Functional analysis of seven genes encoding eight translation initiation factor 4E (eIF4E) isoforms in Drosophila

Greco Hernández\textsuperscript{\textsc{a},\textsc{\textstar}}, Michael Altmann\textsuperscript{b}, José Manuel Sierra\textsuperscript{c}, Henning Urlaub\textsuperscript{d}, Ruth Diez del Corral\textsuperscript{e}, Peter Schwartz\textsuperscript{f}, Rolando Rivera-Pomar\textsuperscript{a,\textsuperscript{1}}

\textsuperscript{a}Max-Planck-Institut für biophysikalische Chemie, Abt. Molekulare Biologie, Am Fassberg 11, Göttingen 37077, Germany
\textsuperscript{b}Institut für Biochemie und Molekularbiologie, Universität Bern, Bühlstrasse 28, 3012 Bern, Switzerland
\textsuperscript{c}Centro de Biología Molecular Severo Ochoa, Universidad Autónoma de Madrid, Cantoblanco, 28049 Madrid, Spain
\textsuperscript{d}Max-Planck-Institut für biophysikalische Chemie, Abt. Zelluläre Biochemie, Am Fassberg 11, 37077 Göttingen, Germany
\textsuperscript{e}School of Life Sciences, University of Dundee. Wellcome Trust Biocenter, Dundee. DD1 5EH, Scotland, UK
\textsuperscript{f}Zentrum Anatomie, Abt. Anatomie-Embryologie, Georg-August-Universität Göttingen, Kreuzbergering 36, 37075 Göttingen, Germany

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Abstract

The Drosophila genome-sequencing project has revealed a total of seven genes encoding eight eukaryotic initiation factor 4E (eIF4E) isoforms. Four of them (eIF4E-1, eIF4E-3, eIF4E-4 and eIF4E-5) share exon/intron structure in their carboxy-terminal part and form a cluster in the genome. All eIF4E isoforms bind to the cap (m\textsuperscript{7}GpppN) structure. All of them, except eIF4E-6 and eIF4E-8 were able to interact with Drosophila eIF4G or eIF4E-binding protein (4E-BP). eIF4E-1, eIF4E-2, eIF4E-3, eIF4E-4 and eIF4E-7 rescued a yeast eIF4E-deficient mutant in vivo. Only eIF4E-1 mRNAs and, at a significantly lower level, eIF4E3 and eIF4E-8 are expressed in embryos and throughout the life cycle of the fly. The transcripts of the remaining isoforms were detected from the third instar larvae onwards. This indicates the cap-binding activity relies mostly on eIF4E-1 during embryogenesis. This agrees with the proteomic analysis of the eIF4F complex purified from embryos and with the rescue of l(3)67Af, an embryonic lethal mutant for the eIF4E-1,2 gene, by transgenic expression of eIF4E-1. Overexpression of eIF4E-1 in wild-type embryos and eye imaginal discs results in phenotypic defects in a dose-dependent manner.

