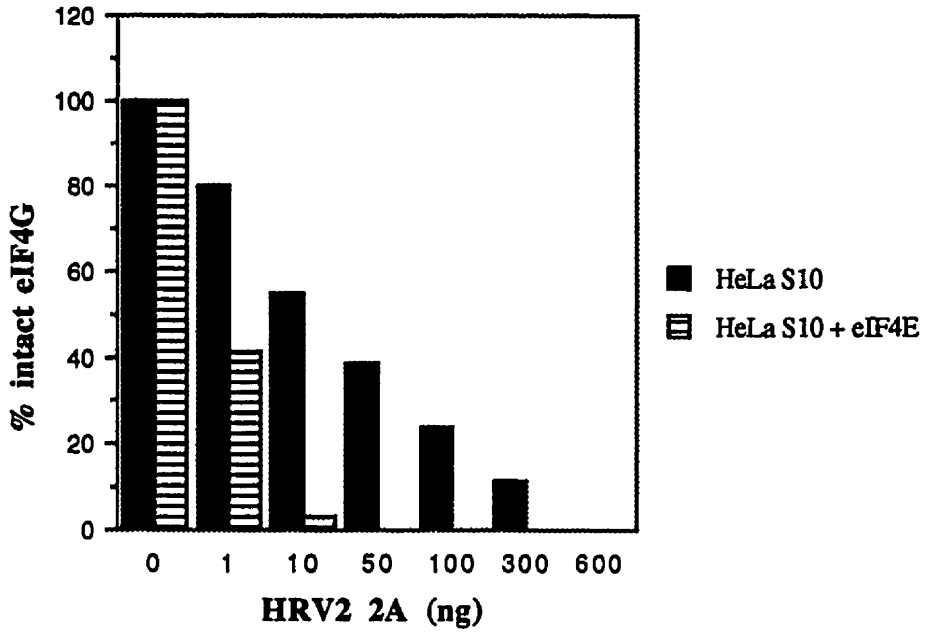
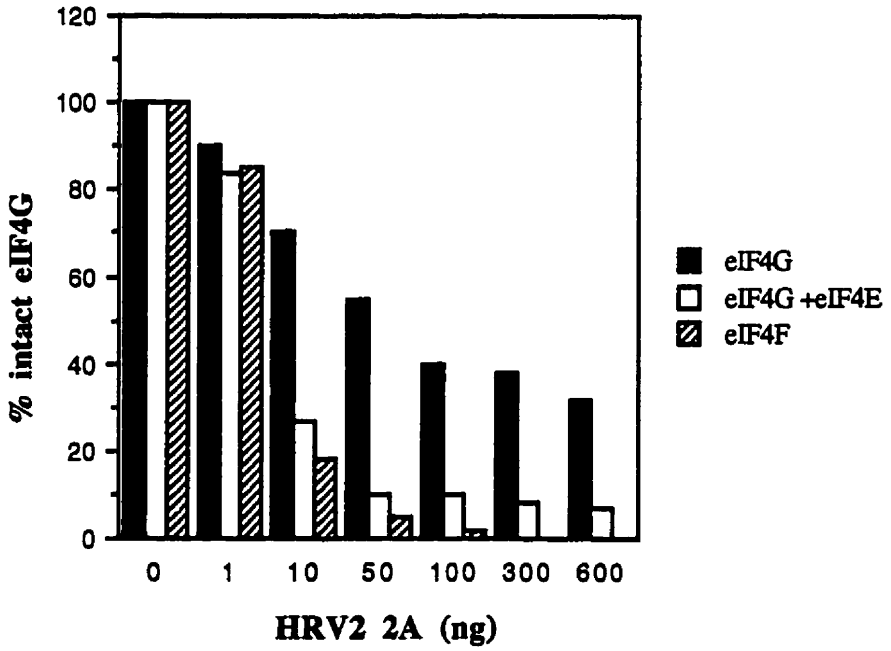
1 2 3 4 5 6 7 8

D


120
100
80
60
40
20
0
% intact eIF4G
0
1
10
50
100
300
600
HRV2 2A (ng)
HeLa S10
HeLa S10 + eIF4E

E


120
100
80
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40
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0
% intact eIF4G
0
1
10
50
100
300
600
HRV2 2A (ng)
eIF4G
eIF4G +eIF4E
eIF4F

To further substantiate this conclusion, a HeLa S10 extract was preincubated with eIF4E and increasing amounts of HRV2 $2A^{pro}$ were added. Incubation with buffer alone did not generate the characteristic cleavage products (Fig. 4.3C, lane 1). Strikingly, in the presence of excess eIF4E, cleavage of the authentic eIF4G was complete with 10 ng of HRV2 $2A^{pro}$ (lanes 2 to 7), compared with more than 300 ng in the absence of exogenous eIF4E (Fig. 4.2A, lanes 2 to 7). These results further confirm the stimulatory effect of eIF4E on the cleavage of eIF4G by HRV2 $2A^{pro}$. The quantitative analysis of the above data is depicted in Fig. 4.3D and E. (Since the antibody does not recognize the cleavage products of flag-eIF4G as efficiently as the intact form, cleavage was calculated as the percentage of intact eIF4G. To ensure that the reduction in intact eIF4G is not due to accidental loss, the experiment was performed three times, with similar results.)

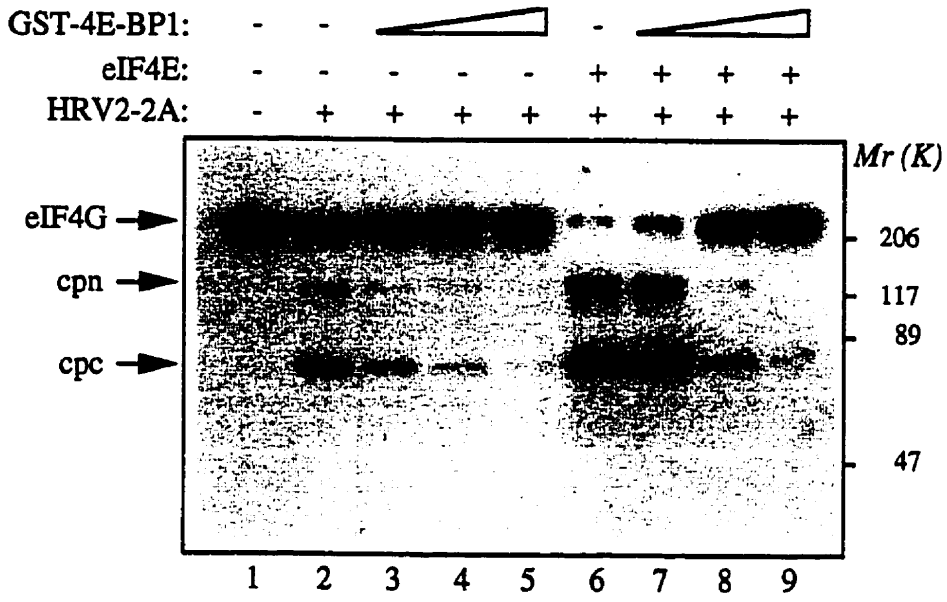
4E-BP1 reverses the stimulatory effect of eIF4E on the cleavage of eIF4G

