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The characterization of translation initiation factor eIF4E in *Drosophila melanogaster*

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Abstract

Protein synthesis is one of the multiple levels at which gene expression is regulated. The rate-limiting steps of protein synthesis occur during initiation. The binding of the ribosome to the mRNA in translation initiation is catalyzed by the proteins of the eukaryotic initiation factor 4 (eIF4) group. In mammals, the mRNA cap-binding protein eIF4E is present in limiting levels and is regulated by several mechanisms. This thesis examines the regulation of eIF4E during the development of the genetically tractable organism, *Drosophila melanogaster*. A *Drosophila eIF4E* gene was cloned, its position was mapped cytologically, and this gene was shown to encode two cap-binding protein isoforms via alternative splicing. Antisera specific to the eIF4E isoforms were raised and purified to characterize the expression of *eIF4E* during development. Several mutant alleles of *eIF4E* were identified and demonstrate that this gene is essential for the viability of *Drosophila*. Furthermore, *eIF4E* mutants arrest in growth during early larval stages. The lethality and growth defects of *eIF4E* mutant alleles were rescued by a transgene containing a wild-type copy of *eIF4E* expressed under the control of its endogenous promoter. Ser251 of *Drosophila* eIF4E is in a sequence context identical to site on which eIF4E is phosphorylated in response to extracellular stimuli in other organisms. To examine the biological significance of the phosphorylation of eIF4E, transgenic flies were generated in which Ser251 was mutated. We show that eIF4E from Ser251 mutant lines cannot incorporate labeled orthophosphate. Interestingly, flies in which the only source of eIF4E is non-phosphorylatable are semi-lethal and escapers are small in size. These results are evidence that Ser251 of eIF4E is required for the normal growth of a multicellular organism.

Résumé

L'expression des gènes est régulé à plusieurs niveaux, incluant la synthèse des protéines. Les étapes limitantes de la synthèse des protéines se manifestent durant l'inititiation. Pendent l'initiation, l'association du ribosome à l'ARN messager est catalyzé par les facteurs d'initiation du groupe eIF4 (eukaryotic initiation factor 4). Cette thèse examine chez *Drosophila melanogaster* la régulation de la protéine eIF4E, qui se lien à la structure coiffe de l'ARN messager. Un gène codant pour un homologue de eIF4E chez la drosophile fut cloné et sa position génétique fut cartographiée. Ce gène produit deux isoformes, eIF4EI et eIF4EII, qui sont codés par différentes formes d'ARN messager qui proviennent du même gène. Des anticorps contre les isoformes d'eIF4E furent générés et purifiés pour charactériser l'expression de ces protéines pendant le développement de la drosophile. Plusieurs allèles mutantes d'*eIF4E* furent identifiées et démontrent que ce gène est essentiel à la survie de la drosophile. En plus, les mouches mutantes pour *eIF4E* sont arrêtés dans leur croissance au stage larvaire. Un transgene composé d'une copie sauvage d'*eIF4E* sous contrôle de son promoteur endogène est capable de redonner la viabilité et d'éliminer la déficiance dans la croissance des allèles d'*eIF4E*. Ser251 chez eIF4E de drosophile est dans un contexte de sequence identique au site phosphorylé en réponse de signaux extracellulaires chez eIF4E dans les mammifères. Pour examiner le rôle biologique de la phosphorylation d'eIF4E, des lignées transgéniques furent générés dans lesquelles l'acide aminé clef fut muté. Le eIF4E dans ces lignés ne peut pas incorporer de phosphate radioactif. En plus, les mouches mutantes, dans lesquelles la seule source d'eIF4E est une forme qui ne peut pas être phosphorylée, sont semi-léthales et les survivants ont une petite taille. Ces résultats démontrent que Ser251 d'eIF4E est requis pour la croissance normale d'un organisme multi-cellulaire.

Acknowledgements

So Moon-Watcher stared at the Monolith with unblinking eyes, while his brain lay open to its still uncertain manipulations. Often he felt nausea, but always he felt hunger.
-Arthur C. Clarke, "2001: A Space Odyssey"

It is somewhat fitting that I am submitting this thesis in 2001 since during my training I have often felt like the Man-Ape, Moon-Watcher, being imparted wisdom and slowly adapting into a more advanced being. I have had the chance to receive excellent supervision and would like to extend my gratitude to Professor Paul Lasko for his unending support, encouragement, and funding this project. Also, I am grateful that Paul encouraged my participation in numerous scientific conferences and defrayed the involved costs. I have had the occasion to grow immensely, both scientifically and personally, during the years spent in the Lasko lab and would like to take this opportunity to thank all the individuals involved.

This project was part of a collaborative effort with Mathieu Miron, from Nahum Sonenberg's laboratory in the Department of Biochemistry. I learned a lot from the scientific discussions and experiments we performed together. I am also grateful for the unpublished results, reagents, and equipment, which they shared.

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Like Moon-Watcher, I have also felt the equivalent of "nausea" at several occasions during the course of this project and I am indebted to the people that helped maintain my sanity. Specifically, I thank Mathieu for the late-night Starcraft games over the internet, Sylvia and Rashmi for the coffee (or scotch) breaks (and numerous enjoyable discussions about music), Aaron Windsor for being a good listener (but am not too grateful for his beating me at pool all the time and I still believe that tortellini is a type of dumpling), Travis for being a great lunch partner over these last couple years, and Andrew Swan for being a good friend, listener, and an unparalleled line-mate on the departmental hockey team.

I must also ask forgiveness to my parents, Sophie, Dan, Marc, and all of my friends and relatives outside of Montréal for neglecting them over the years and I hope to make it up to them in the future. Last but far from least, I would like to thank Tina Hueftlein for her patience through the difficult times and for her unending encouragement.

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Finally, a special mention must be made for all the trees that have sacrificed their lives for the production of this thesis.

Preface

The following statements have been included in accordance with thesis specifications outlined in "Guidelines for thesis preparation".

Candidates have the option of including as part of the thesis, the text of a paper(s) submitted or to be submitted for publication, or the clearly duplicated text of a published paper(s). These texts must be bound as an integral part of the thesis.

If this option is chosen, connecting texts that provide logical bridges between the different papers are mandatory. The thesis must be written in such a way that it is more than a mere collection of manuscripts; in other words, results of a series of papers must be integrated.

The thesis must still conform to all other requirements of the "Guidelines for Thesis Preparation". The thesis must include: A Table of Contents, an abstract in English and French, an introduction which clearly states the rationale and objectives of the study, a comprehensive review of the literature, a final conclusion and summary, and a thorough bibliography or reference list.

Additional material must be provided where appropriate (e.g. in appendices) and in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research in the thesis.

In the case of manuscripts co-authored by the candidate and others, **the candidate is required to make explicit statement in the thesis as to who contributed to such work and to what extent.** Supervisors must attest to the accuracy of such statements at the doctoral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make perfectly clear the responsibilities of all the authors and co-authors of the papers. **Under no circumstance can a co-author of such a thesis serve as an examiner for that thesis.**

Chapter 2 has been published in the following journal:

C. Lavoie, P.E.D. Lachance, N. Sonenberg, and P. Lasko (1996) Alternatively spliced transcripts from the *Drosophila eIF4E* gene produce two different cap-binding proteins. *J. Biol. Chem.* (271) 16393-16398.

The generation and characterization of eIF4E antibodies presented in Chapter 3 have been incorporated in the following publication (for reference, this publication is presented in its entirety in Appendix A):

Sigrist, S.J., P.R. Thiel, D. F. Reiff, P.E.D. Lachance, P. Lasko, and C. M. Schuster (2000) Postsynaptic translation affects the efficacy and morphology of neuromuscular junctions. *Nature*, 405, 1062-1065.

Chapter 4 has been submitted for publication; the manuscript is authored by P. E. D. Lachance, M. Miron, B. Raught, N. Sonenberg, and P. Lasko.

Minor editing changes have been made to the manuscripts and all references have been incorporated at the end of this thesis.

All experiments described in this thesis have been performed by the candidate with the exception of the following. For Chapter 2, Figure 1, the purification of the cap-binding proteins was performed by C. Lavoie. The peptide alignment in Figure 3 was generated by P. Lasko. For Chapter 3, M. Miron performed the ovary stainings in Figure 4 and assisted with the heat shock experiments performed in Figure 6A. For Chapter 4, M. Miron assisted in the generation of transgenic flies and with the imaginal disc dissections and FACS analysis in Figure 4. B. Raught assisted with the metabolic labeling experiments in Figure 2C. The writing and preparation of manuscripts (Chapters 2 and 4) were shared with Prof. P. Lasko and Prof. N. Sonenberg. C. Lavoie participated in the preparation of the manuscript in Chapter 2. All non-authored contributions are acknowledged at the end of the results chapters.

Contributions to Original Knowledge

1- The candidate cloned and sequenced a gene encoding the *Drosophila* homologue of eIF4E, the mRNA cap-binding protein critical to translation initiation, and showed that this gene is alternatively spliced to form two protein isoforms, eIF4EI and eIF4EII.

2- The candidate generated and characterized several antisera for the study of the eIF4E isoforms that were used to describe the expression of eIF4EI and eIF4EII during development. One of the eIF4E antisera was a critical contribution to a study examining the role of translation in plasticity at the *Drosophila* neuromuscular junction (a copy of this manuscript is included in appendix A).

3- The gene encoding for *eIF4E* was mapped to region 67A8-B2 of polytene chromosomes, allowing for the identification of novel recessive lethal alleles of *eIF4E*. Phenotypic characterization of these alleles showed that *eIF4E* mutant larvae have a growth arrest phenotype.

4- eIF4EI from transgenic mutant lines in which Ser251 is altered do not incorporate labeled orthophosphate *in vivo*. Ser251 corresponds to Ser209 of mammalian eIF4E, which is phosphorylated in response to extracellular stimuli.

5- Transgenic *Drosophila* lines were generated to rescue the *eIF4E* mutants and to genetically test the biological significance of phosphorylation of eIF4E on Ser251. Results obtained from these transgenic lines are evidence that Ser251 of eIF4E is required for the normal growth of a multicellular organism.

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List of Abbreviations

ATP	adenosine triphosphate
EDTA	ethylenediaminetetraacetate
eIFs	eukaryotic initiation factors
GDP	guanosine diphosphate
GST	glutathione-S-transferase
GTP	guanosine triphosphate
IPTG	isopropyl-β-D-1-thiogalactopyranoside
kb	kilobase pairs
kDa	kiloDalton
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	Polymerase Chain Reaction
PI3K	phosphatidyl inositol-3-kinase
PMSF	phenyl methane sulphonyl fluoride
SDS	sodium dodecyl sulphate
Tris	tris(hydroxymethyl)aminomethane
UAS	upstream activating sequence
UTR	untranslated region

Chapter 1

Literature Review

1.1 Overview

Initiation of protein synthesis is a highly regulated process mediated by the eukaryotic initiation factors (eIFs). Most of the research on the activity of the eIFs has been performed using mammalian model systems. In contrast, much less is known about the eIFs in *Drosophila melanogaster*. While several of the *Drosophila* eIF homologues have been identified over the years, the recent completion of the *Drosophila* genome project has allowed for a comprehensive annotation of all *Drosophila* eIFs (Adams *et al.*, 2000; Lasko, 2000). Furthermore, recent studies in *Drosophila*, which extend the biochemical data obtained from mammalian cell culture experiments, have phenotypically correlated the control of translation with the regulation of cellular growth (Weinkove and Leevers, 2000). With these molecular and genetic tools becoming available, there has been a dramatic increase in the use of *Drosophila* as a model for the study of translation initiation and the mechanisms by which it is regulated.

Due to the immense scope of knowledge that covers the whole of translation initiation, the body of this review will focus on the activities of factors involved in the process by which the messenger RNA (mRNA) is initially recognized by the eIFs. A particular emphasis will be placed on work performed using *Drosophila melanogaster* as a model for the study of translational regulation.

1.2 Global survey of the initiation of protein synthesis

eIFs catalyze the recognition of the mRNA by the ribosomal subunits and the positioning of the Met-tRNA$_i$ at the initiator codon (reviewed in Merrick and Hershey

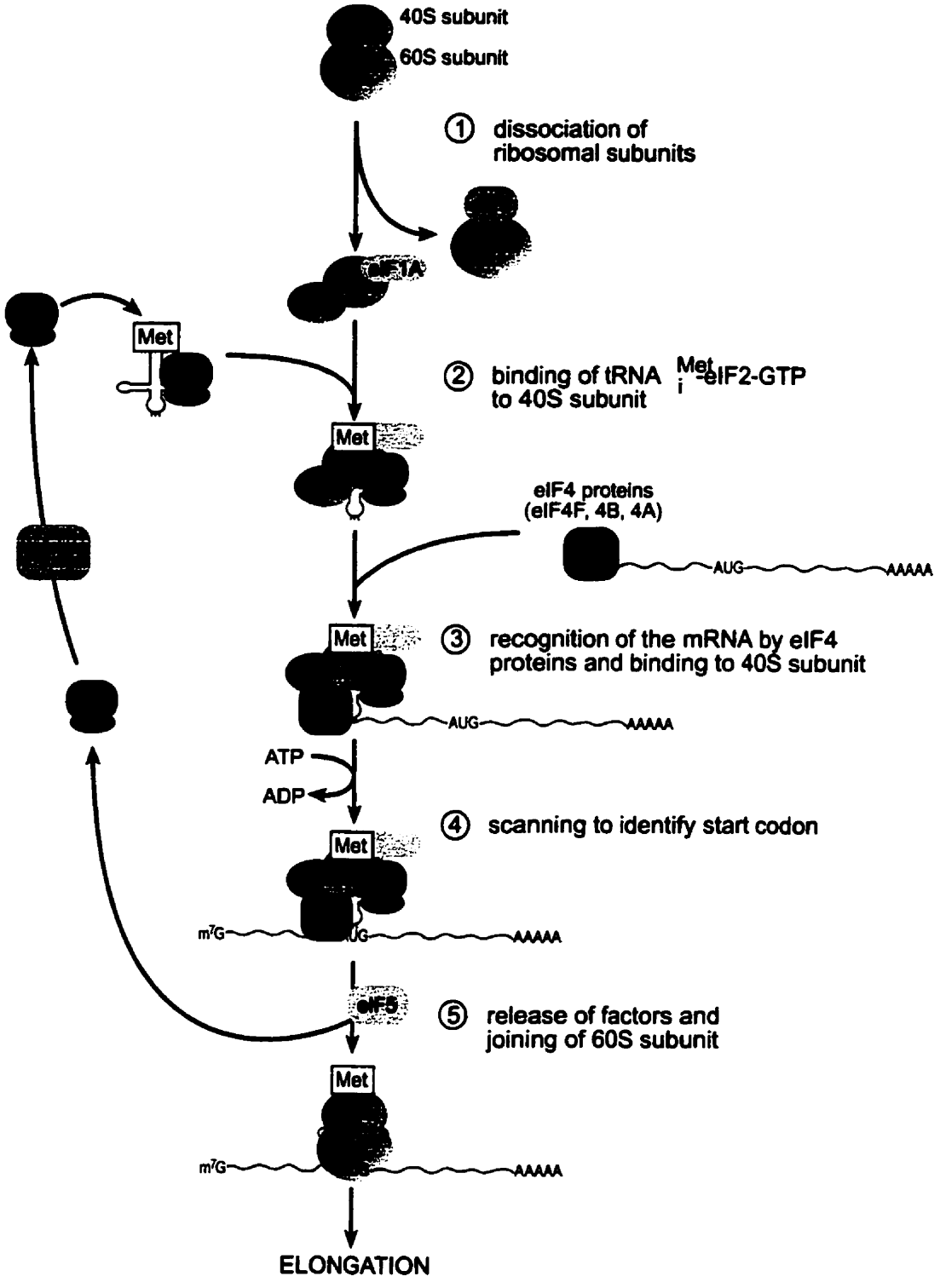
1996). The initiation of translation can be summarized in five major steps (Fig. 1): (1) dissociation of the ribosomal subunits, (2) binding of the Met-tRNA$_i$ to the small (40S) ribosomal subunit, (3) binding of the mRNA, (4) recognition of the initiator codon, and (5) rejoining of small and large (60S) ribosomal subunits.

1.2.1 Dissociation of ribosomal subunits and binding of Met-tRNA$_i$

The activities of eIF1A, eIF3, and eIF6 facilitate the dissociation of the 40S and 60S ribosomal subunits. Before joining with the 40S subunit, Met-tRNA$_i$ forms a ternary complex with eIF2 and GTP. This ternary complex then binds with the 40S subunit to form the 43S pre-initiation complex; the hydrolysis of GTP by eIF2 is required for this step (Rowlands *et al.*, 1988). As the dissociation of GDP from eIF2 is very inefficient, another factor, eIF2B, is needed to catalyze the guanidine nucleotide exchange and to allow the initiation of subsequent rounds of protein synthesis.

eIF2 is one of the key targets for the regulation of protein synthesis. In conditions of cellular stress, the exchange of GDP can be physiologically regulated by the phosphorylation of eIF2. Upon phosphorylation, the affinity of eIF2 for eIF2B is dramatically increased (Clemens, 1996). This effectively suppresses the levels of free eIF2B and results in a fall in the rates of protein synthesis.

Fig. 1. The initiation of metazoan translation is catalyzed by the eukaryotic initiation factors (eIFs). Cap-dependent translation initiation can be summarized in five major steps: (1) the dissociation of the ribosomal subunits, (2) binding of the $tRNA_i^{Met}$-eIF2-GTP ternary complex to the small ribosomal subunit, (3) recognition of the mRNA cap by eIF4 proteins, (4) identification of the start codon by "scanning", and (5) release of factors and joining of the large ribosomal subunit. Initiation of translation is followed by the elongation of the amino acid chain resulting in the production of a protein.


40S subunit
60S subunit
① dissociation of ribosomal subunits
eIF1A
Met
② binding of tRNA $^{Met}_{i}$-eIF2-GTP to 40S subunit
eIF4 proteins (eIF4F, 4B, 4A)
AUG
AAAAA
③ recognition of the mRNA by eIF4 proteins and binding to 40S subunit
ATP
ADP
④ scanning to identify start codon
m7G
⑤ release of factors and joining of 60S subunit
eIF5
ELONGATION

1.2.2 Mechanisms of initiator codon recognition – cap-dependent, cap-independent translation and the shunt mechanism

The recognition of the mRNA by the 43S pre-initiation complex is facilitated by the translation factors of the eIF4 group. There are several mechanisms by which the initiator codon is then detected by the translation machinery. "Cap-dependent" initiation is mediated by the recognition of the mRNA at the 5' cap structure and is followed by the "scanning" of the 43S complex until a start codon in a favorable sequence context is identified (Kozak, 1989). The cap-dependent mechanism of translation initiation and the functions of the eIF4 factors are described in further details in the next sections of this review.

"Cap-independent" initiation involves the direct binding of the 43S complex to an internal site on the mRNA, bypassing the 5'cap structure and parts of the 5'UTR. This type of initiation is facilitated via an internal sequence termed the internal ribosome entry site (IRES), which can be experimentally identified using a bicistronic mRNA assay (Pelletier and Sonenberg, 1988). In most cases, with the exception of the mRNA cap binding protein eIF4E, all of the eIF4 proteins are required for cap-independent initiation. Notable exceptions include the hepatitis A IRES, which requires eIF4E, but not its cap-binding activity, for initiation (Ali *et al.*, 2001), and hepatitis C, in which direct binding of the ribosome to the IRES and initiation of translation requires only eIF2 and eIF3 (Pestova *et al.*, 1998). Messenger RNAs containing an IRES have been described in various organisms including viruses (ex: EMCV (Jang *et al.*, 1989), poliovirus (Pestova *et*

al., 1991)), mammals (ex: *myc* (Nanbru *et al.*, 1997), *fibroblast growth factor-2* (Vagner *et al.*, 1995)), and *Drosophila* (ex: *antennapedia* (Oh *et al.*, 1992)).

Another alternative for translation initiation is the "shunt mechanism" (Futterer *et al.*, 1993). During shunting, the 5'cap structure is still bound by the 43S pre-initiation complex and the activities of the eIF4 proteins are required. However, the ribosome can bypass stretches of the 5'UTR while scanning. The shunting mechanism has been detected during the translation of several viral mRNAs including the 35S mRNA from cauliflower mosaic virus (Futterer *et al.*, 1993), adenovirus major late mRNAs (Yueh and Schneider, 1996), and Sendai virus mRNA (Latorre *et al.*, 1998). The physiological reasons for shunting or the nature of the trans-acting factors that can induce it are unknown. Recent data suggests the involvement of mRNA complementarity to the sequence of the 18S rRNA for shunting during translation of the adenovirus late mRNA (Yueh and Schneider, 2000).

1.2.3 Rejoining of ribosomal subunits

Once the initiator Met-tRNA$_i$ is properly positioned on the start codon, eIF2-GDP is released and the 60S subunit joins the initiation complex. This step is aided by eIF5, which binds the 40S subunit and catalyzes the hydrolysis of eIF2-GTP. The binding of the large ribosomal subunit and the release of initiation factors complete a round of initiation. The elongation of the peptide chain and the production of a protein product then ensue.

1.3 The mRNA cap

The structure at the 5' end of all RNA polymerase II transcripts is critical for cap-dependent translation initiation and can serve as a means of recognition of the mRNA in other cellular processes. The mRNA cap consists of a guanosine residue, methylated at position 7, which is linked to the penultimate nucleotide via a 5'-5' triphosphate bond (Shatkin, 1976). The composition of the mRNA cap can be described generically by the following formula: m^7G-5'-ppp-5'-N_1(m)pN_2(m)pN_3p (where m is a methyl group, N is any nucleotide, and p is a phosphate group). In some mRNAs, the nucleotides at positions N_1 and N_2 can receive 2'-*O*-ribose-methylations. Ribose methylated caps are referred to as type 1 (ribose methylation on N_1) or type 2 (ribose methylation on N_1 and N_2). Caps containing no ribose methylation are type 0. In *Drosophila*, all three types of caps have been identified (Levis and Penman, 1978).

In addition to translation initiation (Shatkin, 1985), the mRNA cap can be directly bound by proteins involved in mRNA splicing (Edery and Sonenberg, 1985), RNA stability (Murthy *et al.*, 1991), and nuclear export (Dostie *et al.*, 2000a; Dostie *et al.*, 2000b; Hamm and Mattaj, 1990). Eukaryotic translation initiation factor 4E (eIF4E) was the first cap-binding protein purified; it was isolated either a monomer or as part of a complex termed eIF4F (Sonenberg *et al.*, 1978; Sonenberg *et al.*, 1979).

eIF4E is present in limiting concentrations in most cell types and its activity can be regulated by several known mechanisms. The recognition of the mRNA cap by eIF4 group proteins (eIF4F, eIF4A, and eIF4B) is thus a rate-limiting step for the initiation of

cap-dependent protein synthesis. The activities and regulation of these factors are detailed in the following section.

1.4 eIF4 group translation factors

The main proteins of the eIF4 group are eIF4F and eIF4B. The heterotrimeric cap-binding complex eIF4F is composed of three proteins: eIF4A, eIF4E, and eIF4G. eIF4A is an RNA-dependent ATPase and helicase (Gingras *et al.*, 1999). The cap-binding activity is provided by eIF4E while eIF4G is a large polypeptide that binds eIF4E, eIF4A, eIF3, and the Poly(A) Binding Protein (PABP). eIF4B is an RNA-binding protein whose role in translation is poorly understood (Gingras *et al.*, 1999).

1.4.1 eIF4A

eIF4A is approximately 46 kDa in size and is the prototype for the DEAD-box family of proteins (Linder *et al.*, 1989; Nielsen *et al.*, 1985). In addition to translation, DEAD-box proteins are implicated in various cellular processes including RNA splicing, and ribosome biogenesis (Linder and Daugeron, 2000). The activity conferred by several motifs within eIF4A or other DEAD-box proteins has been characterized by mutational analysis (Liang *et al.*, 1994; Pause *et al.*, 1993; Pause and Sonenberg, 1992) (Table 1). DEAD-box proteins are most divergent at their N-termini, which provide the specificity for their differing biological functions (Schmid and Linder, 1992).

eIF4A likely functions in unwinding 5'UTR secondary structures during the scanning process (Jaramillo *et al.*, 1991). It is the most abundant of initiation factors and

Table 1. Activity conferred by the conserved motifs of DEAD-box proteins

Motif	Activity	Reference
AXXXXGKT (A motif)	ATP binding	Pause *et al.*, 1992
PTRELA	unknown	
TPGR	unknown	
DEAD	ATP hydrolysis	Pause *et al.*, 1992
SAT	RNA unwinding	Pause *et al.*, 1992
ARGXD	RNA unwinding	Liang *et al.*, 1994
HRIGRXXR	ATP-dependent RNA binding	Pause *et al.*, 1993

appears to function as part of eIF4F and as a monomer (Duncan *et al.*, 1983; Grifo *et al.*, 1983). Mutants in eIF4A that affect ATP metabolism (mutants in the DEAD or HRIGRXXR domains) are dominant-negative inhibitors of cap-dependent and independent translation (Pause *et al.*, 1994b). Addition of exogenous eIF4F but not wild-type eIF4A can alleviate the inhibition caused by the dominant negative eIF4A mutants. It was thus hypothesized that the free form of eIF4A is required for recycling through eIF4F during initiation.

There are three isoforms of eIF4A in vertebrates (eIF4AI, eIF4AII, and eIF4AIII) that are encoded by different genes and which have differential tissue expression patterns (Nielsen and Trachsel, 1988; Weinstein *et al.*, 1997). Two identical genes encoding eIF4A have also been identified in yeast (Prat *et al.*, 1990). The individual role of the eIF4A isoforms is unclear. *Xenopus* eIF4AI and eIF4AII are highly homologous (89% identity) and are functionally interchangeable *in vitro* (Morgan and Sargent, 1997). In contrast, *Xenopus* eIF4AIII cannot functionally substitute for eIF4AI in an *in vitro* assay and it inhibits translation in reticulocyte lysates (Li *et al.*, 1999). Murine eIF4AI is present in eIF4F at levels four times higher than eIF4AII but while eIF4AI can be readily purified in free form, eIF4AII is predominantly found as part of eIF4F (Conroy *et al.*, 1990). This might suggest that eIF4AII preferentially associates into eIF4F and may imply that tissues expressing higher levels of this isoform are more translationally active (assuming that the activity of other translation factors is unchanged).

1.4.2 eIF4B

Mammalian eIF4B is approximately 80 kDa in size and contains two functional RNA-binding domains (Methot *et al.*, 1996b; Milburn *et al.*, 1990; Naranda *et al.*, 1994). The role of eIF4B in translation initiation is not well understood as initiation can proceed in its absence and the yeast eIF4B mutant, *TIF3*, is viable (Altmann *et al.*, 1993). This suggests either that eIF4B is not essential for translation initiation or that another yet unidentified protein can substitute for its activity in these contexts.

eIF4B can stimulate the helicase activity of eIF4A *in vitro* (Altmann *et al.*, 1990) and can complement a temperature-sensitive allele of yeast eIF4A (Coppolecchia *et al.*, 1993). While the C-terminal RRM of eIF4B binds non-specifically to RNA, its N-terminal RRM can associate with an RNA sequence obtained by SELEX that inhibits the binding of eIF4B with the 18S rRNA (Methot *et al.*, 1996a). Also, eIF4B and the yeast homologue TIF3 can stimulate the annealing of RNA duplexes *in vitro* (Altmann *et al.*, 1995). eIF4B can homodimerize via a motif in the center of the protein; this domain also binds the p170 subunit of eIF3 (Methot *et al.*, 1997; Methot *et al.*, 1996b). Given these data, it was postulated that eIF4B could facilitate the binding of the mRNA, rRNA, Met-$tRNA_i$ and the ribosome during the scanning process (Altmann *et al.*, 1995). The activity of eIF4B may also be regulated by phosphorylation. Several isoelectric variants of eIF4B have been identified, all of which are phosphoproteins except for the most basic form (Duncan and Hershey, 1984). Further studies are required to determine the effects of eIF4B phosphorylation on translation initiation.

1.4.3 eIF4G

The largest component of eIF4F is eIF4G. There are two isoforms of eIF4G in mammals and yeast (Goyer *et al.*, 1993; Gradi *et al.*, 1998). The mammalian eIF4Gs possess similar activities suggesting that they both function in translation complexes (Gradi *et al.*, 1998).

eIF4G is a scaffold protein which bridges the interaction between the mRNA bound by eIF4E and eIF4A and other components involved in initiation such as eIF3, PABP, and the eIF4E kinase Mnk1. eIF4G also contains an RRM motif and can directly bind RNA (Goyer *et al.*, 1993). The binding of eIF4E is mediated by the conserved motif YXXXXLΦ (where X is any amino acid and Φ is an aliphatic residue), located in the N-terminal third of eIF4G (Mader *et al.*, 1995; Morino *et al.*, 2000). eIF4A binds sites in the center and C-terminal half of mammalian eIF4G (Imataka and Sonenberg, 1997). The middle domain also binds eIF3, independently of eIF4A, while Mnk1 associates with sequences in the carboxy terminus, and PABP with a region at the N-terminus (Imataka *et al.*, 1998; Imataka and Sonenberg, 1997; Pyronnet *et al.*, 1999).

The N-terminal region of eIF4G, including the eIF4E-binding site, is absolutely necessary for cap-dependent translation (Morino *et al.*, 2000). Several viruses utilize this to favor the translation of their mRNAs by cap-independent mechanisms to the detriment of the host cell. These viruses cleave eIF4G into two stable fragments (Etchison and Fout, 1985), effectively removing the eIF4E binding site and the cap-binding activity of eIF4F (Lamphear *et al.*, 1995). The end result is an enhancement of viral translation

caused by the diversion of the cellular translation machinery towards the internal initiation of viral mRNA.

The cleavage of eIF4G into several fragments also occurs during apoptosis (Clemens *et al.*, 1998; DeGracia *et al.*, 1996; Marissen and Lloyd, 1998). The cleavage of eIF4G during apoptosis appears to be mediated by caspases: caspase inhibitors can prevent cleavage while Caspase-3 was shown to directly cleave eIF4GI *in vitro* (Marissen and Lloyd, 1998).

An inhibitor of translation with a high degree of similarity to eIF4G was cloned by several groups (Imataka *et al.*, 1997; Levy-Strumpf *et al.*, 1997; Shaughnessy *et al.*, 1997; Yamanaka *et al.*, 1997). The p97 gene product (also known as DAP-5 and NAT1) is 28% identical to the carboxy terminus of eIF4G and can bind eIF4A and eIF3 but not eIF4E and PABP (Imataka *et al.*, 1997). The similarity of p97 to eIF4G suggests that it acts by replacing eIF4G. Unlike the C-terminal portion of eIF4G that can still initiate cap-independent translation, p97 is an inhibitor of both cap-dependent and cap-independent protein synthesis (Yamanaka *et al.*, 1997). Reduced expression of p97 during the progression of murine liver cancers (Yamanaka *et al.*, 1997) and its involvement in γ-interferon-induced cell death (Levy-Strumpf *et al.*, 1997) suggest that p97 is an important regulator of translation.

1.4.4 eIF4E

eIF4E, the smallest subunit of eIF4F, was originally identified by its ability to be retained in an m^7GDP-sepharose column (Sonenberg *et al.*, 1979). It is highly conserved

among species but varies in size. The difference in the size of eIF4E proteins from various organisms resides in a highly variable N-terminal region that is dispensable for its cap-binding activity (Marcotrigiano *et al.*, 1997). The remainder of the polypeptide contains several highly conserved residues that have been implicated in cap binding, eIF4G interaction, or eIF4E regulation.

Solving of the eIF4E crystal structure revealed that eIF4E resembles a "cupped hand" in which the mRNA cap interacts with the inner groove while eIF4G binds to the dorsal surface (Marcotrigiano *et al.*, 1997; Marcotrigiano *et al.*, 1999). The inner groove is comprised of eight curved β-sheets in which m^7GDP forms π-π interactions between two invariably conserved tryptophan residues (W56 and W102 in murine eIF4E). The interaction with m^7GDP is strengthened by hydrogen bonds (W102 and E103), a Van der Waals contact (W166), water bridges (W166, R112), and positively charged amine groups that interact with the negative phosphate groups of the cap (R112, R157, R162). The dorsal surface of eIF4E, comprised of three α-helices, can interact with a peptide based on the conserved eIF4E-binding sequence of eIF4G (Marcotrigiano *et al.*, 1999). Again, highly conserved residues participate in this interaction via Van der Waals interactions, salt bridges, and hydrogen bonds (H37, P38, V69, W73, L131, E132, L135, I138, E140, D147). Of these, W73 is absolutely required as interaction with eIF4G can be abolished by substitution to alanine (Marcotrigiano *et al.*, 1999; Ptushkina *et al.*, 1998).

In most cells and tissues examined to date (with the exception of reticulocyte lysates), eIF4E is the lowest abundance translation factor (Duncan *et al.*, 1987; Hiremath

et al., 1985; Rau *et al.*, 1996). This makes eIF4E a good target for the regulation of protein synthesis.

1.5 The regulation of eIF4E activity

Artificially changing the levels of cellular eIF4E results in the deregulation of growth in mammalian cultures (Sonenberg, 1996). The activity of eIF4E is thus tightly controlled by the cell to maintain normal growth. There are at least three mechanisms by which the activity of eIF4E can be modulated: (1) the regulation of transcription of the *eIF4E* gene, (2) phosphorylation at a conserved serine residue, and (3) inhibition by association with the small molecular weight 4E-Binding Proteins (Gingras *et al.*, 1999b). Outlined in this section are the known mechanisms by which eIF4E is controlled and a survey of the effects of deregulating eIF4E activity.

1.5.1 Regulation of eIF4E transcription

The expression of the *eIF4E* gene in mammals is regulated by the transcription factor MYC (Rosenwald *et al.*, 1993). The promoter region of *eIF4E* contains two consensus E-box motifs, which can bind MYC and which are necessary for the activation of *eIF4E* gene expression by this factor (Jones *et al.*, 1996). Indeed, overexpressing MYC in tissue cultures causes increases in the abundance of *eIF4E* mRNA (Rosenwald *et al.*, 1993). Also, there is a correlation between the levels of *eIF4E* mRNA and MYC protein during the S-phase of the cell cycle (Rosenwald *et al.*, 1993). These observations are

interesting as MYC is a strong promoter of cell growth and may thus partly mediate its effects by upregulating translation via increases in *eIF4E* transcription.