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1. Introduction

Translation of eukaryotic mRNAs involves recognition of the cap structure m\textsuperscript{7}GpppN present at the 5′ end of mRNAs by eukaryotic initiation factor 4E (eIF4E). In association with eIF4G, eIF4E forms the eIF4F complex that allows binding of 40S ribosomal subunits probably through association with eIF3. Together with the helicase protein complex formed by eIF4A and eIF4B, eIF4F unwinds the secondary structure at the 5′ untranslated region (UTR) of most mRNAs. This event promotes the landing of 40S ribosomal subunits and the subsequent search of the initiator codon (Gingras et al., 1999). Due to its central role in cap-dependent translation, regulation of eIF4E activity is critical to normal cell growth. Overexpression of eIF4E in cell cycle-sensitized or proto-oncogenic cells results in overgrowth and malignant transformation. eIF4E expression is also significantly increased in many cancers (de Benedetti and Graff, 2004; Dua et al., 2001; Rosenwald, 2004; Sonenberg and Gingras, 1998). The recently discovered structural features of eIF4F shed light on the biological properties of this factor. The three-dimensional structure of yeast, mouse and human eIF4E in complex with different cap analogs has been resolved (Gross et al., 2003; Marcotrigiano et al., 1997;...
Matsuo et al., 1997; Tomoo et al., 2002, 2003). The amino-terminal part of eIF4E is unstructured, highly flexible and is completely separated from the functional moiety of the protein (Gross et al., 2003; Matsuo et al., 1997; Tomoo et al., 2002, 2003). In addition, the amino-terminal moiety is highly divergent across the phyla, is dispensable for cap recognition, for binding to eIF4G and 4E-BP, for stimulation of cap-dependent translation, and for in vivo functionality of the protein (Gross et al., 2003; Marcotrigiano et al., 1997; Robalino et al., 2004; Vasiliscu et al., 1996). Conversely, the sequence and three-dimensional structure of eIF4E carboxy-terminal part is highly conserved and contains all the residues important for its functionality. Its shape resembles a baseball glove in whose cavity the guanine ring of the cap is stacked by π–π interactions between the lateral chains of amino acids W56 and W102 (numbers refer to mouse eIF4E). E103 and W102 as well as the interaction between W166 and the methyl group of the cap structure further stabilize this interaction. Positive charges of R112, R157 and R162 interact with the negatively charged phosphate residues of the cap (Gross et al., 2003; Marcotrigiano et al., 1997; Matsuo et al., 1997; Tomoo et al., 2002, 2003). Additional contacts between the second nucleotide of m’GpppA and the carboxy-terminal part (residues 204–211) of human eIF4E have been recently identified (Tomoo et al., 2002, 2003). Moreover, eIF4E interacts with eIF4G and with the negative regulators eIF4E-binding proteins (4E-BPs) through several conserved residues present in its carboxy-terminal moiety, located in the convex dorsal surface of the protein (Gross et al., 2003; Marcotrigiano et al., 1999; Matsuo et al., 1997; Ptushkina et al., 1998). Phosphorylation of mammalian eIF4E S209 is very well documented to occur in response to extracellular signals (Flynn and Proud, 1995; Joshi et al., 1995; Pyronnet et al., 1999; Waskiewicz et al., 1999). Although the physiological importance of S209 in the activity of mammalian eIF4E has been recently challenged (McKendrick et al., 2001; Scherer et al., 2002), the phosphorylation of an equivalent to S209 in Drosophila eIF4E-1 (S251) has been proven to be critical for growth (Lachance et al., 2002).

The genome-wide sequencing projects have started to reveal the presence of several genes encoding eIF4E in different organisms, but the physiological relevance of this is still not known. Three different eIF4E proteins have been studied in mammals, eIF4E-1 (Rychlik et al., 1987), 4EHP (Rom et al., 1998) and eIF4E-3 (Joshi et al., 2004), and many others genes can be identified from the human raw sequence (A. Andrei and R. Rivera-Pomar, unpublished): three isoforms are known in plants termed eIF4E, eIFiso4E (Browning, 1996) and novel cap-binding protein (nCBP) (Ruud et al., 1998); at least five isoforms of eIF4E exist in C. elegans (Keiper et al., 2000), two in Zebrafish (Fahrenkrug et al., 1999; Robalino et al., 2004), two in Xenopus (Wakiyama et al., 2001), and two in S. pombe (Ptushkina et al., 1996, 2001). In S. cerevisiae only a single essential gene encoding for eIF4E has been identified (Altmann et al., 1987).

In Drosophila, the translational control of maternal genes plays a key role during embryogenesis (Johnstone and Lasko, 2001). On the other hand, eIF4E is an important target of regulatory factors such as BICOID to regulate the cap-dependent translation of caudal mRNA (Niessing et al., 1999, 2002), and CUP for the regulation of oskar mRNA translation (Nakamura et al., 2004; Wilhelm et al., 2004). In order to understand the role of the different eIF4E isoforms in Drosophila embryo, we studied the expression and biochemical properties of all eIF4E genes from Drosophila. We found that Drosophila possesses seven genes encoding eight eIF4E polypeptides, and we established by genetic and biochemical means that during embryogenesis eIF4E-1 plays an essential role and that the cap-binding activity relies only on this isoform.