Additional experiments were designed to demonstrate that the stimulatory effect of eIF4E is a result of complex formation with eIF4G. The activity of eIF4E is modulated by two specific binding proteins (BPs), termed 4E-BP1 and 4E-BP2 (Lin *et al.*, 1994; Pause *et al.*, 1994). 4E-BP1 competes with eIF4G for binding to eIF4E and represses cap-dependent translation (Haghighat *et al.*, 1995). It is therefore predicted that 4E-BP1 would reverse the stimulatory effect of eIF4E on $2A^{pro}$ cleavage. HeLa S10 extract was preincubated with increasing amounts of GST–4E-BP1 before the addition of HRV2 $2A^{pro}$. Buffer alone had no effect on the stability of eIF4G in the extract (Fig. 4.4A, lane 1). Addition of HRV2 $2A^{pro}$ generated the expected cleavage products (lane 2). Significantly, preincubation of the HeLa S10 extract with increasing amounts of GST–4E-BP1 rendered eIF4G more resistant to cleavage by $2A^{pro}$ (lanes 3 to 5). In addition, while exogenous eIF4E enhanced the rate of appearance of the characteristic cleavage products (compare

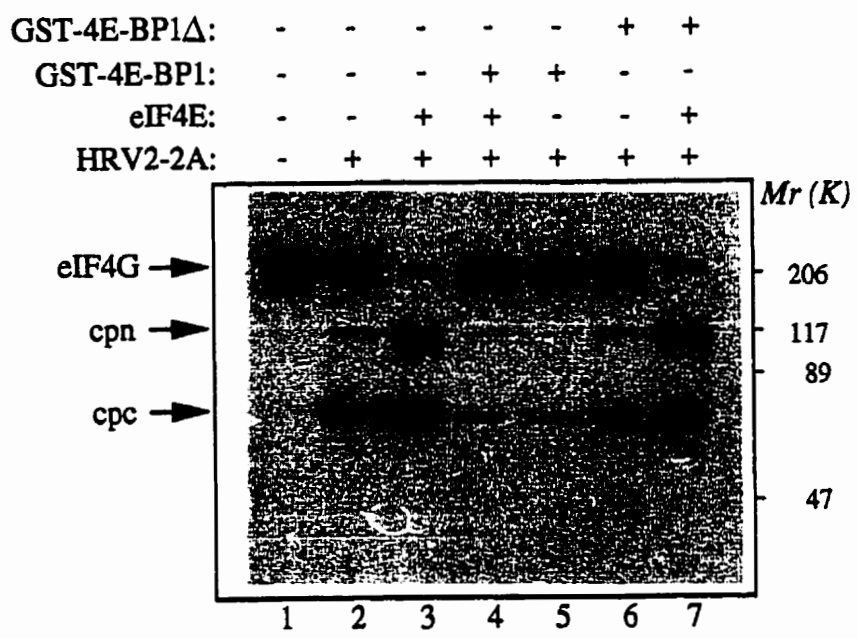
Figure 4.4. Effect of 4E-BP1 on the cleavage of eIF4G *in vitro.*

(A) HeLa S10 extract (12 μg) was preincubated with eIF4E, GST–4E-BP1, or both at 30°C for 5 min before the addition of 10 ng of HRV2 $2A^{pro}$. Lanes: 1, buffer A; 2 to 9, HRV2 $2A^{pro}$. The amounts of GST–4E-BP1 were as follows: lanes 3 to 5, 10, 50, and 100 ng, respectively; lanes 7 to 9, 10, 50, and 100 ng, respectively. Where indicated, 50 ng of eIF4E was added to the extracts. **(B)** As in panel A, except that the deletion mutant GST–4E-BP1Δ was used as a control (lanes 6 and 7). Where indicated, 50 ng of eIF4E, 50 ng of GST–4E-BP1, 50 ng GST–4E-BP1Δ, or a combination of two, was preincubated with the extracts before the addition of HRV2 $2A^{pro}$. **(C)** As in panel B, except that ~80 ng of flag-eIF4G was used. Samples were processed for Western blotting as in Materials and Methods.

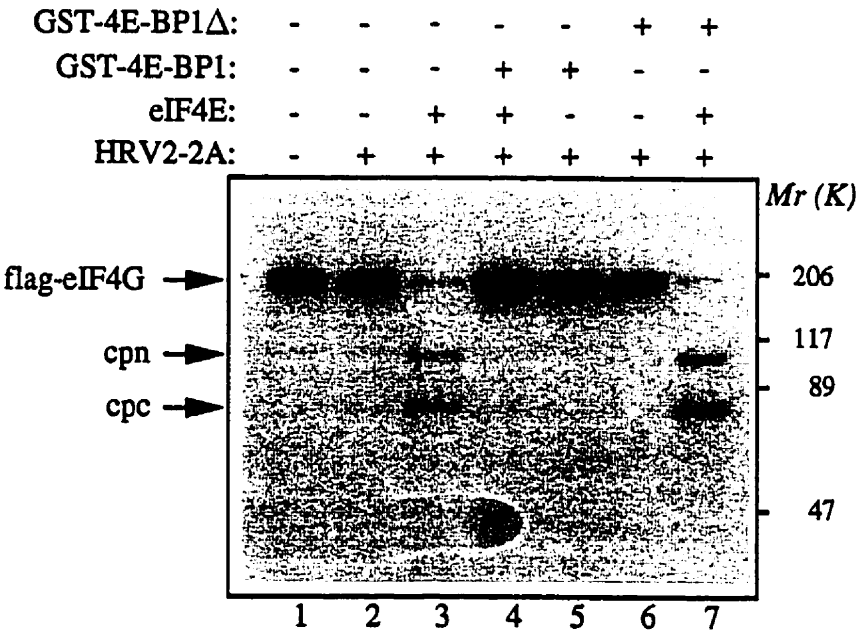
A


GST-4E-BP1: - - - -
eIF4E: - - - - - + + + +
HRV2-2A: - + + + + + + + +
eIF4G
cpn
cpc
Mr (K)
206
117
89
47
1 2 3 4 5 6 7 8 9

B


GST-4E-BP1Δ: - - - - - + +
GST-4E-BP1: - - - + + - -
eIF4E: - - + + - - +
HRV2-2A: - + + + + + +
Mr (K)
eIF4G
cpn
cpc
206
117
89
47
1 2 3 4 5 6 7

C


GST-4E-BP1Δ: - - - - - + +
GST-4E-BP1: - - - + + - -
eIF4E: - - + + - - +
HRV2-2A: - + + + + + +
Mr (K)
flag-eIF4G
cpn
cpc
206
117
89
47
1 2 3 4 5 6 7

lanes 2 and 6), preincubation of the exogenous eIF4E with increasing amounts of GST–4E-BP1 repressed the stimulatory activity of eIF4E (lanes 7 to 9).

To demonstrate the specificity of the effect of GST–4E-BP1 on the cleavage of eIF4G, a mutant of 4E-BP1 containing a deletion of the 4E binding domain (GST–4E-BP1Δ) was used. This mutant does not prevent the interaction of eIF4G with eIF4E and does not repress translation (Haghighat *et al.*, 1995; Mader *et al.*, 1995). Preincubation of GST–4E-BP1Δ with the HeLa S10 extract had no effect on the rate of eIF4G cleavage (Fig. 4.4B, compare lanes 2 and 6). Furthermore, the deletion mutant did not reverse the stimulatory effect of eIF4E (compare lanes 3 and 7), whereas wild-type GST–4E-BP1 prevented the stimulatory activity of eIF4E (lanes 3 and 4).