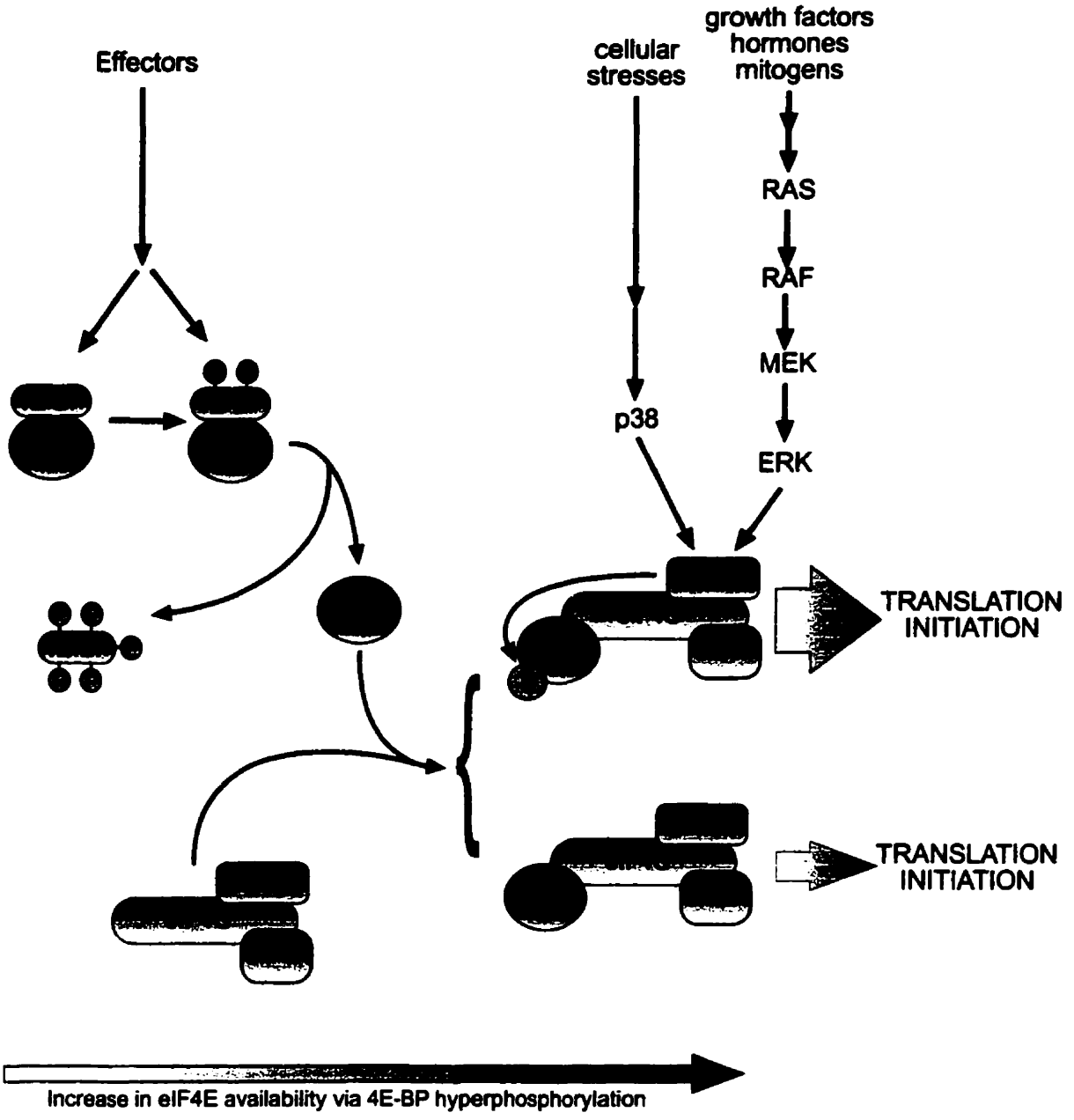
1.5.2 Regulation of eIF4E by phosphorylation

There are good correlations between changes in cell growth induced by various extracellular stimuli and the phosphorylation state of eIF4E such that in most cases phosphorylation is augmented concomitant with elevations in growth rates (Gingras *et al.*, 1999b). Although originally mapped to Ser53, the key phosphorylation site of mammalian eIF4E has been reassigned to Ser209 (Flynn and Proud, 1995; Whalen *et al.*, 1996).

It has been suggested that the phosphorylation of eIF4E can increase its affinity for the mRNA cap (Minich *et al.*, 1994; Shibata *et al.*, 1998). The phosphorylated form of eIF4E has a higher affinity for mRNA caps than the non-phosphorylated form, as measured by fluorescence quenching of tryptophan residues (Minich *et al.*, 1994). Consistent with this, mutants that mimic eIF4E phosphorylation by conversion of Ser209 to aspartic or glutamic acid have a slightly higher affinity for m^7GTP *in vitro* than wild-type or control eIF4E proteins (Shibata *et al.*, 1998). The idea that phosphorylated eIF4E binds stronger to the mRNA cap is supported by the three-dimensional structure of eIF4E (Marcotrigiano *et al.*, 1997). The three-dimensional position of Ser209 by the mRNA binding slot suggests that phosphorylation can create a salt bridge with a lysine residue located on the other side of the slot, thereby clamping the mRNA and increasing the strength of binding. This hypothesis has yet to be directly tested experimentally.

Although the effectors in the pathway leading to eIF4E phosphorylation are largely unknown, several proteins have been implicated in this process (Fig. 2). A good candidate for the kinase that phosphorylates eIF4E is the MAP-kinase-interacting protein kinase-1 (MNK1), which can phosphorylate eIF4E on Ser209 upon activation by either the ERK and p38 map kinases (Fukunaga and Hunter, 1997; Waskiewicz *et al.*, 1997). Support that MNK1 is an *in vivo* kinase for eIF4E comes from a study in which MNK1 mutants were expressed in NIH 3T3 cells (Waskiewicz *et al.*, 1999). Expression of a dominant-negative mutant of MNK1 was shown to inhibit the phosphorylation of eIF4E induced by phorbol esters while a constitutive mutant leads to increased eIF4E phosphorylation. MNK1 physically interacts with the C-terminal region of eIF4G, bringing it in very close proximity to eIF4E *in vivo* (Pyronnet *et al.*, 1999; Waskiewicz *et al.*, 1999). Mutations in the eIF4G-binding sites of eIF4E cause a decrease in the phosphorylation of eIF4E (Pyronnet *et al.*, 1999). Thus, the association of eIF4E with eIF4G appears necessary in mediating eIF4E phosphorylation. Mammalian MNK2 was cloned concurrently with MNK1 (Fukunaga and Hunter, 1997; Waskiewicz *et al.*, 1997) and is capable of phosphorylating eIF4E *in vitro*, albeit to a lesser extent than MNK1 (Waskiewicz *et al.*, 1999). Unlike MNK1, MNK2 is activated by ERKs but not p38 (Waskiewicz *et al.*, 1999). Very little is known on the cellular contribution of MNK2 on eIF4E phosphorylation but its presence raises the strong possibility that multiple kinases can phosphorylate eIF4E and that these may be responsive to different stimuli or may be differentially expressed in tissues. Further work will be required to address these issues.

Fig. 2. eIF4E activity is regulated by two mechanisms: (1) the availability of eIF4E is modulated by association with the 4E-BPs and (2) increased phosphorylation of eIF4E coincides with increased translation. 4E-BPs compete with eIF4G for the binding of eIF4E; when bound to 4E-BP, eIF4E cannot associate with eIF4G to initiate translation. Binding of the 4E-BPs to eIF4E is alleviated by the hyperphosphorylation of 4E-BP. The kinase MNK1, which binds directly to eIF4G phosphorylates eIF4E. MNK1 activation is believed to be mediated by the p38 or ERK Map Kinases. Although phosphorylation of eIF4E correlates with increased translation and elevated cellular growth rates, the non-phosphorylated form of eIF4E can still initiate translation.


Effectors
cellular stresses
growth factors hormones mitogens
RAS
RAF
MEK
p38
ERK
TRANSLATION INITIATION
TRANSLATION INITIATION
Increase in eIF4E availability via 4E-BP hyperphosphorylation

Interestingly, MNK1 may also be a target for the inhibition of protein synthesis. MNK1 can interact with p97, a protein with high similarity to the carboxy-terminal portion of eIF4G (Pyronnet *et al.*, 1999). As p97 cannot bind eIF4E, the binding of MNK1 may serve to inhibit translation via its sequestration from eIF4G. It is also possible that the binding of MNK1 to p97 serves to phosphorylate as yet unidentified targets. Inhibition of protein synthesis may also be achieved by the displacement of MNK1 from eIF4G by the adenovirus 100k protein; this displacement is concomitant with a block in host cell protein synthesis and a reduction in eIF4E phosphorylation (Cuesta *et al.*, 2000). Furthermore, adenovirus infection does not affect the kinase activity of MNK1 while the block in protein synthesis and the reduction in eIF4E phosphorylation are alleviated by inactivation of a temperature-sensitive mutant of 100k protein (Cuesta *et al.*, 2000). Finally, the translation of an eIF4F-dependent cellular mRNA reporter is directly impaired by the dephosphorylation of eIF4E while adenovirus mRNAs are not affected. In addition to being an interesting mechanism for host cell translational inhibition upon viral infection, these data are consistent with the notion that the phosphorylation of eIF4E is important for normal cellular translation.

Several extracellular stimuli, such as serum, phorbol esters, insulin, and growth factors have been shown to induce eIF4E phosphorylation in cell cultures (for a survey of compounds known to affect the phosphorylation state of eIF4E, see Gingras *et al.*, 1999b). Members of the RAS signaling pathway have been shown to mediate the phosphorylation of eIF4E induced by extracellular stimuli. In cells transformed by RAS, the phosphorylation of eIF4E is augmented (Frederickson *et al.*, 1991). The activity of

eIF4E was shown to be required for the RAS-mediated transformation of cell cultures as expression of eIF4E antisense mRNA inhibits this process (Rinker-Schaeffer *et al.*, 1993). In contrast, expression of a dominant-negative form of RAS in PC12 cell cultures prevents the phosphorylation of eIF4E induced by nerve growth factor (Frederickson *et al.*, 1992). The MEK kinase (MAP or ERK Kinase) is downstream of RAS in the pathway (reviewed by Rommel and Hafen, 1998). Consistent with a role of the RAS pathway in eIF4E regulation, expression of dominant-negative MEKs or incubation with inhibitory compounds that prevent ERK activation by MEK, significantly prevents the phosphorylation of eIF4E (Morley, 1997). In conditions of cellular stress in which eIF4E phosphorylation is increased, ERKs are not activated; instead, cellular treatments that induce conditions of stress activate the JNK and p38 MAP kinases (Robinson and Cobb, 1997). Pre-incubation of cells with the p38-specific inhibitor SB203580 prevents the induction of eIF4E phosphorylation by certain cellular stresses (Wang *et al.*, 1998).

Since it can phosphorylate eIF4E on Ser209 *in vitro*, it has been suggested that protein kinase C (PKC) is a physiological kinase for eIF4E (Whalen *et al.*, 1996). In addition, prolonged treatment of cells with phorbol esters, which down regulate PKC, can inhibit the insulin-stimulated phosphorylation of eIF4E (Smith *et al.*, 1991). However, it was observed that in certain cell lines, such as PC12 and CHO cells, that eIF4E phosphorylation is independent of PKC (Flynn and Proud, 1996; Frederickson *et al.*, 1992). It remains unclear how PKC might be implicated in the physiological phosphorylation of eIF4E; it is possible that isoforms of PKC lie upstream in the eIF4E phosphorylation pathway and that they can enhance phosphorylation directly or

indirectly in some cells. Nevertheless, current models would suggest that the MNK proteins are the most important eIF4E kinases in the mammalian cell types examined to date.

1.5.3 Regulation of eIF4E by association with 4E-Binding Proteins

In animals, a family of small molecular weight proteins called the 4E-Binding Proteins (4E-BPs) are emerging as important regulators of eIF4E activity (Fig. 2). 4E-BPs were first identified in a Far-Western screen for proteins that interact with eIF4E (Pause *et al.*, 1994a). Binding of 4E-BPs with eIF4E inhibits cap-dependent translation in cell-free extracts and *in vivo*. The 4E-BPs and eIF4G share the common consensus eIF4E-binding motif (YXXXXLΦ) (Mader *et al.*, 1995) and thus compete for the binding of eIF4E (Haghighat *et al.*, 1995). Interestingly, a homologue of 4E-BP1, called PHAS-I, was originally cloned from rats as a protein that is highly phosphorylated upon treatment of cells with insulin (Hu *et al.*, 1994). The hyperphosphorylation of 4E-BPs upon treatment of cells with insulin causes a decrease in its affinity for eIF4E, which is then free to bind eIF4G and form an active cap-binding complex.

The phosphorylation of 4E-BPs upon treatment of cells with insulin appears to be mediated by effectors in the phosphoinositide 3-kinase (PI3K) pathway. Pretreatment of cells with the PI3K inhibitors wortmannin or LY294002 abrogates the phosphorylation of 4E-BP1 (von Manteuffel *et al.*, 1996) while expression of a constitutively active mutant of the catalytic subunit of PI3K results in an increase in 4E-BP phosphorylation (Gingras *et al.*, 1998). The downstream effectors of the PI3K

pathway, Akt (also known as protein kinase B) and FRAP/mTOR, have also been shown to mediate 4E-BP1 phosphorylation (Burnett *et al.*, 1998; Gingras *et al.*, 1999a; Gingras *et al.*, 1998). Interestingly, through various studies in *Drosophila*, the PI3K pathway is emerging as a key regulator of cell and tissue growth (see section 1.8).

A two-step mechanism has been hypothesized for the release of eIF4E upon hyperphosphorylation of 4E-BP (Gingras *et al.*, 1999a). The phosphorylation of 4E-BP1 first occurs on two residues, T37 and T46. When phosphorylated on T37 and T46, 4E-BP1 can still associate with eIF4E. However, phosphorylation at these two sites is required to prime the subsequent phosphorylation of several carboxyl-end residues, which lead to a decrease in the affinity of 4E-BP1 for eIF4E. The phosphorylation of T37 and T46 appears to be directly performed by the kinase FRAP/mTOR (Burnett *et al.*, 1998; Gingras *et al.*, 1999a). The kinase or kinases responsible for the phosphorylation of the set of residues at the carboxyl end of 4E-BP1 remain elusive.

1.5.4 Changes in eIF4E activity lead to the deregulation of cellular growth

The importance of eIF4E in the regulation of cellular growth was strongly suggested by the fact that the overexpression of eIF4E in mammalian cells is oncogenic (Lazaris-Karatzas *et al.*, 1990). Similarly, overexpression of eIF4E in HeLa cultures results in aberrant growth with cells becoming multinucleate (De Benedetti and Rhoads, 1990) while injection of *eIF4E* mRNA in NIH 3T3 cells induces DNA synthesis (Smith *et al.*, 1990). Furthermore, eIF4E can transform primary cell cultures when expressed in conjunction with immortalizing genes, such as E1A or v-MYC, effectively replacing RAS

in the two-oncogene transformation assay (Lazaris-Karatzas and Sonenberg, 1992). Conversely, down-regulation of eIF4E activity by expressing antisense RNA or by overexpression of 4E-BP1 or 4E-BP2 results in reduced growth or in the inhibition of oncogenic phenotypes (Rinker-Schaeffer *et al.*, 1993; Rousseau *et al.*, 1996a). Consistent with a role in oncogenesis, the expression levels of eIF4E were found to be elevated in various malignant human tumors, including breast cancers and head and neck squamous cell carcinomas (De Benedetti and Harris, 1999).

A mechanism by which eIF4E overexpression may mediate its oncogenic effects has been postulated (Sonenberg, 1996). In this model, an increase of available eIF4F is thought to facilitate the translation of mRNAs containing a high degree of secondary structure in their 5'UTRs. This hypothesis stems from the observation that increases in eIF4E levels facilitate the expression of normally poorly-translated reporter genes fused to highly structured 5'UTRs while the expression rates of reporters with a low secondary structure remain constant (Koromilas *et al.*, 1992). In support of this model, many genes that promote cellular growth contain complex 5'UTRs and several of these genes have been shown to be translationally upregulated when eIF4E activity is increased. Examples include mRNAs encoding ornithine decarboxylase (a polyamine metabolism enzyme), cyclin D1, fibroblast growth factor-2, vascular endothelial growth factor, and c-MYC (De Benedetti *et al.*, 1994; Graff *et al.*, 1997; Kevil *et al.*, 1995; Manzella *et al.*, 1991; Rousseau *et al.*, 1996b).

1.6 Poly(A) Binding Protein (PABP)

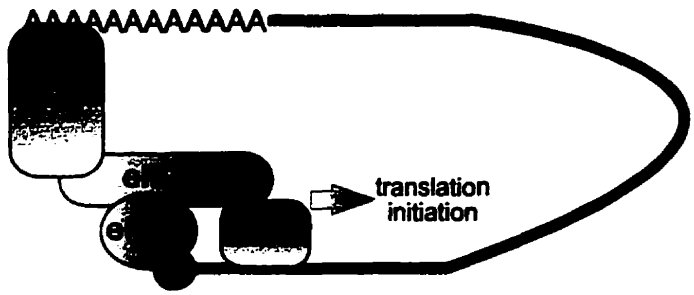
Previous sections focused on the role of the eIFs that interact with the 5' UTR of the mRNA during protein synthesis initiation. The 3' end of eukaryotic mRNAs contains a tract of uninterrupted adenosine residues termed the poly(A) tail (Lim and Canellakis, 1970). The poly(A) tail is added to nascent mRNA chains within the nuclei by cleavage of a highly conserved sequence (AAUAAA) and by action of the Poly (A) Polymerase and other factors (for review, see Wahle and Ruegsegger, 1999). The length of the poly(A) tail correlates with the translational activity of certain mRNAs, a phenomenon that is notable during the early development of *Xenopus, Drosophila*, and mouse embryos (reviewed by Richter, 1996). Thus, the poly (A) tail, a structure at the 3' end of the mRNA, also contributes to translation efficiency.

1.6.1 PABP and the closed-loop mRNA model for translation initiation

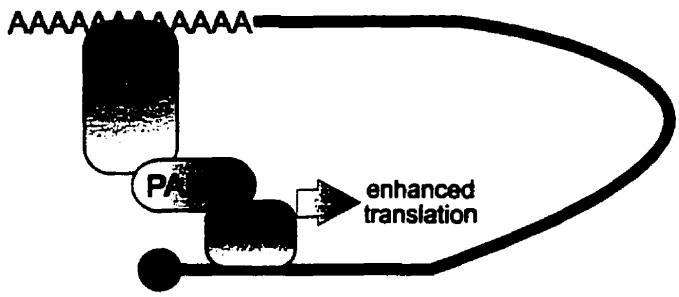
PABP binds the poly(A) tail via four RNA recognition motifs (RRMs) and is implicated in mRNA stability and translation initiation (Adam *et al.*, 1986; Sachs *et al.*, 1986; Sachs and Davis, 1989). PABP mediates the enhancement of translation effected by the poly(A) tail in reticulocyte lysates and yeast (Coller *et al.*, 1998; Munroe and Jacobson, 1990). Mechanistically, PABP stimulates translation initiation by direct interaction with eIF4G (Le *et al.*, 1997; Tarun and Sachs, 1996), thus bringing in close proximity the mRNA cap and the poly(A) tail and effectively circularizing the mRNA (Fig. 3A). The interaction of PABP with mammalian eIF4G was mapped to the N-terminus of eIF4GI and eIF4GII (Imataka *et al.*, 1998).

Fig. 3. The closed-loop mRNA model for translation initiation. **(A)** Poly (A) Binding Protein (PABP) directly interacts with eIF4G to circularize the mRNA and increase the efficiency of translation initiation. **(B)** PAIP-1 is a protein with partial homology to eIF4G that interacts with eIF4A and PABP. Although PAIP-1 enhances cap-dependent translation, it is unclear how this is achieved in the absence of eIF4E. A possible intermediate structure, involving the looping of the mRNA, is shown. **(C)** Another protein with eIF4G homology is p97, which can bind eIF4A but not PABP or eIF4E. p97 is an inhibitor of both cap-dependent and cap-independent translation.

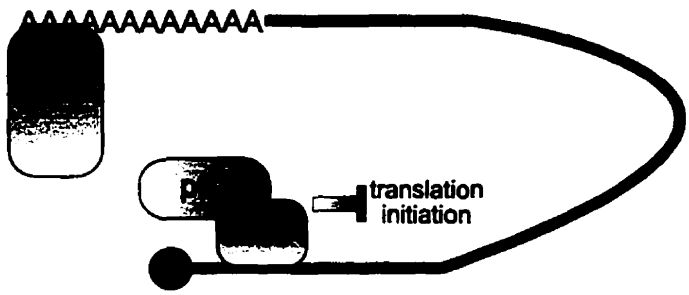
A


translation
initiation

B


enhanced
translation

C


translation
initiation

The closed-loop model for translation initiation is supported by electron micrographs that have detected circular polysomes (Christensen *et al.*, 1987). Furthermore, a circular mRNA can be formed *in vitro* in the presence of recombinant eIF4G, eIF4E, and PABP (Wells *et al.*, 1998). The exact mechanisms by which a circular mRNA can stimulate translation initiation have not been elucidated; current hypotheses are that a closed-loop mRNA facilitates the reinitiation of translation by transfer of the 40S ribosome from the 3' to the 5' UTR (Gingras *et al.*, 1999b).

1.6.2 PABP-interacting protein-1 (PAIP-1)

Since the interaction of PABP with eIF4G enhances translation, other proteins that interfere with this interaction would be predicted to affect the translational efficiency of mRNAs. To isolate new regulators of translation, a two-hybrid screen was performed with PABP as bait; this screen identified the novel PABP-Interacting Protein-1 (PAIP-1) (Craig *et al.*, 1998). Interestingly, the N-terminus of PAIP-1 is homologous to the middle segment of eIF4G (which contains the eIF3 and eIF4A binding regions); a region interacting with PABP was mapped to the C-terminus of PAIP-1 (Craig *et al.*, 1998). As predicted from the sequence similarity with eIF4G, PAIP-1 can interact with eIF4A but not eIF4E (Craig *et al.*, 1998). Surprisingly, PAIP-1 enhances the translation of a reporter RNA when expressed in COS cells (Craig *et al.*, 1998). It is thus possible that PAIP-1 can bridge an interaction between PABP and eIF4A *in vivo* and circularizes the RNA, but it is unclear how this interaction can stimulate cap-dependent translation in the absence of

eIF4E (Fig. 3B). The activity of PAIP-1 contrasts to that of p97, another protein with partial homology to eIF4G, which cannot bind PABP and inhibits translation (Fig. 3C).

1.7 eIF4 initiation factors in Drosophila

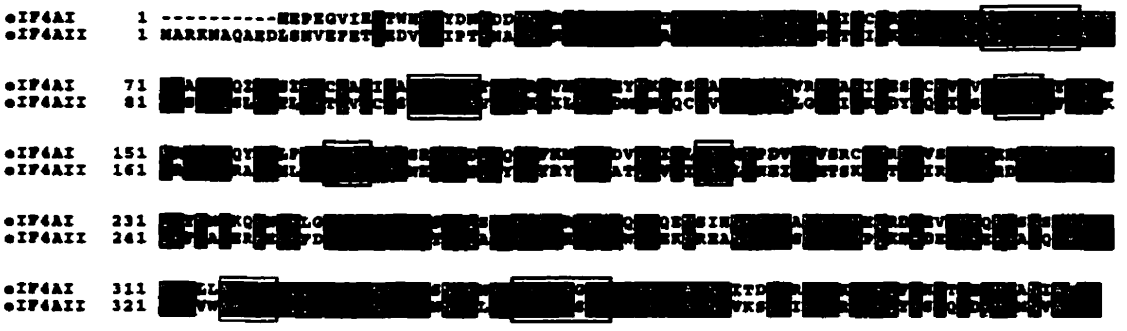
Having established in previous sections the known functions and regulatory mechanisms of proteins in the eIF4 group, this section aims to survey the similarities and differences between the mammalian eIF4 proteins and the homologous factors identified in *Drosophila*.

1.7.1 eIF4A and eIF4B

The *Drosophila* genome contains two genes with similarity to mammalian eIF4A (Lasko, 2000). The *Drosophila eIF4A* isoforms are 70.6% identical in their amino acid sequence (Fig. 4). These isoforms, to be referred to as *eIF4AI* (CG9075) and *eIF4AII* (CG7483), are mapped to chromosomal regions 2L-26B1 and 3R-84F11 respectively (CG numbers are standard identifiers for the genes annotated during the *Drosophila* genome project; these numbers will be indicated where appropriate to maintain this standardization). All eIF4A and DEAD box protein-specific sequences (see Table 1) are conserved in the *Drosophila* isoforms (Fig. 4).

eIF4AI was independently cloned by two groups (Dorn *et al.*, 1993; Verheyen and Cooley, 1994). Mutant alleles of *eIF4AI* are recessive lethal, suggesting that its gene product is essential and that the *eIF4AII* cannot fully compensate for *eIF4AI* function.

Fig. 4. The *Drosophila* genome encodes for two isoform of eIF4A. eIF4AI (CG9075) and eIF4AII (CG7483) are 70.6% identical at the amino acid level and contain all functional domains conserved among the eIF4A proteins from divergent organisms (red boxes). Alignments performed using ClustalW 1.8.



Since mutant alleles have yet to be identified for *eIF4AII*, its role in development is unknown.

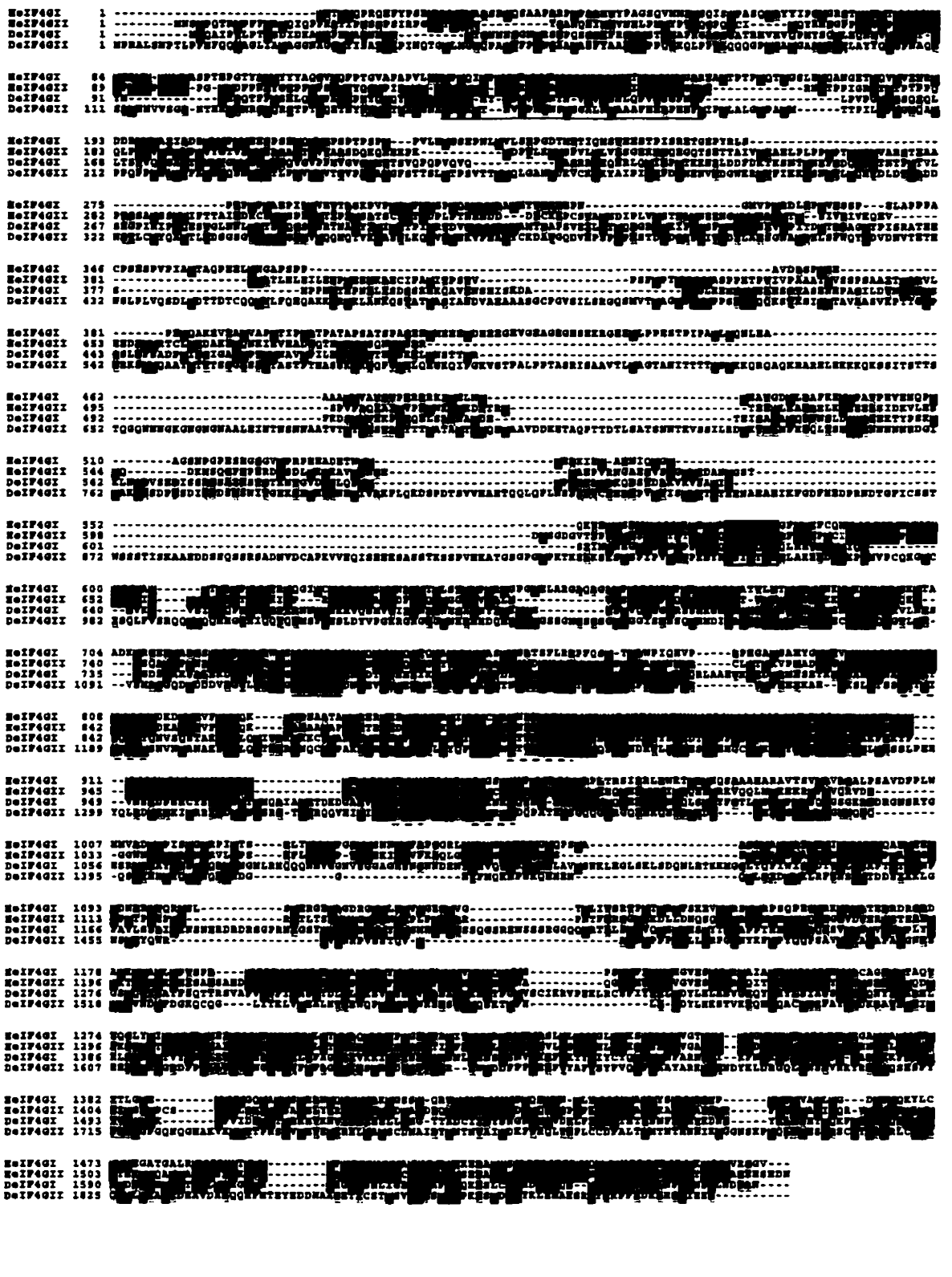
When purified from *Drosophila* or yeast, eIF4F does not include any eIF4A (Goyer *et al.*, 1989; Zapata *et al.*, 1994). In contrast with mammalian eIF4G, which possesses two eIF4A-binding regions, yeast eIF4G lacks the C-terminal binding site (Dominguez *et al.*, 1999). Thus, one possible explanation for the lack of eIF4A in purified yeast eIF4F is that yeast eIF4G has a lower affinity for eIF4A. The presence of a single eIF4A-binding region on the *Drosophila* homologues of eIF4G would also explain the absence of eIF4A in purified eIF4F but this hypothesis remains to be tested experimentally.

A search of the sequenced *Drosophila* genome revealed that no protein with high homology to eIF4B is present. The protein with the best homology to eIF4B is an uncharacterized protein containing RNA recognition motifs (CG10837, Blast score 1e-13, Lasko, 2000). It is unclear whether this protein is the functional homologue of eIF4B in *Drosophila*.

1.7.2 eIF4G and proteins with homology to eIF4G

A *Drosophila* gene encoding a homologue of eIF4G (CG10811, to be referred to as *eIF4GI*) was identified in cytological region 102E of chromosome 4 (Hernandez *et al.*, 1998). Conceptual translation of the cDNA predicts that *Drosophila* eIF4GI is a protein of 184 kDa and is 25% identical at the amino acid level to eIF4Gs from other organisms (Fig. 5). A search of the completed sequence of the *Drosophila* genome revealed the

Fig. 5. There are two genes that encode for eIF4G homologues in the *Drosophila* genome. Presented is an alignment of the human eIF4Gs (HeIF4GI, HeIF4GII) with a predicted translation of the genes encoding for the *Drosophila eIF4Gs* (DeIF4GI, CG10811; DeIF4GII, CG10192). The conserved eIF4E binding-motif (red box) is indicated. The sites in the middle third of mammalian eIF4G required for eIF4A binding are also well conserved in the *Drosophila* homologues (solid blue underline, site shown to be absolutely required for eIF4A binding; dashed blue underline, sites shown to enhance eIF4A binding; according to Imataka *et al.*, 1997). The region in the N-terminus of the mammalian eIF4Gs believed to be required for PABP interaction is not well conserved among the *Drosophila* proteins (Imataka *et al.*, 1998, green underline). Alignments performed using ClustalW 1.8.



presence of a second, previously unidentified, homologue of eIF4G located on the right arm of chromosome 3 at cytological position 95C4-5 (CG10192, to be referred to as *eIF4GII*). *Drosophila* eIF4GII is 1905 amino acids in length compared to 1666 for eIF4GI. These isoforms are 50% homologous to each other (with 34% amino acid identity).

Alignment of the *Drosophila* and human eIF4G isoforms reveals that most but not all of the sequences shown to be important for interaction with other proteins are conserved (Fig. 5). The eIF4E-binding motif (YXXXXLΦ) is conserved in *Drosophila eIF4GI* while *eIF4GII* is slightly divergent in the last position where an Arg is in place of the consensus aliphatic residue. Interestingly, the *Drosophila* homologue of 4E-BP is divergent in this position of the eIF4E-binding motif where it also possesses a positively charged residue and the binding of the *Drosophila* 4E-BP to eIF4E was shown to be weaker than that of the human 4E-BPs (Miron *et al.*, 2001). By extension, it could be hypothesized that *Drosophila* eIF4GII interacts more weakly with eIF4E than eIF4GI.

The middle third of the mammalian eIF4Gs was shown to be important for eIF4A and eIF3 interaction while the C-terminus was shown to bind MNK-1 (Imataka and Sonenberg, 1997; Pyronnet *et al.*, 1999). Homology in these regions of the *Drosophila* eIF4G isoforms is high (Fig. 5). A stretch at the N-terminus of the mammalian eIF4Gs is important for interaction with PABP (Imataka *et al.*, 1998). This region is not well conserved in the *Drosophila* eIF4G isoforms; the sequences required for interaction with PABP would have to be determined experimentally (Fig. 5).

Homologues of p97 and PAIP-1, two proteins that resemble portions of eIF4G, were also identified in a search of the *Drosophila* genome. *Drosophila p97* (CG3845) is encoded on the right arm of chromosome 2 at position 49E1 while *Drosophila PAIP-1* (CG8963) is located at cytological position 2R-53F1. The *Drosophila p97* annotated by the genome project is truncated at the N-terminus and is lacking some important sequences for interaction with eIF4A (data not shown). However, the translation of human p97 was shown to initiate at a GUG start codon (Imataka *et al.*, 1997). When assuming that *Drosophila* p97 also initiates at a GUG, the N-terminus of the protein is extended to include the conserved eIF4A-binding regions. Several expressed sequence tags (ESTs) have been identified for *Drosophila* p97 but only one extends into the N-terminus putatively lengthened by a GUG start codon and none extend all the way to the initiator codon. Although the use of the GUG start codon for the *Drosophila* p97 would have to be demonstrated experimentally, the evolutionary conservation of such an uncommon start of initiation suggests that it is an important mechanism for the regulation of p97 expression.

Although there are two genes encoding isoforms of eIF4G and good homologues of *p97* and *PAIP-1* in the *Drosophila* genome, mutants in none of these genes have been identified. Identification of such mutants and characterization of their phenotypes will be important to further our understanding of how these proteins contribute to the initiation of translation.

1.7.3 Several genes encode homologues of eIF4E in Drosophila

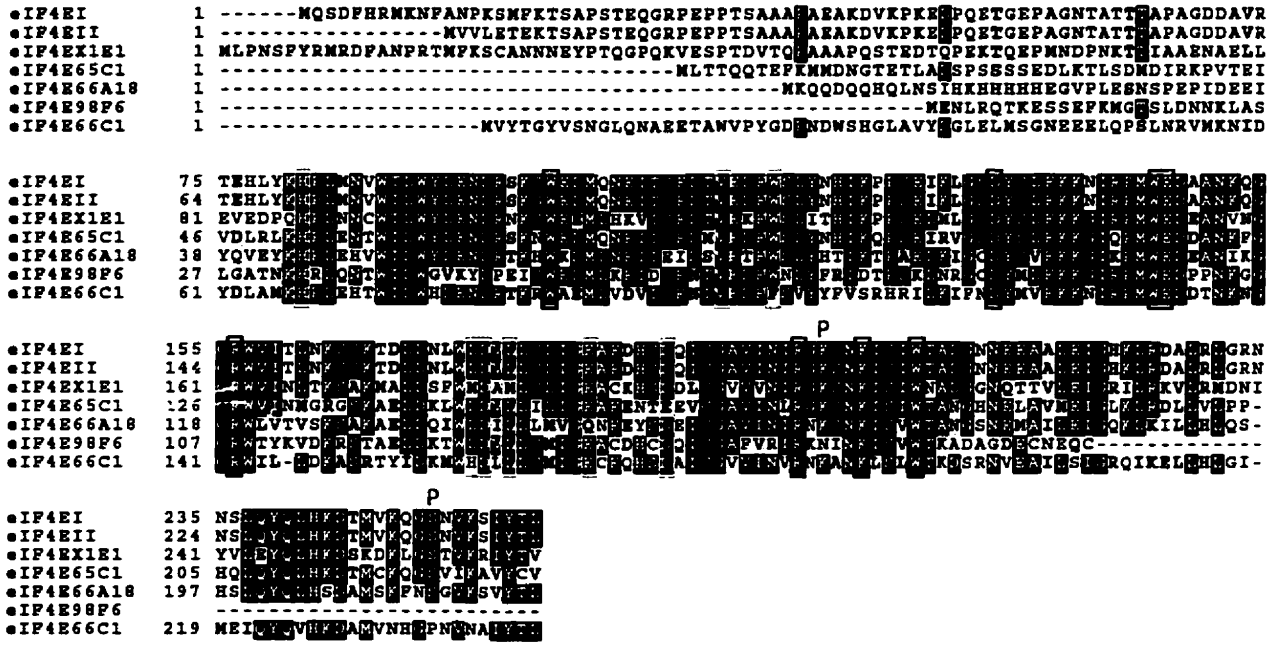
The cloning of two protein isoforms of *Drosophila* eIF4E, encoded by a single gene, is described in chapter 2 of this thesis. Recently, a search of the *Drosophila* genome revealed the presence of six additional genes encoding proteins with homology to eIF4E (Table 2; Lasko, 2000). The expression of three of these genes was confirmed by the identification of expressed sequence tags (EST) by the *Drosophila* genome project (Table 2; Lasko, 2000). The residues required for the interaction of mammalian eIF4E with the mRNA cap, eIF4G, or 4E-BP were mapped via X-ray crystallography; these amino acids are well conserved among eIF4E homologues cloned from different species (Marcotrigiano *et al.*, 1997; Marcotrigiano *et al.*, 1999). An alignment of the *Drosophila eIF4E* cognates shows that all residues shown to be required for binding to the mRNA cap are invariably conserved among all isoforms (Fig. 6A). Amino acids on the dorsal surface of eIF4E required for interaction with eIF4G and 4E-BP are somewhat less conserved in some of the cognates. *eIF4E66C1* (CG8023) is the most divergent with changes in two residues required for interaction with eIF4G, including a conservative change of a tryptophan shown to be absolutely required for this interaction. Whether this change affects the affinity of this isoform for eIF4G or 4E-BP needs to be determined empirically. Most of the *eIF4E* cognates also possess a serine residue in the proper context for regulation by phosphorylation and the lysine proposed to facilitate the formation of a salt bridge with the phosphorylated serine (Fig. 6A). Exceptions are *eIF4E66C1* (CG8023), which possesses a proline in lieu of the serine, and *eIF4E98F6* (CG1442), which encodes a protein truncated at the C-terminus prior to the phosphorylation site. Interestingly,

Table 2. Several genes encode for proteins homologous to eIF4E in *Drosophila*. Indicated are the names, CG numbers, and cytological locations of the *eIF4E* cognates. Expression was confirmed by the Berkeley *Drosophila* Genome Project for some of the *eIF4E* cognates via the identification of expressed-sequence tags (EST).