2. Results

2.1. Seven genes encode eight eIF4E isoforms in Drosophila

Early studies performed with embryos characterized the polypeptide and the cDNA encoding for Drosophila eIF4E-1 (Hernández and Sierra, 1995; Maroto and Sierra, 1989). It was later reported that a single gene, eIF4E-1,2, encodes for two isoforms, eIF4E-1 and eIF4E-2, as a result of alternative splicing (see Fig. 2) (Hernández et al., 1997; Lavoie et al., 1996). The completion of the sequencing project of Drosophila genome (Adams et al., 2000) led to the annotation of five new eIF4E genes (Lasko, 2000) and a seventh one was annotated by the Ensembl Project (Sanger Institute) (Birney, 2004), encoding a total of eight eIF4E isoforms. An important discrepancy, however, was noticed for the cDNA of CG32859 gene (encoding for eIF4E-7) reported by the above sources. Although the predicted sequence of six detected Drosophila eIF4E cDNAs coincided in both analyses, there has been no experimental validation for the sequences and for the functionality of the encoded polypeptides. We cloned the eight Drosophila eIF4E cDNAs from ESTs or from a cDNA library and sequenced both strands, confirming the sequences previously reported by the Ensemble Project (Birney, 2004). The annotated eIF4E genes, their chromosomal location, and the predicted length and molecular mass of the polypeptides are listed in Table 1. An alignment of the Drosophila polypeptides together with the sequence of mouse (Jaramillo et al., 1991) and yeast eIF4E (Altmann et al., 1987) is shown in Fig. 1a. All Drosophila eIF4E5s, in particular eIF4E-7, have long amino-terminal regions (from 31 to 249 amino acids) as compared to eIF4E from other species. This makes eIF4E-7 the largest eIF4E so far known in any organism. The amino-terminal region is highly divergent in all the proteins compared, as well as in eIF4Es from other species. In contrast, all polypeptides share high
sequence similarity in their carboxy-terminal moiety (Fig. 1a,b). *Drosophila* eIF4E isoforms, except for eIF4E-8, contain the residues assumed to be involved in the recognition of the cap structure (Fig. 1a, depicted with lowercase s, r, p and m). However, the stretch of residues identified in human eIF4E to interact with the second nucleotide of m7GpppA (Fig. 1a, black horizontal line), that also contains the phosphorylatable serine (Fig. 1a, second asterisk) is partially conserved in *Drosophila* eIF4Es, and is totally absent in eIF4E-6. *Drosophila* eIF4E-3 also lacks the phosphorylatable serine. The lysine residue involved in the formation of a salt bridge with this serine is also absent in eIF4E-8 (Fig. 1a, first asterisk). Moreover, eIF4E-6 and eIF4E-8 show non-conservative substitutions in some of the residues proven to interact with eIF4G in other organisms (Fig. 1a, dots). The above sequence features found in *Drosophila* eIF4E-6 and eIF4E-8 suggest that these proteins might have an altered function. The identity values for the carboxy-terminal moiety of *Drosophila* eIF4E isoforms are shown in Fig. 1b. Interestingly, *Drosophila* eIF4E-8 shares more identity with human 4EHP (48%) and *C. elegans* IFE-4 (44%) than with other eIF4E isoforms (approximately 30%). In these three proteins together with Arabidopsis nCBP, a tryptophan residue (corresponding to WS6 of mouse eIF4E) involved in cap recognition is substituted by a tyrosine.

When the intron/exon organization of the eight *Drosophila* eIF4E genes was analyzed, it was noticed that genes eIF4E-1,2, eIF4E-3, eIF4E-4 and eIF4E-5, which are clustered on region 65C–67A5 of chromosome 3L (Adams et al., 2000; Hernández et al., 1997; Lavoie et al., 1996) (Table 1), have two conserved exons of 100 and 88 bp or a fusion of them in the case of eIF4E-5 (Fig. 2). These exons encode the highly conserved carboxy-terminal moiety of the proteins. The position of the introns A, B and C (Fig. 2) interrupting these exons is also conserved. Thus, this cluster of genes may have originated by gene duplications from a common ancestor. Interestingly, introns B and D are conserved also in zebrafish eIF4E-1B (B. Joshi, personal communication), pepper, *A. thaliana*, rice, *S. pombe* and human eIF4E-1 genes (Ruffel et al., 2004). By contrast, eIF4E-6, eIF4E-7 and eIF4E-8 do not share any common exon/intron structure with the rest of eIF4E genes and they are scattered along the *Drosophila* genome (Fig. 2 and Table 1).