Similar experiments were extended to the flag-eIF4G preparation to examine the specificity of eIF4E stimulatory effect on the cleavage of flag-eIF4G by $2A^{pro}$. No cleavage products were detected in the presence of either buffer alone (Fig. 4.4C, lane 1) or small amounts of HRV2 $2A^{pro}$ (lane 2). As observed above, eIF4E significantly enhanced the cleavage rate of flag-eIF4G (lane 3). The effect of eIF4E was diminished by GST–4E-BP1 (lane 4), whereas the deletion mutant did not prevent the accelerated cleavage of eIF4G in the presence of eIF4E (lane 7). Taken together, these results and those in Fig. 4.3 demonstrate that eIF4G in a complex with eIF4E is a better substrate for HRV2 $2A^{pro}$ than is free eIF4G.

Both eIF4E and eIF4G are required for restoration of cap-dependent translation following $2A^{pro}$ treatment

The amino terminus of eIF4G is stably associated with eIF4E in picornavirus-infected cells, because it can be purified as a complex by chromatography on an m^7GDP-

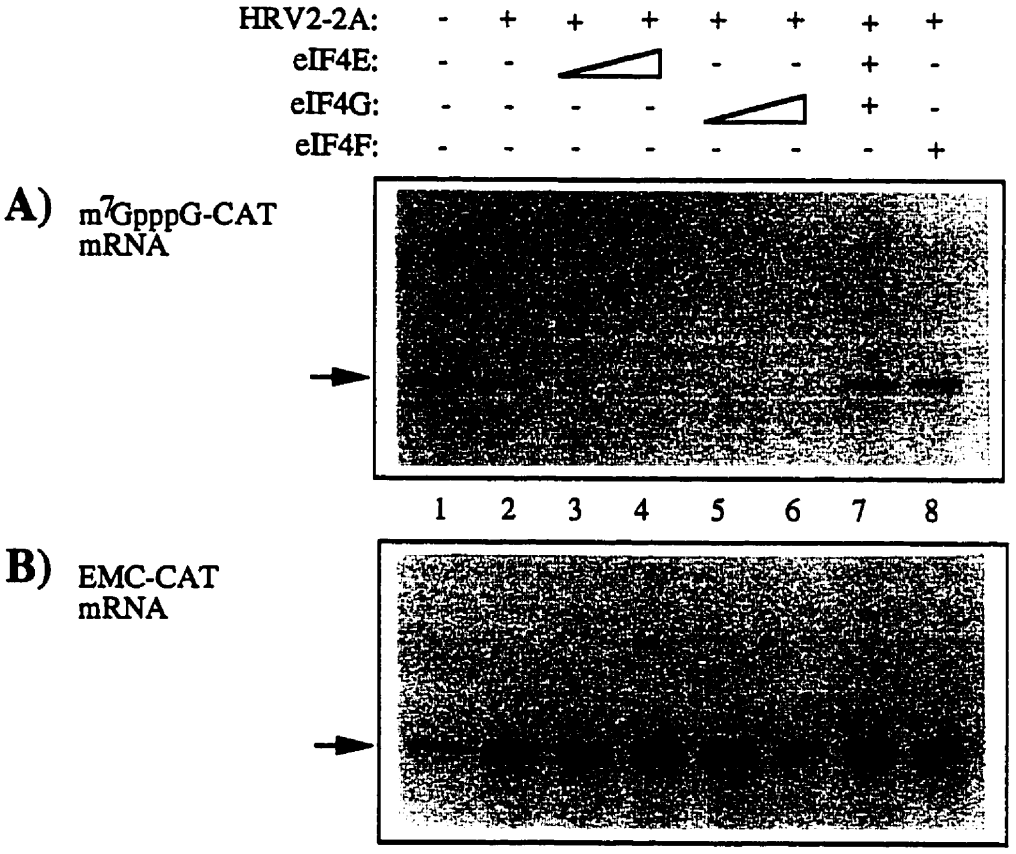
coupled agarose resin (Lee *et al.*, 1985). Consequently, eIF4E is sequestered by the amino-terminal half of eIF4G following cleavage with the picornavirus 2A or L proteinases. It is predicted, therefore, that restoration of cap-dependent translation would require the addition of both eIF4E and eIF4G. The availability of purified eIF4G allowed us to directly address this prediction. Krebs-2 ascites cell extracts were treated with an excess of HRV2 $2A^{pro}$ to ensure a rapid cleavage of eIF4G. Prior to the addition of initiation factors, extracts were treated with elastatinal to inhibit the HRV2 $2A^{pro}$ (Liebig *et al.*, 1993). Extracts were subsequently programmed with a capped transcript (m^7GpppG-CAT). The translation of CAT mRNA in control Krebs-2 ascites cell extracts was efficient (Fig. 4.5A, lane 1). Treatment of the extract with $2A^{pro}$ resulted in complete cleavage of eIF4G (data not shown) and abolished translation, as expected (lane 2). Addition of either eIF4E alone (lanes 3 and 4), or eIF4G alone (lanes 5 and 6) did not restore translation. However, addition of both eIF4E and eIF4G to a $2A^{pro}$-treated extract restored translation to almost control levels (lane 7). eIF4F, used as a positive control, also exhibited similar restoring activity, consistent with earlier results (lane 8) (Edery *et al.*, 1984; Tahara *et al.*, 1981).

To assess the cap specificity of the inhibition, duplicate samples were programmed with an mRNA which initiates translation by a cap-independent mechanism (EMC-CAT; the chloramphenical acetyltransferase (CAT) open reading frame is preceded by the IRES of encephalomyocarditis virus). Similar to the results with the capped mRNA, CAT was efficiently translated in the control extract (Fig. 4.5B, lane 1). Treatment of the extracts with $2A^{pro}$ enhanced translation (lane 2), in agreement with earlier results (Hambidge and and P, 1992; Liebig *et al.*, 1993; Ohlmann *et al.*, 1995). Addition of eIF4E and eIF4G, either alone (lanes 3 and 4 and lanes 5 and 6, respectively) or together (lane 7), had no effect on the translation of EMC-CAT mRNA in the treated extracts (the inhibition by the larger amount of eIF4G, lane 6, was not reproducible). eIF4F did not further stimulate translation, either (lane 8). Taken together, these results and those in Fig. 4.4 directly support the hypothesis that eIF4E is sequestered by the amino terminus of eIF4G

following cleavage by the HRV $2A^{pro}$. Furthermore, an intact eIF4E-eIF4G complex is required for restoration of cap-dependent translation in picornavirus-infected cells.

Figure 4.5. Restoration of cap-dependent translation in HRV2 $2A^{pro}$-treated Krebs-2 ascites cell extracts.

Extracts were treated with either buffer B (lane 1) or HRV2 $2A^{pro}$ (lanes 2 to 8) for 4 min at 30°C followed by a 10 min incubation on ice in the presence of 0.7 mM elastatinal. Extracts were then supplemented with the purified initiation factors and mRNA as indicated. **(A)** Translation of m^7GpppG-CAT mRNA. **(B)** Translation of EMC-CAT mRNA. Initiation factors were added as follows: lanes 1 and 2, buffer alone; lanes 3 and 4, 0.2 and 0.4 μg of eIF4E; lanes 5 and 6, ~0.2 and ~0.4 μg of flag-eIF4G; lane 7, 0.2 μg of eIF4E and ~0.4 μg of flag-eIF4G; lane 8, 0.75 μg of eIF4F. The position of the CAT product is indicated by an arrow to the left of each panel.