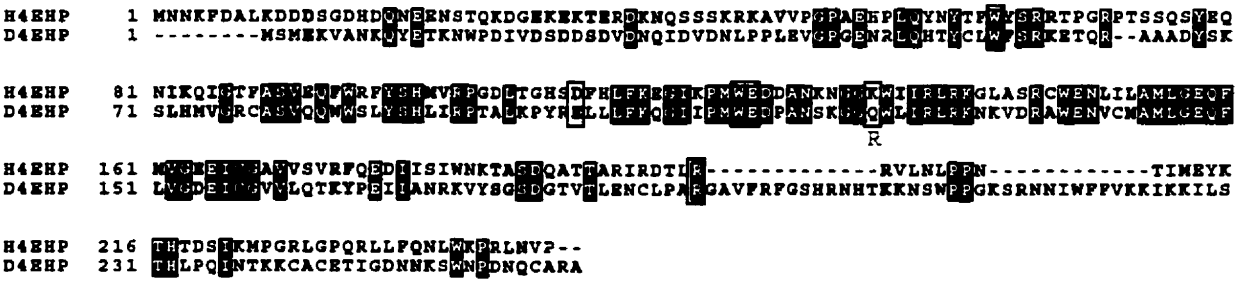
Gene	CG#	Cytology	EST support?	Remarks
eIF4E	CG4035	3L-67A8	yes	Produces 2 isoforms – eIF4EI and eIF4EII
eIF4EX1E1	CG11392	X-1E1	no	
eIF4E65C1	CG10124	3L-65C1	yes	
eIF4E66A18	CG8277	3L-66A18	no	
eIF4E98F6	CG1442	3R-98F6	no	Truncated at C-terminus
eIF4E66C1	CG8023	3L-66C1	yes	Lacks conserved phosphorylation site
D4E-HP	CG10716	3R-95D9	yes	

Fig. 6. Alignment of the *Drosophila* proteins homologous to eIF4E. **(A)** Alignment of the *Drosophila eIF4E* cognates. Residues in mammalian eIF4E shown to be required for binding the mRNA cap (red boxes), for binding of eIF4G or 4E-BP (green boxes), and for regulation of eIF4E by phosphorylation (P) are indicated. **(B)** Alignment of human 4E-HP (H4E-HP) with the *Drosophila* homologue (D4E-HP, CG10716). Residues required for mRNA cap binding conserved among eIF4Es are highly divergent in 4E-HP (red boxes). Note that only 4 residues are conserved in the 4E-HPs while eIF4Es have 8 invariably conserved residues involved in cap-binding (Panel A). Amino acids required for eIF4G/4E-BP interaction are absent in the 4E-HPs. Alignments performed using ClustalW 1.8.

A


eIF4EI
eIF4EII
eIF4EX1E1
eIF4E65C1
eIF4E66A18
eIF4E98F6
eIF4E66C1
P
P

B


H4EHP
D4EHP
R

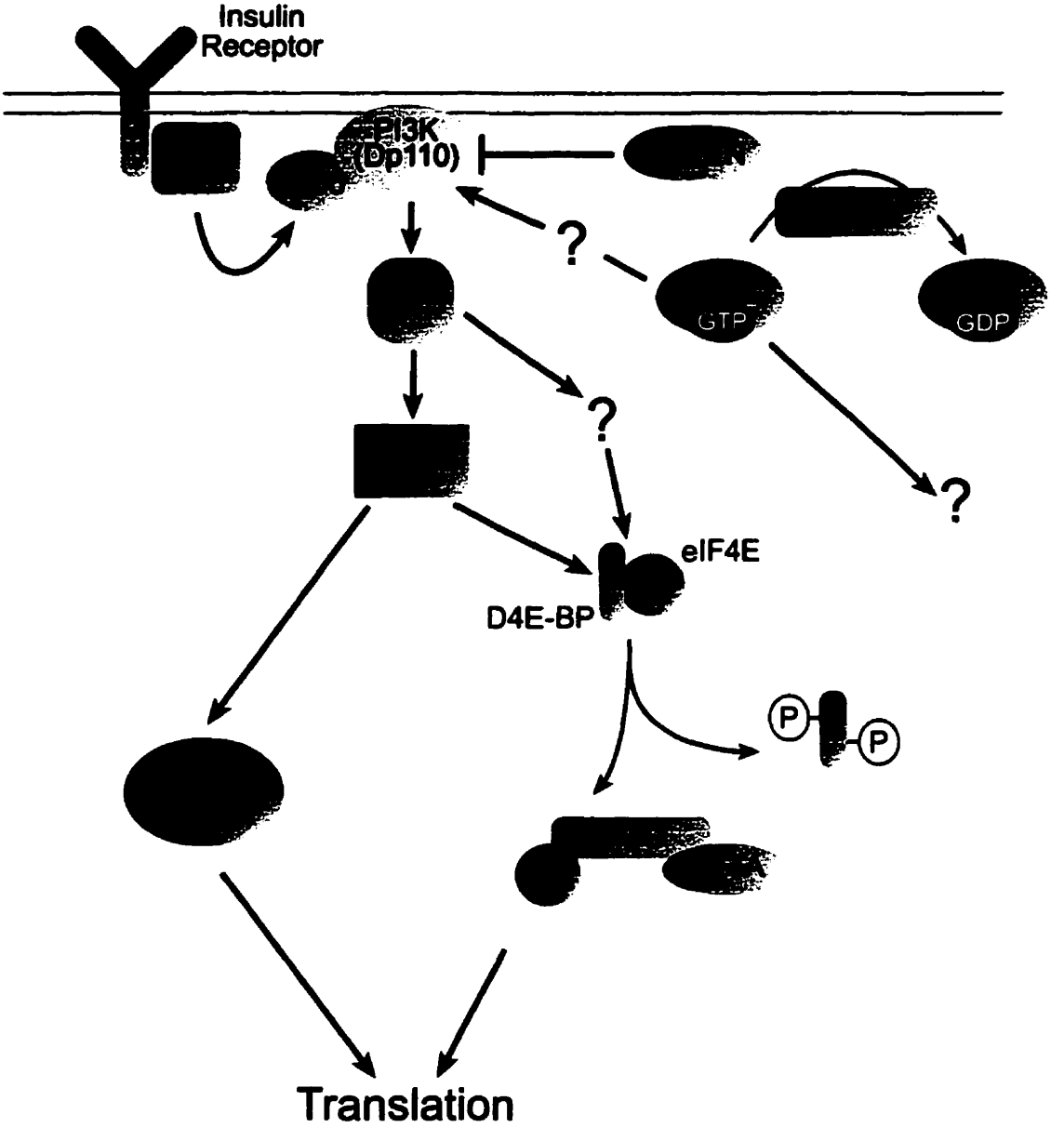
eIF4E66C1 is one of the genes from which an EST was identified, indicating that its gene product is expressed and suggesting that its protein is not regulated by phosphorylation.

The final *Drosophila eIF4E* cognate (CG10716) appears to be the ortholog of the mammalian eIF4E-Homologous Protein (4E-HP) (Fig. 6B). 4E-HP homologues have previously been identified in humans, rats, plants, and *C. elegans*, but not in yeast (Keiper *et al.*, 2000; Rom *et al.*, 1998). Although they contain residues divergent from those in eIF4E shown to interact with the mRNA cap (Marcotrigiano *et al.*, 1997), 4E-HP can bind m^7GpppG but not GpppG capped mRNA *in vitro* (Rom *et al.*, 1998). However, 4E-HP cannot bind eIF4G or 4E-BP1 (Rom *et al.*, 1998), which is not surprising since residues required for these interactions are not conserved (Fig. 6B). 4E-HP appears to localize to the cytoplasm in mammalian cells but its function is unknown.

1.8 Effectors in the PI(3)kinase (PI3K) signal transduction pathway regulate growth and lead to the phosphorylation of 4E-BP

Recent studies in *Drosophila* have shown that the regulation of cell growth and proliferation are uncoupled. Genetic manipulation of the cell cycle in imaginal discs results in tissue compartments with an increased number of small cells or with fewer large cells but never changes the overall size of the tissue (Neufeld *et al.*, 1998). In contrast, members of the phosphoinositide 3-kinase (PI3K) pathway are emerging as key effectors for extracellular signals that control growth (Fig. 7). Interestingly, this signaling pathway leads to the regulation protein synthesis via at least two mechanisms: the phosphorylation of the ribosomal protein S6 kinase $p70^{s6k}$ and of 4E-BP (Gingras *et al.*,

Fig. 7. The phosphoinositide-3 kinase (PI3K) pathway in *Drosophila* controls growth in part by signaling to two regulators of protein synthesis: D4E-BP and DS6K. Most of the evidence for the existence of this pathway comes from mammalian studies although genetic analysis is consistent with its conservation in *Drosophila*. The analysis of phenotypes from *Drosophila* mutants or from overexpression of the genes encoding for Insulin Receptor, Chico, Dp60, Dp110, DPTEN, Dakt1, dTOR, and DS6K are consistent with a function in growth control (see text). In mammals, the kinase Target-of-Rapamycin (TOR, dTOR in *Drosophila*) is central to this pathway as it directly phosphorylates S6K and 4E-BP. The phosphorylation at two residues of 4E-BP by TOR is required as a priming event. This priming event allows the subsequent hyperphosphorylation of 4E-BP, by as yet unidentified kinase(s), and leads to the release of eIF4E. Ras and RasGAP have also been implicated in growth control but it is unclear whether they signal to effectors of the PI3K pathway or whether their effects on growth are mediated through parallel pathways.


Insulin
Receptor
PI3K
(Dp110)
?
GTP
GDP
?
?
eIF4E
D4E-BP
P
P
Translation

1998; von Manteuffel *et al.*, 1997). This section will survey the members of the PI3K pathway and their contribution to the regulation of growth in *Drosophila*.

1.8.1 Insulin receptor and chico

One of the first indications that effectors of insulin signaling influence growth control came from the study of mutations in *Inr*, the gene encoding the *Drosophila* insulin receptor (Chen *et al.*, 1996). Hypomorphic *Inr* mutations lead to the production of adult flies that are small in size. Similarly, a mutation in *chico*, a gene encoding a homologue of insulin receptor adaptor proteins IRS1-4, leads to adults which are less than 50% the size of normal flies and which are delayed in development by 2-3 days (Bohni *et al.*, 1999). The smaller size of *chico* flies and tissues results from the presence of fewer and smaller cells. The reduction in cell number in *chico* tissues is not due to increased apoptosis suggesting that it is pleiotropic and has an effect on proliferation in addition to its function in growth regulation (Bohni *et al.*, 1999).

1.8.2 PI3K

PI3K is downstream of *Inr* and *chico*. The catalytic subunit of *Drosophila* PI3K is encoded by the gene *Dp110* (Leevers *et al.*, 1996). Overexpression of *Dp110* in eye and wing imaginal discs leads to enlarged tissues while expression of a dominant negative form results in smaller organs. Further, mitotic clones of a null mutation in *Dp110* are smaller than their heterozygous or wild-type neighbors indicating that it regulates cell growth in a cell-autonomous fashion (Weinkove *et al.*, 1999). Signals from the insulin

receptor to Dp110 are mediated through the adaptor protein Dp60 (Weinkove *et al.*, 1997). Consistent with this function, mitotic clones of *Dp60* contain cells smaller than those from control clones and tissue specific overexpression of wild-type or dominant negative *Dp60* alters organ size (Weinkove *et al.*, 1999).

1.8.3 DS6K and dAkt1

Similar effects on growth were obtained for the downstream effectors *dAkt1* and *DS6K*, which encode for *Drosophila* homologues of Akt and $p70^{s6k}$ respectively (Montagne *et al.*, 1999; Verdu *et al.*, 1999). RNA interference (RNAi) studies in *Drosophila* S2 cells confirm the relationship of *dAkt1* with other members of the PI3K pathway; activation of *dAkt1* depends on *chico* and is negatively regulated by the antagonist *DPTEN* (Clemens *et al.*, 2000).

Homozygous mutants of the partially viable hypomorphic allele $ds6k^{l-1}$ are delayed in development for about 5 days and are reduced in size by 40% (Montagne *et al.*, 1999). While $ds6k^{l-1}$ flies have smaller cells, unlike *chico* mutants, $ds6k^{l-1}$ tissues do not have a reduction in cell number. One model for the effects of *DS6K* on growth is that phosphorylation of ribosomal protein S6 upregulates the translation of the subset of mRNAs that contain 5' pyrimidine tracts (Jefferies *et al.*, 1997). Thus, *DS6K* mutants may alter organ size by affecting the translation of specific messages that regulate ribosome biogenesis (Weinkove and Leevers, 2000).

1.8.4 DPTEN

The mammalian tumor suppressor protein PTEN (phosphatase and tensin homologue deleted on chromosome 10) antagonizes PI3K signaling (Maehama and Dixon, 1999). Consistent with a negative effect on the PI3K pathway, tissues mutant in *Drosophila PTEN* (*DPTEN*) have large cells and are overgrown while overexpression results in opposite phenotypes (Gao *et al.*, 2000; Goberdhan *et al.*, 1999). The loss-of-function effects of *DPTEN* are suppressed by mutations in *dAkt1* and translation factor *eIF4A*, which supports a role of DPTEN in PI3K signaling and suggests that its effects are at least in part mediated through the deregulation of translation (Gao *et al.*, 2000). However, *DPTEN* alleles also produce an increase in proliferation suggesting additional functions (Gao *et al.*, 2000; Goberdhan *et al.*, 1999).

1.8.5 dTOR

A key component of the PI3K pathway in mammals and yeast is the kinase Target of Rapamycin (TOR, mTOR in mammals; also referred to as FRAP in the literature) (Dennis *et al.*, 1999). The yeast homologues, *TOR1* and *TOR2* were originally identified because they confer resistance to the immunosuppressant drug rapamycin (Heitman *et al.*, 1991). Both *TOR1* and *TOR2* positively regulate protein synthesis in yeast (Barbet *et al.*, 1996). Loss of *TOR* activity in yeast produces a phenotype similar to that of nutrient starvation, suggesting that it is part of a nutrient sensing machinery. In mammals, mTOR is upstream of two branching pathways that regulate ribosome biogenesis and cap-dependent protein synthesis: those leading to $p70^{s6k}$ and 4E-BP

phosphorylation (von Manteuffel *et al.*, 1997). Mutants in the *Drosophila* homologue of TOR (*dTOR*) were independently isolated by two groups (Oldham *et al.*, 2000; Zhang *et al.*, 2000). The stimulation of growth by *DPTEN* requires *dTOR* activity supporting the notion that *dTOR* is an effector in the PI3K pathway (Zhang *et al.*, 2000). In addition, mutants in *dTOR* have phenotypes consistent with a function in amino acid sensing. Loss of *dTOR* function causes a reduction in the growth of endoreplicating tissues (Oldham *et al.*, 2000), the aggregation of lipid vesicles in the fat body, a reduction in size of nucleoli, and induces cell cycle arrest that can be rescued by expression of S-phase cyclins (Zhang *et al.*, 2000). Overexpression of *DS6K* can restore viability to *dTOR* mutants but the rescued mutants are smaller than control animals (Zhang *et al.*, 2000). In addition to phosphorylating $p70^{s6k}$ in mammals, upon activation via the PI3K pathway, TOR directly phosphorylates 4E-BP1; however at least one additional kinase is required to phosphorylate 4E-BP and induce its release of eIF4E (Burnett *et al.*, 1998; Gingras *et al.*, 1999a). The partial rescue of *dTOR* by *DS6K* may be explained by the fact that *4E-BP* is also phosphorylated by *dTOR* in *Drosophila*. Analysis of phenotypes of *dTOR* mutants rescued by *DS6K* overexpression in the background of a *4E-BP* mutation would address this hypothesis genetically.

1.9 Other genes that regulate growth in Drosophila – further links with the control of protein synthesis

In addition to effectors of the PI3K pathway, several other genes that regulate biosynthesis have been shown to have phenotypes consistent with a function in the

control of tissue growth. The subsets of these genes that have a direct function in regulating translation initiation are described in this section.

1.9.1 Nutrition, growth, and eIF4A

When wild-type larvae are given a diet deficient in amino acids, they exhibit an arrest or delay in their growth but continue to live for extended periods of time (Britton and Edgar, 1998). This observation was used as a basis for a screen to identify lethal alleles of genes that phenocopy the larval growth arrest (Galloni and Edgar, 1999). In this screen, an allelic series of translation initiation factor *eIF4A* were found to delay larval development. *eIF4A* mitotic clones induced in imaginal discs also grow slowly but the final size of the disc is not affected. A similar phenotype was described for the gene *bonsai,* which encodes a homologue of the mitochondrial ribosomal protein S15 (Galloni and Edgar, 1999). However, the mutations in *bonsaï* were non cell-autonomous which lead to the suggestion that specialized organs rich in mitochondria coordinate the control of growth in the organism.

1.9.2 Minutes

Heterozygous *Minute* mutants are delayed in their growth but still achieve normal adult body size (Morata and Ripoll, 1975). Several *Minute* genes have been cloned and found to encode cytoplasmic ribosomal proteins or genes involved in ribosome biogenesis (Saeboe-Larssen *et al.*, 1998). *Minute* mitotic clones are out-competed by their wild-type neighbors while the final size of the tissue is not affected (Morata and Ripoll, 1975). The

phenotypes of *Minute* class genes and of *eIF4A* suggest that altering the levels of global protein synthesis affects the growth rate tissues but does not necessarily affect their final size.

1.9.3 eIF2α

The phosphorylation of mammalian eIF2 is an important mechanism for regulating translation initiation. Deregulation of translation was achieved in *Drosophila* by overexpression of phosphorylation mutants of *eIF2α* (Qu *et al.*, 1997). eIF2α mutant proteins in which Ser50 was converted to Ala or Asp and overexpressed via the *hsp70* promoter marginally affected global protein synthesis. However, the Ser50Asp mutant significantly decreased the time of development and caused a reduction in the final body size of the flies. Conversely, overexpression of the Ser50Ala protein induced an increase in the growth rate of flies but only females exhibited increases in body size. These results support the notion that the regulation of translation is required for normal development and that its deregulation leads to aberrant growth.

1.9.4 Ras, dMyc, and Ras-GAP

Drosophila homologues of the genes encoding Myc (*dMyc*) and Ras, both of which were shown to regulate eIF4E activity in mammals, are also implicated in tissue growth control (Johnston *et al.*, 1999; Prober and Edgar, 2000). In line with a role in regulating biosynthesis, *dMyc* can promote the progression from the G_1 to S-phase of the cell cycle but not of the G_2/M transition (Johnston *et al.*, 1999). A putative target for the

dMyc transcription factor that also promotes growth is the gene *pitchoune*, which encodes a DEAD-box family RNA helicase (Zaffran *et al.*, 1998). Mutants in *pitchoune* have a larval growth defect phenotype similar to that described for *eIF4A*.

In mammals, Ras can directly upregulate PI3K activity (Rodriguez-Viciana *et al.*, 1996). Although the involvement of Ras in the PI3K pathway has not been shown in *Drosophila*, *Ras* mitotic clones and overexpression of dominant negative or constitutive Ras proteins clearly demonstrate a function in the regulation of growth (Prober and Edgar, 2000). Furthermore, overexpression in wing imaginal discs of the *Drosophila* homologue of the p120 Ras-GTPase-Activating Protein (RasGAP), which stimulates the GTPase activity of Ras, results in adult wings that are only 55% the size of wings from control flies (Feldmann *et al.*, 1999). The relationship between growth regulation, the Ras pathway, and the regulation of eIF4E activity remains to be demonstrated directly in *Drosophila*.

1.10 Research objectives and rationale for experimental design

At the onset of this project, surprisingly little was known about the genes encoding eIF4 group initiation factors in *Drosophila*. The only member of this group that was cloned was *eIF4A*, the DEAD-box RNA helicase and subunit of eIF4F (Dorn *et al.*, 1993; Verheyen and Cooley, 1994). The ongoing objective of the laboratory was to identify the genes encoding the eIF4 group initiation factors in *Drosophila* since it is a genetically tractable organism well suited to study the regulation of these factors in a multicellular organism.

In mammals, eIF4E is the rate-limiting component of eIF4F and its activity is subject to regulation by phosphorylation and by association with the 4E-BPs. In *Drosophila*, a candidate protein for eIF4E was identified from embryos and *Drosophila* Schneider S2 cells (Duncan *et al.*, 1995; Maroto and Sierra, 1989; Zapata *et al.*, 1994). In S2 cells, the putative eIF4E was shown to be a phosphoprotein; the phosphorylation of eIF4E in S2 cells was reduced upon heat shock, similar to what has been previously described for eIF4E in mammalian cells (Duncan and Hershey, 1984; Duncan *et al.*, 1995). It was thus hypothesized that the activity of eIF4E is also regulated in *Drosophila* and that these mechanisms can be studied using the genetic techniques available to this organism.

The original objective of this project was the cloning of the *Drosophila* homologue of *eIF4E*, for the purpose of characterizing the regulation of this factor in a multicellular organism. At the beginning of this project, Cynthia Lavoie, a previous student in the lab, isolated two mRNA cap-binding proteins by m^7GDP-Sepharose chromatography and obtained microsequence data for the N-termini of these proteins. Concurrently, a cDNA encoding a *Drosophila* homologue of eIF4E was independently identified by another group (Hernandez and Sierra, 1995). The predicted amino acid translation of this *eIF4E* cDNA-matched the sequence of one of the proteins isolated by m^7GDP chromatography. However, the second cap-binding protein remained unknown. We rationalized that it may be a second form of *eIF4E* and we set out to clone the gene and cDNAs encoding this protein. The cloning and characterization of the *Drosophila* gene encoding *eIF4E* is described in Chapter 2.

Upon cloning *eIF4E*, we set out to characterize its regulation during the development of *Drosophila*. Several polyclonal antibodies were generated to characterize the expression of the eIF4E isoforms. Furthermore, since eIF4E overexpression leads to oncogenesis in mammalian tissue cultures, we were also interested in examining the effects of eIF4E overexpression in *Drosophila* in the hope of using potential phenotypes as a tool to identify proteins that regulate eIF4E activity. The results of eIF4E overexpression in *Drosophila* tissues and the characterization of eIF4E isoform expression in different stages of *Drosophila* development are discussed in Chapter 3.

To study eIF4E genetically, we identified mutant alleles of *Drosophila eIF4E*. The identification, molecular characterization, and phenotypic analysis of *Drosophila eIF4E* mutants are described in Chapter 4. We also wished to examine the regulation of eIF4E by phosphorylation. Although mammalian studies had shown a correlation between the phosphorylation state of eIF4E and the growth status of a cell (Raught *et al.*, 2000a), a direct link between eIF4E phosphorylation and growth control had never been established. Via the use of transgenic flies, we generated mutants in the putative phosphorylation site of *Drosophila eIF4E* to (i) identify the site for eIF4E phosphorylation in *Drosophila* and (ii) characterize the phenotypes of these mutants. These results are the first demonstration that the phosphorylation of eIF4E is required for the normal growth of a multicellular organism.

Chapter 2

Alternatively spliced transcripts from the *Drosophila eIF4E* gene produce two different cap-binding proteins

Eukaryotic initiation factor 4E (eIF4E) is the subunit of eIF4F which binds to the cap structure at the 5' end of messenger RNA, and is a critical component for the regulation of translation initiation. Using 7-methyl-GTP-Sepharose affinity chromatography, two distinct cap-binding proteins that migrate on SDS-PAGE at approximately 35 kDa were purified from *Drosophila* adults. Peptide microsequence analysis indicated that these two proteins differ at their amino termini. Analysis of a set of cDNA clones encoding eIF4E led to the conclusion that the two different protein isoforms, which we term eIF4EI and eIF4EII, result from three alternatively spliced transcripts from a single *eIF4E* gene which maps to region 67A8-B2 on polytene chromosomes. The three *eIF4E* transcripts also vary greatly in the lengths of their 5' UTRs, suggesting the possibility of complex translational control of expression of the two eIF4E isoforms.

Introduction

Translation of eukaryotic mRNAs is a complex process that involves numerous components and is regulated at many steps (Merrick and Hershey, 1996). A critical point in the initiation of translation is the binding of the mRNA to the 43S pre-initiation complex, which requires the initiation factor eIF4F. In mammals eIF4F consists of three subunits, eIF4E, eIF4A, and eIF4G (Edery *et al.*, 1983; Grifo *et al.*, 1983). The eIF4E subunit binds the cap structure, $m^7G(5')ppp(5')N$ (where N is any nucleotide), which is found at the 5' end of all cellular eukaryotic mRNAs (Shatkin, 1976; Sonenberg *et al.*, 1979). Among the initiation factors participating in this step, eIF4F, consistent with the low abundance of its eIF4E subunit (Duncan *et al.*, 1987; Hiremath *et al.*, 1985), is a key factor in modulating the rate of ribosome binding to mRNAs.

A single gene encoding eIF4E has been cloned in the following organisms: yeast, *Drosophila*, and three mammalian species (Altmann *et al.*, 1987; Altmann *et al.*, 1989; Hernandez and Sierra, 1995; Metz *et al.*, 1992; Rychlik *et al.*, 1987; Rychlik and Rhoads, 1992). While the mammalian proteins differ in just a few residues, yeast eIF4E is only 33% identical to the mammalian, yet the murine eIF4E can function *in vivo* in yeast, albeit when expressed from a multicopy plasmid (Altmann *et al.*, 1989). The polypeptide compositions of cap-binding complexes (or eIF4F) differ in various experimental systems. Mammalian eIF4F is composed of three distinct polypeptides: eIF4E, eIF4A, and eIF4G (Edery *et al.*, 1983; Grifo *et al.*, 1983; Tahara *et al.*, 1981), but the yeast and *Drosophila* eIF4F proteins lack the eIF4A polypeptide (Goyer *et al.*, 1993; Goyer *et al.*, 1989; Zapata *et al.*, 1994). Wheat germ has two cap-binding complexes: eIF4F resembles its

yeast and *Drosophila* counterparts and contains subunits of 26 and 220 kDa, while the second cap-binding complex, called eIF(iso)4F, is composed of two polypeptides of 82 and 28 kDa (Allen *et al.*, 1992; Browning *et al.*, 1992). The 28 kDa wheat germ protein is approximately 50% identical in amino acid sequence to the 26 kDa subunit of eIF4F.

In mammals, the *eIF4E* gene has been demonstrated to be oncogenic, as overexpression of eIF4E in the murine NIH 3T3 cell line or in Rat 2 fibroblasts causes malignant transformation, and microinjection of eIF4E into quiescent NIH 3T3 cells activates DNA synthesis (Lazaris-Karatzas *et al.*, 1990; Smith *et al.*, 1990). These effects have been shown to be mediated by the Ras proto-oncogene (Lazaris-Karatzas *et al.*, 1992). Additionally, eIF4E can co-operate with the nuclear oncogenes c-*myc* and *E1A* in transformation of primary cultured cells (Lazaris-Karatzas and Sonenberg, 1992). A role for eIF4E in development is also supported by the demonstration that injection of eIF4E into *Xenopus laevis* animal pole explants leads to mesoderm induction (Klein and Melton, 1994).

As part of an effort to understand the mechanisms underlying the initiation of translation in *Drosophila melanogaster*, we are studying translation initiation factors. A 35 kDa cap-binding protein resembling eIF4E has previously been purified from *Drosophila* (Maroto and Sierra, 1989; Zapata *et al.*, 1994). Its gene has been recently identified and shown to encode a protein with extensive sequence similarity to eIF4E (Hernandez and Sierra, 1995). In this report we show that the 35 kDa cap-binding activity is composed of two distinct isoforms of eIF4E, with different amino-terminal

ends, which we term eIF4EI and eIF4EII. These isoforms result from alternative splicing of a single primary transcript.

Experimental Procedures

Cap-column chromatography

Oregon-R adults were collected and frozen at -70°C. 12 grams of thawed material was lysed using a polytron (Brinkmann) at 10,000 rpm in 200 ml buffer A (50 mM HEPES, pH 7.6; 70 mM KCl; 2 mM DTT; 10% glycerol; 0.1 mM EDTA; 5 mM magnesium acetate; 40 mg/l PMSF; 50 mg/l TLCK; 0.5 mg/l aprotinin). The unlysed material was pelleted for 15 min at 5000 x g in a Sorvall SS-34 rotor and the supernatant was further purified of particulate matter by passage through nylon mesh (Nitex). The supernatant was spun two times at 40,000 x g for 25-30 min in a Beckman 45Ti rotor. *Drosophila* eIF4E has previously been shown to be enriched in the post-ribosomal supernatant compared with ribosomal high salt wash (Maroto and Sierra, 1989). Post-ribosomal supernatants were prepared essentially as described previously (Mateu and Sierra, 1987; Webster *et al.*, 1991). Briefly, the supernatant was spun for 2 hr at 260,000 x g in a Beckman 70Ti rotor. A 0-70% ammonium sulfate fraction of the post-ribosomal supernatant was then dialysed against buffer B (20 mM HEPES, pH 7.6; 120 mM KCl; 1 mM DTT; 3% glycerol; 0.1 mM EDTA; 40 mg/l PMSF; 50 mg/l TLCK; 0.5 mg/l aprotinin). All steps were performed at 4°C.

Cap column chromatography was carried out on post-ribosomal supernatants as in Maroto and Sierra (1989) using m^7GTP-Sepharose (Pharmacia) and the cap analogue

m^7GDP (Sigma). A total of 22 mg of protein from the post-ribosomal supernatant was added to 0.5 ml m^7GTP-Sepharose and incubated for 2.5 hours at 4°C. The beads and protein were then poured onto a disposable column (BioRad) and washed with three 10 ml volumes of buffer B. The second wash contained 0.1 mM GTP. Elution volumes of 0.5 ml were collected using 75 μM m^7GDP in buffer B.

Analysis of proteins and preparation for microsequencing

Proteins (10 μl) were analysed on silver-stained 12% SDS-polyacrylamide gels. For microsequencing, elutions from cap-binding columns were concentrated by lyophilization, then run on several lanes of a 12% SDS- polyacrylamide gel and transferred to Millipore PVDF filters. The filters were stained with 0.25% Coomassie blue (Sigma) and destained using 90% methanol, 7% acetic acid. The bands of interest were excised from the filters and kept at -20°C until processing. Amounts analysed by microsequencing were 13 and 4 pmol of the faster and slower migrating 35 kDa proteins, respectively.

Isolation of Drosophila* eIF4E *clones

A fragment of the *Drosophila eIF4E* gene was amplified by PCR from *Drosophila* genomic DNA using 250-300 pmol of sense (5'-AAACACCC GCTCATGAA-3') and antisense (5'-CAGCTTGTGACCAATCTC-3') primers; the primer sequences were obtained from the previously published *eIF4E* (Hernandez and Sierra, 1995). PCR buffer

(Gibco-BRL) was supplemented with 1.5 mM $MgCl_2$, 0.4 mM each dNTP, and 2.5 units of Taq DNA polymerase (Gibco-BRL). Thermocycling was performed in a Perkin-Elmer-Cetus instrument using the following conditions: 2 cycles of [95°C for 2 minutes, 46°C for 2 minutes, and 72°C for 4 minutes] followed by 20 cycles of [95°C for 40 seconds, 46°C for 1 minute, and 72°C for 50 seconds]. Reactions were then supplemented with 4 mM EDTA and precipitated in one volume of 7.5 M ammonium acetate and 2 volumes of ethanol and resuspended in water. A second round of PCR was performed with conditions as above except that one twenty-fifth of the ammonium acetate precipitated material was used as template. A 700 base pair product was gel purified and confirmed as a fragment of the *eIF4E* gene by direct sequencing. This fragment was labeled with α-^{32}P-dCTP by random priming (Oligolabeling Kit, Pharmacia) and used to screen 150,000 individual plaques of a 0-2 hr embryo cDNA library constructed in λZAP (Beat Suter, unpublished results). Hybridization was performed using standard techniques (Sambrook *et al.*, 1989). Nine positively hybridizing clones were obtained. The pBluescript phagemid was excised from λZAP using the ExAssist helper phage/SOLR cell system (Stratagene). The clones were then sequenced on both strands with the double-stranded dideoxynucleotide method using oligonucleotide primers. Genomic DNA clones that include the *eIF4E* gene were isolated by screening approximately 240,000 individual plaques from a *Drosophila* genomic DNA library constructed in the vector λFIXII (Beat Suter, unpublished results). To screen the genomic library, a 1.4 kb fragment from the *Drosophila eIF4E* gene was amplified by

PCR using sense (5'-TGTTGGAGACGGAGAAG-3') and antisense (5'-GTTCACCAGTCTCCTG-3') primers and labeled with α-^{32}P-dCTP as described above. Five positively hybridizing clones were obtained and subcloned in pBluescript. Two of the clones were then sequenced on both strands as described above.

Obtaining the 5' sequence of the 2.0 kb* eIF4E *transcript

As the only cDNA clone representing the 2.0 kb transcript was truncated at the 5' end, we obtained 5' terminal sequence by PCR as described above, using as template DNA prepared from a 0-4 hr embryo cDNA library (Brown and Kafatos, 1988). Amplification primers were 5'-CGATTTAGGTGACACTATAG-3' (SP6 Primer, sense) and 5'-CGCGGTGTTTGTGATAG-3' (primer A, antisense). A second round of PCR using the same primers was done using 1/25th of the ammonium-precipitated material from round one. To specifically amplify the 5' end of the 2.0 kb *eIF4E* transcript, a PCR experiment was performed using as template the product of the above reaction and as primers the SP6 primer and a second one, 5'-ACTCGTTAAACTTGTTG-3' (primer B, antisense), within exon 1A of *eIF4E*. In this reaction a 450 bp product was amplified. Further amplification reactions were done using this product as template with primers 5'-TGTTGGAGACGGAGAAG-3' (primer C, sense) and either primer B or 5'-ATGGTGTTGAGTATCC-3' (primer D, antisense) and products of 160 bp and 220 bp were obtained. These products were sequenced directly on both strands.

Nucleic acid hybridizations

In situ hybridizations to salivary gland chromosomes were carried out using biotinylated probes essentially as described in Ashburner, 1989. Southern hybridization to genomic DNA was done using GeneScreen Plus filters (Dupont) at high stringency according to the manufacturer's instructions. Poly A^+ RNA samples isolated from 0-3 hr embryos were separated by formaldehyde gel electrophoresis and transferred to a GeneScreen Plus membrane (DuPont). The membrane was incubated for 3-4 hr at 42°C in hybridization solution [5X SSPE, 50% deionized formamide, 5X Denhardt's (0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 1% SDS, 10% dextran sulfate]. The solution was then replaced with fresh hybridization solution supplemented with $5x10^5$ cpm/ml of α-^{32}P-dCTP labeled probe and incubated overnight at 42°C. The membrane was then washed twice for 15 minutes in 2X SSPE at room temperature, twice for 45 minutes in 2X SSPE/2% SDS at 60°C, twice for 15 minutes in 0.1X SSPE at room temperature, and autoradiographed. Between hybridizations probe was removed from the filter by boiling for 30 min in 10 mM Tris-HCl pH 7.5, 1 mM EDTA, 1% SDS.