### 2.2. Functional properties of *Drosophila* eIF4Es

We tested the ability of the proteins synthesized in vitro to bind to the cap structure by affinity chromatography on m7GTP-sepharose (Fig. 3a). All *Drosophila* eIF4E isoforms were able to bind to the cap. The translation of eIF4E-7 gave rise to several bands, probably due to the use of different methionines to initiate translation in our in vitro assay. All of them bound to the cap, in particular one at 37 kDa. The apparent lower cap-binding capacity of eIF4E-6 and eIF4E-8 might be explained because eIF4E-8 possesses three conservative (Y68, E102 and K164) and two non-conservative (Q124 and S169) substitutions in residues involved in cap recognition, while eIF4E-6 possesses a truncated carboxy-terminal part (Fig. 1a). Molecular modeling indicated that the cloud of positive charges formed by R157, K15 and K162 (for mouse eIF4E) surrounding the three phosphates of the cap is lost in *Drosophila* eIF4E-8, which only possesses one positive residue (K164) (not shown).

The ability of eIF4E isoforms to bind *Drosophila* eIF4G (Hernández et al., 1998) and 4E-BP (Bernal and Kimbrell, 2000) was then investigated by using the yeast two-hybrid system (Bartel et al., 1993) (Fig. 3b). A strong interaction of eIF4G with eIF4E-1, eIF4E-2 and eIF4E-4 was observed, while an apparently weaker interaction of eIF4G with eIF4E-3, eIF4E-5 and eIF4E-7 was detected. We could not detect any interaction between eIF4G and eIF4E-6 or eIF4E-8. The same pattern of interactions was obtained with 4E-BP (Fig. 3b). The lack of interaction of eIF4E-6 and eIF4E-8 is in agreement with the existence of non-conservative substitutions in some of the residues shown to interact with eIF4G in other organisms (Fig. 1a, dots). Molecular modeling showed that P38 of mouse eIF4E which by hydrophobic interactions docks a pocket in the 4E-binding site of eIF4G is replaced by an arginine in *Drosophila* eIF4E-6 and eIF4E-8, losing in this way such contacts (not shown).

For a functional approach, we analyzed whether *Drosophila* eIF4E isoforms either alone or in combination with *Drosophila* eIF4G are able to complement the lack of eIF4E in a CDC33-knockout yeast strain (Altmann et al., 1989a). *Drosophila* eIF4E-1, eIF4E-3 and eIF4E-7 with the highest efficiency, and eIF4E-2 and eIF4E-4 with lower but significant efficiency, rescued the growth of the yeast mutant. In contrast, eIF4E-5, eIF4E-6, and eIF4E-8 were not able to support growth of the yeast mutant (Fig. 3c, upper panel). The failure of eIF4E-5, eIF4E-6, and eIF4E-8 to complement the yeast eIF4E mutant could be due to their inability to interact with yeast eIF4G (TIF4631) as shown by two-hybrid system experiments (Fig. 3c, lower panel). It is possible that these *Drosophila* proteins are not able to