HRV2-2A: - + + + + + + +
eIF4E: - - - +
eIF4G: - - - +
eIF4F: - - - - - - - +
A) m7GpppG-CAT mRNA
1 2 3 4 5 6 7 8
B) EMC-CAT mRNA

4.4 DISCUSSION

The cleavage of eIF4G during the replication of certain picornaviruses has been well documented (Ehrenfeld, 1996). However, there has been much debate on the possible involvement of a cellular proteinase and other translation initiation factors such as eIF3 (Ehrenfeld, 1996). In addition, the use of eIF4G alone or as a complex with eIF4E has led to different conclusions with regard to the substrate requirements.

Recently, the availability of pure recombinant $2A^{pro}$ from HRV2 and coxsackievirus B4 and the leader proteinase of FMDV allowed the demonstration that they cleave eIF4G as part of the eIF4F complex without a requirement for cellular proteins (Kirchweger *et al.*, 1994; Liebig *et al.*, 1993). Moreover, cleavage of eIF4G by the two different proteinases takes place at sequences determined to be optimal for 2A cleavage (Lamphear *et al.*, 1993; Sommergruber *et al.*, 1994), further strengthening the idea of a direct mechanism of cleavage. It is also of interest in this regard that the cleavage activity in infected cells exhibits an almost identical inhibitor profile to both poliovirus and rhinovirus $2A^{pro}$. In particular, *N*-ethylmaleimide and iodoacetamide but not E64 inhibit the activity in infected cells (Sommergruber *et al.*, 1992; Wyckoff *et al.*, 1992).

In this work, we have expressed human flag-eIF4G by using a baculovirus expression system, and immunopurified the recombinant protein on an anti-flag column. The ability of flag-eIF4G to act as a substrate for HRV2 $2A^{pro}$ and to restore cap-dependent translation in HRV2 $2A^{pro}$-treated extracts was then examined. The cleavage of flag-eIF4G alone by HRV2 $2A^{pro}$ was inefficient (Fig. 4.2). However, addition of exogenous eIF4E to a molar ratio of 4:1 increased the cleavage efficiency by at least 50-fold (Fig. 4.3E). Furthermore, complete cleavage of flag-eIF4G was obtained only after isolation of the eIF4G-eIF4E complex (Fig.4.3B). These results indicate that eIF4E binding to eIF4G

changes the conformation of eIF4G, rendering it more susceptible to cleavage by $2A^{pro}$. Furthermore, the data suggest that the cleavage region functions as a hinge between the amino and carboxy terminal fragments of eIF4G. Stimulation of eIF4G cleavage in a HeLa S10 extract by eIF4E was also clearly evident (Fig. 4.3C), indicating that the stimulation is not restricted to the recombinant eIF4G that is produced in baculovirus.

The data presented here could explain the discrepancies in the literature concerning the mechanism of cleavage of eIF4G. Recombinant $2A^{pro}$ of HRV2 and CVB4 expressed in *E. coli* directly cleaved rabbit reticulocyte eIF4F to produce the characteristic cleavage products that are observed *in vivo* (Lamphear *et al.*, 1993; Sommergruber *et al.*, 1994). However, poliovirus $2A^{pro}$ has not been tested on intact eIF4F in a similar fashion. Instead, the activity of poliovirus $2A^{pro}$ has been tested on eIF4G alone that has been separated from eIF4E during the purification (Wyckoff *et al.*, 1992). Addition of eIF3 was required for cleavage of eIF4G by poliovirus $2A^{pro}$ (Wyckoff *et al.*, 1992). eIF3 preparations have been shown to contain eIF4E (Sonenberg *et al.*, 1978), and it is possible that eIF4E in the eIF3 preparation formed a complex with eIF4G to provide a preferable substrate for poliovirus $2A^{pro}$.

The data shown in this paper provide the strongest evidence yet that rhinovirus $2A^{pro}$ can cleave directly, and without intermediates, the cap-binding protein complex eIF4F. Because previous studies were performed with eIF4F purified from rabbit reticulocyte lysate, it could be argued that this complex contained some trace amounts of other initiation factors or other proteins that could promote or catalyze the proteolytic cleavage. In this study, all components tested in the reactions were recombinants except for eIF3. eIF4G can be cleaved by $2A^{pro}$. However, complex formation between eIF4G and eIF4E enhanced the reaction rate and decreased the amount of uncleaved material. These results show that the eIF4G-eIF4E complex is the preferred substrate for $2A^{pro}$.

Cleavage of eIF4G by picornavirus $2A^{pro}$ yields an amino-terminal fragment of about 50 kDa (which migrates as a set of two or three polypeptides of 110 to 130 kDa) that is bound to eIF4E and a carboxy-terminal cleavage product of about 100 kDa that binds eIF4A and eIF3 and associates with ribosomes (Lamphear *et al.*, 1995; Mader *et al.*, 1995; Rau *et al.*, 1996). Thus, cleavage of eIF4G leads to the uncoupling of the cap recognition function of eIF4E from the helicase and ribosome-binding activities of eIF4A and eIF3. Moreover, eIF4E remains sequestered by the amino terminus of eIF4G. The outcome of the cleavage of eIF4G is the specific inactivation of eIF4F function and inhibition of cap-dependent translation. In contrast, translation via internal ribosome binding to the IRES is stimulated (Liebig *et al.*, 1993; Ohlmann *et al.*, 1995). It has been suggested that the stimulation is effected by the carboxy-terminal two-thirds of eIF4G, which has a higher affinity for the IRES than does intact eIF4G (Ohlmann *et al.*, 1995). This is consistent with the idea that cleavage of eIF4G by some picornaviruses is a strategy for stimulating their IRES-driven translation.

4.5 MATERIALS AND METHODS

Cell culture, protein factors and enzymes

Spodoptera frugiperda (Sf9) insect cells were cultured in Grace medium (GIBCO-BRL) supplemented with 10% fetal calf serum, TC Yeastolate, lactalbumin hydrolysate, 50 μg of gentamicin sulfate per ml, 2.5 μg of amphotericin B (Fungizone) per ml in either T flasks or spinner flasks at 27^{o}C as described previously (Summers and Smith, 1987). Glutathione-S-Sepharose (GST) fusion proteins of HMK-4E-BP1 and HMK-4E-BPΔ1 were expressed in *E. coli* BL21 and purified as described previously (Pause *et al.*, 1994). Murine eIF4E protein was expressed in *E. coli* K38, and purified as described previously (Edery *et al.*, 1988). HRV2 2A^{pro} was expressed in *E. coli* BL21(DE3)pLysE and purified as described previously (Liebig *et al.*, 1993). m^{7}GDP column chromatography was performed as previously described (Edery *et al.*, 1988). Polyclonal antibody to eIF4G was as described previously (Aldabe *et al.*, 1996).

Generation of recombinant baculovirus

To generate a flag-HMK fusion of eIF4G in the baculovirus expression system, we first constructed a new baculovirus transfer vector, pVL1392flagHMK, derived from pVL1392 (Pharmingen). This vector contains the flag-HMK epitope (Blanar and Rutter, 1992) at an *EcoRI* site. The *EcoRI* fragment of eIF4G was excised from plasmid pSK(-)HFC1 (a kind gift from R. E. Rhoads, Yan *et al.*, 1992) and inserted blunt into the EcoRI site of pVL1392flagHMK, creating pVL1392flagHMK-eIF4G. Recombinant baculovirus was subsequently generated with the BaculoGold expression system (Pharmingen). At 5 days posttransfection, the virus released into the media was collected and amplified. The resulting high-titer virus was used for preparation of recombinant protein. The flag

epitope-tagged protein was subsequently immunopurified on a commercial anti-flag affinity column (Kodak). flag-eIF4G was eluted with flag peptide (100 μg/ml) in TEN buffer (20 mM Tris-HCl [pH 7.5], 0.1 mM EDTA, 150 mM NaCl). The eluate was then dialyzed against buffer A (20 mM Tris-HCl [pH7.5], 100 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 5% glycerol).