Results

Two distinct 35 kDa cap-binding proteins in Drosophila *adults*

Using extracts prepared from *Drosophila* adults, we purified cap-binding proteins by m^7GTP-Sepharose column chromatography. In accordance with previous reports (Duncan *et al.*, 1995; Maroto and Sierra, 1989; Zapata *et al.*, 1994), the major

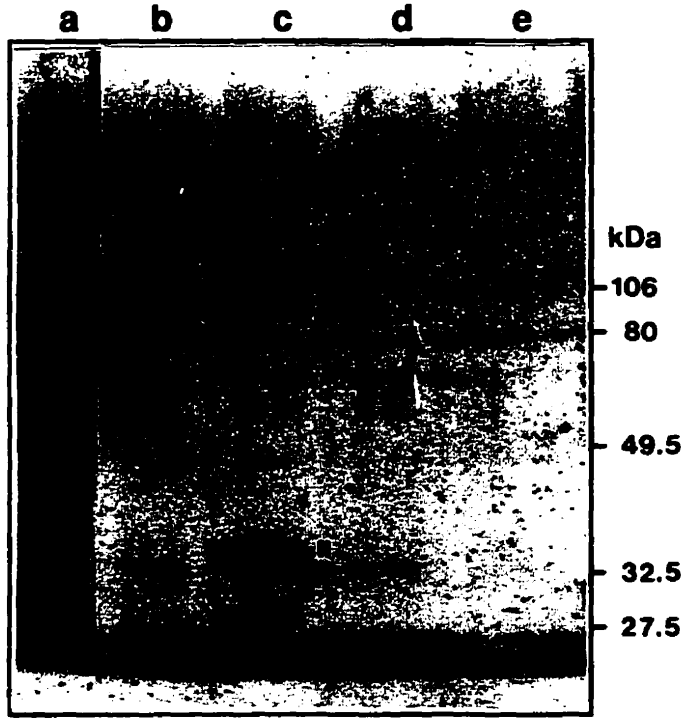
cap-binding activity migrates at approximately 35 kDa on SDS-PAGE; however, our gels resolved two distinct polypeptide bands (Fig. 1A, *lane c*). As a wash containing unmodified GTP elutes at most a small proportion of these two proteins (Fig. 1A, *lane a*), their binding to the column is specific to the methylguanosine cap. The results of N-terminal microsequencing of the two cap-binding proteins is shown in Fig. 1B. The sequence of the faster migrating form matches well (9/10 identities) with residues 24-33 of the eIF4E protein sequence reported by Hernández and Sierra (1995), but the sequence of the slower migrating form does not correspond to the previously reported sequence.

Different cDNA clones encode different eIF4E proteins

Since we purified two distinct cap-binding proteins and since the *eIF4E* gene produces three different transcripts (Hernandez and Sierra, 1995), we reasoned that different isoforms of eIF4E might be produced from different RNAs. To test this idea we isolated nine independent eIF4E clones from a 0-2 hr embryonic cDNA library (B. Suter, unpublished observations). We found that five of the nine clones were colinear with the sequence reported by Hernández and Sierra and would be predicted to encode the same eIF4E protein they described. However, these clones indicated that this *eIF4E* transcript has a substantially longer 5' untranslated region (UTR) than has been previously recognized. Two other clones (1.4A1 and 1.4D2; Fig. 2) differed from the others in that they lacked a segment of 330 nucleotides from the 5' UTR and extreme 5' end of the predicted open reading frame. Conceptual translation of these clones resulted in a predicted second eIF4E protein (which we term eIF4EII) in which the N-terminal 19

Fig. 1. **(A)** Post-ribosomal supernatant, processed as described in Experimental Procedures was adsorbed to a column of m^7GDP-Sepharose. A wash containing 0.1mM GTP was analysed on a silver-stained dried SDS-PAGE (lane a). Proteins were obtained using 75 μM m^7GDP. Four elutions were analyzed for the presence of cap-binding proteins (lanes b-e). **(B)** N-terminal peptide sequence obtained from **i)** the faster-migrating 35 kDa cap-binding protein (eIF4EI); **ii)** the slower-migrating 35 kDa cap-binding protein (eIF4EII).

A

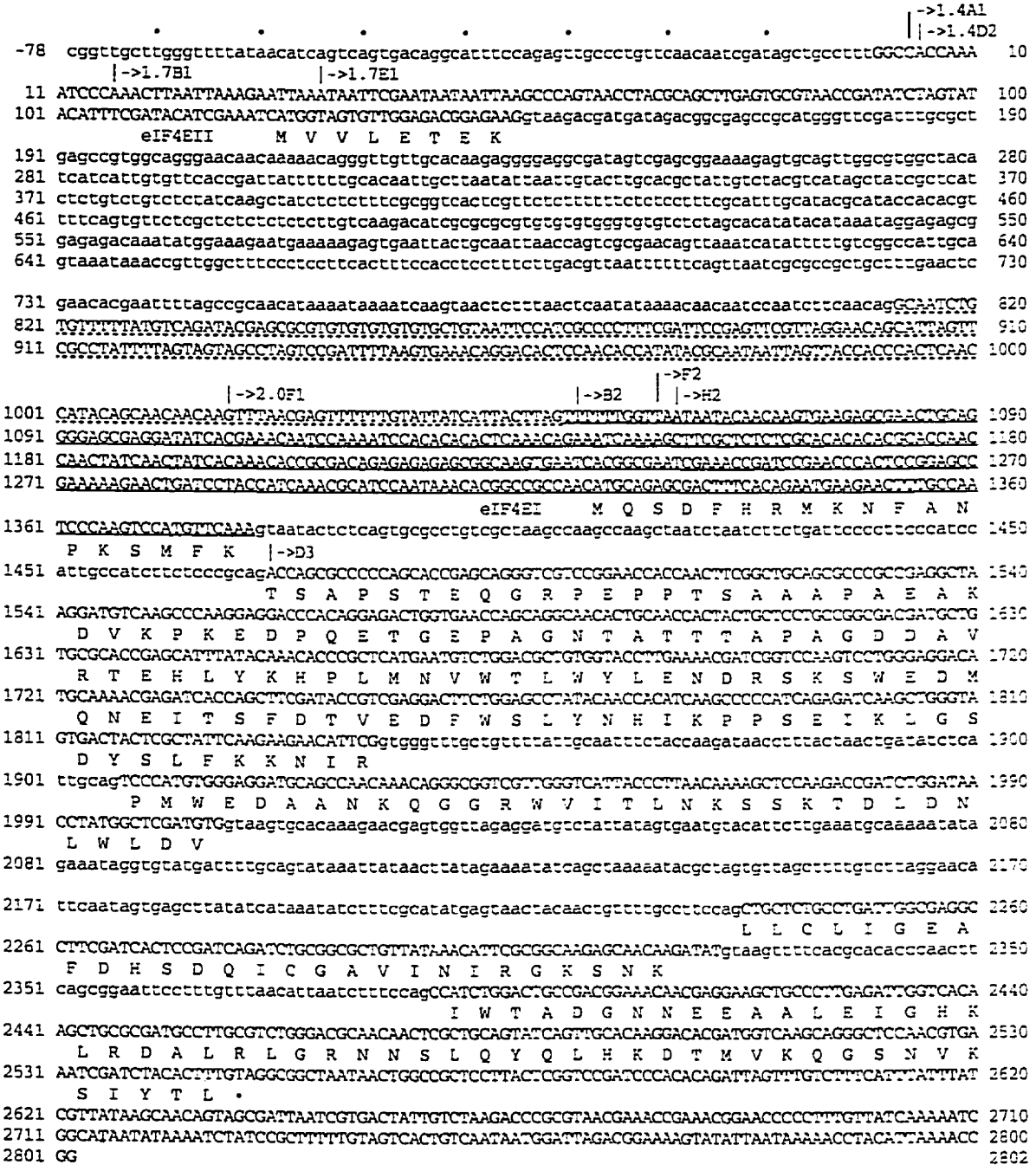


B

i) KTEQPRPEPP
S A T
G
A

ii) VLETEKTSA

Fig. 2. Nucleotide sequencing of *Drosophila* genomic DNA containing the *eIF4E* gene. Nucleotide +1 is defined as the first nucleotide of the 1.4A1 cDNA clone; all other cDNA clone start sites are indicated. Intron sequences are in lower case. The 3' ends of the cDNAs are all identical. Clones prefixed 1.7 encode eIF-4EI and lack nucleotides 146-1049 (intron 1) and 1380-1470 (intron 2), while clones prefixed 1.4 lack nucleotides 146-1470 (intron 1, exon 1B, and intron 2 are removed as a single intron) and encode eIF4EII. The truncated clones B2, F2, and H2 could arise from either the 1.7 or 2.0 kb transcript. Clone 2.0F1 results from use of an alternative intron 1 splice acceptor site and retains nucleotides 813-1049 (exon 1A). The truncated clone D3 contains only sequences common to all three transcripts. The 5' end of the clone described in Hernández and Sierra (1995) is at nucleotide 1152, and this clone is of the 1.7 or 2.0 type.


-78 cggttgcttgggttttataacatcagtcagtgacaggcatttccagagttgccctgttcaacaatcgatagctgcctttGGCCACCAAA 10
|->1.7B1
|->1.7E1
11 ATCCCAAACTTAATTAAAGAATTAAATAATTCGAATAATAATTAAGCCCAGTAACCTACGCAGCTTGAGTGCGTAACCGATATCTAGTAT 100
101 ACATTTCGATACATCGAAATCATGGTAGTGTTGGAGACGGAGAAGgtaagacgatgatagacggcgagccgcatgggttcgatttgcgct 190
eIF4EII M V V L E T E K
191 gagccgtggcagggaacaacaaaaacagggttgttgcacaagaggggaggcgatagtcgagcggaaaagagtgcagttggcgtggctaca 280
281 tcatcattgtgttcaccgattatttttgcacaattgcttaatattaattgtacttgcacgctattgtctacgtcatagctatcgctcat 370
371 ctctgtctgtctctatcaagctatctctctttcgcggtcactcgttctctttttctctcctttcgcatttgcatacgcatacccacacgt 460
461 tttcagtgttctcgctctctctctcttgtcaagacatcgcgcgcgtgtgtgtgggtgtgtctctagcacatatacataaataggagagcg 550
551 gagagacaaatatggaaagaatgaaaaagagtgaattactgcaattaaccagtcgcgaacagttaaatcatatttttgtcggccattgca 640
641 gtaaataaaccgttggctttccctccttcactttccacctcctttcttgacgttaatttttcagttaatcgcgccgctgctttgaactc 730

731 gaacacgaattttagccgcaacataaaataaaatcaagtaactctttaactcaatataaaacaacaatccaatcttcaacagGCAATCTG 820
821 TGTTTTTATGTCAGATACGAGCGCGTGTGTGTGTGTGCTGTAATTCCATCGCCCCTTTCGATTCCGAGTTCGTTAGGAACAGCATTAGTT 910
911 CGCCTATTTTAGTAGTAGCCTAGTCCGATTTTAAGTGAAACAGGACACTCCAACACCATATACGCAATAATTAGTTACCACCCACTCAAC 1000
|->2.0F1
|->B2
|->F2
|->H2
1001 CATACAGCAACAACAAGTTTAACGAGTTTTTTGTATTATCATTACTTAGTTTTTTGGTTAATAATACAACAAGTGAAGAGCGAACTGCAG 1090
1091 GGGAGCGAGGATATCACGAAACAATCCAAAATCCACACACGACTCAAACAGAAATCAAAAGCTTCGCTCTCTCGCACACACGCCACCAAC 1180
1181 CAACTATCAACTATCACAAACACCGCGACAGAGAGAGAGCGGCAAGTGAATCACGGCGAATCGAAACCGATCCGAACCCACTCCGGAGCC 1270
1271 GAAAAAGAACTGATCCTACCATCAAACGCATCCAATAAACACGGCCGCCAACATGCAGAGCGACTTTCACAGAATGAAGAACTTTGCCAA 1360
eIF4EI M Q S D F H R M K N F A N
1361 TCCCAAGTCCATGTTCAAAgtaatactctcagtgcgcctgtcgctaagccaagccaagctaatctaatcttctgattcccctttcccatcc 1450
P K S M F K |->D3
1451 attgccatctttctcccgcagACCAGCGCCCCCAGCACCGAGCAGGGTCGTCCGGAACCACCAACTTCGGCTGCAGCGCCCGCCGAGGCTA 1540
T S A P S T E Q G R P E P P T S A A A P A E A K
1541 AGGATGTCAAGCCCAAGGAGGACCCACAGGAGACTGGTGAACCAGCAGGCAACACTGCAACCACTACTGCTCCTGCCGGCGACGATGCTG 1630
D V K P K E D P Q E T G E P A G N T A T T T A P A G D D A V
1631 TGCGCACCGAGCATTTATACAAACACCCGCTCATGAATGTCTGGACGCTGTGGTACCTTGAAAACGATCGGTCCAAGTCCTGGGAGGACA 1720
R T E H L Y K H P L M N V W T L W Y L E N D R S K S W E D M
1721 TGCAAAACGAGATCACCAGCTTCGATACCGTCGAGGACTTCTGGAGCCTATACAACCACATCAAGCCCCCATCAGAGATCAAGCTGGGTA 1810
Q N E I T S F D T V E D F W S L Y N H I K P P S E I K L G S
1811 GTGACTACTCGCTATTCAAGAAGAACATTCGgtgggtttgctgttttattgcaatttctaccaagataacctttactaactgatatctca 1900
D Y S L F K K N I R
1901 ttgcagTCCCATGTGGGAGGATGCAGCCAACAAACAGGGCGGTCGTTGGGTCATTACCCTTAACAAAAGCTCCAAGACCGATCTGGATAA 1990
P M W E D A A N K Q G G R W V I T L N K S S K T D L D N
1991 CCTATGGCTCGATGTGgtaagtgcacaaagaacgagtggttagaggatgtctattatagtgaatgtacattcttgaaatgcaaaaatata 2080
L W L D V
2081 gaaataggtgtatgattttgcagtataaattataacttatagaaaatatcagctaaaaatacgctagtgttagcttttgtcttaggaaca 2170

2171 ttcaatagtgagcttatatcataaatatcttttcgcatatgagtaactacaactgttttgccttccagCTGCTCTGCCTGATTGGCGAGGC 2260
L L C L I G E A
2261 CTTCGATCACTCCGATCAGATCTGCGGCGCTGTTATAAACATTCGCGGCAAGAGCAACAAGATATgtaagttttcacgcacacccaacttt 2350
F D H S D Q I C G A V I N I R G K S N K
2351 cagcggaattcctttgtttaacattaatcttttccagCCATCTGGACTGCCGACGGAAACAACGAGGAAGCTGCCCTTGAGATTGGTCACA 2440
I W T A D G N N E E A A L E I G H K
2441 AGCTGCGCGATGCCTTGCGTCTGGGACGCAACAACTCGCTGCAGTATCAGTTGCACAAGGACACGATGGTCAAGCAGGGCTCCAACGTGA 2530
L R D A L R L G R N N S L Q Y Q L H K D T M V K Q G S N V K
2531 AATCGATCTACACTTTGTAGGCGGCTAATAACTGGCCGCTCCTTACTCGGTCCGATCCCACACAGATTAGTTTGTCTTTCATTTATTTAT 2620
S I Y T L •
2621 CGTTATAAGCAACAGTAGCGATTAATCGTGACTATTGTCTAAGACCCGCGTAACGAAACCGAAACGGAACCCCCTTTGTTATCAAAAATC 2710
2711 GGCATAATATAAAATCTATCCGCTTTTTGTAGTCACTGTCAATAATGGATTAGACGGAAAAGTATATTAATAAAAACCTACATTAAAACC 2800
2801 GG 2802

amino acids present in the previously reported polypeptide (eIF4EI) are replaced with 8 new amino acids encoded by sequences included in the 5' UTR of the unspliced transcript (Fig. 3). The two final clones (2.0F1 and D3; Fig. 2) will be discussed below.

When we compared the predicted eIF4EII sequence to that of the N-terminal peptide sequence we obtained from the slower migrating 35 kDa cap-binding protein (Fig. 1B, *sequence ii*), we found a perfect match (9/9) with predicted amino acids 3-11, which span the putative splice junction. The concordance between the peptide sequence we obtained and the structure of the 1.4A1 and 1.4D2 clones indicates that both alternative splice forms are present and actively translated in *Drosophila* adults, resulting in two distinct eIF4E proteins differing at their amino-terminal ends. *Drosophila* eIF4EI differs from other related proteins by a unique N-terminal extension (Hernandez and Sierra, 1995). The alternative N-terminus found in the eIF4EII sequence more closely resembles those of other eIF-4E proteins (Fig. 3).

A single* eIF4E *gene is located in polytene chromosome region 67A8-B2

To distinguish whether these different transcripts were the products of a single *eIF4E* gene, or whether there are multiple copies of *eIF4E,* we carried out hybridizations using the cDNAs as probes to total genomic DNA and to larval polytene chromosomes. *In situ* hybridizations indicated the *eIF4E* gene maps to region 67A8-B2 on the left arm of chromosome 3 (Fig. 4A). Genomic Southern blots also only indicated bands predicted from our genomic clones (Fig. 4B). Furthermore, nucleotide sequencing of the 67A8-B2 genomic DNA showed that all nine cDNA clones represent transcripts originating from

Fig. 3. Alignment of the polypeptide sequences of *Drosophila* eIF4EI and eIF4EII with related cap-binding proteins from mouse, wheat (eIF4E and p28) and yeast. Identities are labeled with a •. Underline indicates amino acids identified by N-terminal peptide microsequencing of purified cap-binding proteins (Fig. 1B).

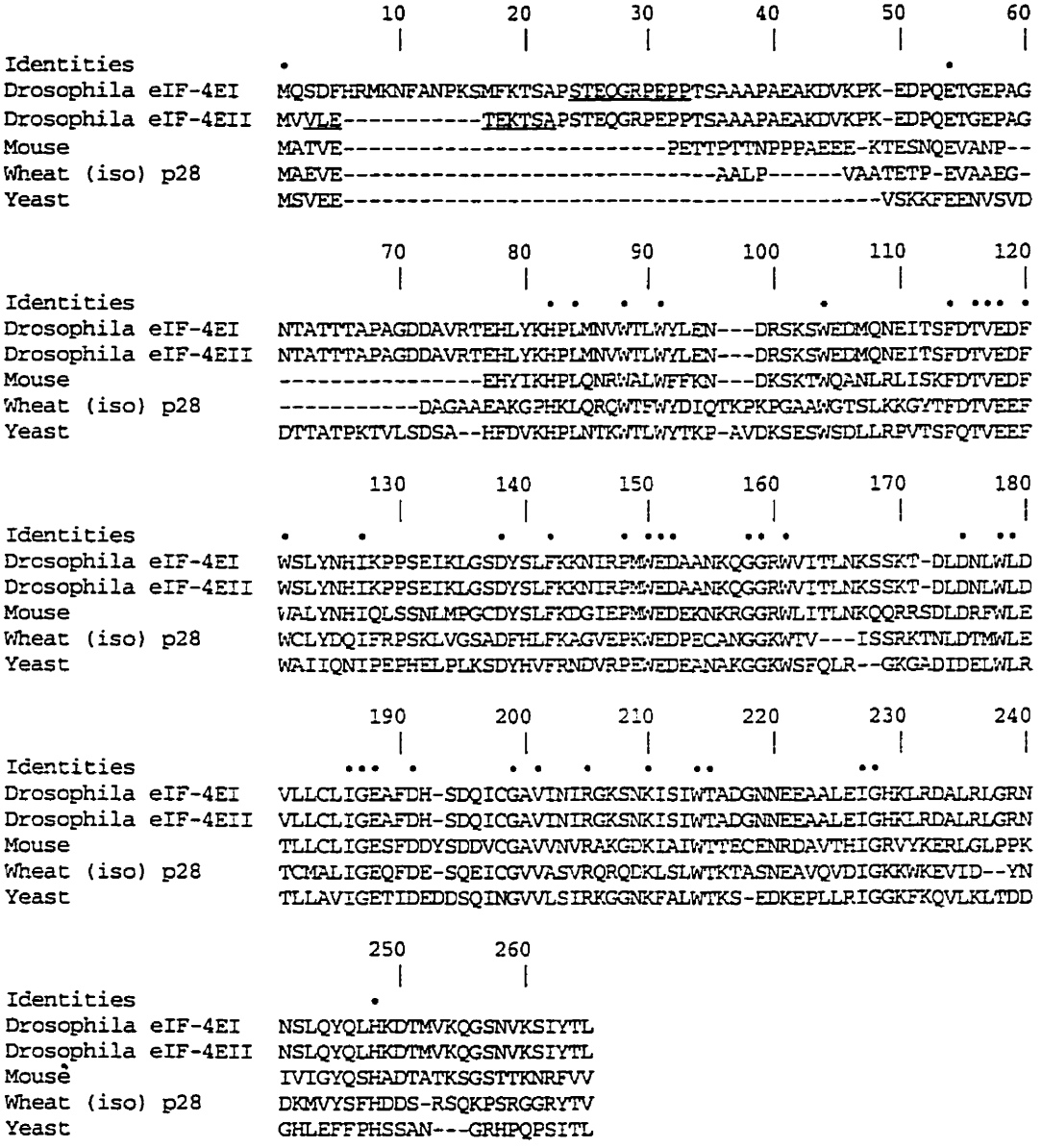
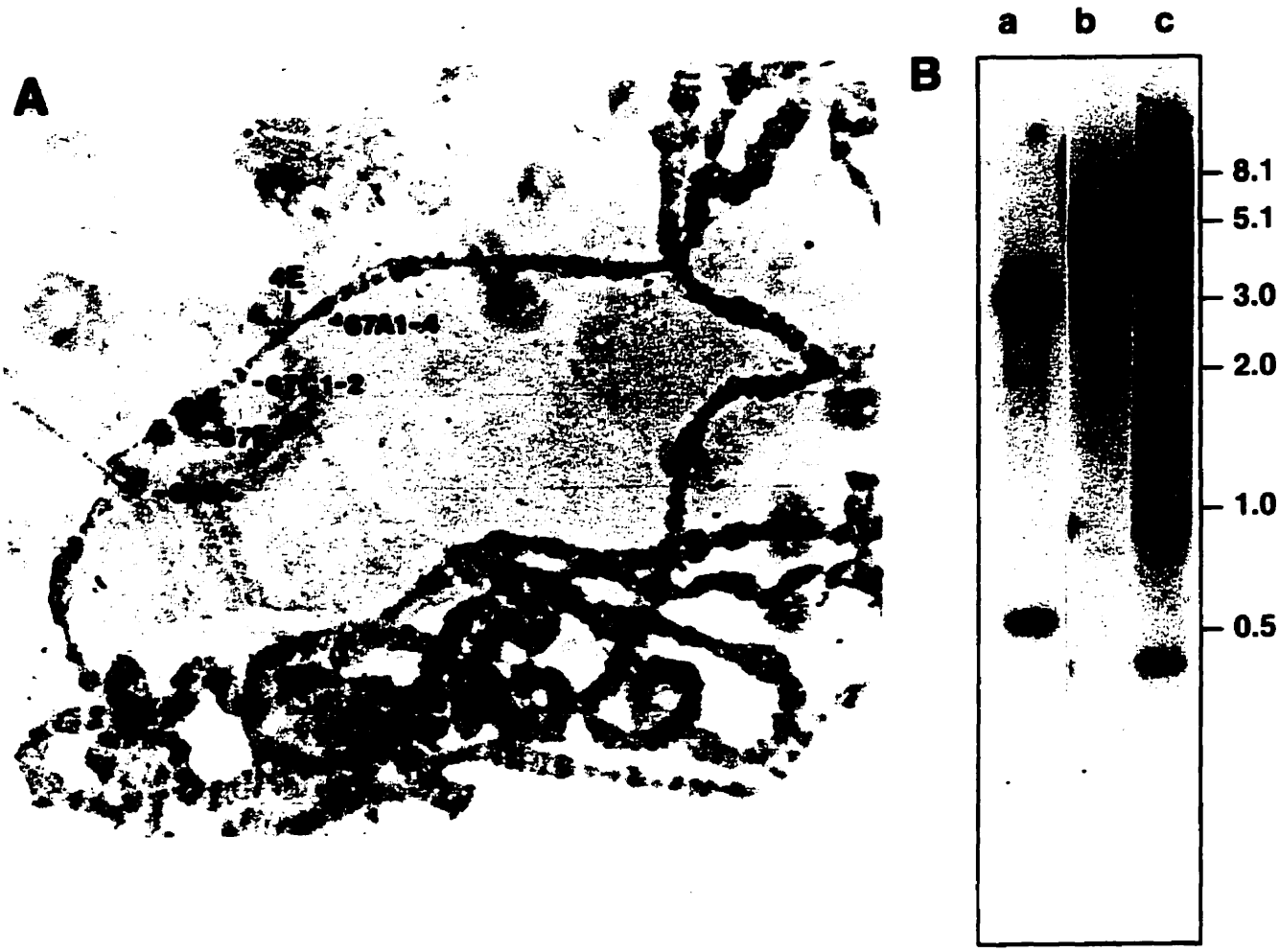

10 20 30 40 50 60
Identities
Drosophila eIF-4EI MQSDFHRMKNFANPKSMFKTSAPSTEQGRPEPPTSAAAPAEAKDVKPK-EDPQETGEPAG
Drosophila eIF-4EII MVVLE----------TEKTSAPSTEQGRPEPPTSAAAPAEAKDVKPK-EDPQETGEPAG
Mouse MATVE----------PETTPTTNPPPAEEE-KTESNQEVANP--
Wheat (iso) p28 MAEVE----------AALP------VAATETP-EVAAEG-
Yeast MSVEE----------VSKKFEENVSVD
70 80 90 100 110 120
Identities
Drosophila eIF-4EI NTATTTAPAGDDAVRTEHLYKHPLMNVWTLWYLEN---DRSKSWEDMQNEITSFDTVEDF
Drosophila eIF-4EII NTATTTAPAGDDAVRTEHLYKHPLMNVWTLWYLEN---DRSKSWEDMQNEITSFDTVEDF
Mouse ----------EHYIKHPLQNRWALWFFKN---DKSKTWQANLRLISKFDTVEDF
Wheat (iso) p28 ----------DAGAAEAKGPHKLQRQWTFWYDIQTKPKPGAAWGTSLKKGYTFDTVEEF
Yeast DTTATPKTVLSDSA--HFDVKHPLNTKWTLWYTKP-AVDKSESWSDLLRPVTSFQTVEEF
130 140 150 160 170 180
Identities
Drosophila eIF-4EI WSLYNHIKPPSEIKLGSDYSLFKKNIRPMWEDAANKQGGRWVITLNKSSKT-DLDNLWLD
Drosophila eIF-4EII WSLYNHIKPPSEIKLGSDYSLFKKNIRPMWEDAANKQGGRWVITLNKSSKT-DLDNLWLD
Mouse WALYNHIQLSSNLMPGCDYSLFKDGIEPMWEDEKNKRGGRWLITLNKQQRRSDLDRFWLE
Wheat (iso) p28 WCLYDQIFRPSKLVGSADFHLFKAGVEPKWEDPECANGGKWTV---ISSRKTNLDTMWLE
Yeast WAIIQNIPEPHELPLKSDYHVFRNDVRPEWEDEANAKGGKWSFQLR--GKGADIDELWLR
190 200 210 220 230 240
Identities
Drosophila eIF-4EI VLLCLIGEAFDH-SDQICGAVINIRGKSNKISIWTADGNNEEAALEIGHKLRDALRLGRN
Drosophila eIF-4EII VLLCLIGEAFDH-SDQICGAVINIRGKSNKISIWTADGNNEEAALEIGHKLRDALRLGRN
Mouse TLLCLIGESFDDYSDDVCGAVVNVRAKGDKIAIWTTECENRDAVTHIGRVYKERLGLPPK
Wheat (iso) p28 TCMALIGEQFDE-SQEICGVVASVRQRQDKLSLWTKTASNEAVQVDIGKKWKEVID--YN
Yeast TLLAVIGETIDEDDSQINGVVLSIRKGGNKFALWTKS-EDKEPLLRIGGKFKQVLKLTDD
250 260
Identities
Drosophila eIF-4EI NSLQYQLHKDTMVKQGSNVKSIYTL
Drosophila eIF-4EII NSLQYQLHKDTMVKQGSNVKSIYTL
Mouse IVIGYQSHADTATKSGSTTKNRFVV
Wheat (iso) p28 DKMVYSFHDDS-RSQKPSRGGRYTV
Yeast GHLEFFPHSSAN---GRHPQPSITL

Fig. 4. (A) *In situ* hybridization of biotin-labelled *eIF4E* cDNA clone 1.7E1 to larval salivary gland chromosomes. Chromosomes were stained with Trypan blue to visualize banding, and the reddish-black hybridization signal corresponding to *eIF4E* is marked with an arrow and labelled 4E. Prominent nearby bands are identified. **(B)** Southern hybridization of *eIF4E* cDNA clone 1.7E1 to genomic DNA digested with (a) *Eco*RI, (b) *Sst*I, (c) *Pst*I. There are three *Pst*I sites, one *Eco*RI site, and no *Sst*I sites within the *eIF4E* gene. A 7.5-kb genomic *Pst*I fragment which contains sequences from the extreme 5' end of the cDNA (nucleotides 16-145 and 1050-1090, Fig. 2) is not apparent on this exposure, but is readily detected when a fragment of an *eIF4E* genomic clone containing intron 1 is used as probe (data not shown).


A
B
a
b
c
8.1
5.1
3.0
2.0
1.0
0.5

the single *eIF4E* gene (Fig. 2). In the five clones which are colinear with the previously reported sequence (and thus encode eIF4EI), nucleotides 146-1049 are removed as an intron, nucleotides 1050-1379 remain in place as exon 1B, and nucleotides 1380-1470 are removed as an intron. Clones 1.4A1 and 1.4D2 (Fig. 2), which encode eIF4EII, represent an alternative splicing event, in that exon 1B is missing and nucleotides 146-1470 are removed as a single intron. An eighth clone, 2.0F1 (Fig. 2), has at its 5' end 33 nucleotides of sequence corresponding to the 3' end of the first intron in the 1.7 series clones (nucleotides 1017-1049). This clone suggests a third alternative splicing event in which a different acceptor site is utilized for intron 1 in the mature message leaving behind additional exon sequences which we term exon 1A. This alternatively-spliced transcript would be predicted to encode eIF4EI, but would have a longer 5' UTR than the other eIF4EI clones. The final clone, D3, begins at nucleotide 1471 (Fig. 2) and probably represents an aberrant splicing event in which the more 5' exons were lost.

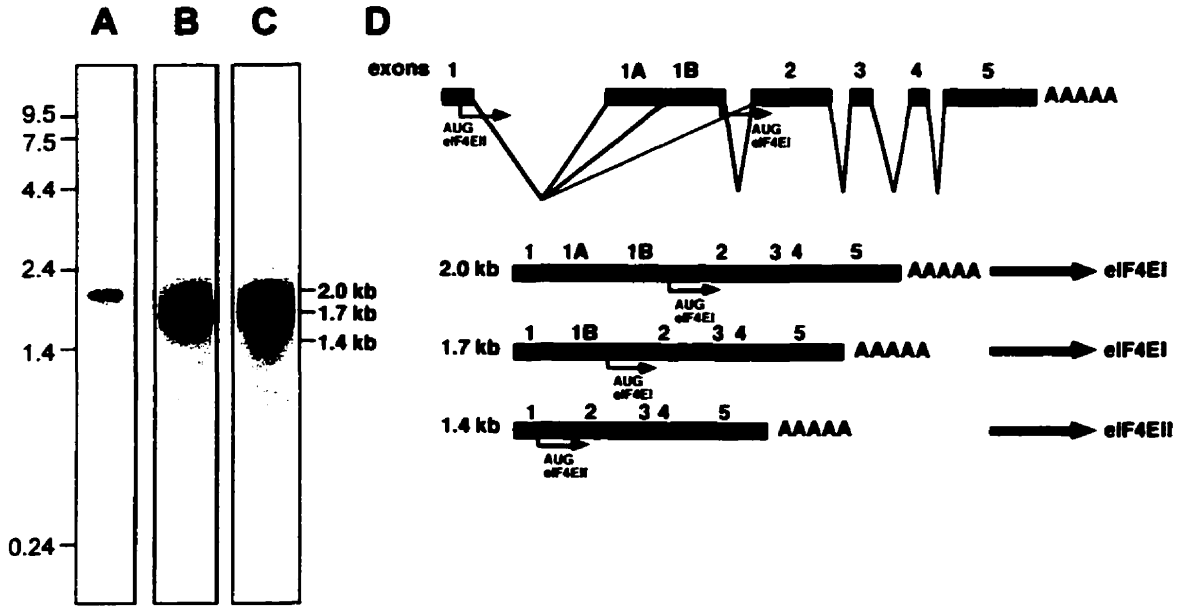
The two larger transcripts are composed of six exons while the 1.4 kb transcript contains five. Three introns (introns 3-5; Fig. 2) are spliced identically in all transcripts; these introns are 65, 231, and 61 nucleotides long, respectively. In the longest transcript, the first intron is 667 nt long and the second is 91 nt long; to form the 1.7 kb transcript a different acceptor site is utilized for the first intron, such that 904, rather then 667 nucleotides are excised from the primary transcript. Finally, to form the smallest transcript the entire sequence from the donor site of intron 1 to the acceptor site of intron 2 is excised as a single 1265-nucleotide intron. While all three transcripts contain the eIF4EII initiation codon (Fig. 2, nucleotides 122-124), the splicing events that result in

the larger two transcripts place in-frame termination codons relatively nearby: from the eIF4EII initiator the 2.0 kb transcript only encodes a 24 amino acid open reading frame before reaching a stop codon at nucleotides 861-863 (Fig. 2), while from the eIF4EII initiator the 1.7 kb transcript only encodes a 12 amino acid open reading frame before reaching a stop codon at nucleotides 1059-1061 (Fig. 2). For these two longer transcripts, the first long open reading frame extends from the initiation codon at nucleotides 1323-1325 (Fig. 2).

Three* eIF4E *transcripts encode two protein isoforms

Hernández and Sierra (1995) reported the expression of three *eIF4E* transcripts. We wished to determine how these transcripts correlate to our various cDNA clones, and, more specifically, which transcripts encode eIF4EI and which encode eIF4EII. Fig. 5 illustrates the results of a series of Northern hybridizations using portions of the *eIF4E* gene as probes. A probe which includes exon 1A sequences hybridizes only to the largest transcript (2.0 kb on our gels; Fig. 5A) and a probe specific to exon 1B hybridizes to both the largest (2.0 kb) and the intermediate-sized (1.7 kb) transcripts (Fig. 5B). A common probe containing sequences from exons 2-5 hybridizes to all three transcripts (Fig. 5C). The relative intensities of the three *eIF4E* transcripts is similar to those previously reported (Hernandez and Sierra, 1995). These results indicate that the 2.0 and 1.7 kb transcripts encode eIF4EI, while the 1.4 kb transcript encodes eIF4EII. As our sole cDNA clone which represents the 2.0 kb transcript (and retains exon 1A) is not full-length, we confirmed the 5' end of this largest transcript by sequencing an amplification

Fig. 5. Northern hybridizations mapping the three *eIF4E* transcripts. Polyadenylated RNA (15 μg) was separated by agarose gel electrophoresis, transferred to a filter, probed with **(A)** a probe specific to intron 1 and exon 1A (nucleotides 340-1027); **(B)** a probe specific to exon 1B (nucleotides 1049-1376); **(C)** the entire 1.7E1 cDNA, and autoradiographed. The probes in **(A)** and **(B)** were generated by PCR using appropriate primers, and the same filter was used for all three hybridizations. **(D)** Diagram of the alternative splicing events that produce the three *eIF4E* transcripts.


A
B
C
D
9.5
7.5
4.4
2.4
1.4
0.24
2.0 kb
1.7 kb
1.4 kb
exons 1
1A
1B
2
3
4
5
AAAAA
AUG eIF4EII
AUG eIF4EI
2.0 kb
1.7 kb
1.4 kb
eIF4EI
eIF4EII

product produced by PCR on a 0-4 hr embryonic cDNA library (Brown and Kafatos, 1988). The alternative splicing events which produce the three different *eIF4E* transcripts are schematically diagrammed in Fig. 5D.