### Table 1

<table>
<thead>
<tr>
<th>Gene</th>
<th>Chromosome localization</th>
<th>Isoform encoded</th>
<th>Length (aa)</th>
<th>Predicted M. W. (Da)</th>
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<td>eIF4E-8</td>
<td>233</td>
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* a Gene represented in genome annotation by CG4035, and previously located in the 67A8 or 67A2-B1 region by Lavoie et al. (1996) or Hernández et al. (1997), respectively.
(a)
form a functional Dm-eIF4E/y-eIF4G complex in vivo. In an attempt to overcome this problem, we performed cotransfections of the yeast mutants to simultaneously express *Drosophila* eIF4E isoforms and eIF4G. The expression of *Drosophila* eIF4G did not modify the results obtained using endogenous yeast eIF4GI (not shown). Finally, we transformed the yeast mutant strain CBY19, which carries a double knockout of eIF4G1 and eIF4GI1 genes (Berset et al., 2003) with a plasmid carrying *Drosophila* eIF4G. Neither *Drosophila* eIF4G alone nor in conjunction with any of the *Drosophila* eIF4Es complemented the lack of eIF4G in yeast, suggesting that the *Drosophila* eIF4E and eIF4G proteins fail to produce a functional complex for the yeast translational machinery. Altogether, the above results allow us to conclude that, at least, five *Drosophila* eIF4Es isoforms, namely eIF4E-1, eIF4E-2, eIF4E-3, eIF4E-4 and eIF4E-7 are functional in vivo.

2.3. Developmental expression of *Drosophila* eIF4E genes

We studied the expression of *eIF4E* genes throughout *Drosophila* development by Northern blot experiments using isoform-specific probes and total RNA. In contrast to eIF4E-1 and eIF4E-2 mRNAs, that were detected throughout the life cycle of *Drosophila* (Hernández and Sierra, 1995), single RNA transcripts of approximately 1.2, 1.2, 1.1 and 1.35 kb for eIF4E-3, eIF4E-4, eIF4E-5 and eIF4E-7, respectively, were found only from the third larva instar onward. No transcript of eIF4E-6 was detected by this method. Two mRNAs (1.15 and 1.2 kb approximately) of eIF4E-8 were detected at early embryonic stage (Fig. 4a). This analysis was validated by quantitative, real-time RT-PCR using total RNA derived from different life stages and specific oligonucleotide primers for each mRNA except eIF4E-2 mRNA (Fig. 4b). eIF4E-1 polypeptide is encoded by two mRNAs (Hernández et al., 1997; Lavoie et al., 1996) and we used primers that recognize the two transcripts together. The mRNAs of eIF4E-1 were detected during all the developmental stages studied and at a higher level than the RNAs encoded by the other genes, particularly in early embryo stage. eIF4E-8 transcript was also detected in all stages but at a much lower level. We found eIF4E-3 transcript at very low levels from late embryos that increased significantly from the third instar larvae. The transcripts of eIF4E-4, eIF4E-5 in particular, and of eIF4E-6 and eIF4E-7 at much lower levels, were detected from the third instar larvae onwards (Fig. 4b). In situ hybridization of eIF4E-3 and eIF4E-8 in embryos detected a very weak and ubiquitous signal in both cases (data not shown).

2.4. Cap-dependent translation relies on eIF4E-1 in embryos

The above results support the notion that only the transcripts of eIF4E-1, eIF4E-3 and eIF4E-8 are present in *Drosophila* embryos. To examine whether the corresponding proteins were present in an active eIF4F complex, we isolated such a complex from embryos 0–18 h-old by affinity chromatography on a m7GTP-Sepharose column (Zapata et al., 1994), analyzed it by SDS/PAGE and the protein bands were identified by MALDI-TOF mass spectroscopy. As already described (Zapata et al., 1994) *Drosophila* eIF4F consists of eIF4E-1 and eIF4G polypeptides, although traces of additional polypeptides, namely HSP70, PABP and a putative RNA-binding protein encoded by the gene CG2950 were also detected (Fig. 5a). When the amount of eIF4F analyzed was scaled up 30 fold, traces of additional polypeptides were detected and further identified by LC-MSMS (see Fig. 5b). However, only eIF4E-1 and a trace amount of eIF4E-8 were found. In *Drosophila* only one eIF4G protein has been characterized (Hernández et al., 1998; Zapata et al., 1994), which is present in the eIF4F complex from embryos (Fig. 5a,b). Lately another two putative eIF4G genes (CG10192 and CG3845) were identified in the *Drosophila* genome (Adams et al., 2000), but none of them was detected in the embryos eIF4F. The above results suggest that the bulk of cap-recognition activity in embryos relies on eIF4E-1.