HRV2 2A proteinase cleavage assays

Incubation of either HeLa S10 cell extracts or purified flag-eIF4G with the HRV2 $2A^{pro}$ took place in buffer B (50 mM NaCl, 50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 5 mM dithiothreitol, 5% glycerol) in a final volume of 12 μl at 30^{o}C for 30 min. Reactions were terminated by the addition of Laemmli sample buffer. Cleavage products of eIF4G were resolved on sodium dodecyl sulfate (SDS) 8% polyacrylamide gels and analyzed by immunoblotting with a rabbit anti-eIF4G polyclonal antibody (Aldabe *et al.*, 1996).

Western Blotting

Nitrocellulose membranes were incubated for 90 min at room temperature in Tris-buffered saline containing 0.2% Tween 20 (TBST) and 5% dry milk. Next, membranes were incubated with rabbit anti-eIF4G polyclonal antibody overnight at 4^{o}C. After extensive washing with TBST, the membranes were incubated with ^{125}I-protein A for 2 h, washed with TBST, and exposed to Dupont reflection film.

In vitro Transcription and Translation

The plasmids pSP64-CAT and pEMC-CAT were linearized with *BamHI*. Transcription was performed with SP6 RNA polymerase as previously described (Pelletier

and Sonenberg, 1985). Capped transcripts were obtained in a reaction mixture containing 50 μM GTP and 500 μM m^7GpppG. The integrity of RNAs was analyzed on a formaldehyde-agarose gel and amounts were quantitated by spectrophotometry. Translations were performed in Krebs-2 ascites cell extracts as described previously (Svitkin and Agol, 1978) in a final volume of 14 μl. Where indicated, extracts were treated with HRV2 $2A^{pro}$ or buffer B for 4 min at 30°C and then incubated for 10 min on ice in the presence of 0.7 mM elastatinal (Sigma). Initiation factors were then added, followed by the mRNA (200 ng) and other translation ingredients. Translation reaction mixtures were incubated at 30°C for 90 min and subsequently analyzed by SDS-polyacrylamide gel electropheresis. Gels were fixed, treated with En^3Hance and processed for autoradiography.

4.6 ACKNOWLEDGMENTS

We thank Greg Cosentino, Andrew Craig, and Nathalie Méthot for critical reading of the manuscript and Martin Langer for preparing recombinant HRV2 $2A^{pro}$. This work was supported by a grant from the Medical Research Council of Canada to N.S., grant P-09885 from the Austrian Science Foundation (to E.K.), and a grant from the Josefine Hirtl Foundation for medical research (to T.S.).

CHAPTER V

General Discussion

5.1 Cap-dependent mRNA binding to ribosomes in eukaryotes

In eukaryotes, translational regulation is predominantly exerted at the level of initiation, which is usually the rate-limiting step in protein synthesis (Hershey, 1991). One particular step of the initiation pathway which is a frequent target for translational regulation is the initial binding of the 43S preinitiation complex to mRNA. The binding of mRNA to ribosomes has the potential for controlling both the global rate of translation, as well as the selective regulation of different mRNAs in response to various stimuli. In eukaryotes, where most cellular mRNAs (except organellar) are posttranscriptionally modified to harbour a cap structure, the initiating 40S ribosomal subunit binds at the 5' end of the mRNA before migrating to the appropriate initiation codon.

The mRNA binding step is catalyzed by several initiation factors, including eIF4F and eIF4B. The concerted action of these initiation factors is believed to direct the ribosome to the 5' end of the mRNA, and unwind secondary structures that would otherwise impede ribosome migration along the mRNA. Although the importance of these initiation factors in effecting the mRNA-ribosome interaction is well established, detailed consideration of the mechanism of this step has revealed uncertainties *vis à vis* the exact order of association and dissociation events involved in this complex process. Expectedly, several alternative models for the mechanism of mRNA binding to ribosomes have been reported. The discrepancies between the models revolve around a key question of whether the different components of eIF4F bind to the mRNA independently or they preassemble into eIF4F before interacting with mRNA.

All components of eIF4F complex can be cross-linked to capped mRNAs. The specific cap binding activity of eIF4F is mediated by its eIF4E subunit. eIF4E is isolated in both a free form and in a complex with the other eIF4F subunits, eIF4G (p220) and eIF4A.

eIF4E can interact with eIF4G *in vitro* in the absence of RNA, or any posttranslational modification of the proteins (chapters II and III; Mader *et al.*, 1995). A 49-amino-acid hydrophobic region of eIF4G, located in the amino terminus, has been shown to be both necessary and sufficient for interaction with eIF4E (Mader *et al.*, 1995). Mutational analysis in this region has identified a 12-amino-acid region conserved in mammals and yeast. eIF4E binds specifically to the cap structure *in vitro* by itself. However, the cross-linking of eIF4E alone to the cap structure is very inefficient (chapter III and references therein). The finding that the interaction of eIF4E with the cap structure is significantly enhanced when in a complex with eIF4G (chapter III) provides additional support for the model which posits that a preassembled eIF4F initially interacts with the mRNA cap structure.

It is important to note that much of the evidence for the working model of mRNA-ribosome binding is primarily based on *in vitro* studies with purified components. However, the assays tend to be performed in the absence of some key initiation factors such as eIF3, or even ribosomal subunits. Indeed, these components play influential roles *in vivo*. In mammalian cells, eIF3 is a multimeric complex of at least eight polypeptides that exhibits pleiotropic effects. Although it has long been determined that eIF3 stabilizes 43S preinitiation complexes, and to be essential for binding of the latter complex to mRNA, the molecular basis of its role in effecting mRNA-ribosome interactions remains unclear. eIF3 interacts with mRNA, presumably through its p66 subunit (Naranda *et al.*, 1994). A potentially important interaction between eIF3 and the carboxy terminal half of eIF4G has also been suggested, which could juxtapose the 40S ribosome next to 5' end of mRNA (Lamphear *et al.*, 1995). An eIF3•eIF4F complex can indeed be isolated from cell extracts. This eIF3•eIF4F interaction is disrupted under conditions of high salt concentration. Despite these observations, it remains unclear which subunit(s) of eIF3 mediates the interaction with eIF4F, and whether or not this interaction is direct or RNA

mediated. Detailed analysis of this association could yield important insights into the mechanism of mRNA binding to ribosomes.

Equally important is the recent demonstration that eIF4B interacts directly with the p170 subunit of eIF3 (Methot *et al.*, 1996). eIF4B plays an important role in mRNA binding to ribosomes. It interacts with the mRNA cap structure in an eIF4F-dependent fashion (chapter III), and appears to form an RNA-mediated complex with eIF4F (Jaramillo *et al.*, 1991). An attractive scenario emerges where an eIF4B•eIF4F complex in the 5' UTR of mRNAs promotes the binding of ribosomes to the mRNA through a direct interaction between eIF3 and eIF4B, and possibly eIF4G.

Furthermore, recruitment of poly(A)+ mRNA to ribosomes in eukaryotes is mediated by the poly A binding protein, Pab1p. Recent experiments have demonstrated an RNA-dependent interaction between Pab1p and the yeast homologue of eIF4G, p150 (Tarun and Sachs, 1996). On these lines, it has been suggested that eIF4G could integrate the functions of the 5' and 3' ends of the mRNA. Such an interaction has not been detected in mammalian systems (A. Craig, unpublished). However, proteins that specifically interact with mammalian Pab1p have been identified, which may mediate similar functions (A. Craig, unpublished data). Characterization of the molecular interaction between these proteins are currently under research, and could offer invaluable information on the mechanisms of translational regulation in eukaryotes.