Discussion

We present evidence that two isoforms of eIF4E, differing at their amino-termini, are produced from a single *Drosophila* gene by alternative splicing. Our data further indicate that mRNAs for both isoforms are expressed throughout *Drosophila* development, and that both protein isoforms can be identified from *Drosophila* adults. Earlier investigations (Maroto and Sierra, 1989) reported only one eIF4E isoform in extracts prepared from *Drosophila* embryos; our differing results from adults may reflect differential expression of eIF4EII in various developmental stages. While this is the first example of different eIF4E proteins arising from alternatively spliced transcripts, it is possible that multiple isoforms of eIF4E exist in other organisms as well. In *Xenopus*, two different eIF4E cDNAs have been isolated which encode products of 213 and 231 amino acids (Wakiyama *et al.*, 1995). These clones differ by a 54-nt segment which is present in one copy in the shorter clone, but in two copies in the longer clone. As genomic clones have not yet been characterized in *Xenopus* it is unclear whether these transcripts arise from the same or from different genes. In wheat germ two forms of eIF4E of 26 and 28 kDa are present (and the gene encoding p28 is duplicated), but these two proteins share only 50% amino acid identity and are found in different cap-binding complexes (Allen *et al.*, 1992; Browning *et al.*, 1987; Metz *et al.*, 1992).

The peptide sequencing data we presented above unambiguously support the existence *in vivo* of the novel eIF4EII isoform, but the amino acid sequence we obtained from the faster-migrating isoform is not N-terminal to either predicted protein, and is in fact present internally in both. It is possible that the more abundant faster-migrating protein, which we believe to be the product of the two larger transcripts, is degraded in our extracts, as degradation of *Drosophila* eIF4E *in vitro* has previously been reported as particularly problematic (Duncan *et al.*, 1995). However, the ratio of eIF4EI to eIF4EII is relatively constant in numerous extracts we have prepared, with eIF4EI always the more intense band. Furthermore, any degradation must be specific to eIF4EI, as our extraction conditions lead to the recovery of full-length eIF4EII. The difference between the N-terminal sequence we determined and that predicted by the nucleotide sequence may also result from specific post-translational processing *in vivo*. In this context it is noteworthy that the first 23 amino acids of the predicted eIF4EI polypeptide which our N-terminal sequencing predicts are absent in the mature protein are residues which are not conserved in eIF4E proteins in species other than *Drosophila* (Fig. 3). It is also possible that the first AUG in the eIF4EI open reading frame is not the true initiation codon, as in the eIF4EI sequence there are in-frame initiator codons at positions 8 and 17 in addition to the AUG at codon 1. The AUGs at codons 1 and 8 (but not 17) are in a favorable context for translation initiation (Brown *et al.*, 1994; Cavener, 1987)

While the multiple transcripts from the *eIF4E* gene result in the production of two different protein isoforms, they differ most strikingly by the lengths of their 5' UTRs. The 1.4 kb transcript which encodes eIF4EII has a relatively short 5' UTR of

approximately 110 nt, but the two eIF4EI transcripts have much longer 5' UTRs of 451 and 687 nt, respectively. Translation of mRNAs with long 5' UTRs is typically highly regulated and frequently such transcripts are not abundantly expressed (Cavener, 1987; Sonenberg, 1996). It is possible that the translation of the *Drosophila* eIF4EI transcripts is more tightly controlled than that of the eIF4EII transcript as the ratio of eIF4EII to eIF4EI protein recovered in our affinity purification (approximately 1:3; Fig. 1) is much greater than the ratio of 1.4 kb transcript to the sum of the 1.7 kb and 2.0 kb transcripts (Fig. 4A). Alternatively, this could rather reflect a greater affinity of eIF4EII as compared with eIF4EI to the cap-binding column used in our purification. Further direct analysis of the expression of the two eIF4E protein isoforms in various tissues and developmental stages, and analysis of other initiation factors with which they co-purify, should provide insight into their respective functions.

Acknowledgments

We are grateful to William S. Lane and the Harvard Microchemistry Facility for peptide sequence analysis, to Akira Nakamura for assistance with preparing Northern blots, and to the Sheldon Biotechnology Centre for synthesis of oligonucleotides. We also thank Michel Harvey for assistance with sequencing and Beat Suter for the gift of cDNA and genomic DNA libraries. This work was supported by a grant from the Medical Research Council of Canada to the laboratories of Paul Lasko and Nahum Sonenberg.

Chapter 3

Characterization of *Drosophila* eIF4E protein expression

A *Drosophila* gene mapped to cytological region 67A8-B2 encodes two protein isoforms of eIF4E via alternative splicing. To characterize the expression of *eIF4E* during the development of *Drosophila*, antisera specific for eIF4EI and eIF4EII were generated. eIF4EI is expressed at similar levels throughout development but accumulates in subcellular regions of egg chambers. In contrast, eIF4EII is expressed at lower levels during oogenesis and embryogenesis while it increases to levels comparable to eIF4EI in larvae, pupae, and adults. However, despite the distinct developmental expression pattern of eIF4EII, mutant flies expressing only eIF4EI are viable and develop normally. To assess the effects of deregulating *eIF4E* expression, eIF4EI was overexpressed in various tissues. Overexpression of eIF4EI results in an increase in the levels of its inhibitor, 4E-BP. Nonetheless, flies overexpressing eIF4EI in wild-type or in 4E-BP null mutant backgrounds exhibit normal growth and are viable.

Introduction

eIF4E is a subunit of the heterotrimeric complex eIF4F, which binds the mRNA cap during the initiation of translation. We identified a *Drosophila eIF4E* gene that produces via alternative splicing two protein isoforms, eIF4EI and eIF4EII (Chapter 2 of this thesis; Hernandez *et al.*, 1997; Lavoie *et al.*, 1996). Multiple isoforms of eIF4E have also been identified in various organisms including humans (Gao *et al.*, 1998), *C. elegans* (Jankowska-Anyszka *et al.*, 1998; Keiper *et al.*, 2000), *Xenopus* (Wakiyama *et al.*, 1995), zebrafish (Fahrenkrug *et al.*, 1999; Fahrenkrug *et al.*, 2000), and plants (Browning *et al.*, 1987; Browning *et al.*, 1992; Carberry and Goss, 1991).

The expression levels of eIF4E varies during the development of certain organisms. For instance, eIF4E is expressed in the post-meiotic germ cells of rat testes at levels 50 times higher than in other tissues (Miyagi *et al.*, 1995) while the zebrafish isoforms of eIF4E are expressed in dynamic and asymmetric patterns during embryogenesis (Fahrenkrug *et al.*, 1999). In addition, the biological and biochemical functions of ostensibly similar eIF4E isoforms may differ. The *C. elegans* isoforms of eIF4E have differing preferences for the mono- and tri-methylated mRNA caps found in this organism (Jankowska-Anyszka *et al.*, 1998). Furthermore, RNAi studies suggest that the eIF4E isoforms of *C. elegans* cannot fully compensate for loss of function in some of these genes (Keiper *et al.*, 2000).

eIF4E is a rate-limiting component of initiation and its activity and expression are tightly regulated in normal cells (reviewed by Raught *et al.*, 2000a). In vertebrates,

deregulation of eIF4E expression has been shown to influence cellular growth and development. Overexpression of eIF4E in mammalian cell cultures leads to oncogenesis while eIF4E expression is increased in several human carcinomas (for reviews, see Sonenberg, 1996 and De Benedetti and Harris, 1999). In addition, a direct role in development for eIF4E was demonstrated via injection of *eIF4E* mRNA into animal cap explants of *Xenopus* embryos, which results in the induction of mesoderm (Klein and Melton, 1994).

To characterize the expression of eIF4EI and eIF4EII during *Drosophila* development, affinity-purified antisera against these isoforms were generated and characterized. The effects of increasing *eIF4E* activity in a variety of *Drosophila* tissues, via overexpression of eIF4EI, were also investigated.

Materials and Methods

Expression and purification of eIF4EI-GST

A fragment of the eIF4EI cDNA 1.7E1 (Chapter 2, Lavoie *et al.*, 1996) was amplified by PCR using the primers 4EGEX1 (CCCGGATCCTAAACACGGCCGCCAAC) and T7 (GTAATACGACTCACTATAGGGC). The pGEX-eIF4EI vector was constructed by insertion of this fragment into the BamHI/XhoI sites of pGEX-5X-2 (Pharmacia). *E. coli* (strain DH5α) transformed with pGEX-eIF4EI were grown for 2-3 hrs at 37°C and expression of eIF4EI-GST was induced by incubation for an additional 2 hrs in the presence of 100 μM IPTG (isopropyl-β-D-1-thiogalactopyranoside). Harvested bacterial cells were resuspended in ice-cold lysis buffer (25μg/ml lysozyme, 1 mM

αPMSF, 1 μg/ml aprotinin, 10 μg/ml pepstatin, and 1 mM EDTA in PBS), sonicated, incubated for 30 min at 4°C in the presence of 0.8% Triton X-100, and centrifuged at 10,000g for 10 min at 4°C. The supernatant was added to glutathione-Sepharose 4B (Pharmacia) and incubated overnight at 4°C. The beads were washed four times with ice-cold PBS (supplemented with 1 mM αPMSF, 1 mM EDTA) and eIF4EI-GST was eluted with 3 ml Elution Buffer (20 mM Glutathione, 100 mM Tris-HCl pH 8.0, 120 mM NaCl, 1 mM αPMSF, 1 mM EDTA). To remove degradation products, eIF4EI-GST was further purified using the PrepCell system (Bio-Rad). Briefly, the eIF4EI-GST sample was boiled in Laemmli buffer and subjected to SDS-PAGE on a 6 cm 8% acrylamide tube gel and eluted fractions were collected. Fractions containing eIF4EI-GST, as determined by SDS-PAGE and Coomasie-Blue staining, were pooled and concentrated with Centriprep-30 (Amicon), diluted in PBS, and concentrated a second time to remove as much SDS as possible. An estimated 800-1000 μg of eIF4EI-GST in a final volume of 150 μl was purified.

Immunization of rabbits with eIF4EI-GST and affinity purification of antiserum

Two rabbits (rabbits #1739 and #1740) were injected intramuscularly in each hind quadricep with 100 μg eIF4EI-GST in TiterMax adjuvant (CytRx). Subcutaneous immunizations were performed at intervals of three weeks with 100 μg eIF4E-GST in Freund's incomplete adjuvant (Sigma). The crude antisera were obtained two weeks following the second immunization. On Western blots, the crude antisera from rabbits #1739 and #1740 can detect the bacterially-expressed eIF4EI-GST and bands of

approximately 35 kDa from adult *Drosophila* protein extracts (data not shown). Antiserum #1739 was further purified by affinity chromatography on an Affigel-15 column (Bio-Rad) coupled with an eIF4EI-HMK fusion protein (a gift from M. Miron and N. Sonenberg). The affinity-purified antiserum from rabbit #1739 is referred to as αeIF4E.

eIF4EI and eIF4EII peptide antisera

Rabbits were immunized with the eIF4EI (MQSDFHRNKNFANPKSMF) or eIF4EII (MVVLETEKTS) peptides and crude antisera were obtained (Research Genetics). The eIF4EI serum (rabbit #35630, 10 week bleed) was affinity purified against an Affigel-15 column coupled with the eIF4EI peptide and concentrated to a final volume of 200 µl using Centricon-10 (Amicon). The affinity-purified eIF4EI antiserum is referred to as αeIF4EI. The eIF4EII serum (rabbit #35632, 4 week bleed) is referred to as αeIF4EII.

Preparation of protein extracts and immunoblotting

Protein extracts were prepared from frozen or fresh *Drosophila* tissues by homogenization in RIPA Buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 0.5% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 1 mM αPMSF, 1 µg/ml aprotinin, 10 µg/ml pepstatin, and 1 mM EDTA), followed by centrifugation at 14,000g for 5 min at 4°C, and boiling of the supernatant in Laemmli buffer. Western blotting and detection was

performed according to the manufacturer's protocol (Renaissance chemiluminescence reagent, DuPont NEN).

Immunoprecipitation from Drosophila *extracts using αeIF4E*

Drosophila extracts prepared in 1 ml RIPA buffer were pre-incubated with Protein-A Sepharose for 1 hr at 4°C and immunoprecipitation was performed in the presence of 1 μl αeIF4E for 2 hrs. Beads were washed five times with RIPA buffer and the immunoprecipitate was eluted by boiling in 30 μl Laemmli buffer.

Immunocytochemistry

Drosophila ovaries were fixed for 20 min in PP solution (4% paraformaldehyde in PBS), rinsed three times with 0.2% Tween-20 in PBS, and washed with PBSBT (0.2% Tween-20, 0.2% Triton X-100, and 1% BSA in PBS). Ovaries were then blocked for 4 hrs in PBSBT and incubated overnight at 4°C with αeIF4E, αeIF4EI, or an antibody against *Drosophila* 4E-BP (α4E-BP, a gift from M. Miron and N. Sonenberg) in PBSBT at dilutions of 1:1000. Following primary antibody incubation, ovaries were washed four times for 5 min and four times for 1 hr with 0.2% Tween-20 in PBS, and were incubated overnight with a rhodamine-coupled anti-rabbit secondary antibody (1:1000) at 4°C. Ovaries were then washed four times for 1 hr with 0.2% Tween-20 in PBS and were mounted in 70% glycerol. Samples were analyzed with a Leica Confocal Laser Scanning Microscope (McGill Department of Biology Electron Microscopy Facility).

Fly strains

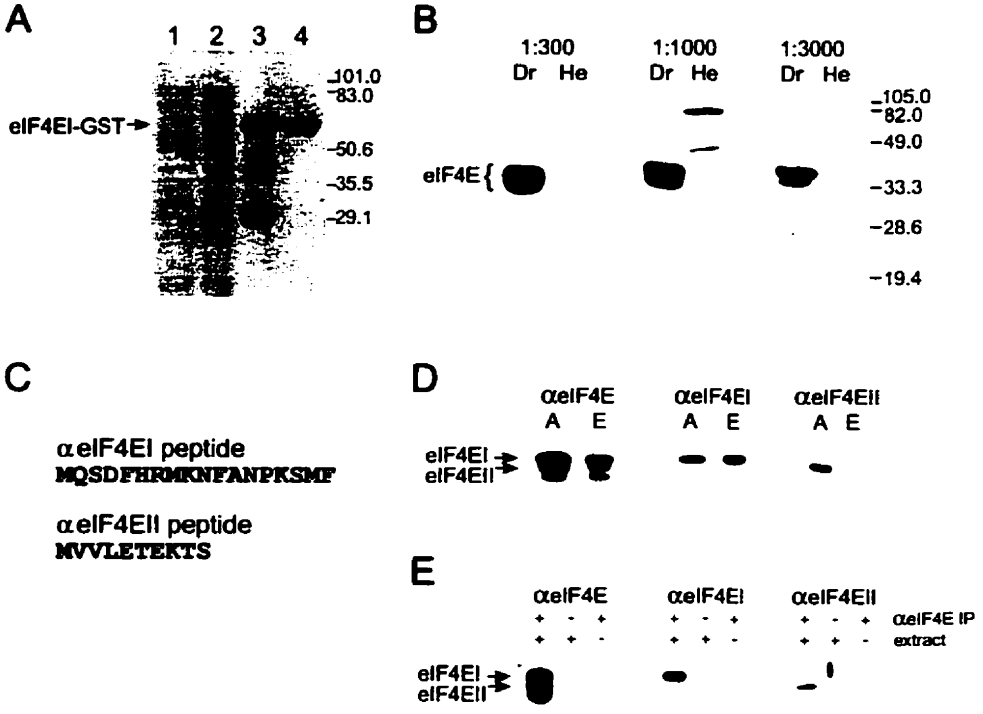
Wild-type stock used was the strain Oregon-R. The isolation and characterization of the *eIF4E* mutant alleles ($eIF4E^{589/11}$, $eIF4E^{67Af}$) are described in Chapter 4. The UAS-eIF4EI transgenic lines were constructed using the eIF4EI cDNA 1.7E1 (Lavoie *et al.*, 1996) and were a generous gift from S. Datar and B. Edgar. The *Thor* (4E-BP) null allele was generated by Bernal and Kimbral (2000). GAL4 lines were obtained from the Bloomington stock center.

Results and Discussion

Characterization of polyclonal antisera specific for the Drosophila *eIF4E isoforms*

A polyclonal antiserum was generated against bacterially expressed and purified eIF4EI-GST (Fig. 1A). The affinity purified anti-eIF4E serum (αeIF4E) detects two bands of approximately 35 kDa from *Drosophila* adult extracts but does not cross-react with the 24 kDa mammalian eIF4E (Fig. 1B). To distinguish between eIF4EI and eIF4EII, polyclonal antisera were raised against peptides based on the unique N-terminal sequences of these isoforms (Fig. 1C). Characterization of the peptide antibodies by Western blotting of *Drosophila* extracts and αeIF4E immunoprecipitations show that eIF4EI is the slowest migrating band detected by αeIF4E while eIF4EII is the second band (Fig. 1D,E).

Fig. 1. Characterization of *Drosophila* eIF4E antibodies. **(A)** Coomasie-Blue stained SDS-PAGE of *E. coli* extracts that were uninduced (lane 1) or induced for eIF4EI-GST expression for 2 hrs (lane 2). eIF4EI-GST from induced *E. coli* extracts was affinity purified by gluthathione-sepharose chromatography (lane 3). Degradation products were removed under denaturing conditions using a Bio-RAD PrepCell (lane 4) and purified eIF4EI-GST was used to immunize rabbits for the production of a polyclonal antiserum. **(B)** Titration of affinity-purified polyclonal serum (αeIF4E) from rabbits injected with eIF4EI-GST. Western blot containing *Drosophila* adult (*Dr*) and HeLa cell (He) extract was probed with indicated dilution of αeIF4E. **(C)** Peptides used for the production of rabbit polyclonal antibodies specific to eIF4EI and eIF4EII. **(D)** Western blot of *Drosophila* adult (A) and embryo (E) extracts probed with αeIF4E (1:1000 dilution), affinity-purified eIF4EI antibody (αeIF4EI, 1:1000 dilution), or eIF4EII serum (αeIF4EII, 1:300). **(E)** Imunoprecipitations from adult extracts were performed using αeIF4E (1:1000 dilution), were transferred to a Western blot, and were probed with the indicated antibodies. Control immunoprecipitations performed in the absence of αeIF4E or adult extract are also shown.


A
1 2 3 4
eIF4EI-GST
101.0
83.0
50.6
35.5
29.1
B
1:300
Dr He
1:1000
Dr He
1:3000
Dr He
eIF4E
105.0
82.0
49.0
33.3
28.6
19.4
C
αeIF4EI peptide
MQSDFHRMKNFANPKSMF
αeIF4EII peptide
MVVLETEKTS
D
αeIF4E
A E
αeIF4EI
A E
αeIF4EII
A E
eIF4EI
eIF4EII
E
αeIF4E
αeIF4EI
αeIF4EII
αeIF4E IP
extract
eIF4EI
eIF4EII

Cross-reacting product detected by αeIF4E

In certain lanes, αeIF4E cross-reacts with a product whose migration is faster than the eIF4E isoforms (ex: Fig. 1D,E, Fig. 2 lane 5). This band may represent an N-terminal degradation product of eIF4EI since it co-migrates with one of the purified and micro-sequenced proteins isolated by cap-column chromatography (see Chapter 2, Fig. 1). Consistent with this, neither of the isoform-specific N-terminal peptide antibodies can detect the cross-reacting product (Fig. 1D,E). The possibility that this band is a degradation product is also supported by the fact that it is more abundant when extract preparation times are longer (for example, the band is more prominent during immunoprecipitations (Fig. 1E) which require 3 hrs of incubation prior to the preparation of the protein extract). The degradation of *Drosophila* eIF4E during the preparation of protein extracts has also been suggested elsewhere (Duncan *et al.*, 1995). However, it is not ruled out that the cross-reacting band is a product from one of other genes homologous to eIF4E that were recently identified in the *Drosophila* genome (Lasko, 2000). This other possibility can be addressed when antisera against the eIF4E cognates become available or upon the identification of null mutant alleles in these genes. Since the identity of the fastest-migrating product that cross-reacts with αeIF4E is unclear, this characterization of eIF4E expression will focus on the products that are detected by αeIF4EI and αeIF4EII.

Expression of eIF4EI and eIF4EII during development

The expression levels of the eIF4E isoforms at different stages of *Drosophila* development were examined by Western blotting (Fig. 2). eIF4EI is expressed at constant levels throughout development while eIF4EII is expressed at low levels in ovaries and embryos (Fig. 2, lanes 1-4, 8) but increases to levels similar to eIF4EI in larvae, pupae, and adults (Fig. 2, lanes 5-7). The reduced expression of eIF4EII in embryos is also consistent with the results from αeIF4E immunoprecipitations performed from embryonic extracts (Fig. 1E). No difference was observed in the levels of eIF4EI and eIF4EII expression in males and females or in the different larval instars (data not shown).

Prior reports described the presence of a single *Drosophila* eIF4E protein rather than two isoforms (Maroto and Sierra, 1989; Zapata *et al.*, 1994). As these studies were performed using embryonic tissue, they are consistent with our finding that eIF4EI is the predominantly expressed isoform during embryogenesis.

Dynamic localization of eIF4EI in the developing egg chamber

The expression of the eIF4E isoforms was further characterized via immunocytochemistry. In ovaries, αeIF4E and αeIF4EI produce identical staining patterns (Fig. 3). In addition to a ubiquitous cytoplasmic immunoreactivity in all cells, eIF4EI is concentrated in oocytes as early as one is detectable in the germarium (Fig. 3A) and continues to accumulate there until stages 7-8 where it begins to concentrate at the cortex of the oocyte (Fig. 3B-D). At stages 9-10, eIF4EI transiently accumulates at the posterior pole of the oocyte (Fig. 3E). In addition, perinuclear concentrations of eIF4EI

Fig. 2. Expression of the eIF4E isoforms during development. Protein extracts (approx. 30 μg) isolated from (1) 0-2 hr embryos, (2) 2-6 hr embryos, (3) 6-12 hr embryos, (4) 12-20 hr embryos, (5) larvae, (6) pupae, (7) adults, and (8) ovaries were subjected to SDS-PAGE and analyzed by Western blotting using αeIF4E (1:1000 dilution). The bands representing eIF4EI and eIF4EII are shown (arrows). Marker sizes (kDa) are indicated on the left.

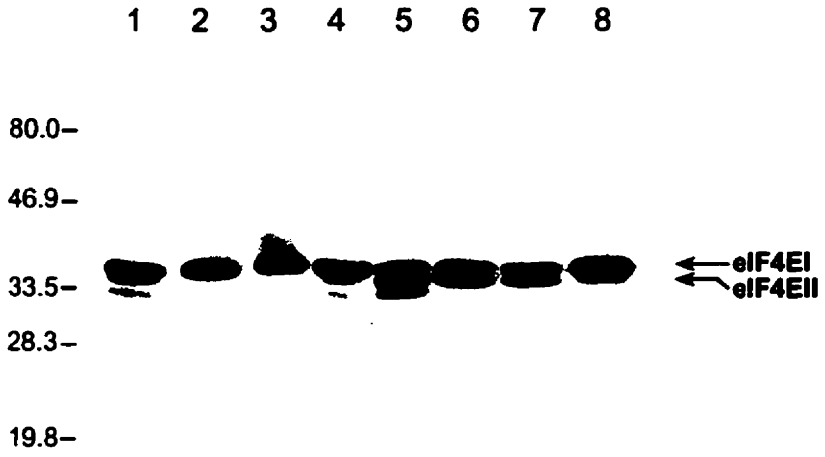
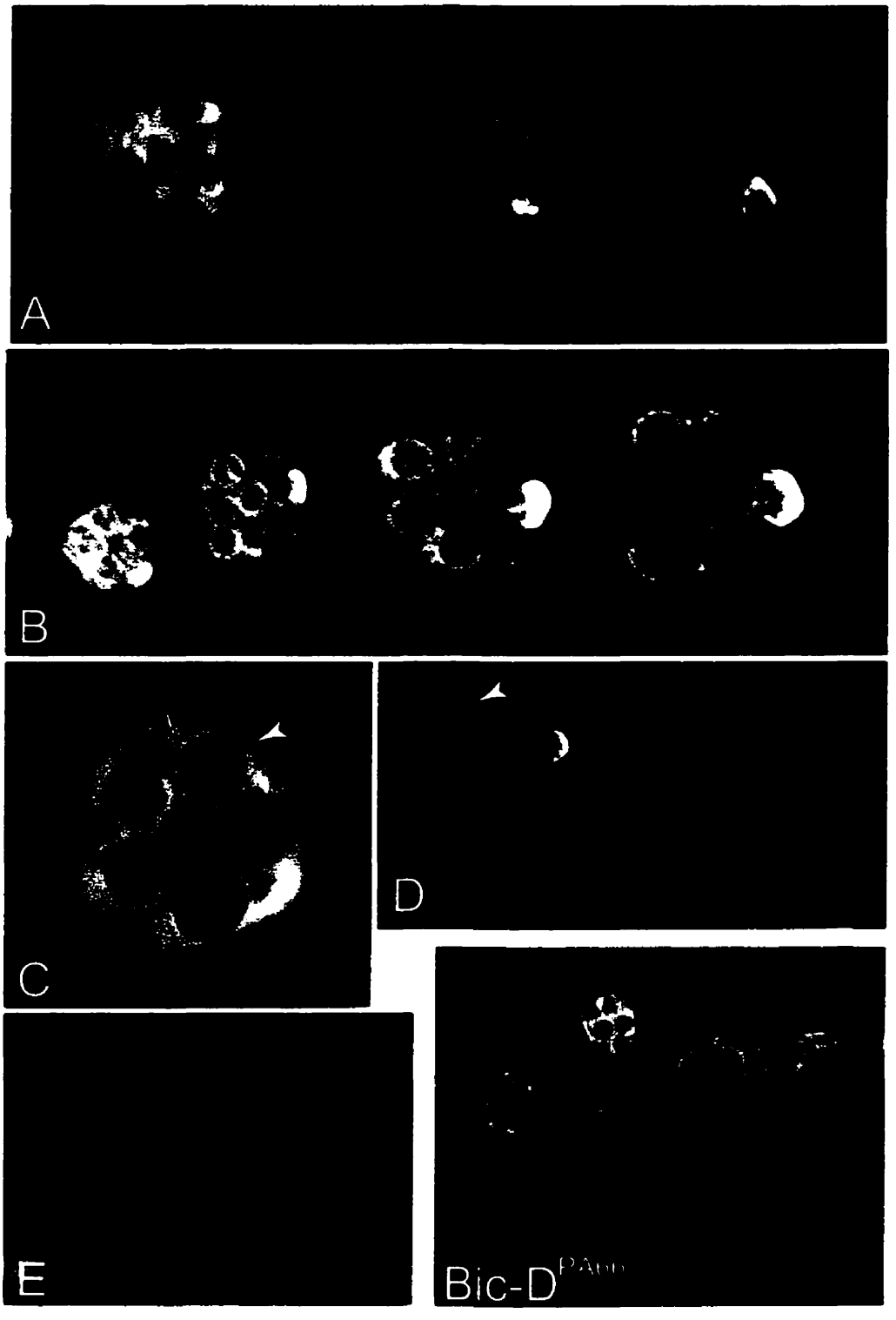

1 2 3 4 5 6 7 8
80.0–
46.9–
33.5–
28.3–
19.8–
eIF4EI
eIF4EII

Fig. 3. eIF4EI dynamically concentrates to subcellular locations in the ovary. (A-E) Confocal laser imaging of wild-type *Drosophila* ovaries stained with αeIF4E or αeIF4EI. **(A)** germarium, **(B)** stages 3-6, **(C)** stage 6, **(D)** stages 7-8, and **(E)** stages 9-10. Perinuclear concentration of eIF4EI in nurse cells is indicated (white arrow). Ovaries from *Bic-D*$^{P.A66}$ mothers were stained as a control for oocyte localization; *Bic-D*$^{P.A66}$ ovaries do not produce an oocyte.


A
B
C
D
E
Bic-D$^{\Delta Adn}$

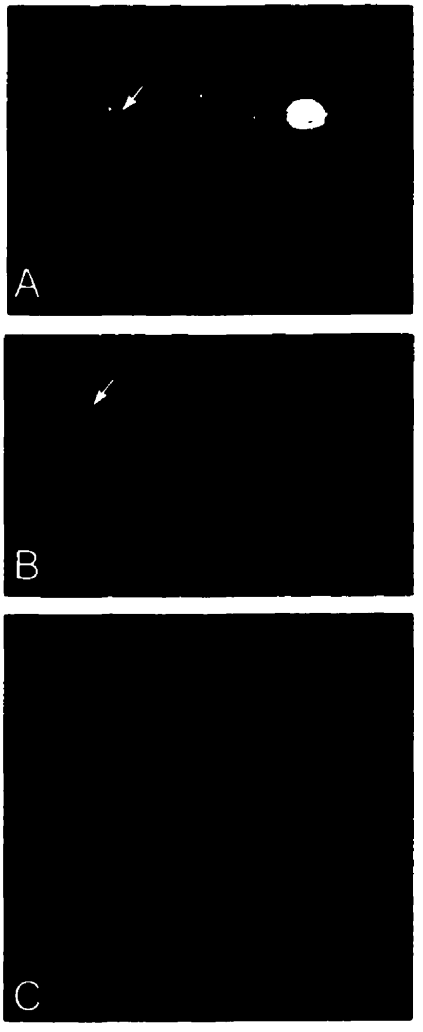
are observed in nurse cells (Fig. 3C,D, white arrows). Localized immunoreactivity in ovaries was not detected by αeIF4EII (data not shown).

A previous report showed that during embryogenesis *eIF4E* mRNA accumulates in pole cells, and is concentrated in the midgut, mesoderm, and in the somatic musculature (Hernandez *et al.*, 1997). To determine whether the localization of *eIF4E* mRNA results in localized eIF4E protein, immunostaining of *Drosophila* embryos was performed with the eIF4E antisera. Despite the previously reported accumulations of *eIF4E* mRNA in embryonic tissues, a ubiquitous staining pattern with αeIF4E and αeIF4EI was observed in all stages of embryogenesis (data not shown).

Immunostainings were also performed with antibodies directed against the inhibitor of eIF4E, 4E-BP (Fig. 4). Like eIF4EI, 4E-BP is found perinuclearly in nurse cells and is cortical in the oocyte at stages 9-10. However, 4E-BP does not accumulate to the oocyte cytoplasm like eIF4EI.

The tissue-specific and subcellular aggregation of translation factors may mediate site-specific protein synthesis. This phenomenon has best been described in synapses, where polyribosomes concentrated post-synaptically have the ability to direct local protein synthesis (reviewed by Schuman, 1999). These locally synthesized proteins are thought to contribute to synaptic modifications or plasticity (Kang and Schuman, 1996; Martin *et al.*, 1997; Sigrist *et al.*, 2000). eIF4E itself is localized post-synaptically at the *Drosophila* neuromuscular junction (Sigrist *et al.*, 2000). Changes in the levels of post-synaptically localized eIF4E, as detected by αeIF4E, directly mediates the plasticity of the neuromuscular junction, affects the efficacy of neurotransmission, and regulates the

Fig. 4. Localization of 4E-BP in ovaries. Confocal laser imaging of *Drosophila* ovaries stained with α4E-BP serum (1:1000 dilution). **(A, B)** In early stage egg chambers, 4E-BP accumulates at the perinuclear region of nurse cells (white arrows) and to the oocyte nucleus (dark arrow). **(C)** In stage 10 egg chambers, 4E-BP localizes to the cortex of the oocyte (dark arrowheads).


A
B
C

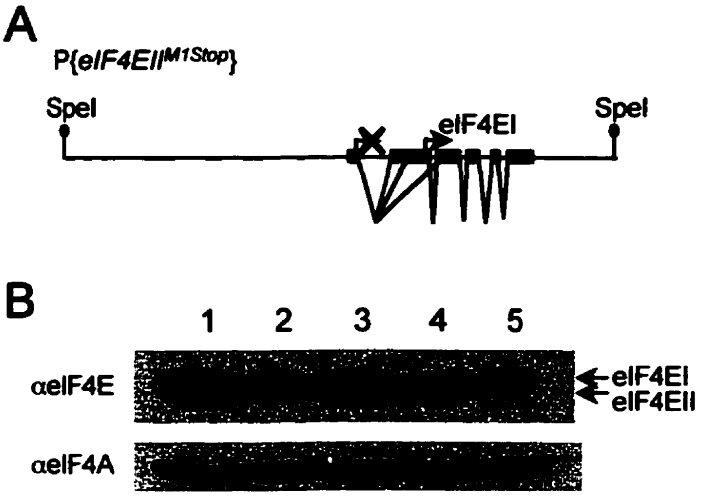
translation of an mRNA encoding a glutamate receptor subunit (Sigrist *et al.*, 2000, see Appendix A).

The dynamic expression patterns of eIF4EI and 4E-BP in ovaries may thus suggest that the subcellular localization of the translational machinery provides an additional level of regulation for the expression of localized messages during oogenesis.

eIF4EII is not required for viability

The observation that eIF4EII expression varies during development while that of eIF4EI is constant may suggest that eIF4EII is required for discrete developmental processes. To address the necessity of the eIF4EII isoform, specific *eIF4EII* mutant alleles were generated. *eIF4EII* mutants were created by germ line transformation (Spradling and Rubin, 1982) of an *eIF4E* genomic fragment in which the eIF4EII start codon (AUG) was altered to a stop (UAG), thereby producing a transgene that can only express eIF4EI (P{$eIF4EII^{M1Stop}$}, Fig. 5A). Normally, the *eIF4E* transheterozygote $eIF4E^{589/11}$/ $eIF4E^{67Af}$ is larval lethal. The larval lethality of $eIF4E^{589/11}$/ $eIF4E^{67Af}$ can be rescued by a genomic fragment encoding wild-type *eIF4E* (P{$eIF4E^{WT}$}; see Chapter 4 for the characterization of *eIF4E* mutant alleles and transgenic rescue constructs). Interestingly, flies carrying P{$eIF4EII^{M1Stop}$}in the background of the otherwise lethal *eIF4E* alleles are also fully viable and have no growth or morphological defects. The absence of eIF4EII in the viable transgenic mutants was confirmed by Western blotting (Fig. 5B). These data indicate that despite its distinct expression pattern, eIF4EII is not required for the normal growth and development of flies.

Fig. 5. *Drosophila* eIF4EII is not required for viability. **(A)** Schematic representation of transgene used to generate flies that can express only eIF4EI (P{*eIF4EII*$^{\Delta 1 Stop}$}). This transgene was generated by converting the start codon of eIF4EII (AUG) to a stop (UAG). **(B)** Protein extracts (approx. 30 μg) from adult (1) wild-type, (2) *eIF4E*$^{589/11}$/*TM3*, (3) *eIF4E*^{67Af}/*TM3*, (4) P{*eIF4E*^{wt}}; *eIF4E*$^{589/11}$/ *eIF4E*^{67Af}, and (5) P{*eIF4EII*$^{\Delta 1 Stop}$}; *eIF4E*$^{589/11}$/ *eIF4E*^{67Af} probed with αeIF4E or an antiserum directed against eIF4A (αeIF4A, Styhler *et al.*, 1998). All genotypes are viable. Note the absence of eIF4EII in the *eIF4E* mutant rescued by P{*eIF4EII*$^{\Delta 1 Stop}$}(lane 5).


A
P{elF4EII^M1Stop}
SpeI
eIF4EI
SpeI
B
1
2
3
4
5
αeIF4E
eIF4EI
eIF4EII
αeIF4A

This result was not unexpected given that eIF4EI and eIF4EII are very similar at the amino acid level, differing only at sequences at their N-termini. The N-termini of eIF4E homologues from different organisms are the most variable regions of the protein and are dispensable for eIF4E function (Marcotrigiano *et al.*, 1997; Marcotrigiano *et al.*, 1999). Therefore, since eIF4EI is ubiquitously expressed, it might be expected that it is capable of compensating for the absence of eIF4EII function. However, another possibility is that the function of eIF4EII is redundant with one of the other *Drosophila* eIF4E cognates (Lasko, 2000).

Overexpression of eIF4EI in* Drosophila *tissues

As upregulation of eIF4E in mammalian tissue cultures leads to oncogenesis (Lazaris-Karatzas *et al.*, 1990) and eIF4E injection in *Xenopus* animal caps promotes mesoderm induction (Klein and Melton, 1994), we were interested in determining whether the deregulation of eIF4E in *Drosophila* could produce overgrowth phenotypes.