To confirm this hypothesis we analyzed the lack of eIF4E-1 function in embryos in the context of the whole
organism. We used the six non-identified lethal alleles obtained by Leicht and Bonner (Leicht and Bonner, 1988) in the region 67A2-B1 of the third chromosome, where this gene was mapped (Hernández et al., 1997). One of these alleles, namely h(3)67Af, was later identified to be an embryonic null mutant of the eIF4E-1,2 gene (Hernández et al., 2004b; Lachance et al., 2002). We then investigated whether these mutants could be rescued by the expression of the transgene UAS-eIF4El under the control of the T80-Gal4 driver, which drives the expression of the transgene from stage 11 onwards in a weak but ubiquitous way (Hrdlicka et al., 2002). We performed the phenotypic rescue experiments of the mutants in transheterozygosis with the deficiency Df(3L)29A6(66F3; 67B1), ri' / p' (Leicht and Bonner, 1988) in order to avoid possible lethality due to the homozygosis of other mutations in essential genes in each mutant not detected previously. In this way, the lethality by homozygosis is in each case unambiguously attributed to the mutant analyzed. The rescue was determined as the number of individuals that reached pupae or adulthood. As shown in Table 2, the expression of the transgenic UAS-eIF4El has the ability to rescue the h(3)67Af mutant until adulthood. None of the other five alleles in this region were rescued, meaning a specific complementation of the eIF4E function by the UAS-eIF4El activity. These results support an essential role for eIF4E-1 in embryos that is not redundant with the activity of other eIF4E genes found in Drosophila, in particular with genes whose expression was detected during embryonic stages, namely eIF4E-3 and eIF4-8.