5.2 Regulation of eIF4F complex assembly

In mammals, eIF4E has been the focus of intensive study in recent years. It is generally believed that the availability of eIF4E limits translation in eukaryotic cells. eIF4E is present in low abundance relative to other initiation factors and ribosomes, which renders it an excellent candidate for the regulation of translation (Sonenberg, 1996).

Initially, artificial manipulation of eIF4E levels demonstrated dramatic effects on protein synthesis and cell growth. Overexpression of eIF4E in several cell lines has led to aberrant growth control, and the ability to induce tumor formation in nude mice. Additionally, depletion of eIF4E in HeLa cells reduced drastically protein synthesis to very low levels. One may reason that these observations are consistent for a factor that is implicated in the initial step of mRNA recruitment to ribosomes. The cellular concentrations of eIF4E can also be physiologically regulated under various conditions. For example, expression of eIF4E mRNA is upregulated in cells transformed with c-myc (De Benedetti *et al.*, 1994), and during T-cell activation, suggesting that the amount of eIF4E is modulated with respect to the proliferation capacity of the cell.

Recently, we have revealed a novel mechanism for regulating the availability of eIF4E in eukaryotic cells (chapter II). Two polypeptides termed 4E-binding protein (BP)-1 and 2, have been identified on the basis of their specific interaction with eIF4E. The interaction of eIF4E and 4E-BP1 is relatively strong with a 1:1 stoichiometry. Overexpression of 4E-BPs inhibited specifically cap-dependent translation *in vivo* and *in vitro* (Pause *et al.*, 1994a). This led to the proposal that 4E-BPs sequester eIF4E and prevent eIF4F complex formation. Consistent with this hypothesis, experiments demonstrated that 4E-BP1 exerted its inhibitory effect by preventing mRNA-ribosome interactions. Furthermore, using purified recombinant components, we provided evidence for a competitive scenario where the binding of eIF4G and 4E-BP1 to eIF4E is mutually exclusive. Interestingly, sequence comparisons between 4E-BPs and the 49-aa 4E-binding region in eIF4G revealed a similar sequence motif (Mader *et al.*, 1995). Deletion of the 9-aa conserved motif in the 4E-BPs, or simply a double point mutation of two conserved leucines abrogated the interaction of 4E-BP1 and 4E-BP2 with eIF4E (Mader *et al.*, 1995). Consistently, removal of the interaction motif of 4E-BP1 abolished the competition with

eIF4G for binding to eIF4E, and did not inhibit translation. These results (data presented in chapter II) have subsequently been confirmed by other groups using co-immunoprecipitation to study molecular interactions between eIF4G, eIF4E, and 4E-BP1 in reticulocyte lysate (Rau *et al.*, 1996).

In responsive cells, the activity of 4E-BP1 is regulated through its reversible phosphorylation. 4E-BP1 has a predicted molecular weight of 12 kDa. Typically, however, several forms of the protein migrate anomalously with a M_r of ~20 kDa when subjected to SDS-PAGE. Phosphorylation of 4E-BP1 in response to insulin and growth factors decreases its electrophoretic mobility. The underphosphorylated forms of 4E-BP1 possess a high affinity for eIF4E, whereas none of the more highly phosphorylated species copurify with eIF4E. Therefore, phosphorylation of 4E-BP1 disrupts its association with eIF4E, liberating eIF4E to bind eIF4G, thus forming a functional mRNA cap binding complex. As the dependence on the availability of eIF4E correlates strongly with the degree of secondary structure in the 5'UTR, one may predict that the subset of mRNAs with excessively structured 5'UTRs may benefit more significantly form the consequences of 4E-BP1 phosphorylation. For example, while global translational rates are only marginally increased in response to insulin treatment, translation of ornithine decarboxylase mRNA, which possesses a highly structured 5'UTR, is enhanced 30-fold (Manzella *et al.*, 1991). Phosphorylation of 4E-BP1 and release of eIF4E may explain the increase in translational rates in response to insulin. Indeed, it has been demonstrated that more active eIF4F facilitates expression of mRNAs that contain excessive secondary structure in their 5' non-coding region that are otherwise discriminated against by the translational apparatus (Koromilas *et al.*, 1992). Therefore, characterization of the relationship between secondary structure and 4E-BP1 would be of great interest.

It is clear that identification of 4E-BPs, and the elucidation of their function has greatly fortified our understanding of the mechanism by which insulin and growth factors elicit a rapid increase in protein synthesis. As such, much effort has been directed towards the characterization of the signal transduction pathways, and identification of the kinase(s) responsible for the modulation of 4E-BP1 activity. The several species of 4E-BP1 detected by SDS-PAGE is indicative of multiple phosphorylation sites. The major site of phosphorylation has been assigned to Ser-64 (Lin *et al.*, 1994). The ERK1 and ERK2 isoforms of *mitogen*-activated protein (MAP) kinase were initially shown to copurify with the insulin-stimulated activity that phosphorylated 4E-BP1 on Ser-64. Consistent with a role for MAP kinase in regulating the activity of 4E-BP1, numerous stimulators of cell growth, such as insulin, increase MAP kinase activity. Recently, it has been shown that prolonged incubation of MAP kinase with 4E-BP1 *in vitro* also phosphorylated Thr-36, Thr-45, Thr-69, and Ser-82 to various degrees (Fadden *et al.*, 1997). With the identification of these novel phosphorylation sites, the relative contribution of each site to the interaction between 4E-BP1 and eIF4E has been addressed. Based on these *in vitro* experiments, Ser-64 phosphorylation is neither necessary nor sufficient for inhibiting the association of 4E-BP1 with eIF4E. In addition, phosphorylated Thr-36 retains its binding affinity for eIF4E. From this study, Thr-45 and Thr-69 have emerged as candidates for the important regulatory sites on 4E-BP1. It appears, therefore, that several sites may contribute to the regulation of 4E-BP1 interaction with eIF4E.

The legitimate involvement of MAP kinases in the cascade of events that lead to the modulation of 4E-BP1 activity has recently been challenged, however, based on several observations. While 4E-BP1 remains phosphorylated in the continued presence of insulin, MAP kinase activity tends to decline to pre-treatment levels. Recently, it was demonstrated that the immunosuppressant drug rapamycin, which does not inhibit MAP kinase activity, prevented the phosphorylation of 4E-BP1 and, in parallel, $p70^{s6k}$ (Beretta

et al., 1996). Rapamycin inhibits the function of FRAP, a protein kinase that regulates $p70^{s6k}$ activation. Although neither $p70^{s6k}$ nor FRAP phosphorylate 4E-BP1 directly *in vitro* (Brown *et al.*, 1995), the FRAP rapamycin-sensitive pathway has been implicated in the control of 4E-BP1 phosphorylation (Von Manteuffel *et al.*, 1996). As such, although activation of a phosphatase in this signaling pathway cannot be ruled out, the identity of the potential kinase that phosphorylates 4E-BP1 remains unknown.