Overexpression in different developmental contexts and tissues was performed using UAS-eIF4EI lines induced by GAL4 drivers (Brand and Perrimon, 1993; Table 1). None of the GAL4 lines we used to drive eIF4EI overexpression resulted in overgrowth phenotypes or in a reduction in viability (Table 1).

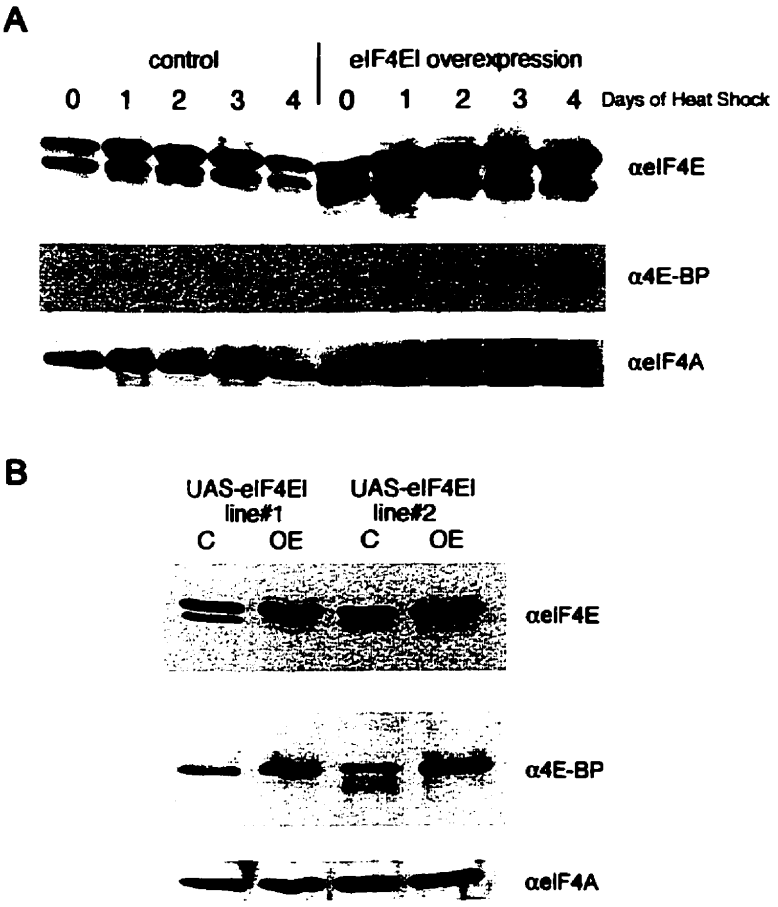
However, we observed that eIF4EI overexpression results in the upregulation in the levels of 4E-BP (Fig. 6). A similar negative feedback loop between increased eIF4E levels and 4E-BP activity was previously observed in mammalian cells (Khaleghpour *et al.*, 1999). The mammalian eIF4E/4E-BP homeostasis is achieved via the

Table 1. GAL4 lines used to drive UAS-eIF4EI overexpression.

Line	Tissue/Stage of Expression	Remarks
act5C-GAL4*	ubiquitous	Actin 5C promoter drives expression. Examined adults for reduced viability, altered wing size, aberrant eye growth. Used 2 independent Act5C-GAL4 lines.
Hsp70-GAL4*	ubiquitous, inducible by heat shock	Induced overexpression by 37°C heat shocks in adults and examined DAPI-stained ovaries for morphological defects. Overexpression also induced by growth at 29°C. Used 2 independent *Hsp70*-GAL4 lines. Note: induction of *Hsp70*-GAL4 during larval development by 37°C heat shocks, in the absence of UAS transgenes, is lethal.
arm-GAL4	Expressed ubiquitously during development	Uses *armadillo* promoter. Examined adults for reduced viability.
GMR-GAL4	Strong expression in all cells behind morphogenetic furrow of eye imaginal disc	Uses *glass* promoter. Examined adults for aberrant eye growth.
2x*sev*-GAL4	Precursors of photoreceptor cells in eye imaginal disc	Expression driven by 2 copies of *sevenless* promoter. Examined adults for aberrant eye growth.
en-GAL4	Expressed in parasegments, CNS of embryo and in posterior compartment of imaginal discs	Uses *engrailed* promoter. Examined adults for reduced viability and quantified wing areas to identify changes in the size of posterior compartment.
71B-GAL4*	Imaginal discs	P{GAWB}enhancer trap drives expression. Quantified adult wing areas and examined for changes in size.
MS1096-GAL4*	Wing imaginal disc with slightly higher expression in dorsal cell layer	Quantified adult wing areas to identify changes in size. Also examined adults for a "curved wing" phenotype that would suggest larger and/or more cells in dorsal layer.
198Y-GAL4*	Expressed in ovary nurse cells after stage 6; expression stronger in border cells and follicle cells covering oocyte.	P{GAWB} enhancer trap drives expression. Examined DAPI-stained ovaries for aberrant growth or morphology.
dpp-GAL4*	Expressed in embryonic tissues, anterior-posterior margin of imaginal disc in larva and pupa	Uses *decapentaplegic* promoter. Quantified adult wing areas to identify changes in size of anterior-posterior compartment.

*Indicates that driver was also used to overexpress eIF4EI in *Thor* (4E-BP) null genetic background.

Fig. 6. Overexpression of eIF4EI results in an increase in the levels of 4E-BP. Expression of UAS-eIF4EI was induced by **(A)** hsp70-GAL4 and **(B)** Act5C-GAL4 and levels of eIF4E (αeIF4E), 4E-BP (α4E-BP), and eIF4A (αeIF4A) were examined by Western blotting. **(A)** Expression was induced in UAS-eIF4EI/hsp70-GAL4 flies; control samples are the UAS-eIF4EI/CyO siblings. To induce hsp70-GAL4, 37°C heat shocks were performed twice per day for 45 min each. Samples were collected daily following the second heat shock. **(B)** Extracts were collected from UAS-eIF4EI/CyO (C) and UAS-eIF4EI/Act5C-GAL4 (OE); two independent UAS-eIF4EI lines are shown.


A
control
eIF4EI overexpression
0 1 2 3 4 0 1 2 3 4 Days of Heat Shock
αeIF4E
α4E-BP
αeIF4A
B
UAS-eIF4EI line#1
UAS-eIF4EI line#2
C OE C OE
αeIF4E
α4E-BP
αeIF4A

hypophosphorylation 4E-BP upon increases in eIF4E levels. It was thus hypothesized that overexpression of *Drosophila* eIF4EI in a background incapable of expressing 4E-BP might result in overgrowth phenotypes. Null mutations in *Thor*, the gene encoding *Drosophila* 4E-BP, are viable and do not have visible phenotypes when flies are grown under normal conditions (Bernal and Kimbrell, 2000). Furthermore, *Thor* is the only gene in the *Drosophila* genome encoding a homologue of 4E-BP (Lasko, 2000). Despite the absence of 4E-BP expression, overexpression of eIF4EI does not result in overgrowth phenotypes, developmental defects, or reduced viability (Table 1). It is thus concluded that flies can physiologically compensate for increased eIF4EI levels under most conditions. Similar observations for *eIF4E* overexpression in *Drosophila* were also recently reported elsewhere (Zhang *et al.*, 2000).

Several possibilities may explain why *Drosophila* can withstand increases in eIF4EI, even in the absence of 4E-BP. It may be that our treatments did not increase the activity of *eIF4E* to a level at which a phenotype can be observed. Although NIH 3T3 cells require only a two- to threefold increase in eIF4E expression to exhibit overgrowth phenotypes (Rousseau *et al.*, 1996b), other mammalian cultures, such as CHO cells, require a 7-fold increase (De Benedetti *et al.*, 1994). For primary cell cultures, cooperation with other proto-oncogenes is necessary for transformation (Lazaris-Karatzas and Sonenberg, 1992). Hence, an overgrowth phenotype resulting from increased *Drosophila eIF4E* activity may only be possible with higher expression than achieved in our experiments or by co-overexpression with other oncogenes. Another explanation is that regulators of eIF4E activity that do not share homology with 4E-BPs

are present within the *Drosophila* genome. One such candidate is Myst, a *Drosophila* protein that can interact with eIF4E but whose biological function is unknown (M. Miron and N. Sonenberg, personal communication).

Although the GAL4 lines used to drive eIF4EI overexpression did not produce the expected effects, other drivers and the quantification of different phenotypes may nevertheless result in phenotypes indicative of eIF4E-induced increases in growth. Indeed, overexpression of eIF4EI using the muscle driver *Mhc*-GAL4 (myosin heavy chain promoter) results in an elevated number of presynaptic specializations at the neuromuscular junction while the nervous system driver *elav*-GAL4 does not (Sigrist *et al.*, 2000, see Appendix A). These observations suggest that while the whole fly can readily deal with increases in eIF4E activity, certain developing tissues can be susceptible to localized changes in *eIF4E* expression.

Acknowledgements

I would like to thank Mathieu Miron and Nahum Sonenberg for sharing unpublished results and providing the 4E-BP antiserum, D. Kimbrall for the *Thor* alleles, and S. Datar and B. Edgar for the *UAS-eIF4EI* lines. I would also like to acknowledge Sylvia Styhler for technical assistance with immunostaining and the use of the confocal laser microscope and Weihua Li for assistance with automated sequencing.

Chapter 4

Phosphorylation of eukaryotic initiation factor 4E (eIF4E) is critical for growth

Eukaryotic translation initiation factor 4E (eIF4E) binds to the cap structure at the 5' end of messenger RNAs and is a critical target for the control of protein synthesis. eIF4E is phosphorylated in many systems in response to extracellular stimuli, but biochemical evidence to date has been equivocal as to the biological significance of this modification. Here we use a genetic approach to the problem. We show that in *Drosophila melanogaster*, homozygous *eIF4E* mutants arrest growth during larval development. In *Drosophila* eIF4EI, Ser251 corresponds to Ser 209 of mammalian eIF4E, which is phosphorylated in response to extracellular signals. We find that *in vivo*, eIF4EI Ser251 mutants cannot incorporate labeled phosphate. Furthermore, transgenic *Drosophila* expressing $eIF4E^{Ser251Ala}$ in an *eIF4E* mutant background have reduced viability. Escapers develop more slowly than control siblings and are smaller in size. These genetic data provide evidence that eIF4E phosphorylation is biologically significant, and is essential for normal growth and development.

Introduction

eIF4E is a rate-limiting component of translation initiation and its activity is tightly regulated in cells (Gingras *et al.*, 1999b; Raught *et al.*, 2000a). Regulation of eIF4E activity is critical to normal cell growth as overexpression of eIF4E in rodent cells is oncogenic (Lazaris-Karatzas *et al.*, 1990), while injection of eIF4E into quiescent NIH 3T3 cells induces DNA synthesis (Smith *et al.*, 1990). In addition, *S. cerevisiae* cells carrying the temperature-sensitive *eIF4E* allele *cdc33ts4-2* at the non-permissive temperature arrest at the G_1 to S transition of the cell cycle (Brenner *et al.*, 1988), further implicating eIF4E in the regulation of growth.

eIF4E functions as a subunit of a complex, eIF4F, that associates with the 5' end of the mRNA and facilitates the binding of the small ribosomal subunit and associated factors. In mammals, eIF4F consists of three subunits: eIF4E, eIF4A, and eIF4G (Raught *et al.*, 2000a). eIF4E binds to the 7-methyl-guanosine cap structure at the 5' end of the mRNA. The activity of eIF4E protein is a key target for the regulation of translation by two known mechanisms. The inhibitory eIF4E-binding proteins (4E-BPs) control the availability of eIF4E by competing for its binding with eIF4G (Haghighat *et al.*, 1995; Mader *et al.*, 1995) while phosphorylation of eIF4E at a conserved serine is hypothesized to control its mRNA cap-binding activity (Raught *et al.*, 2000a).

Unlike the 4E-BPs, the function of eIF4E phosphorylation is poorly understood. Unphosphorylated eIF4E can stimulate translation *in vitro* and bind the mRNA cap or cap analogues suggesting that phosphorylation is not strictly required for eIF4E function (Raught *et al.*, 2000a). However, when translation activity is altered by treatments with

various extracellular stimuli, the phosphorylation state of eIF4E changes; in most cases, increased eIF4E phosphorylation correlates with increased translational activity. Furthermore, eIF4E is hypophosphorylated during mitosis when the translation rate of mRNAs is low (Bonneau and Sonenberg, 1987) and, various cellular stresses such as heat shock and viral infection are correlated with reduced eIF4E phosphorylation (Raught *et al.*, 2000a). The key phosphorylation site of mammalian eIF4E is Ser209 (Flynn and Proud, 1995; Whalen *et al.*, 1996). Structural studies suggest that phosphorylation at Ser209 might allow tighter binding of the mRNA cap by formation of a salt bridge with a lysine residue on the other side of the mRNA trajectory, thereby clamping the mRNA (Marcotrigiano *et al.*, 1997).

Work in invertebrate systems also supports a link between eIF4E phosphorylation and translation efficiency. Using an antibody specific to the phosphorylated form of *Aplysia* eIF4E, a significant correlation between translation rates and increases in eIF4E phosphorylation in ganglia preparations was shown (Dyer and Sossin, 2000). In *Drosophila*, a gene encoding eIF4E was identified and mapped to polytene chromosome region 67A on the left arm of chromosome 3 (Hernandez *et al.*, 1997; Lavoie *et al.*, 1996). As is the case in mammals, the phosphorylation of *Drosophila* eIF4E decreases upon heat shock concomitant with a decrease in translation rates (Duncan *et al.*, 1995).

The correlation between increased eIF4E phosphorylation and elevated growth rates suggests that phosphorylation is important for the regulation of eIF4E activity. It was therefore critical to examine the importance of eIF4E phosphorylation in a

genetically-tractable multicellular organism. To do this, we identified the major phosphorylation site of *Drosophila* eIF4E, and found it to correspond to the site that is phosphorylated in the mammalian protein. By mutating this site, we demonstrated that phosphorylation of eIF4E is necessary for the efficient growth and development of *Drosophila.*

Materials and Methods

Fly work

Alleles *l(3)589/11* ($eIF4E^{589/11}$) and *l(3)715/13* ($eIF4E^{715/13}$) were provided by Kim Kaiser and originated in a screen for 3rd chromosome lethal lines (Deak *et al.*, 1997). All other strains were provided by the Bloomington Stock Center. Phenotypic characterization of the larval growth defect of *eIF4E* mutant alleles was performed as previously described (Britton and Edgar, 1998; Galloni and Edgar, 1999; Migeon *et al.*, 1999) with some modifications. We used *Tubby (Tb)* on the TM6B *Tb* balancer to identify larvae with a wild-type copy of *eIF4E*, and for the larval growth assays we examined hemizygotes for an *eIF4E* allele and a deficiency that includes *eIF4E* to rule out the effects of unknown second-site recessive mutations. The deficiencies used were *Df(3L)AC1*/TM6B *Tb* or *Df(3L)29A6*/TM6B *Tb* . Embryos were collected on standard apple juice egg-lay medium for 1-2 hr at 24°C. The number of living Tb^- (*eIF4E* transheterozygotes) and Tb^+ (control siblings) were counted at 24 hr intervals. Control experiments with the wild-type strain Oregon-R were performed in parallel, with results identical to the TM6B *Tb* control siblings.

P-element constructs and generation of transgenic flies by germ-line transformation

An 8.9 kb *Spe*I genomic fragment that includes *eIF4E* was obtained from a genomic clone (Lavoie *et al.*, 1996) and was subcloned into pCaSpeR-4. This fragment contains approximately 4.9 kb of 5' flanking DNA upstream of *eIF4E* and about 1.0 kb of 3' flanking DNA. Codon 251 (TCC) was changed to GCC (Ser251Ala) and GAC (Ser251Asp) using the *Pfu* high-fidelity polymerase and verified by sequencing. The three constructs, referred to as P{$eIF4E^{WT}$}, P{$eIF4E^{Ser251Ala}$} and P{$eIF4E^{Ser251Asp}$}, were transformed into *yw* flies by standard germ-line transformation techniques (Spradling and Rubin, 1982) using the *mini-white*$^+$ selection marker and the pTurbo helper plasmid as source of transposase. Two independent transformation lines were characterized for each of the constructs.

Antisera

Protein blots were probed using a rabbit polyclonal anti-serum generated against a peptide derived from N-terminal sequence of eIF4EI (amino acid sequence MQSDFHRMKNFANPKSMF). The eIF4EI serum was affinity purified against the peptide and used at a dilution of 1:1000 in all our experiments. An affinity-purified eIF4E antiserum, directed against the whole protein (Sigrist *et al.*, 2000), was used at a 1:1000 dilution.

In vivo *metabolic labeling of eIF4E*

Twenty pairs of *Drosophila* ovaries were dissected into 1 ml of phosphate-free Schneider's cell culture media (Biofluids) and were incubated for 2 hr at room temperature in the presence of 0.1 mCi [^{32}P] orthophosphate. The ovaries were then washed three times with PBS, homogenized in 1 ml lysis buffer [10% glycerol, 50 mM Tris-HCl pH7.5, 60 mM KCl, 2mM CDTA (trans-1,2-diaminocyclohexane-*N,N,N',N'*-tetraacetic acid), 1% Triton X-100, 2 mM DTT, 50 mM β-glycerophosphate, 5 mM NaF, 0.1 mM $NaVO_3$], and extracts were frozen at –20°C until immunoprecipitations were performed. Immunoprecipitations using the eIF4EI antiserum were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Xymotech). Membranes were autoradiographed and analyzed by Western blotting.

Analysis of adult eyes

Whole flies were dehydrated in an ethanol series. The ethanol was then gradually replaced with Freon-113 by incubation in increased concentrations of Freon-113:ethanol in 24 hr increments. Flies were mounted and scanning electron microscopy was performed to obtain photographs of eyes for five individuals of each genotype and gender examined. All micrographs were obtained at identical magnifications (160 x). Analysis of individual ommatidia areas was performed by scanning the micrographs into Adobe Photoshop and using the histogram function. The ommatidia size for each compound eye is an average of the areas for N=5 ommatidia near the center of the eye. The average

number of ommatidia per compound eye was counted manually (N=5 for each genotype and gender examined).

Flow Cytometry

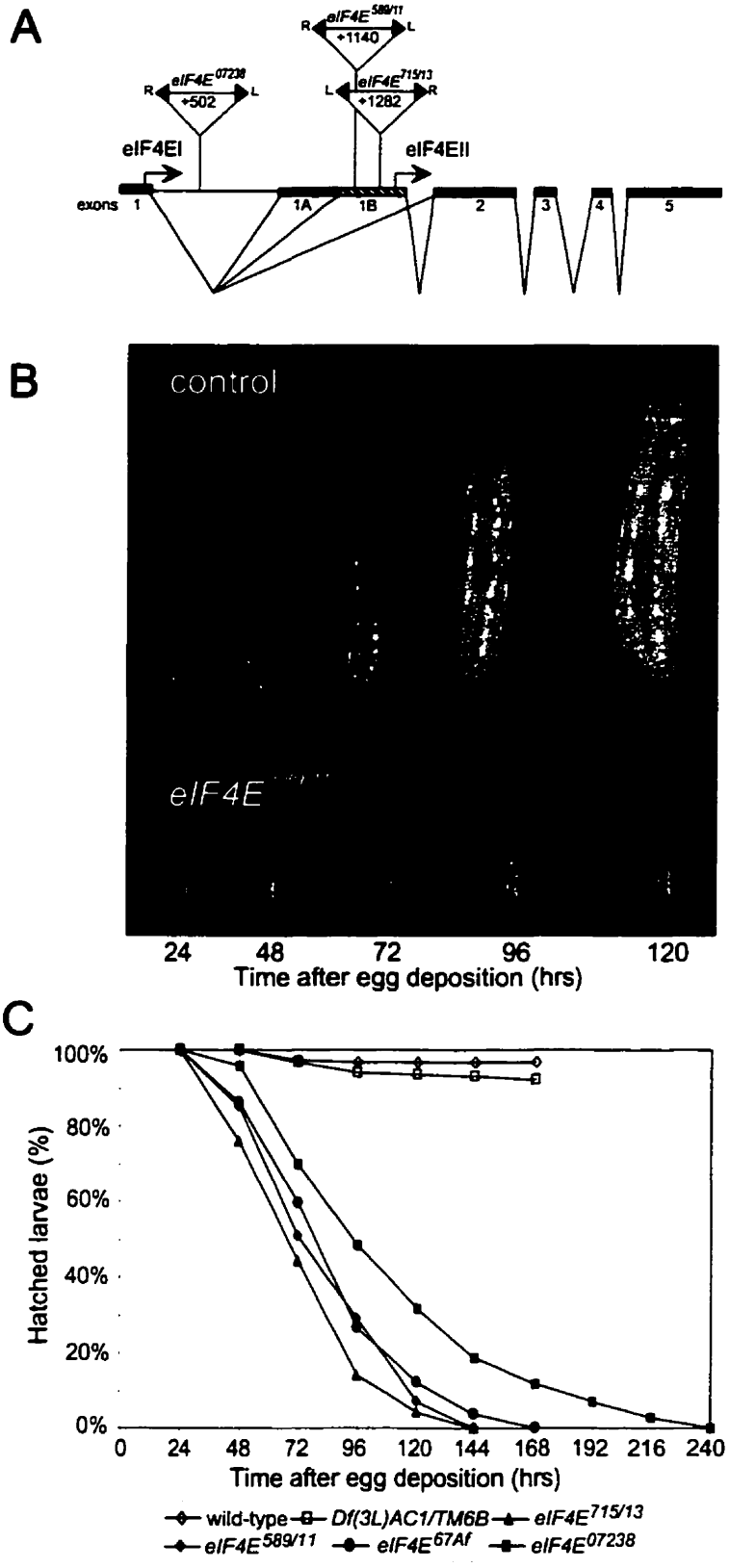
Larvae from 2 hr collections were aged at 24°C for a total of 116 hours after egg deposition. To compensate for the developmental delay in the $eIF4E^{Ser251Ala}$, larvae of this genotype were aged longer and used when they reached the third instar wandering stage. Wing imaginal discs were dissected in Schneider's medium (Gibco-BRL) and subjected to flow cytometry as previously described (Neufeld *et al.*, 1998) using a Becton Dickinson FACScan. Data was analyzed using WinMDI 2.8 software.

Results

Isolation and molecular characterization of eIF4E *mutant alleles*

Mutants in *eIF4E* were identified by probing a plasmid-rescue library generated from third chromosome lethal lines (Guo *et al.*, 1996) with a radiolabelled eIF4EI cDNA (Lavoie *et al.*, 1996). Plasmids corresponding to three P-element lethal lines hybridized to the probe. By sequencing we determined that alleles $eIF4E^{07238}$, $eIF4E^{589/11}$, and $eIF4E^{715/13}$ have P-element insertions at nucleotide positions +502, +1140, and +1282, respectively, all of which are within the large first intron of the gene (Fig. 1A). Nucleotide positions are in accordance with an earlier description of the *eIF4E* gene (Lavoie *et al.*, 1996). These lines all failed to genetically complement one another. A recessive lethal mutation, *l(3)67Af*, generated in a screen for EMS-induced lethal lines

Fig. 1. Recessive lethal mutant alleles of *eIF4E* have a larval growth arrest phenotype. (**A**) Schematic representation of the insertion site of the P-elements in the *eIF4E* alleles. Insertion position is indicated with respect to the previously published *eIF4E* genomic sequence (Lavoie *et al.*, 1996). (**B**) The growth of $eIF4E^{589/11}$ is arrested at the first instar larval stage whereas control siblings continue to develop. $eIF4E^{589/11}$ and control larvae are shown at 24, 48, 72, 96, and 120 hours after egg deposition (AED). Similar growth arrests were observed for all *eIF4E* alleles examined. (**C**) Lifespan of growth-arrested *eIF4E* mutants (solid shapes) in the different alleles of *eIF4E*. The survival rates of wild-type larvae and control siblings (open shapes) are also shown.


A
eIF4E 589/11
+1140
eIF4E 07238
+502
eIF4E 715/13
+1282
eIF4EI
eIF4EII
exons 1
1A
1B
2
3
4
5
B
control
eIF4E
24
48
72
96
120
Time after egg deposition (hrs)
C
100%
80%
60%
40%
20%
0%
Hatched larvae (%)
0
24
48
72
96
120
144
168
192
216
240
Time after egg deposition (hrs)
wild-type
Df(3L)AC1/TM6B
eIF4E 715/13
eIF4E 589/11
eIF4E 67Af
eIF4E 07238

(Leicht and Bonner, 1988), was also found to be allelic to *eIF4E* and will subsequently be referred to as $eIF4E^{67Af}$. Since portions of *eIF4E* remain in all of these loss-of-function alleles, we cannot rule out the possibility that they can still direct low levels of gene expression that are not sufficient to sustain viability. Viability and fertility were completely restored to the *eIF4E* alleles by introducing in trans an 8.9 kb *SpeI* genomic fragment that includes the *eIF4E* gene (P{$eIF4E^{WT}$}).

eIF4E *mutants have a larval growth arrest phenotype*

Since lethal alleles in *eIF4A* are deficient in growth and arrest during larval development (Galloni and Edgar, 1999), we examined our *eIF4E* alleles for similar phenotypes. The growth defect phenotype differs from simple larval lethality in that growth-defective larvae never reach the normal third instar larval size, or else reach it after a substantially longer time than wild-type larvae yet survive for a minimum of four days after hatching from the egg (Galloni and Edgar, 1999). Wild-type larvae reach the second larval instar in approximately 24 hours and reach the third in another 24 hours. *eIF4E* mutants have a larval growth arrest phenotype (Fig. 1B,C). Maternally contributed wild-type *eIF4E* likely participates in the early development of these alleles, allowing them to survive through embryogenesis in the absence of zygotic *eIF4E* expression. Interestingly, three alleles ($eIF4E^{715/13}$, $eIF4E^{589/11}$, and $eIF4E^{67Af}$) arrest development in the first instar larval stage, but survive for several days, while $eIF4E^{07238}$ arrests its growth in the second instar. For the weakest allele, $eIF4E^{07238}$, second instar arrested larvae can live up to 10 days after egg deposition (Fig. 1C). In addition, we observed

some embryonic lethality with one allele, $eIF4E^{715/13}$ (18% of embryos fail to hatch). No other morphological defects were observed for any of the *eIF4E* alleles.

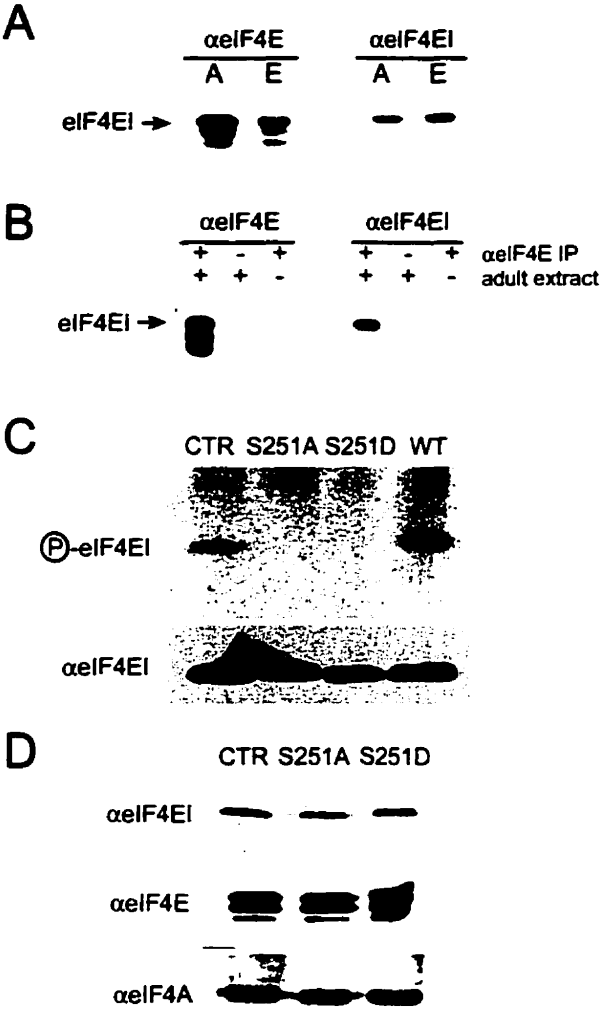
***Ser251 mutants do not incorporate labeled orthophosphate* in vivo**

In mammalian cells, Ser209 of eIF4E is the major site of phosphorylation (Flynn and Proud, 1995; Whalen *et al.*, 1996). A serine residue in a similar sequence context (Ser251 in eIF4EI) is present near the C-terminus of *Drosophila* eIF4E. To study whether *Drosophila* eIF4E is phosphorylated on this site *in vivo*, and to study the function of this residue in development, we generated transgenic *Drosophila* lines expressing eIF4E under its own promoter, in which Ser251 altered to Ala (P{$eIF4E^{Ser251Ala}$}) or Asp (P{$eIF4E^{Ser251Asp}$}).

To analyze eIF4E, we used an eIF4EI antiserum generated against a peptide limited to the unique N-terminal sequence of this isoform, a region that is highly variable in all eIF4E cognates (Lasko, 2000). The affinity purified eIF4EI antibody binds to the slowest migrating eIF4E isoform detected by an antiserum against all forms of eIF4E (Sigrist *et al.*, 2000) (Fig. 2A,B). Thus, the eIF4EI antiserum is specific for this isoform.

To assess whether eIF4EI was phosphorylated when Ser251 was replaced by Ala or Asp, we immunoprecipitated eIF4EI from *Drosophila* ovaries that were metabolically labeled with [^{32}P] orthophosphate (Fig. 2C). While eIF4EI was immunoprecipitated from all genotypes examined (Fig. 2C, bottom panel), eIF4EI was labeled with [^{32}P] orthophosphate only in wild-type ovaries and in transgenic ovaries expressing wild-type eIF4EI (Fig. 2C, top panel). eIF4EI immunoprecipitated from mutant ovaries, in which

Fig. 2. Mutants at Ser251 do not incorporate [^{32}P]orthophosphate *in vivo*. (**A**) Characterization of the affinity purified antiserum raised and purified against the eIF4EI N-terminal peptide MQSDFHRMKNFANPKSMF. A western blot containing *Drosophila* embryo (E) and adult (A) extracts was probed with a general eIF4E antiserum (αeIF4E) or the eIF4EI peptide serum (αeIF4EI). Both antisera were used at 1:1000 dilutions. (**B**) Immunoprecipitations performed with αeIF4E were transferred to a Western blot and probed with αeIF4E or αeIF4EI. These data indicate that the eIF4EI antiserum is specific for the slowest migrating form of eIF4E. (**C**) Immunoprecipitation of eIF4EI from extracts of *Drosophila* ovaries metabolically labeled with [^{32}P] orthophosphate (top panel, autoradiography; bottom panel, Western blot with αeIF4EI). Immunoprecipitations from control sibling (CTR), $eIF4E^{Ser251Ala}$ (S251A), $eIF4E^{Ser251Asp}$ (S251D), and wild-type (WT) ovary extracts are shown. (**D**) Immunoblot depicting the levels of eIF4E (detected by αeIF4E or αeIF4EI) and, as loading control, of eIF4A (αeIF4A, Styhler *et al.*, 1998) in adult extracts from control siblings (CTR) and from the *eIF4E* phosphorylation mutants (S251A, S251D).


A
αeIF4E
αeIF4EI
A E
A E
eIF4EI
B
αeIF4E
αeIF4EI
+ - +
+ + -
+ - +
+ + -
αeIF4E IP
adult extract
eIF4EI
C
CTR S251A S251D WT
Ⓟ-eIF4EI
αeIF4EI
D
CTR S251A S251D
αeIF4EI
αeIF4E
αeIF4A

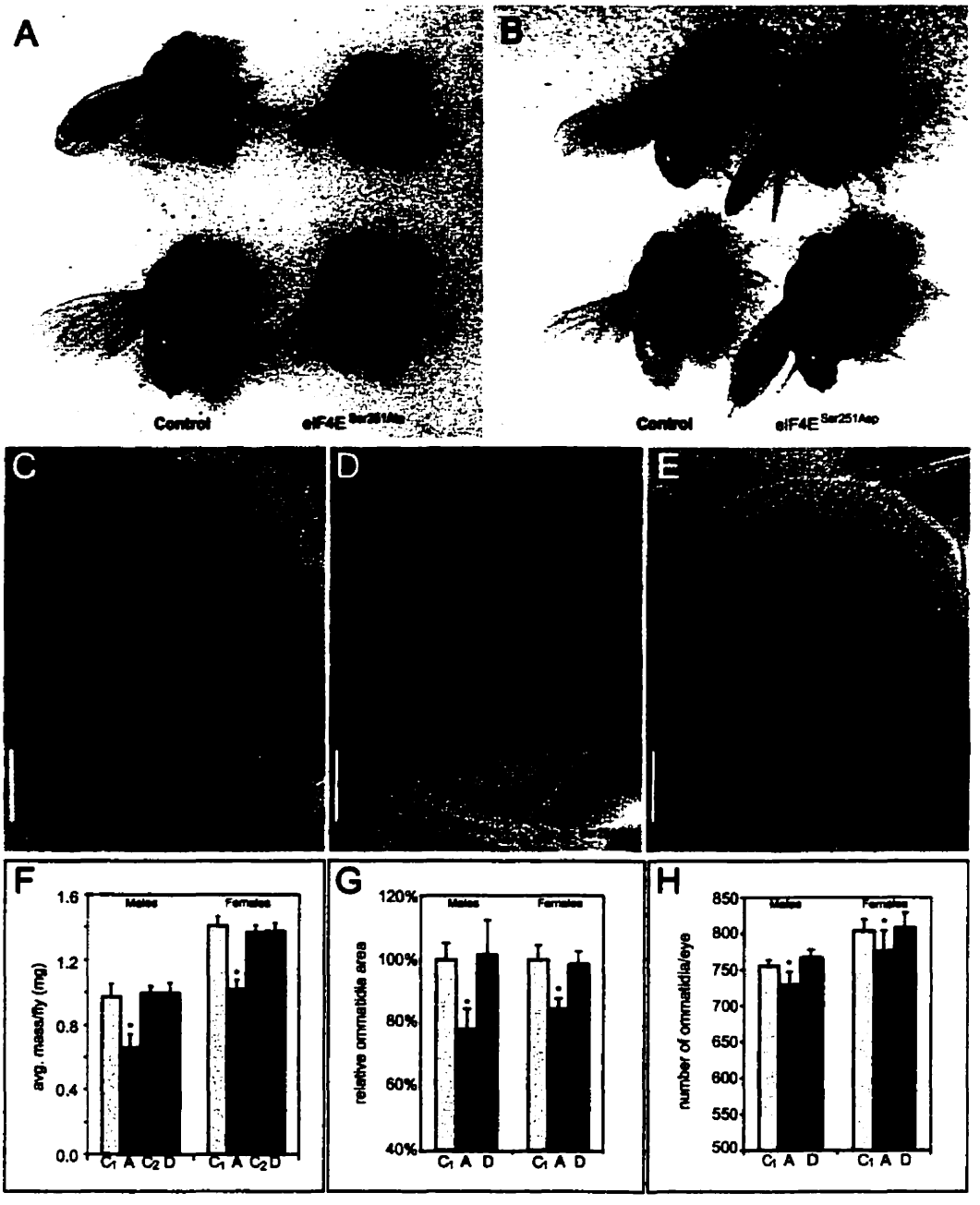
the only source of eIF4EI is from either of the P{*eIF4E*^{Ser251Ala}}or P{*eIF4E*^{Ser251Asp}} transgenes, does not detectably incorporate [^{32}P] orthophosphate. These results suggest that *Drosophila* eIF4EI is phosphorylated *in vivo* on Ser251.