2.5. Overexpression of eIF4E-1 causes phenotypic defect in a dose-dependent manner

We also studied the effect of overexpression of eIF4E-1 in embryos using the transgenic flies UAS-eIF4El and different Gal4 drivers. No phenotypic effect was obtained with the T80-Gal4, which drives the expression of the transgene from stage 11 onwards (Hrdlicka et al., 2002). We then used the NGT40-Gal4 (Li and Gergen, 1999) to overexpress UAS-eIF4El from the moment of oocyte fecundation. When we allowed the first 8 h of development to occur at 17 °C, and then either switched the embryos to 29 °C or permitted to continue at 17 °C (control), no mortality was detected and adults had no phenotype defects. When the embryos where allowed to grow throughout at 29 °C to overexpress the UAS-eIF4El transgene from the beginning of development, we obtained 20% embryo mortality. Cuticle preparation of first instar larva NGT40-Gal4/UAS-eIF4El showed defects in segmentation (Fig. 6a). A more dramatic effect was observed when UAS-eIF4El was overexpressed early in development by using the en-Gal4 driver. The growth of the flies at 17 °C during the complete life cycle (control) produced normal adults. When the eggs were allowed to grow at 25 or 29 °C from the moment of fertilization, all animals died in early syncitial blastoderm stage. When the eggs en-Gal4/UAS-eIF4El were grown for the first 72 h at 17 °C and then switched to 25 or 29 °C, all eggs reached adulthood with no phenotypic defects. As shown in Fig. 4b, the highest amount of eIF4E-1 mRNA was found in 0–3 h-old embryos. We then performed Western blot experiments to analyze the relative amounts of eIF4E-1 protein throughout the embryogenesis of Drosophila. In agreement with mRNA amounts observed in Fig. 4b, the highest abundance of eIF4E-1 protein is detected in embryos 0–3 h-old and decreases as embryogenesis proceeds (Fig. 6b). A likely explanation for the observed phenotype upon overexpression of eIF4E-1 only in early embryonic stages with the en-Gal4 is that there is a threshold in the amount of eIF4E-1 to be surpassed in order to produce phenotypic effects. We then analyzed the overexpression of eIF4E-1 in a later stage of development. By using GMR-Gal4 (Freeman, 1996), sev-Gal4 (Reiter et al., 1996) and ey-Gal4 (Halder et al., 1995) drivers, we overexpressed the UAS-eIF4El
Fig. 3. Functionality of eIF4E genes. (a) Binding to cap of eIF4E isoforms. Autoradiography of \(^{35}\)S-labeled proteins subjected to cap-binding analysis by affinity chromatography on a m\(^7\)GTP-Sepharose column. Specific cap-bound proteins were eluted with buffer containing 100 \(\mu\)M m\(^7\)GTP and further resolved by SDS-PAGE (12.5% acrylamide), detected and quantified with the help of a phosphorimager. For each case, one-tenth of the analyzed input was loaded on the gel. Molecular mass markers are indicated on the left. (b) Interaction of Drosophila eIF4E isoforms with Drosophila eIF4G and 4E-BP observed when using the yeast two-hybrid. Bait eIF4E (4E) isoforms were fused to the Gal4-binding domain (BD). Prey eIF4G and 4EBP were fused to the Gal4-activator domain (AD). The relative strength of the interactions is indicated. (c, upper) Phenotypic rescue of a null yeast eIF4E mutant by Drosophila eIF4E isoforms. The S. cerevisiae strain T93C (Altmann et al., 1989b), a conditionally lethal allele of yeast CDC33 (eIF4E) gene, was transformed with DNA constructs expressing different Drosophila eIF4E isoforms and tested for complementation of endogenous eIF4E. Transformed yeast cells were streaked on YPGal (2% galactose)-agar plates and colony growth was analyzed at 30 °C. eIF4E-1, eIF4E-2, eIF4E-3, eIF4E-4 and eIF4E-7 supported growth of the mutant yeast. eIF4E-5, eIF4E-6 and eIF4E-8 did not support growth. Same results were obtained when the yeast mutant was cotransformed with eIF4E constructs in conjunction with Drosophila eIF4G (not shown). (c, lower) Interaction of Drosophila eIF4E isoforms fused to the Gal4-binding domain (BD) with yeast TIF4631 (eIF4GI) fused to the Gal4-activator domain (AD) as studied by the yeast two-hybrid system. The relative strength of the interactions is shown.
Expression of elf4E genes during Drosophila life cycle. (a) Northern blot detection of elf4E-3, elf4E-4, elf4E-5, elf4E-6, elf4E-7 and elf4E-8 mRNAs using isoform specific [32P]-labeled probes on total RNA derived from early embryos (0–3 h), embryos (0–18 h), first (1), second (2) and third (3) instar larvae, pupae (P) and adults (A). The blots for elf4E-6, elf4E-7 and elf4E-8 were exposed 5 times longer than the rest. (b) Relative levels of elf4E isoform mRNAs measured by quantitative real time RT-PCR of total RNA from the same stages as displayed in (a). Data represent the average of three independent experiments with a variance of 5%.

When the transgene was present in two copies, cell proliferation, abnormal growth, severe disturbance of the periodic pattern of ommatidia lattice and the presence of extra chaete were observed (Fig. 6c). These results support our hypothesis that a threshold of elf4E-1 level has to be surpassed in order to produce phenotypic effects in the fly.
As assessed by Western blot, at least four times more eIF4E-1 protein on eyes in transgenic flies is required to produce a phenotypic effect (Fig. 6d).