Reversible phosphorylation in response to numerous growth factors, mitogens and hormones appears to also play a role in the regulation of eIF4E activity. Phosphorylation of eIF4E appears to enhance its activity, since only phosphorylated eIF4E associates with 48S complexes (Joshi-Barve *et al.*, 1990). Phosphorylation of eIF4E following induction corresponds to the recovery of increased levels of eIF4E as part of the eIF4F complex. In addition, the phosphorylated form of eIF4E interacts with the mRNA cap structure with higher affinity relative to its unphosphorylated form. Recently the major phosphorylation site has been conclusively re-assigned to Ser-209 (Whalen *et al.*, 1996). However, the physiological kinase of eIF4E remains unidentified. Although PKC phosphorylates eIF4E at Ser-209 *in vitro*, there is evidence for and against the involvement of this kinase *in vivo*. Recently, it has been shown that Mnk1, a member of a new subfamily of murine serine/threonine kinases, can also phosphorylate eIF4E at Ser-209 *in vitro* (Waskiewicz *et al.*, 1997). Phosphorylation of eIF4E by PKC is more efficient when the protein is in the eIF4F complex (Tuazon *et al.*, 1990). Furthermore, although an efficient and seemingly stable interaction between recombinant eIF4E and eIF4G is observed *in vitro* (chapter II), phosphorylation of the eIF4G subunit of eIF4F by PKC *in vitro* has been reported to increase its affinity for eIF4E and stabilize this interaction. What is interesting, however, is the finding that *in vitro* phosphorylation of eIF4E by PKC is greatly diminished when eIF4E

is in a complex with 4E-BP1. These results suggest a temporal relationship between eIF4E•4E-BP1 complex formation and eIF4E phosphorylation.

Based on the observations described above, we have proposed a model (Figure 5.1) which outlines the cascade of events that emanate from growth factors at the cell surface and leads to regulated ribosome binding to capped mRNAs in the cytoplasm. In resting cells it is predicted that a large portion of eIF4E is restrained by 4E-BP1. eIF4E in a complex with 4E-BP1 is unphosphorylated and interacts weakly with the mRNA cap structure (chapter III). Stimulation of cells by growth factors and hormones results in the phosphorylation of 4E-BP1 (1) and hence the release of eIF4E (2). eIF4E can then associate with eIF4G and eIF4A to form an active cap-binding protein complex (3), which can bind efficiently to mRNA cap structure (4). It is important to highlight the dual role played by eIF4G in the mRNA binding process. eIF4G significantly enhances the binding of eIF4E to the mRNA 5' cap structure (chapter III). Furthermore, interaction of eIF4G with eIF4E appears to render the latter a better substrate for phosphorylation, which in turn further enhances the binding of eIF4E to the cap structure, and increases translation (5). Therefore, it appears that regulation of eIF4F complex assembly depends strongly on the concentrations and relative affinities of eIF4E, eIF4G and 4E-BP1.

To accurately assess the contribution of 4E-BPs to the regulation of eIF4F complex assembly in cell, it will be pertinent to quantitate the relative concentrations of 4E-BPs and eIF4E in various cell lines. The pressing importance of the answers of this assessment is in light of data that has provided eIF4E:4E-BP1 ratios as varied as 1:20 to 1:1 (Pause *et al.*, 1994a; Rau *et al.*, 1996). Another important issue is the ratio of eIF4E•4E-BP1 complex to eIF4F *in vivo* under different conditions. Although it has been demonstrated that eIF4F precludes the association of 4E-BP1 *in vivo* (Haghighat *et al.*, 1995), the

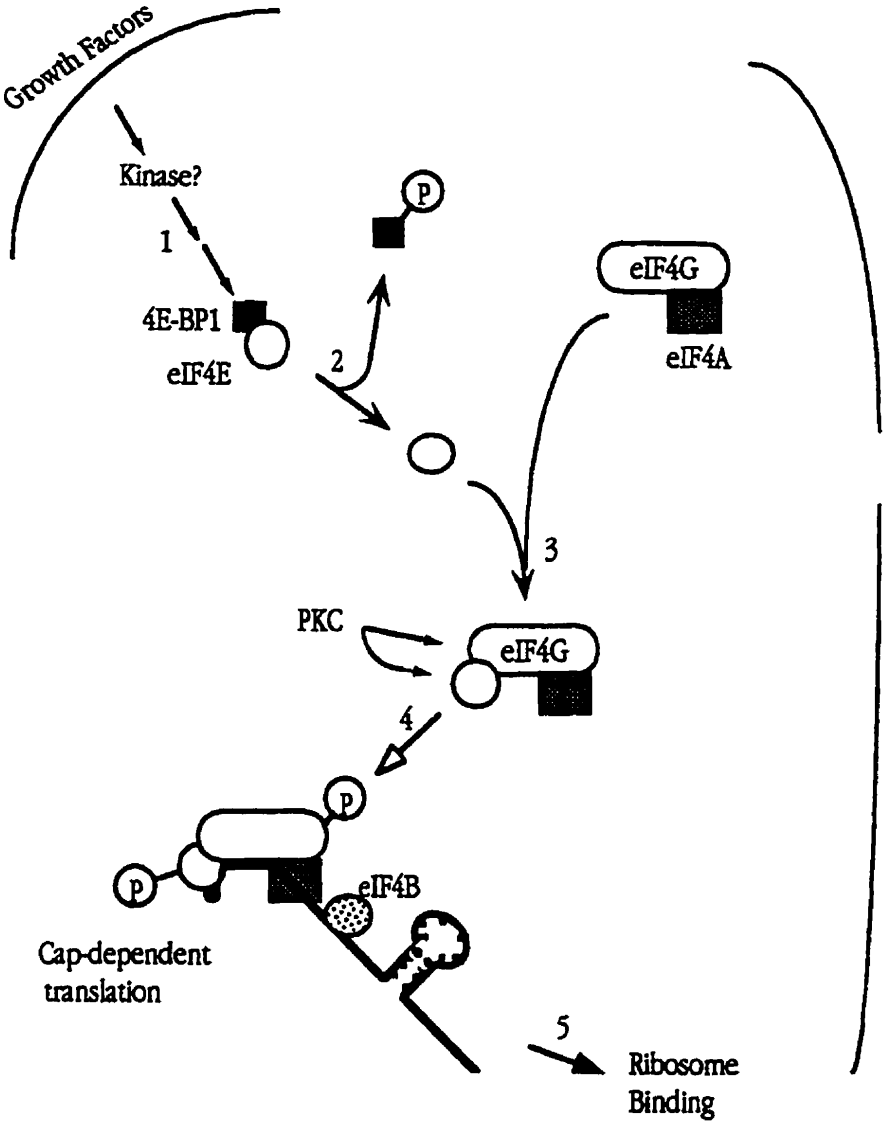


Figure 5.1. Model for the cascade of events leading to cap-dependent mRNA binding to ribosomes in eukaryotes.

redistribution of eIF4E between an inactive eIF4E•4E-BP1 complex and an active eIF4F cap-binding complex in response to various physiological stimuli needs to be illustrated.

Emerging evidence has rendered this simplistic view of eIF4F complex formation more complicated. For example, mammalian eIF4G, which migrates as a cluster of polypeptides between 200 -220 kDa., may be encoded by several genes (N. Sonenberg, unpublished). Similarly, eIF4AI and eIF4AII are the product of two closely related genes in mammals. Recently, a closely related form of mammalian eIF4E has also been identified (E. Rom, personal communication). Furthermore, additional members of the 4E-binding protein family (e.g.: 4E-BPII) are widely distributed among tissues and cell types. A similar scenario exists in the yeast *S. cerevisiae* where the homologues of eIF4G are encoded by two genes TIF4631 and TIF4632 (Goyer *et al.*, 1993). In plants the situation appears even more complex as two different eIF4F complexes have been identified. One is a complex of p220 and p26, which are homologues of mammalian eIF4G and eIF4E, respectively. The other complex, called eIF(iso)4F, contains p82 and p28 (Browning *et al.*, 1990). The relative contribution of each of these gene products to translation initiation and ribosome binding remains uncertain.