We used an antibody to compare the expression of eIF4E in our transgenic lines, and found that P{*eIF4E*^{Ser251Ala}} and P{*eIF4E*^{Ser251Asp}} express eIF4E at similar levels to wild-type (Fig. 2D). A western blot using anti-eIF4A (Styhler *et al.*, 1998) confirms that equivalent amounts of protein are present in each genotype. Thus the phenotypes we describe cannot be attributed to a dosage effect.

eIF4E^{Ser251Ala} *mutants are delayed in development and are small*

Flies carrying the P{*eIF4E*^{Ser251Ala}} transgene in the background of *eIF4E* mutant alleles have reduced viability (35% lethality), take longer to develop to adulthood, and have blistered wings (Fig. 3A). The viability of *eIF4E*^{Ser251Ala} is lower in males than females, and the developmental delay varies depending on the strength of the *eIF4E* alleles used as genetic background, varying from 1-2 additional days for weaker alleles (*eIF4E*^{07238} and *eIF4E*^{67Af}) to 3-4 additional days for stronger alleles (*eIF4E*$^{589/11}$ and *eIF4E*$^{715/13}$). Strikingly, when P{*eIF4E*^{Ser251Ala}} is placed in the background of stronger *eIF4E* alleles, such as *eIF4E*$^{589/11}$, surviving females and males are smaller and their weight is reduced by 29% (one-tailed *t*-test, $P < 0.001$) and 32% (one-tailed *t*-test, $P < 0.01$) respectively, as compared to control siblings grown under identical conditions (Fig. 3F). Although smaller, the body parts of *eIF4E*^{Ser251Ala} flies are appropriately proportioned and no patterning defects are observed except for the wings. Wings of *eIF4E*^{Ser251Ala} are

Fig. 3. (**A**) Rescue of $eIF4E^{589/11}$ allele with the P{$eIF4E^{Ser251Ala}$} transgene results in adult flies with small body size and aberrant wing growth. (**B**) Flies carrying the P{$eIF4E^{Ser251Asp}$} transgene in the background of the $eIF4E^{589/11}$ allele. Males (top row) and females (bottom row) are shown. The control flies shown are siblings grown under conditions identical to the transgenic mutants. Adult compound eyes of (**C**) control flies, (**D**) $eIF4E^{Ser251Ala}$, and (**E**) $eIF4E^{Ser251Asp}$ in the background of the $eIF4E^{589/11}$ allele. Note reduction in size of $eIF4E^{Ser251Ala}$ compared to control and $eIF4E^{Ser251Asp}$ flies. Control shown is from a male sibling grown under conditions identical to that of the $eIF4E^{Ser251Ala}$ mutants. Similar results are obtained with female eyes (data not shown). All electron micrographs taken at 160x magnification, Bar = 100 μm. (**F**) Average mass of $eIF4E^{Ser251Ala}$ and $eIF4E^{Ser251Asp}$ mutants compared to their respective control siblings (N=60). (**G**) Relative area of individual ommatidia and (**H**) average number of ommatidia per compound eye of males and females (N=5 individuals for each genotype). C_1, control sibling of $eIF4E^{Ser251Ala}$, C_2, control sibling of $eIF4E^{Ser251Asp}$, A, $eIF4E^{Ser251Ala}$, D, $eIF4E^{Ser251Asp}$. (*) Significant *P* values, as calculated using a one-tailed Student's *t*-test comparing the average mass of male and female mutants to their control siblings. Data represent mean ± standard deviation.


A
Control
eIF4E Ser251Asp
B
Control
eIF4E Ser251Asp
C
D
E
F
avg. mass/fly (mg)
1.6
1.2
0.8
0.4
0.0
Males
Females
C1 A C2 D
C1 A C2 D
G
relative ommatidia area
120%
100%
80%
60%
40%
Males
Females
C1 A D
C1 A D
H
number of ommatidia/eye
850
800
750
700
650
600
550
500
Males
Females
C1 A D
C1 A D

blistered, smaller than wild type and are occasionally clipped whereas the size of adult wings from *eIF4E*^{Ser251Asp} mutants is comparable to that of control flies (data not shown).

Transgenic lines in which Ser251 was mutated to Asp were generated to test the effects of mimicking constitutive phosphorylation. The P{*eIF4E*^{Ser251Asp}} transgene can fully rescue the lethality of all *eIF4E* transheterozygote combinations tested (Fig. 3B). No morphological defects or change in size and weight were observed in any of the *eIF4E*^{Ser251Asp} alleles (Fig. 3F). The ability of the P{*eIF4E*^{Ser251Asp}} transgene to fully rescue *eIF4E* alleles suggests that mutating the residue at Ser251 does not necessarily alter the three-dimensional structure and functionality of eIF4E.

The adult eye of* eIF4E^{Ser251Ala} *mutants have smaller and fewer ommatidia

The phenotypes of *eIF4E*^{Ser251Ala} mutants suggest that phosphorylation of eIF4E is important for the normal growth of *Drosophila*. The *eIF4E*^{Ser251Ala} phenotypes are similar to those described for genes that influence growth, such as *Dmyc* and *Dras1*, and from genes of the insulin receptor pathway (Weinkove and Leevers, 2000). These genes influence final body size by affecting the size and number of cells in specific tissues. We thus examined whether cell growth is affected in *eIF4E* alleles rescued by the phosphorylation mutant transgenes.

The *Drosophila* compound eye is a highly precise hexagonal array of units termed ommatidia. The female wild-type eye is composed of approximately 800 ommatidia while the male counterpart has on average 50 fewer ommatidia (Wolff and Ready, 1993). Due to the readily quantifiable architecture and size of the eye, we opted to use this adult

structure to examine whether cell size and number was affected in our various *eIF4E* transgenic lines. While control and $eIF4E^{Ser251Asp}$ flies have normal size eyes (Fig. 3C,E), the compound eye of transgenic $eIF4E^{Ser251Ala}$ mutants is markedly reduced in size (Fig. 3D). The reduction in size of the $eIF4E^{Ser251Ala}$ compound eye is caused mostly by a reduction in the area of individual ommatidia and only slightly by a reduction in their number. The average area of individual ommatidia in the center of the eye in both male and female $eIF4E^{Ser251Ala}$ mutants is significantly reduced (males, 206.51 ± 16.16 μm^2; females, 246.60 ± 10.80 μm^2) compared to the ommatidia of control siblings (males, 265.00 ± 13.17 μm^2; females, 293.24 ± 12.46 μm^2) (Fig. 3G), a difference of 22.1% for males (one-tailed *t*-test, $P < 0.001$) and 15.9% for females (one-tailed *t*-test, $P < 0.001$). The area of individual $eIF4E^{Ser251Asp}$ ommatidia (males, 269.25 ± 28.50 μm^2; females, 289.02 ± 11.51 μm^2) is essentially the same as that of the controls (Fig. 3G). A slight reduction in ommatidia number is also observed in $eIF4E^{Ser251Ala}$ mutants (Fig. 3H). This reduction is small but statistically significant for males (729 ± 19 ommatidia in the mutant compared to 759 ± 8 in controls; one-tailed *t*-test, $P < 0.01$) and females (777 ± 28 ommatidia as compared to 805 ± 14 in controls; one-tailed *t*-test, $P < 0.05$). Again, no difference is observed in the number of ommatidia in $eIF4E^{Ser251Asp}$ (males, 766 ± 12 ommatidia; females, 808 ± 21 ommatidia) when compared to the controls. These data argue that the overall reduction in size of the compound eye observed in $eIF4E^{Ser251Ala}$ mutants mostly results from reduced cell size, with a minor contribution from a reduction in cell numbers.

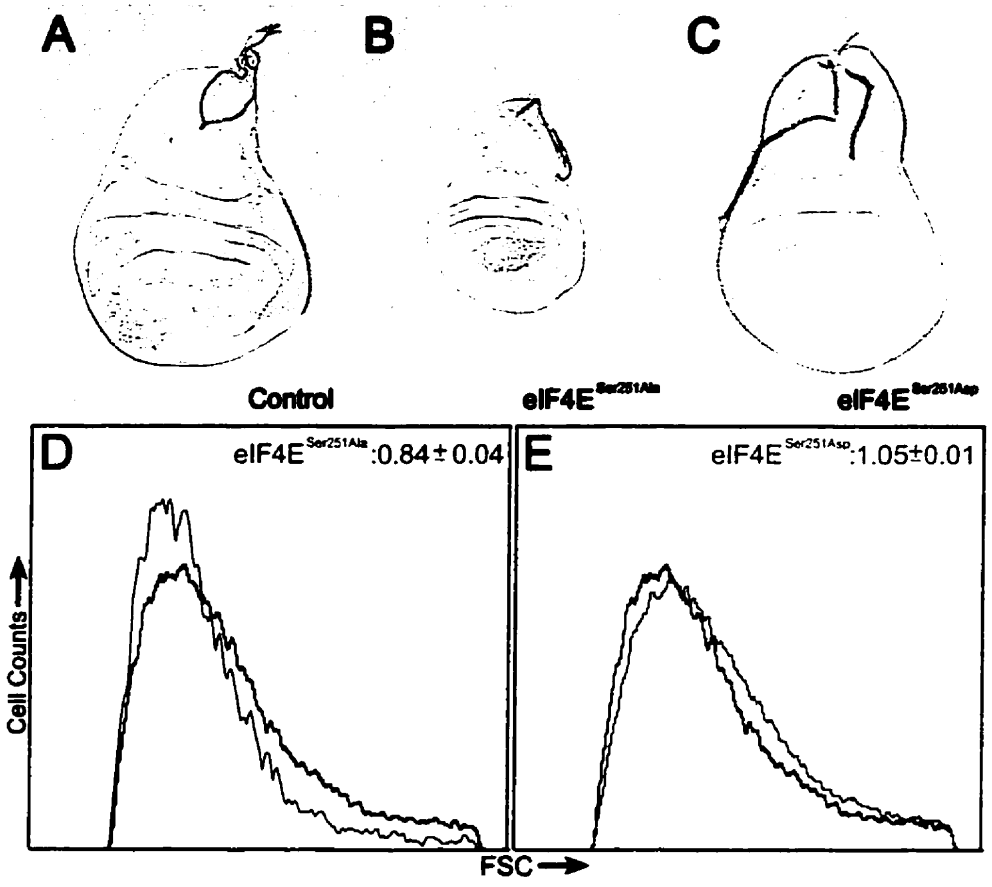
Wing imaginal disc cells of eIF4E^{Ser251Ala} *mutants are smaller than wild-type cells*

The larval precursors of *Drosophila* adult structures are the imaginal discs. We examined wing imaginal discs from late stage third instar *eIF4E*^{Ser251Ala} larvae, and found that they are markedly reduced in size compared to control and *eIF4E*^{Ser251Asp} discs (Fig. 4A,B,C). The decrease in size of the *eIF4E*^{Ser251Ala} wing disc suggests that growth may be reduced at the level of individual wing disc cells. To examine the relative sizes of individual cells from wing discs, imaginal discs were dissociated and their cells were analyzed by flow cytometry (Fig. 4D,E). The forward light scatter value obtained by flow cytometry is a measure of cell size. The mean forward light scatter value of cells from *eIF4E*^{Ser251Ala} imaginal discs is decreased by 16% compared to cells from control discs (Fig. 4D). Also, a slight increase in size of 5% was observed for cells from *eIF4E*^{Ser251Asp} discs (Fig. 4E), suggesting that constitutive phosphorylation of eIF4E has a detectable effect on growth during the development of *Drosophila* wing imaginal discs. Since inhibition of proliferation in *Drosophila* increases rather than decreases the size of cells (Neufeld *et al.*, 1998), these data suggest that eIF4E phosphorylation functions in the regulation of growth.

Discussion

In mammals, increased phosphorylation of eIF4E has been correlated with increased cellular growth; however, unphosphorylated eIF4E can still bind the mRNA cap and initiate translation (Raught *et al.*, 2000a). However, biochemical analyses have never

Fig. 4. Phenotypic analysis of wing imaginal discs in the *eIF4E* phosphorylation mutants. Dissected wing imaginal discs from (**A**) control, (**B**) $eIF4E^{Ser251Ala}$, and (**C**) $eIF4E^{Ser251Asp}$ larvae. Note the reduction in size of $eIF4E^{Ser251Ala}$ imaginal discs compared to control and $eIF4E^{Ser251Asp}$ discs. (**D**) Forward light scatter (FSC) analysis of cells dissociated from $eIF4E^{Ser251Ala}$ (red line) and (**E**) from $eIF4E^{Ser251Asp}$ (blue line) compared to cells from control discs (black line). Numbers represent ratio of mean FSC value of wing disc cells from phosphorylation mutants compared to the control cells. Control animals have wild-type *eIF4E* and were raised under conditions identical to the phosphorylation mutants.


A
B
C
Control
eIF4E Ser251Ala
eIF4E Ser251Asp
D
eIF4E Ser251Ala :0.84 ± 0.04
E
eIF4E Ser251Asp :1.05±0.01
Cell Counts
FSC

resolved the question of the biological importance of eIF4E phosphorylation. Increased phosphorylation of mammalian eIF4E is reported to increase its affinity for mRNA caps (Minich *et al.*, 1994). However, the nature of the eIF4E preparations used in this study was not precisely determined. Others have shown that bacterially-expressed wild-type eIF4E is active in translation, indicating that phosphorylation is not absolutely necessary for eIF4E activity (Morino *et al.*, 2000). Consistent with this result, flies in which eIF4E can no longer be phosphorylated on Ser251 are viable. However, the flies are delayed in development and are smaller in size than control animals. This genetic work thus provides evidence that Ser251 of eIF4E is important for regulating growth of a multicellular organism.

Although phenotypes affecting viability and growth were observed for *eIF4E*^{Ser251Ala}, we did not obtain strong phenotypes suggestive of overgrowth in an *eIF4E* mutant in which constitutive phosphorylation is mimicked by conversion of Ser251 to Asp. The only phenotype observed was a small increase in cell size in wing imaginal discs from third instar larvae, as detected by flow cytometry. We also attempted to increase eIF4E activity by performing overexpression studies. Overexpression of eIF4E in cultured mammalian cells results in increased growth (Lazaris-Karatzas *et al.*, 1990; Smith *et al.*, 1990). Since in mammalian cells a negative feedback loop between increased eIF4E activity and 4E-BP hypophosphorylation has been observed (Khaleghpour *et al.*, 1999), overexpression of *eIF4E*^{wt} was also performed in the background of a *Drosophila 4E-BP* (*d4E-BP*) null mutant allele (Bernal and Kimbrell, 2000). Wild-type *d4E-BP* expression levels do not appear to influence growth rates as *d4E-BP* null alleles raised

under normal conditions (Bernal and Kimbrell, 2000) and overexpression of *d4E-BP*^{wt} (Miron *et al.*, 2001) and indistinguishable from controls. Similarly, we did not observe any overgrowth phenotypes upon overexpression of *eIF4E*^{wt} in various tissues in the background of the *d4E-BP* null allele (P.E.D.L. and P.L., unpublished results). The absence of phenotypes upon overexpression of *Drosophila eIF4E* was also reported elsewhere (Zhang *et al.*, 2000). Since *eIF4E*^{Ser251Asp} or *eIF4E*^{wt} overexpression did not result in increased growth, we conclude that *Drosophila* can physiologically withstand increases in *eIF4E* activity more readily than decreases, although it is possible that our treatments did not increase the activity of *eIF4E* to a level at which a phenotype can be observed. Alternatively, elevated *eIF4E* activity may only result in increased growth and proliferation if other genes are also overexpressed. In primary mammalian cell cultures, cooperation with other proto-oncogenes is necessary for eIF4E-mediated transformation (Lazaris-Karatzas and Sonenberg, 1992).

It was originally believed that the phosphorylation of mammalian eIF4E occurred on Ser53 and mutations in this site were generated to examine the role of eIF4E phosphorylation in mediating the oncogenic transformation of mammalian cells (reviewed in Raught *et al.*, 2000a). However, the three-dimensional structure of eIF4E shows that Ser53 resides within the protein and thus alterations in this residue likely affect protein folding rather than phosphorylation (Marcotrigiano *et al.*, 1997). Later studies showed unambiguously that the phosphorylation of eIF4E occurs on Ser209 *in vivo* (Waskiewicz *et al.*, 1997; Whalen *et al.*, 1996). The three-dimensional position of Ser209 near the mRNA binding slot (Marcotrigiano *et al.*, 1997) is consistent with results showing that

phosphorylation increases the affinity of eIF4E for mRNA caps (Minich *et al.*, 1994). Since Ser209 resides on the surface of eIF4E and since the *eIF4E*^{Ser251Asp} can fully substitute for wild-type eIF4E, we believe that the mutations we generated in Ser251 do not alter the three-dimensional structure of eIF4E.

Although phosphorylated eIF4EI was not detected in the *in vivo* metabolic labeling experiments (Fig. 2C), we cannot rule out the possibility that low levels of phosphorylated eIF4E are produced by the loss-of-function alleles used as genetic background or are contributed maternally. The phenotypes presented here for *eIF4E*^{Ser251Ala} should therefore be interpreted as resulting from reduced eIF4E phosphorylation and not from its complete absence. Similarly, it is possible that the absence of phenotypes in *eIF4E*^{Ser251Asp} results from residual wild-type eIF4E activity.

We also show that lethal *eIF4E* mutations result in growth arrest during larval development. Similar phenotypes were previously described for mutations in *eIF4A* (Galloni and Edgar, 1999). In addition to *eIF4A*, several genes implicated in biosynthesis of proteins and nucleic acids have been shown to possess a larval growth deficiency. These include a mutation in the mitochondrial ribosomal protein S15 gene *bonsai*, the Myc-regulated DEAD-box RNA helicase *pitchoune*, and the DNA replication regulator *peter pan* (Galloni and Edgar, 1999; Migeon *et al.*, 1999; Zaffran *et al.*, 1998). *eIF4E* thus appears to be part of a growing class of genes which have a larval growth defect phenotype and regulate macromolecular synthesis. It is possibile that the larval growth arrest phenotypes defined by Galoni and Edgar (1999) are a result of ecdysone signaling defects, as this steroid hormone is required to direct the molts between larval instars

(Riddiford, 1993). Ecdysone signaling may in fact also function in growth control, as it was hypothesized that hormones synthesized in the ring gland and fat body function in coordinating the growth of the organism (Galoni & Edgar, 1999).

In mammals, the best candidate for the eIF4E kinase is the MAP-kinase-interacting protein kinase-1 (MNK1) which phosphorylates eIF4E on Ser209 upon activation by either the ERK and p38 MAP kinases (Fukunaga and Hunter, 1997; Waskiewicz *et al.*, 1997). MNK1 physically interacts with eIF4G, bringing it in close proximity to eIF4E *in vivo* (Pyronnet *et al.*, 1999). The *Drosophila* protein most similar to MNK1 is the microtubule-associated protein kinase Lk6 (Kidd and Raff, 1997). It will be of interest to determine whether Lk6 interacts with *Drosophila* eIF4G (Hernandez *et al.*, 1998), and if disruption of that interaction results in a phenotype similar to that described here for $eIF4E^{Ser251Ala}$.

A role for the ras/raf/ERK signaling cascade in eIF4E phosphorylation is consistent with the finding that mammalian cells transformed by *ras* or *src* have increased eIF4E phosphorylation (Frederickson *et al.*, 1991; Rinker-Schaeffer *et al.*, 1992). In this respect, *ras* has been shown to regulate cellular growth in the *Drosophila* wing (Prober and Edgar, 2000). Althought *ras* has never been shown to be upstream of eIF4E phosphorylation in *Drosophila*, it is possible that a portion of the effect on growth exhibited by *Drosophila ras* is mediated through changes in eIF4E phosphorylation. Nevertheless, the effects of *Drosophila ras* are likely to be pleiotropic since genetic manipulations of *ras* leads to changes in the activity of the *Drosophila* homologues of *myc* and cyclin E (Prober and Edgar, 2000). Phenotypes consistent with a role in growth

control have not been described for any other *Drosophila* homologue of the ras/raf/ERK pathway.

The insulin signaling pathway in *Drosophila* has been implicated in the control of organismal size (Brogiolo *et al.*, 2001; Weinkove and Leevers, 2000). In mammalian cells, this signal transduction pathway leads to the activation of the kinase FRAP/mTOR that in turn leads to the activation of translation via at least two mechanisms: the phosphorylation of S6K and of 4E-BP (Raught *et al.*, 2000a). *DS6K* is a critical component of this pathway in *Drosophila* as its overexpression can rescue the lethality of *dTOR* mutants (Zhang *et al.*, 2000). However, the effects on growth that result from mutations in genes of the insulin signaling pathway appear at least in part mediated by the regulation of eIF4E availability, as regulated by 4E-BP, although these proteins lie on a branch of the pathway independent of *DS6K* (Miron *et al.*, 2001). The results presented here indicate that phosphorylation of eIF4E, which is believed to be independent of the insulin signaling pathway, is also a biologically significant mechanism of regulating growth.

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Chapter 5

General Discussion

5.1 Overview

This work is part of an ongoing effort to characterize all translation initiation factors in *Drosophila melanogaster*, with the ultimate goal of understanding their regulation during the development of a multicellular organism. This thesis describes several advances made towards an understanding of *Drosophila eIF4E* regulation. In particular, this analysis has shown a gene that encodes an essential *Drosophila* eIF4E homologue is alternatively spliced to produce two protein isoforms. Since no gene is functionally redundant with *eIF4E*, it was surprising that several other genes homologous to *eIF4E* were identified in the *Drosophila* genome (Lasko, 2000). Through the generation of transgenic fly lines, the biological relevance of eIF4E phosphorylation was also examined. These experiments provide the first direct evidence that eIF4E phosphorylation is essential for the normal growth of a multicellular organism. Binding with the inhibitory 4E-BPs also regulates eIF4E. Interestingly, effects on growth were also recently observed from increased *Drosophila* 4E-BP activity (Miron *et al.*, 2001). These results may provide a basis for the future identification of new effectors of eIF4E activity.

5.2 What are possible functions of the new Drosophila *eIF4E cognates?*

Several experiments describing the cap-binding activity of *Drosophila* eIF4E, its ability to associate within eIF4F, and its regulation were performed by examining 35 kDa cap-binding proteins (Duncan *et al.*, 1995; Maroto and Sierra, 1989; Zapata *et al.*, 1994). We described that a single gene located at cytological position 67A8-B2 encodes two 35

kDa isoforms of eIF4E (Chapter 2). The purification of eIF4E by cap-column chromatography suggests that the isoforms originating from the gene at 67A8-B2 are the predominant forms in *Drosophila* tissues (Chapter 2, Fig. 1; Maroto and Sierra, 1989). Furthermore, the finding that mutations in *Drosophila eIF4E* are lethal (Chapter 4) implies that this gene provides an essential biological function. Nevertheless, several *Drosophila* genes encoding additional eIF4E homologues were identified upon the completion and annotation of the *Drosophila* genome sequence (Adams *et al.*, 2000; Lasko, 2000).

What are the roles of the multiple eIF4E cognates given that their functions are not redundant with that of the originally described *Drosophila eIF4E*? One possibility is that the function of the eIF4E homologues is identical to that of *eIF4E* but their expression levels are not capable of rescuing the lethality of *eIF4E* alleles. A thorough examination of the expression of proteins encoded by the eIF4E cognates may support this hypothesis. However, this idea implies that flies already have in place the genes necessary to compensate for the loss of *eIF4E* but have not evolved the mechanisms to take advantage of them. Therefore, another option is that the new eIF4E cognates have biological functions distinct from the *eIF4E* at 67A8-B2.

Insights into the functions of the *Drosophila* eIF4E cognates can be derived from studies performed in *C. elegans*. The *C. elegans* genome encodes five isoforms of eIF4E (Jankowska-Anyszka *et al.*, 1998; Keiper *et al.*, 2000). Although one of these isoforms (encoded by the gene *ife-3*) preferentially binds normal monomethylated mRNA caps, three other genes (*ife-1*, *ife-2*, and *ife-5*) encode for proteins with a binding preference for

trimethylated mRNA caps (Jankowska-Anyszka *et al.*, 1998; Keiper *et al.*, 2000). RNA interference (RNAi) studies show that the elimination of either *ife-3* alone or of *ife-1*, *ife-2*, and *ife-5* in combination is lethal (Keiper *et al.*, 2000). Parallels can be drawn between the results showing that *ife-3* is necessary for viability and our observations that mutations in *Drosophila eIF4E* are lethal. Both *ife-3* and the protein isoforms produced by *Drosophila eIF4E* (eIF4EI and eIF4EII) are good interactors of monomethylated mRNA caps. However, since the presence of mRNAs with trimethylated caps is unique to SL trans-spliced mRNAs in *C. elegans* and are not found in *Drosophila*, the question remains as to the function of the other *Drosophila* eIF4E cognates. One possibility is that rather than binding trimethylated caps, the *Drosophila* eIF4E cognates are required to bind mRNA caps that contain *O*-ribose methylations. *Drosophila* is one of the few organisms where mRNA species with all types of *O*-ribose cap methylations (see Chapter 1, section 1.3) are represented (Levis and Penman, 1978).

The isolation of mutations in the genes encoding the *Drosophila* eIF4E cognates or the use of RNAi techniques would address the necessity of these genes during development. Based on the *C. elegans* results (Keiper *et al.*, 2000), the generation of flies that are deficient for multiple eIF4E cognates may be required to assess the roles of these genes. Biochemical analysis of the binding affinities of the eIF4E cognates for different mRNA cap analogues will be necessary to address the possibility that these proteins distinguish between different types of caps.

One of the *Drosophila* genes similar to eIF4E appears to be the ortholog of the mammalian 4E-Homology Protein (4E-HP) (Lasko, 2000). Although 4E-HP can associate

with mRNA cap analogues *in vitro* (Rom *et al.*, 1998), the biological function of this protein is unknown. It is apparent from the amino acid sequence of the 4E-HPs that these proteins cannot associate with eIF4G or be regulated by 4E-BPs (see Chapter 1, Fig. 6). Since RNAi experiments performed with the *C. elegans* homologue (encoded by *ife-4*) suggest that 4E-HP does not provide an essential biological function (Keiper *et al.*, 2000), it is unclear whether the isolation of mutants in *Drosophila 4E-HP* would help to elucidate the biological function of these proteins. However, one cannot exclude the possibility that the RNAi results are an artefact of the technique until they are confirmed by the characterization of *4E-HP* null mutant alleles, either in *Drosophila* or *C. elegans.*

5.3 Regulation of translation by multiple subunits of eIF4F

In addition to the multiple isoforms of eIF4E, the *Drosophila* genome encodes two isoforms of both eIF4G and eIF4A (Chapter 1, section 1.7). The presence of multiple isoforms of eIF4F subunits appears to be a conserved occurrence. In addition to *Drosophila*, multiple forms of these factors exist in organisms spanning all phyla, including mammals (Gao *et al.*, 1998; Gradi *et al.*, 1998; Nielsen *et al.*, 1985), *C. elegans* (Jankowska-Anyszka *et al.*, 1998; Keiper *et al.*, 2000), *Xenopus* (Li *et al.*, 1999; Morgan and Sargent, 1997), yeast (Goyer *et al.*, 1993; Linder and Slonimski, 1989), and plants (Browning *et al.*, 1987; Browning *et al.*, 1992; Carberry and Goss, 1991). Since protein synthesis is essential for viability, the presence of multiple isoforms may provide a functional redundancy. This appears to be the case in yeast where either of the isoforms of eIF4A or eIF4G is sufficient for viability (Goyer *et al.*, 1993; Linder and Slonimski,

1989). Nevertheless, a subtle functional difference exists between the yeast eIF4Gs as gene disruptions in one isoform exhibit slow growth phenotypes whereas mutants in the other do not (Goyer *et al.*, 1993). Work on the eIF4As of *Xenopus* and on the eIF4Es of *C. elegans* supports the notion that these isoforms have distinct functions (Keiper *et al.*, 2000; Li *et al.*, 1999). Although it is difficult to speculate on the individual functions of all of these isoforms, their presence suggests that multiple eIF4Fs can form *in vivo* and points to an additional level of complexity in the control of translation that must be considered in future studies.

5.4 Effectors in the signal transduction pathway leading to eIF4E phosphorylation

The physiological kinase for eIF4E in mammalian cells appears to be MNK1, a protein that associates directly with eIF4G (Pyronnet *et al.*, 1999; Waskiewicz *et al.*, 1997; Waskiewicz *et al.*, 1999). The best candidate for a *Drosophila* MNK1 homologue is the protein Lk6 (Kidd and Raff, 1997). Although the N-terminal portion of Lk6 is highly homologous to MNK1 (55% identity and 71% homology), it possesses a long C-terminus that extends the protein to more than twice the size of MNK1 (MNK1 comprises 424 amino acids while Lk6 has 1150). Lk6 was originally identified in a screen for microtubule associating proteins and is thought to exist in two isoforms. The 185 kDa form of Lk6 is the most abundant while the rarer 220 kDa protein interacts with microtubules (Kidd and Raff, 1997). Lk6 is rapidly turned over *in vivo* (Kidd and Raff, 1997), as would be expected by the presence of PEST-motifs (Tyystjarvi *et al.*, 1994). Furthermore, overexpression of Lk6 is lethal, producing embryos with microtubule

defects (Kidd and Raff, 1997). Results presented in this thesis suggest that *in vivo*, *Drosophila* eIF4EI is phosphorylated on Ser251; this residue is in a context identical to the site phosphorylated by MNK1 in mammalian eIF4E (Chapter 4). Further work is required to determine if, like MNK1, Lk6 can bind the *Drosophila* eIF4Gs and/or p97 and whether it can phosphorylate eIF4E on Ser251 *in vivo*. The phenotypes of as yet unidentified mutants in *Lk6* may resemble those described here for $eIF4E^{Ser251Ala}$ (Chapter 4), which would be consistent with Lk6 functioning as an eIF4E kinase. However, the observation of such phenotypes may be unlikely given that *Lk6* was shown to function in a cellular process distant from the regulation of translation (microtubule formation, Kidd and Raff, 1997) and would thus be pleiotropic if it also acts as an eIF4E kinase. Furthermore, it cannot be ruled out that Lk6 (or MNK1 for that matter) phosphorylate proteins other than eIF4E.

The identities of other proteins affecting the phosphorylation of eIF4E are less well established. Work in mammalian cells supports the involvement of Ras and the Erk or p38 MAP Kinase pathways in regulating eIF4E phosphorylation (Reviewed in Raught *et al.*, 2000a). A genetic screen for dominant enhancers or suppressors of an hypomorphic allele of *Drosophila eIF4E* could be used to identify additional genes participating in eIF4E regulation. Since they cannot be phosphorylated *in vivo*, the *eIF4E* phosphorylation mutants (Chapter 4) would not be a good choice as the hypomorphic allele for this screen. Rather, new *eIF4E* hypomorphs would have to be identified. The generation of transgenic flies using randomly mutagenized *eIF4E* constructs and the analysis of their phenotypes may yield these new hypomorphic alleles. Another

possibility is to generate an allele based on the yeast temperature-sensitive *eIF4E* mutant, *cdc33*$^{ts4\text{-}2}$, which contains the substitution of a conserved glycine (Gly113 in yeast; Gly154 in *Drosophila* eIF4EI) to an aspartic acid (Altmann *et al.*, 1989). At the non-permissive temperature, *cdc33* $^{ts4\text{-}2}$ binds weakly to mRNA cap analogues and is linked to a reduction in overall protein synthesis. Transgenic flies carrying a construct with this Gly to Asp substitution may thus produce the hypomorphic allele required to perform the screen to isolate enhancers/suppressors of eIF4E activity.

5.5 Regulation of growth by Drosophila 4E-BP

Null mutants in *Thor*, the *Drosophila* gene encoding 4E-BP, are fully viable and do not have phenotypes indicative of a function in growth control (Bernal and Kimbrell, 2000). Similarly, overexpression of wild-type 4E-BP using various GAL4 drivers has no effect on cellular growth (Miron *et al.*, 2001). However, the *Drosophila* homologue of 4E-BP (d4E-BP) has an eIF4E-binding motif that diverges from the consensus sequence (YXXXXLΦ, Mader *et al.*, 1995) in which the two leucines are replaced by a methionine and a lysine. As a consequence, wild-type d4E-BP binds weakly to eIF4E whereas a mutant d4E-BP, in which the eIF4E-binding motif is reverted to the consensus sequence (d4E-BP^{LL}), has a 3-fold increased affinity for eIF4E (Miron *et al.*, 2001). Interestingly, unlike wild-type d4E-BP, overexpression of d4E-BP^{LL} in compartments of the wing imaginal disc results in reduced size (Miron *et al.*, 2001). Thus, reduction of the cellular fraction of eIF4E available for association into eIF4F, by overexpression of d4E-BP^{LL}, results in phenotypes similar those described here for the non-phosphorylatable eIF4E

mutant (Chapter 4). Taken together, these results support the idea that the regulation of eIF4E activity, whether by modulating its availability (via 4E-BP) or by changing its phosphorylation status, is important for the control of normal cellular growth.

Unlike the pathway leading to eIF4E phosphorylation, the pathway regulating 4E-BP activity is established (reviewed in Raught *et al.*, 2000a; see Chapter 1 section 1.5.3). As in mammals, the *Drosophila* PI3K/Akt pathway appears to regulate 4E-BP activity as co-expression of d4E-BP together with PI3K or dAkt1 results in a suppression of the phenotypes elicited by these kinases (Miron *et al.*, 2001). The kinase TOR phosphorylates two residues of mammalian 4E-BP as a priming event for the subsequent phosphorylation of three residues by an as yet unknown kinase or kinases (Gingras *et al.*, 1999a). Only upon the phosphorylation of these three residues is 4E-BP released from eIF4E. Although all of the key amino acids are conserved in d4E-BP, it remains to be determined whether its phosphorylation is regulated by similar mechanisms. If the regulation of d4E-BP phosphorylation is conserved as predicted, a genetic screen for enhancers/suppressors of the d4E-BP^{LL} phenotype could serve to identify the elusive 4E-BP kinase(s).

How do the effects on growth elicited by a reduction in eIF4E activity relate to those caused by the gene encoding the kinase DS6K? Mutants in *DS6K* are deficient in growth owing only to a reduction in cell size and not to reduced cell number (Montagne *et al.*, 1999). Although the reductions in eIF4E activity via d4E-BP^{LL} (Miron *et al.*, 2001) or in the phosphorylation mutants (Chapter 4) have a major effect on cell size, a reduction in cell number is also observed. DS6K phosphorylates ribosomal protein S6

(RpS6). The activity of DS6K is directly modulated by dTOR *in vitro* (Zhang *et al.*, 2000). In mammals, the phosphorylation by TOR of S6K and 4E-BP are believed to be on parallel pathways (von Manteuffel *et al.*, 1997). Therefore, the differences in phenotypes observed for the reduction of eIF4E activity and those for DS6K may be explained by the fact that they reside on these different branches of the PI3K pathway. The phosphorylation of RpS6 is believed to facilitate the translation of mRNAs containing polypyrimidine tracts at their 5' ends (also termed 5' TOP mRNAs) (reviewed in Edgar, 1999). 5' TOP mRNAs appear to encode for ribosomal proteins or genes involved in ribosome biogenesis. Thus, a hypothesis was put forth suggesting that the phenotypes of *DS6K* mutants can be explained by selective changes in the translation rates of mRNAs regulating ribosome biogenesis and not cell division (Thomas, 2000). Since a reduction in eIF4E activity also causes a slight reduction in cell number, it can be hypothesized that mRNAs involved in proliferation are also be affected in this situation. For 4E-BP^{LL} overexpression, an increase in apoptosis contributes to the observed reduction in cell numbers, but this is likely a consequence of the proliferative disadvantage of the 4E-BP^{LL} mitotic clones (Miron *et al.*, 2001). One way to address the differences in growth control between changes in eIF4E or DS6K/RpS6 activity would be to catalogue all mRNAs whose translation is selectively modulated in each genetic context. The newly emerging field of functional genomics will undoubtedly assist in such an endeavor.

5.6 The study of translation in the era of functional genomics

The recent years have witnessed the completion of genome sequencing projects for most of the common model organisms and have made available technologies, such as DNA microarrays, which allow for the solving of problems using larger scale approaches than previously possible. To date, most of the work performed using DNA microarrays examined the differential expression of mRNAs – in other words, these studies focus on gene expression at the level of transcription.

But can DNA microarrays be used to study gene expression at the level of translation? One way this can be achieved is by generating the pools of cDNA probes from the cellular fraction of mRNAs being actively translated. Actively translated cellular mRNAs are part of a polysome fraction that can be purified by sucrose gradient centrifugation (Aroskar *et al.*, 1980). The cDNA probes generated from polysomal mRNAs would thus detect genes being actively translated in a given cellular context. This type of microarray screening technique was successfully used in the Sarnow lab on two reported occasions. First, in a screen for mRNAs being translated by cap-independent mechanisms during poliovirus infection (Johannes *et al.*, 1999), and a second time to characterize genes differentially translated when yeast are switched from a fermentable to a non-fermentable carbon source (Kuhn *et al.*, 2001).

One way this technique could be applied to follow up on the work presented in this thesis is to address the possibility that changes in eIF4E activity affect the specific translation of a subset of cellular mRNAs. In mammals, several mRNAs are differentially translated when the activity of eIF4E is altered (Reviewed in De Benedetti and Harris,

1999). Changes in eIF4E activity and the subsequent effects on a specific subset of mRNAs may be an important step mediating oncogenesis in mammalian cells (De Benedetti and Harris, 1999; Sonenberg, 1996). If the translation of a subset of messages is also affected by $eIF4E^{Ser251Ala}$ (Chapter 4), a comprehensive survey of all of genes affected may help elucidate the molecular mechanisms underlying cellular growth. To examine the differential translation of specific mRNAs during growth, polysomal cDNAs generated from $eIF4E^{Ser251Ala}$ mutant flies could serve to probe *Drosophila* DNA microarrays. In addition, generation of target samples from multiple polysomal fractions would assist in the detection of genes that are subject to more moderate shifts in translation efficiency. A survey of mRNAs actively translated in the mutants compared to those translated in control flies could thus help catalogue the genes that are differentially expressed in conditions of reduced cell growth.

5.7 The regulation of eIF4G and its effect on translation

eIF4E may not be the sole component of eIF4F whose activity is regulated post-translationally. eIF4G phosphorylation may contribute an added level of complexity in the regulation of eIF4F activity. In *Xenopus*, eIF4G phosphorylation increases upon meiotic maturation of the oocyte (Morley and Pain, 1995). Several kinases can phosphorylate eIF4G *in vitro* (Tuazon *et al.*, 1989) and various extracellular stimuli lead to an increase in the incorporation of labeled phosphate (Bu *et al.*, 1993). Recently, it was shown that a subset of eIF4G phosphorylation sites is regulated by the PI3K pathway (Raught *et al.*, 2000b). Inhibition of PI3K (with wortmannin or LY294002) or

of FRAP/mTOR (with rapamycin) reduces the incorporation of radiolabeled phosphate at these sites in eIF4G. The MEK inhibitor PD98059 can also inhibit the phosphorylation of eIF4G but on a different subset of residues (Raught *et al.*, 2000b). It thus appears that the phosphorylation of eIF4G is regulated via more than one signal transduction pathway and that distinct subsets of residues are responsive to different signals.

To date, the effects of the phosphorylation of eIF4G are unknown but it can be hypothesized that it serves to regulate its activity. Since eIF4G binds numerous proteins (eIF4E, eIF4A, PABP, MNK1, components of eIF3), phosphorylation could selectively modulate its affinity for any number of these proteins or their isoforms. Alternatively, the phosphorylation of eIF4G could regulate its RNA-binding activity. Determination of the effects of eIF4G phosphorylation will best be dissected using *in vitro* biochemical assays. However, the analysis of *Drosophila* eIF4G phosphorylation mutants can help elucidate the biological consequences of altering these activities, much in the way that *eIF4E* phosphorylation mutants were examined here (Chapter 4).

5.8 Synopsis

During the course of this project, many genes encoding the *Drosophila* homologues of the eIF4 proteins were identified either by screens in various laboratories or upon the completion of the *Drosophila* genome project (Adams *et al.*, 2000; Lasko, 2000). The ongoing objective of the laboratory is to examine the regulation of these factors during the development of a genetically tractable multicellular organism. This body of work contributes to the survey of the *Drosophila* eIF4 proteins by providing

new insights on the regulation of *Drosophila eIF4E* and its contribution to the control of cellular growth. Work conducted elsewhere during the course of this project has also shown the involvement of *eIF4A* in growth control (Galloni and Edgar, 1999). Mutants in genes encoding the other eIF4 proteins, and their multiple isoforms, remain to be isolated so that their contribution to the development of *Drosophila* can be established. *Drosophila* will also prove to be a good model organism to determine the biological functions of the homologues of proteins such as PAIP-1 (Craig *et al.*, 1998) and p97 (Imataka *et al.*, 1997; Levy-Strumpf *et al.*, 1997; Yamanaka *et al.*, 1997), which were recently identified as regulators of eIF4F activity in mammals. Furthermore, the growth deficiency phenotypes described in this thesis may serve in genetic screens to identify new regulators of protein synthesis and growth. Thus, based on the studies performed here or by the numerous other sources discussed in this thesis, *Drosophila* should prove to be an excellent genetic model to ask questions regarding the regulation of translation factors during the development of a multicellular organism.

Appendix A

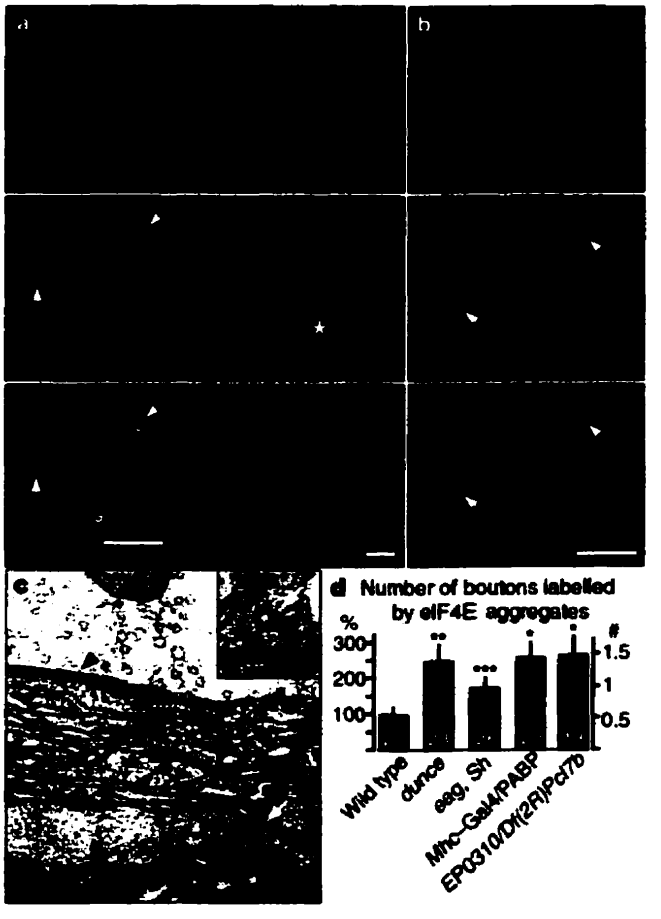
Postsynaptic Translation Controls Efficacy and Morphology of *Drosophila* Neuromuscular Junctions

Stephan J. Sigrist, Philippe R. Thiel, Dierk F. Reiff, Pascal E.D. Lachance, Paul Lasko and Christoph M. Schuster (2000) *Nature* 405: 1062-1065.

Recent evidence suggests that long-term synaptic plasticity is associated with structural rearrangements within the neuronal circuitry (Engert and Bonhoeffer, 1999; Maletic-Savatic *et al.*, 1999). While the molecular mechanisms governing such activity-controlled morphological alterations are largely elusive, polysomal accumulations at the base of developing dendritic spines (Steward and Falk, 1986) as well as the activity-induced synthesis of synaptic components suggest a role of localised translation during synaptic plasticity (Kiebler and DesGroseillers, 2000; Schuman, 1999). Here we show that large aggregates of translational components as well as mRNAs coding for the postsynaptic glutamate receptor subunit DGluR-IIA (Schuster *et al.*, 1991) are localised within subsynaptic compartments of larval neuromuscular junctions of *Drosophila melanogaster* (NMJs). Both, genetic models of junctional plasticity (Budnik *et al.*, 1990) and genetic manipulations using the translation initiation factors eIF4E (Sonenberg, 1996) and PABP (Gallie, 1998) showed an increased occurrence of subsynaptic translation aggregates. This was associated with a significant increase in the postsynaptic DGluR-IIA levels and a reduction of the junctional expression of the cell adhesion molecule Fasciclin II (FasII). In addition, the efficacy of junctional neurotransmission and the size of larval NMJs were significantly increased. Thus, our results provide evidence for a postsynaptic translational control of long-term junctional plasticity.

Translational control is primarily exerted by regulation of the initiation step of translation (Mathews *et al.*, 1996), which appears to be controlled by the rate-limiting initiation factor eIF4E (Sonenberg and Gingras, 1998). In addition, the interaction of the 5'-cap bound eIF4E with the 3'-end of mRNAs via a complex of further initiation factors and the poly(A)-binding protein (PABP)(Gallie, 1998) has been shown to synergistically facilitate translation initiation (Craig *et al.*, 1998). To assess the potential role of regulated translation during the development of the larval NMJs in *Drosophila*, we analysed the subcellular expression pattern of eIF4E and PABP in filet preparations of third instar larvae. On top of a weak and ubiquitous expression in the cytoplasm of all larval cells both antigens colocalised (inset in Fig. 1a) in up to 2µm large and strongly immunopositive aggregates (red panels in Fig. 1a, b) close to NMJs. The latter have been highlighted by the immunodetection of the junctionally expressed cell adhesion molecule FasII (Schuster *et al.*, 1996) (green panels in Fig. 1a, b). The specific localisation of eIF4E/PABP aggregates close to and partially overlapping with junctional profiles (arrows and arrowheads in Fig. 1a, b) revealed that eIF4E/PABP aggregates are positioned subsynaptically within or adjacent to the subsynaptic reticulum (SSR). We did not find evidence for presynaptic or axonal localisation of such aggregates. Therefore, the almost exclusive subsynaptic distribution of the eIF4E/PABP aggregates within larval muscles suggests a functional relationship between NMJs and the appearance of nearby eIF4E/PABP aggregates.

Fig. 1: Subsynaptic translation aggregates and their regulation. a, b Confocal images of third instar larval NMJs at bodywall muscles 4, which have been fluorescently labelled with antibodies against the indicated proteins. Lower panels combine the individual upper channels recorded from the same NMJ. **a** On top of a weak ubiquitous immunoreactivity surrounding nuclei (*) of all larval cells PABP forms strongly immunopositive aggregates of variable size which are located close to (arrow), or overlapping (arrowhead), with FasII-labelled junctional profiles. **insets** Double-labelling of a larval filet preparation with PABP- and eIF4E-specific antibodies revealed that both proteins colocalise in the same junctional aggregates (yellow colour in bottom panel). **b** eIF4E shows a similar expression profile as PABP (a) including the strongly immunopositive aggregates of variable size in close association to FasII-labelled junctional profiles. Scale bars: 5 μm. **c** Representative electron micrograph of an ultrathin section through a type Ib bouton of muscle 6/7. Arrowhead marks an electron-dense area (synapse) with multiple presynaptic vesicles (above) and the membranous network of the subsynaptic reticulum (SSR; below). Polysomes are localised within (arrows) and close to the SSR. **inset** higher magnification of a circular polysomal profile within the SSR. Scale bars: 200 nm. **d** Quantification of the average number of boutons per NMJ (muscle 6/7, abdominal segments 2-5), which were labelled by one or more eIF4E aggregates. The average number of boutons per NMJ labelled by subsynaptic translation aggregates (#) was significantly increased in animals, which represent both genetic gain-of-function (*Mhc-Gal4/PABP*) and loss-of-function (*EP0310/Df(2R)Pcl7b*) conditions of *pabp* (Student's t-test: *: $p << 0.0005$). A similar increase was observed in the mutants *eag Sh* and *dnc*, which both have been previously implicated in the control of activity dependent junctional plasticity at *Drosophila* NMJs (Budnik *et al.*, 1990; Schuster *et al.*, 1996) (t-test: **: $p < 0.001$; ***: $p < 0.005$). Data are plotted as the mean ± SDM; n within bars: number of analysed animals.


a
b
c
d Number of boutons labelled by eIF4E aggregates
%
300
200
100
#
1.5
1
0.5
Wild type
dunce
eag, Sh
Mhc-Gal4/PABP
EP0310/Df(2R)Pcl7b

Ultrastructural examinations of larval NMJs revealed polysomal accumulations within and close to the SSR (arrows in Fig. 1c). According to their variable size, subsynaptic location and their frequency of detection, these polysomal clusters are likely to represent the eIF4E/PABP aggregates detected by light microscopy (white arrows in Fig. 1c). In addition, smaller polysomal aggregates were found to be widely distributed in discrete membranous compartments throughout the SSR (black arrow in Fig. 1c), while presynaptic and axonal profiles were free of polysomes. We therefore conclude that mRNAs are translated within subsynaptic compartments of larval NMJs (see Fig. 2d) and that local centers of concentrated, subsynaptic translation are identified by large junctional eIF4E/PABP-aggregates.

To assess whether junctional translation is subject to regulation, we quantified the number of synaptic specialisations (boutons) per NMJ, which were labelled by one or more translation aggregates (Fig. 1d). Both, animals which overexpressed PABP in larval muscles and larvae which were mutant in *pabp*, showed a significantly increased occurrence of subsynaptic eIF4E/PABP aggregates (right two bars in Fig. 1d) on top of an unaltered muscular PABP staining level. In support of the latter finding, the total PABP levels in crude larval protein extracts were unaltered in all analysed genotypes, even when PABP mRNA levels were significantly increased or reduced under genetic gain-of-function or loss-of-function conditions, respectively (Fig. 2g). Such a homeostasis of total PABP levels is a well described phenomenon for PABP (Wu and Bag, 1998) and it could mask in crude protein extracts the significant local changes in PABP levels observable within subsynaptic compartments of NMJs (Fig. 1d). While the exact reason

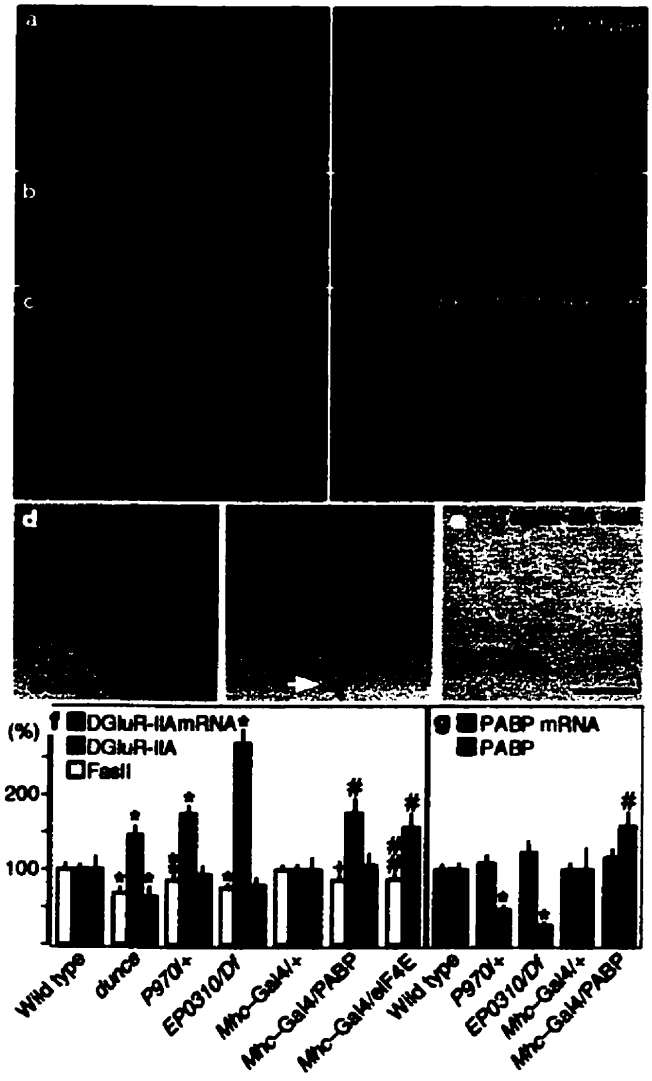
for this increase in eIF4E/PABP-aggregate occurrence remains to be investigated, it appears conceivable that a local perturbation of PABP levels (due to a previously described overshooting compensation of the PABP-homeostasis mechanism (Wu and Bag, 1998)) could cause a facilitation of subsynaptic translation-aggregate formation. Strikingly, a similar increase in the frequency of postsynaptic translation aggregates was also observed in two mutants, which represent well established genetic models of long-term synaptic plasticity in *Drosophila* (Balling *et al.*, 1987; Budnik *et al.*, 1990; Schuster *et al.*, 1996) (Fig. 1d), the hyperactive K^+-channel mutant *eag,Sh* and the cAMP-phosphodiesterase mutant *dunce*. Thus, increased neuronal activity levels (in *eag, Sh*) as well as elevated cellular cAMP levels (in *dunce*) are capable of inducing subsynaptic translation aggregate formation. These findings are consistent with the hypothesis that synaptic activity can control synaptic translation (Nayak *et al.*, 1998; Weiler *et al.*, 1997; Wu *et al.*, 1998).

To identify potential substrates and targets of subsynaptic translation at larval NMJs, we performed quantitative immunostainings of several synaptically expressed proteins, including the synaptic vesicle protein synaptotagmin, the junctional anti-HRP epitope, the cell adhesion molecule Fasciclin II (FasII), the postsynaptic glutamate receptor subunit DGluR-IIA, and the conventional myosin as a nonsynaptic protein. We did not detect obvious differences in the expression levels of myosin, synaptotagmin and the junctional anti-HRP-immunoreactivity in all analysed genotypes (data not shown). However, animals which showed elevated numbers of subsynaptic translation aggregates

(see Fig. 1d) consistently displayed increased junctional levels of DGluR-IIA (red panels in Fig. 2b, c) and an altered junctional distribution of FasII (green panels in Fig. 2b, c), which was associated with a reduction of synaptic FasII levels compared to control animals (white bars in Fig. 2f). A similar FasII phenotype has been previously observed in the above-mentioned plasticity models *eag, Sh* and *dunce* and it has been shown, that such a presynaptic FasII downregulation is essential for increased junctional outgrowth (Schuster *et al.*, 1996). Intriguingly, in *Aplysia* the FasII homologue apCAM has been observed to be presynaptically downregulated following treatments, which increase synaptic efficacy and growth of new synaptic connections (Mayford *et al.*, 1992). This synaptic apCAM regulation is thought to be achieved via a protein-synthesis dependent activation of an endocytic apCAM internalisation (Bailey *et al.*, 1992). These observations might suggest that subsynaptic protein synthesis affects junctional FasII levels through similar mechanisms as seen in *Aplysia.*

The postsynaptic DGluR-IIA immunoreactivities were found to be significantly stronger in translationally sensitised animals (red channels in Fig. 2b, c and hatched bars in Fig. 2f). This strong synaptic expression increase of DGluR-IIA was not due to a transcriptional upregulation of *dglur-IIA*, since the total amounts of DGluR-IIA mRNAs were unaltered or even reduced in the analysed genotypes compared to controls (black bars in Fig. 2f). In situ hybridisation experiments revealed, that DGluR-IIA mRNA surrounds individual type-I boutons with prominent labelling of terminal and branchpoint boutons (large arrows in Fig. 2d) and weak or absent staining within the SSR of inter-bouton connectives (small arrows in Fig. 2d). Thus, the subsynaptically localised

Fig. 2: Increased DGluR-IIA and decreased FasII immunoreactivities upon genetic facilitation of subsynaptic translation. a-c Quantitative confocal images of third instar larval NMJs at bodywall muscles 4, which have been double-labelled with antibodies recognising FasII (green channel) and the postsynaptic glutamate receptor subunit DGluR-IIA (red channel). Synaptic DGluR-IIA immunoreactivity is significantly increased in the cAMP-phosphodiesterase mutant dnc^{M14} (b) and the *pabp* mutant combination *EP0310/Df(2R)Pcl7B* (c). Note that the extrasynaptic unspecific staining is unaltered in all red channel images. Junctional FasII expression is significantly reduced in both mutants compared to wildtype animals. **d** mRNAs coding for DGluR-IIA (Schuster *et al.*, 1991) were detected by *in situ* hybridisation within the cytoplasm of all larval bodywall muscles and in a characteristic pattern within subsynaptic compartments of NMJs (black arrows, see text). Nerve profiles were free of staining (white arrow). **e** DGluR-IIA specific *in situ* signals were not detectable in the transcriptional null mutant $dgluR\text{-}IIA^{g9}$ */Df(2L)clh4*. Scale bars: 10 µm. **f** Quantification of the junctional expression levels of FasII (open bars) and DGluR-IIA (hatched bars) after normalisation to the invariant junctional anti-HRP immunoreactivity. Black bars represent the relative DGluR-IIA mRNA content of the indicated genotypes, as determined by quantitative RT-PCR. The junctional DGluR-IIA immunoreactivities were significantly increased and FasII levels were significantly decreased in animals of the indicated genotypes (Student's t-test as compared to wt: *: $p << 0.0001$, **: $p < 0.03$; compared to Mhc-Gal4/+: #: $p < 0.0005$, ##: $p < 0.005$, +: $p < 0.02$). **g** Quantification of PABP mRNA and protein levels in *pabp* mutants and PABP overexpressing transgenic lines. Quantitative RT-PCR revealed significantly reduced *pabp* mRNA levels in total RNA extracts of *pabp* mutants and increased mRNA levels in overexpressing transgenic animals (filled bars; Student's t-test: compared to wt: *: $p << 0.0001$; compared to Mhc-Gal4/+: #: $p < 0.007$). The PABP protein content of crude larval extracts was unaltered in all analysed genotypes compared to control (hatched bars) revealing a strong homeostasis of general PABP levels. All data are plotted as the mean ± SEM.

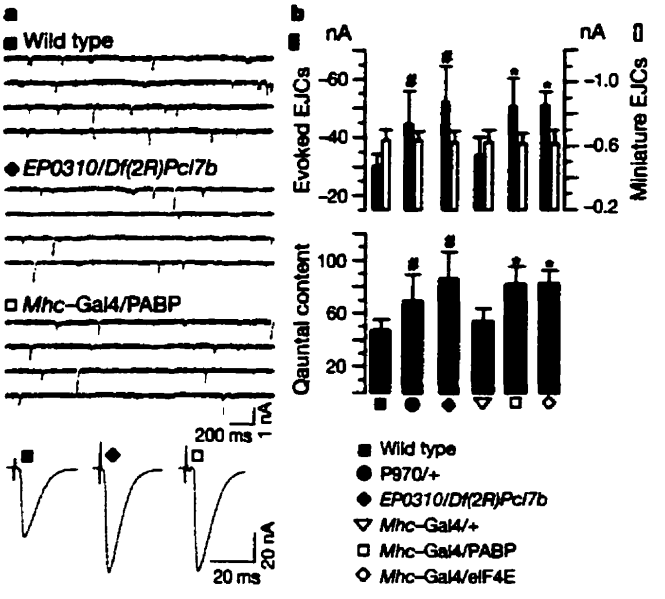

a
b
c
d
e
f
DGluR-IIAmRNA*
DGluR-IIA
FasII
(%)
200
100
g
PABP mRNA
PABP
Wild type
dunce
P970/+
EP0310/Df
Mhc-GalA/+
Mhc-GalA/PABP
Mhc-GalA/eIF4E
Wild type
P970/+
EP0310/Df
Mhc-GalA/+
Mhc-GalA/PABP

DGluR-IIA mRNA represents a direct substrate for the junctional translation machinery. While these results can not exclude an extrajunctional contribution to the observed synaptic DGluR-IIA increase, they strongly suggest that this observation is due to an increased subsynaptic synthesis of DGluR-IIA in genotypes with a higher occurrence of junctional eIF4E/PABP aggregates.

To analyse the functional consequences of modified translational sensitivity we assessed the strength of neurotransmission at NMJs on muscle 6 of third instar larvae (Fig. 3). The average miniature excitatory junctional current amplitudes (mEJCs) and thus the quantal sizes were indistinguishable among all analysed genotypes (open bars in Fig. 3b). This finding suggests that the additional receptor subunits that are synaptically localised (red panels in Fig. 2a-c) are either functionally silent (e.g. through physiological silencing (Davis *et al.*, 1998) or intracellular localisation (Shi *et al.*, 1999)) or that the amount of glutamate released from an individual quantum is not sufficient to saturate the postsynaptic receptors (Liu *et al.*, 1999). In contrast to the mEJC-recordings, postsynaptic responses evoked by suprathreshold stimulation of motor nerve axons (bottom traces in Fig. 3a) were substantially larger in all mutants exhibiting increased levels of subsynaptic translation (filled bars in top panel of Fig. 3b). Thus, the derived quantal content was significantly increased over controls (Fig. 3b), suggesting that the observed larger amplitudes of evoked junctional responses arise from an increased number of released presynaptic vesicles per action potential.

To investigate whether the increase in junctional efficacy was due to a change in the number of synaptic specialisations, we quantified the number of junctional boutons

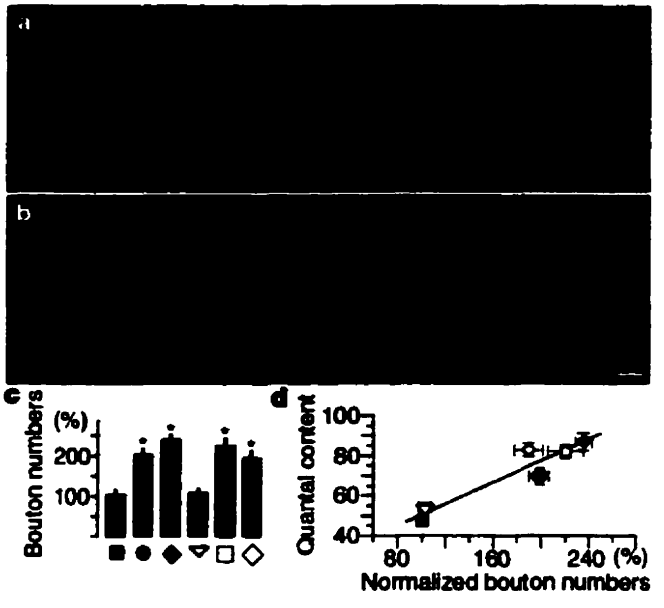
Fig. 3: Sensitised initiation of translation in larval muscles increases junctional efficacy. **a** Representative traces of miniature postsynaptic current recordings (mEJCs, upper panels) and average-traces of 10 consecutively recorded evoked EJCs (bottom panel) of the indicated genotypes. **b** The mean amplitudes of the mEJCs are indistinguishable among all analysed genotypes (open bars). eEJCs are significantly increased in *pabp*-mutants and in animals overexpressing PABP or eIF4E in muscles compared to wt (Student's t-test: #: $p << 0.0001$) or control (*: $p << 0.0001$). The derived quantal content and thus the junctional efficacy shows similarly significant increases in the analysed genotypes compared to controls. n: number of individual cells and animals from which eEJCs and mEJCs were recorded. All data are plotted as the mean ± SDM.


Wild type
EP0310/Df(2R)Pcl7b
Mhc-Gal4/PABP
200 ms
1 nA
20 ms
20 nA
nA
Evoked EJCs
-60
-40
-20
nA
Miniature EJCs
-1.0
-0.6
-0.2
Qauntal content
100
60
20
Wild type
P970/+
EP0310/Df(2R)Pcl7b
Mhc-Gal4/+
Mhc-Gal4/PABP
Mhc-Gal4/eIF4E

per NMJ (Fig. 4). Genotypes, which displayed an increased occurrence of subsynaptic translation aggregates, had significantly larger NMJs (Fig. 4c) and reduced junctional FasII levels (Fig. 4b, 2f). In addition, the junctional sizes of the analysed animals correlated in a highly significant manner with their estimated quantal contents (Fig. 4d), suggesting that junctional efficacy and the morphological elaboration of NMJs were tightly coupled. Based on our light microscopic examinations of DGluR-IIA labelled NMJs, the density of synapses within NMJs of all mutant animals appeared similar to controls or even higher, indicating that the total number of synapses increased proportionally with the junctional size. This finding supports the idea that the increased quantal content in animals with facilitated subsynaptic translation may be based on an increase in the number of vesicle release sites per given stimulus.

In summary, we have shown that translational machinery and mRNAs are associated with the subsynaptic reticulum of NMJs and that genetic manipulations, which affect the occurrence of subsynaptic translation aggregates are accompanied by changes in the levels of the synaptic proteins DGluR-IIA and FasII. These same manipulations also affected the function and morphology of NMJs. Thus, our results demonstrate that subsynaptic translation can instruct junctional growth and synaptic reorganisation. They further suggest that subsynaptic translation can be regulated by altered levels of neuronal activity, indicating that the regulation of postsynaptic translation participates in activity-dependent junctional plasticity. The involvement of localised protein synthesis in a synapse specific stabilisation of long-term facilitation has been recently suggested in in-vitro and in-vivo preparations of *Aplysia* neurons (Casadio

Fig. 4: Subsynaptic translation instructs morphological and functional growth of NMJs. a, b Confocal images of third instar larval NMJs innervating bodywall muscles 6/7, which have been fluorescently labelled with an antibody recognising FasII. The junctional size and complexity is increased in animals, which overexpress PABP in larval muscles (b) compared to wildtype (a). Scale bar: 10 µm. **c** Genetic manipulations, which increase the occurrence of subsynaptic translation aggregates, either through a series of *pabp* alleles (●: $pabp^{P970}$/+ [n=50], ◆: $pabp^{EP0310}$/*Df(2R)Pcl7b* [n=37]) or through the targeted overexpression of PABP or eIF4E in larval muscles (□: Mhc-Gal4/PABP [n=20], ○: Mhc-Gal4/eIF4E [n=10]), result in significant size increases of larval NMJs compared to controls (■: wildtype [n=36], Δ: Mhc-Gal4/+ [n=27]; Student's t-test: *: $p << 0.0001$). Normalised junctional sizes were obtained by quantifying the number of boutons and relating them to the measured muscle surface area of the innervated muscle (Schuster *et al.*, 1996) (average of all scored muscles sizes in all genotypes: 47 ± 8.5 square scale units, n = 333). Data are plotted as the means ± SEM. **d** The quantal content of the analysed genotypes is plotted as a function of junctional size. A linear regression analysis of the data reveals a highly significant correlation between junctional size and junctional efficacy ($R = 0.955$; $p < 0.001$).


a
b
c
Bouton numbers
(%)
200
100
d
Quantal content
100
80
60
40
80
160
240 (%)
Normalized bouton numbers

et al., 1999; Sherff and Carew, 1999). In addition, ultrastructural examinations of vertebrate brains have provided evidence for polysomal aggregates at the bases of developing spines (Steward and Falk, 1986). Furthermore, the activity dependent synthesis of several molecules, including AMPA-type glutamate receptor subunits, has recently been reported (Nayak *et al.*, 1998; Weiler *et al.*, 1997; Wu *et al.*, 1998). Thus, postsynaptic translational control of synaptic plasticity appears to represent a common principle of long-term alterations of neuronal function and connectivity.

Methods

Molecular Genetics. *Df(2R)Pcl7B* removes the PABP coding region and 3' UTR. $pabp^{k10109}$ (P970) is a P-element insertion into the exon preceding the first coding exon of *pabp*. *P970/Df(2L)Pcl7B* is lethal, a precise excision of *P970* restored viability and reverted the morphological phenotypes of *P970* /+ close to wildtype, while an imprecise excision of *P970*, which removed the transcription start site of *pabp*, phenocopied *P970*. The P-element EP0310 is inserted into the first 3'-non-coding exon of *pabp*. We also generated an UAS-*pabp* transgene containing the PABP coding region without 5'- and 3'-UTRs (to avoid PABP autoregulation) inserted into pUAST for Gal4-controlled expression. The UAS-*eIF4E* transgenic flies were generously provided by Dr. R. Rivera-Pomar. The *Mhc*-Gal4 and *elav*-Gal4 lines allowed the expression of UAS transgenes in all muscles or all neurons, respectively. Mutations in the *dglur-IIA* locus ($dglur\text{-}IIA^{g9}$ and *Df(2L)clh4*) have been previously described (Petersen *et al.*, 1997). To control for the

junctional effects of generally reduced translation we similarly examined four *Minute*-mutants (M(2)58F, M(2)36F, M(2)53-1, M(2)24F-1), which are defective in constitutive components of ribosomal subunits (Berkeley *Drosophila* Genome Project). These *Minutes* caused a significant developmental delay and somewhat smaller larval body sizes, but no obvious molecular or morphological junctional phenotypes.

Quantitative RT-PCR. Total RNA was extracted from 20 to 70 early to mid third instar larvae. Two independent RNA preparations per genotype were transcribed and each cDNA was subjected to multiplex PCR (Steinbach and Rupp, 1999) 6 to 15 times. As an internal standard we used oligonucleotides specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), whose invariant expression in all analysed genotypes was confirmed by comparison to the myosin heavy chain mRNA level. The amount of PABP- or DGluR-IIA specific PCR product was quantified and normalised to the GAPDH-specific PCR product.

Antibodies. The rabbit anti-PABP antiserum was raised against the peptide L_{535}-K_{552}, affinity purified and specificity controlled by peptide competition experiments and comparison to a previously characterised anti-PABP serum (Dr. R. Rivera-Pomar). The rabbit anti-eIF4E antiserum was raised against a bacterially expressed GST-eIF4E fusion protein. The affinity-purified antiserum detects the two isoforms of eIF4E (Lavoie *et al.*, 1996) on Western blots. The following antibodies were generous gifts of C.S. Goodman (FasII [1D4], myosin [FMM5]), J. Kidokoro (DGluR-IIA [DM2]) and T. Littleton

(synaptotagmin). The anti HRP-antibody recognises a neural epitope in insects (Gorczyca *et al.*, 1993) and was purchased from Sigma.

Immunofluorescence Quantification. All larvae used in this study were raised under normalised culture conditions (25^{o}C, 65% humidity, high animal density). Mid-third instar larvae of similar age and body size were processed for immunofluorescent detection as previously described (Schuster *et al.*, 1996). Junctional immunoreactivity levels of DGluR-IIA and FasII were quantified in triple-labelled larval preparations with the invariant anti-HRP immunoreactivity at NMJs as an internal staining standard. 5-9 type Ib boutons (muscle 6/7, abdominal segment 2) were selected in the anti-HRP channel of a recorded confocal image stack and the average fluorescence signal of this selection was determined for all three channels. The signal ratios DGluR-IIA/HRP and FasII/HRP of at least 2 non-overlapping areas per NMJ were accumulated from the indicated number of animals.

PABP-Protein Quantification. 20 male larvae of the indicated genotypes were homogenised, the crude protein extract equivalent of 2 animals was immunoblotted and probed with affinity purified anti-PABP serum and anti-tubulin antibody (Amersham). The anti-PABP immunoreactivity was quantified and normalised to the anti-tubulin reaction based on two to four independent extracts.

Junctional Size Quantification. Since none of the here used genotypes showed systematic alterations of larval muscle sizes (average of all scored muscles of all genotypes: 47 ± 8.5 square scale units, n = 333), we used the measured muscle surface area as a fine-scale staging criterion to normalise the bouton counts per muscle 6/7 NMJ (abdominal segment 2).

Electrophysiology. Third instar larvae were dissected and prepared for intracellular recordings as described (Schuster *et al.*, 1996). Miniature and evoked postsynaptic currents were recorded from muscle fiber 6 of abdominal segments 2 and 3 in TEVC mode (Axoclamp 2B, Axon Instruments). Stimulation: the cut end of the intersegmental nerve was placed into a suction electrode and suprathreshold positive current pulses were applied at 0.1 Hz. Recordings: muscle cells were impaled with two 15-30 MΩ microelectrodes filled with 2M KCl (the resistance of the current passing electrode was usually 5-10 MΩ lower than that of the voltage sensing electrode). Cells with a resting potential of less than -60 mV in HL3-solution (1 mM Ca^{2+}) were selected for further analysis. Clamp settling times in response to voltage steps from -60 to -70 mV were 300-600 µsec and voltage errors were up to 4 mV when eEJCs were close to 100nA. eEJCs (VC at -60 mV) were low-pass filtered at 2 kHz, mEJCs (VC at -70 mV) at 500Hz and subsequently digitised. 30-50 eEJCs and 90 sec of mEJCs recordings were used per cell for off-line analysis (pClamp6, Axon Instruments; Jaejin Software, Leonia). Estimates of the quantal content were derived from these data by dividing the mean eEJC through the mean mEJC of each analysed cell.

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