5.3 Cap-independent initiation of translation

The radical mechanism of internal initiation of translation constitutes one of many deviations from the more orthodox 5' cap-dependent binding of ribosomes to mRNA, as evident in eukaryotes. Internal initiation is characterized by the fact that a minimal element, known as "Internal ribosome entry segment" (IRES), promotes direct binding of initiation-competent ribosomes to internal sites that are far removed from the 5' end of the mRNA. Picornaviruses are the paradigm for this mode of translation (section 1.5.1.1).

Despite the mechanistic differences between the alternative modes of ribosome binding, remarkably little difference exists between the requirements for canonical initiation factors to sustain internal initiation compared to cap-dependent initiation. Surprisingly, this conclusion also holds for eIF4F.

As described in Chapter IV, following infection of cells with enteroviruses, rhinoviruses, and FMDV, the intact eIF4F holoenzyme complex becomes the target for direct cleavage by viral proteases. Targeting the active cap-binding protein complex, eIF4F, is a cunning strategy adopted by these members which leads to a dramatic and rapid shut-off of cellular protein synthesis. Under these conditions, a modified eIF4F is sufficient to sustain efficient IRES-mediated translation . In fact, the C-terminal cleavage product of eIF4G with its associated eIF4A, appears to drive cap-independent translation more effectively than intact eIF4F (chapter IV, and references therein). Therefore, although an intact eIF4F may not be required for the translation of certain picornaviral mRNAs, the helicase activity of eIF4A is essential or important in this process. Indeed, studies with dominant negative mutants of eIF4A has provided evidence in support of this conclusion (Pause *et al.*, 1994b). These mutants inhibit the translation of all mRNAs. eIF4A recycles through the eIF4F complex, and its helicase function is significantly enhanced as a subunit of eIF4F (Rozen *et al.*, 1990; Pause *et al.*, 1994b). It is thought that eIF4G functions to stabilize eIF4A in the vicinity of mRNA and enhance its unwinding activity. Consistent with this belief, eIF4A exhibits very weak RNA binding activity, whereas eIF4G can interacts avidly with RNA (Pestova *et al.*, 1996). The inhibitory effect of eIF4A dominant negative mutants seems to result from stable interaction with eIF4G[1]. Interestingly, these eIF4A mutants also inhibit internal initiation of translation in poliovirus-infected HeLa cell extracts, where the eIF4G component of eIF4F is cleaved, lending support to a positive role mediated by the C-terminal cleavage product of eIF4G[1] .

In contrast to the foregoing, cardioviruses do not induce cleavage of eIF4G. Recent evidence suggests that eIF4F function might be modulated in EMCV-infected cells. There is a temporal correlation between 4E-BP1 dephosphorylation and inhibition of cellular protein synthesis in EMCV-infected Krebs-II ascites cells (Gingras *et al.*, 1996). Thus, dephosphorylation of 4E-BP1, and the restraining of eIF4E that ensues, could potentially diminish specifically cellular cap-dependent translation in infected cells. Therefore, it appears that modification of eIF4F complex assembly could also constitute one operative mechanism of shut-off following infection by cardioviruses. It is not clear to what extent this mechanism contributes to the shut-off of host protein synthesis. As such, it remains uncertain whether or not intact eIF4F is still present in EMCV-infected cells at the time of shut-off.

It is interesting to note that during infection by cardioviruses, the modified eIF4F does not seem to enhance IRES-driven translation of the virus. Meanwhile, induced cleavage of eIF4G (e.g. in $2A^{pro}$-treated extracts; see figure 4.5) results in the stimulation of EMCV IRES function. Overexpression of 4E-BP1 inhibits cap-dependent translation. Under these conditions, only a marginal effect on EMCV IRES-mediated translation (which has thus far been interpreted as insignificant) has been observed. These observations may indeed indicate that intact eIF4G that is dissociated from eIF4E exhibits reduced activity as compared to an eIF4G•eIF4E complex. It would follow that complex formation with eIF4E, or its cleavage by picornavirus proteases, provides a more active form of the protein, with the cleavage product being active only in cap-independent initiation of translation. In support of this hypothesis, exogenous recombinant eIF4E stimulates both cap-dependent and cap-independent translation[1] . It has also been reported that the C-terminal cleavage fragment of eIF4G enhances translation of uncapped mRNAs (Ohlmann *et al.*, 1997; A. Haghighat, unpublished data). In addition, the enhancing activity of the recombinant C-terminal fragment of eIF4G is more pronounced as compared to intact

recombinant eIF4G[1] . As suggested above, stimulation of eIF4A helicase activity by eIF4G may explain these observations. Reconstitution of eIF4F helicase activity (intact and modified forms) is currently under research, and may illustrate in more detail the role of the various subunits of eIF4F in promoting mRNA binding to ribosomes.

Given the efficiency of the scanning mechinsm in eukaryotes, the reasons for the evolution of internal initiation remain an enigma. This is specially true in the case of cellular mRNAs that have an IRES. Obviously, these mRNAs are expected to be translated efficiently under conditions where the function of eIF4F is impaired. The advantages that accrue from a more efficient translation of such cellular mRNAs (BiP, fibroblast growth factor-2) under conditions such as infection or heat shock are obscure. Perhaps a larger number of such examples need to be discovered before a regulatory pattern will be discernable. This challenge, combined with further studies on viral systems will continue to provide opportunities to probe translational control mechanisms in eukaryotes.

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[1] (A. Haghighat et al., unpublished results).

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ORIGINAL CONTRIBUTION TO KNOWLEDGE

Initiation of translation is mediated by the multi-subunit initiation factor eIF4F, which binds the cap structure via its eIF4E subunit and facilitates the binding of mRNA to ribosomes. Collectively, the studies presented in this thesis reveal novel mechanisms of regulating eIF4F function.

Chapter II) We have studied the mechanism by which 4E-BP1 inhibits translation. We show that 4E-BP1 inhibits 48S 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 compete with p220, and do not inhibit 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.

Chapter III) We used recombinant proteins to reconstitute the cap recognition activity of eIF4F *in vitro*. We demonstrate that the interaction of eIF4E with the mRNA 5' cap structure is dramatically enhanced by eIF4G, as determined by a UV-induced cross-linking assay. Furthermore, assembly of the eIF4F complex at the cap structure, as well as ATP hydrolysis, is shown to be a requisite for the cross-linking of another initiation factor, eIF4B, to the cap structure. In addition, the stimulatory effect of eIF4G on the cap recognition of eIF4E is inhibited by the translational repressor, 4E-BP1. These results suggest that eIF4E initially interacts with the mRNA cap structure as part of the eIF4F complex.

Chapter IV) The 2A proteinases ($2A^{pro}$) of certain picornaviruses induce the cleavage of the eIF4G subunit of the cap binding protein complex, eIF4F. We report that eIF4G alone is a relatively poor substrate for cleavage by the rhinovirus $2A^{pro}$. However, an eIF4G-eIF4E complex is cleaved efficiently by the $2A^{pro}$, suggesting that eIF4F is a preferred substrate for cleavage by rhinovirus $2A^{pro}$. Furthermore, $2A^{pro}$ drastically reduced the translation of a capped mRNA. An eIF4G-eIF4E complex, but not eIF4G alone, was required to restore translation.