### 3. Discussion

#### 3.1. Functional diversity of Drosophila eIF4E isoforms

The ability of the eight Drosophila eIF4E isoforms to bind to cap, to eIF4G and to 4E-BP, and to support cell growth in a yeast mutant deficient for eIF4E, demonstrate that eIF4E-1, eIF4E-2, eIF4E-3, eIF4E-4 and eIF4E-7 support translation initiation and suggest that they may be functional equivalents.
Fig. 6. Effect of overexpression of eIF4E-1 in embryo and eyes. Cuticles of wild type and UAS-eIF4E1/NGT40-Gal4 first instar larvae. Thoracic (T1–T3) and abdominal (A1–A8) segments, as well as the antennal-maxillary complex (amx) and posterior spiracles (ps) are pointed. Fusions of segments are pointed by a triangle. (b) Analysis of eIF4E-1 in embryos 0–2, 0–12 and 0–18 h-old by Western blot. 5 μg of total protein extracts of embryos was loaded per lane, resolved by SDS-PAGE, blotted and analyzed with an anti-eIF4E-1 antibody (Maroto and Sierra, 1989). (c) Scanning electronic microscopy of adult eyes surfaces from wild type flies (400×), or flies overexpressing two copies of the UAS-eIF4E1 transgene on the developing eye under the Gal4 drivers Glass (GMR) (400×), sevenless (sev) (400×) or eyeless (ey) (500×). (d) Western blot to detect eIF4E-1 and eIF4A (control) in total extracts of five heads of white (1) or flies carrying two copies of transgenic eIF4E-1 (2).
in vivo. On contrary, eIF4E-6 and eIF4E-8 do recognize cap with lower efficiency than other eIF4Es, and did not interact with eIF4G nor were able to support cell growth in the yeast mutant. They are the unique isoforms carrying non-conservative amino acid substitutions (two in the case of eIF4E-6 and six in the case of eIF4E-8) at important residues for the interaction of eIF4E with eIF4G. In conclusion, eIF4E-6 and eIF4E-8 may be either negative regulators of translation or simply non-functional proteins. On the other hand, serine 251 of eIF4E-1 is critical for the function of eIF4E-1 in Drosophila (Lachance et al., 2002), but eIF4E-3 that lacks this serine is able to support cell growth in the yeast eIF4E-mutant. Since yeast eIF4E also lacks this serine we suppose there is no requirement for it in the function of eIF4E from this organism.

In spite of the existence of eight isoforms for eIF4E in Drosophila, here we have shown that in Drosophila embryos the cap-dependent translation relies mainly on eIF4E-1 and that the activity of this factor is essential throughout embryogenesis. This has important implications for development. During oogenesis, the repression of oskar mRNA in the posterior pole of the oocyte is essential for germ line formation and patterning (Johnstone and Lasko, 2001). During early embryogenesis, repression of caudal mRNA expression in the anterior part of the embryo is required for appropriate assembly of the head segments (Niessing et al., 1999). Both maternal mRNAs are regulated by the interaction of their repressors CUP and BICOID with eIF4E-1 (Nakamura et al., 2004; Niessing et al., 1999, 2002; Wilhelm et al., 2004). Here we showed that the likely isoform to be involved in translation of early mRNAs is eIF4E-1. The analysis of the Me31B complex from ovary extracts presented by Nakamura et al. (2004) showing that only eIF4E-1 is enriched in the oskar mRNA repression complex also supports the idea of a key role for eIF4E-1 in early embryogenesis.

3.2. Chimeric translational machineries

In spite of the evolutionary conservation of the translational machinery across the eukaryotic phyla, only some eIF4E isoforms can complement for the lack of the yeast eIF4E. They include human eIF4E-1 (Altmann et al., 1989a), A. thaliana eIF4E (Rodriguez et al., 1998), zebrafish eIF4E-1A (Robalino et al., 2004), and five Drosophila eIF4E isoforms (this study). In contrast, other initiation factors involved in mRNA recruitment and scanning do not allow for cross-complementation: neither mouse elf4A (Prat et al., 1990) nor Drosophila factors elf4A, elf4B (Hernández et al., 2004c) or elf4G (this study) substitute for their yeast counterparts. This suggests that the pathways regulating eIF4E activity may be universally more conserved than those for other factors. It will be worthwhile to test whether other initiation factors (e.g. elf3, elf2, etc.) are able to substitute for their homologs, and whether eIF4E is interchangeable between phyla other than yeast (e.g. between plants and mammals).

3.3. Is eIF4E a limiting factor during initiation of translation?

Increased eIF4F formation is closely related to enhanced protein synthesis and thus to cell growth. eIF4E is referred to as the limiting factor in the formation of eIF4F complex (Sonenberg and Gingras, 1998) because it is less abundant in some mammalian cells than eIF4G (Duncan et al., 1987; Hiremath et al., 1985). However, in most cases, overexpression of eIF4E in mammalian cells leads to malignant transformation and non-controlled cell proliferation only when overexpressed in cell cycle-sensitized cells or when co-expressed together with other proto-oncogenes (de Benedetti and Graff, 2004; Dua et al., 2001; Rosenwald, 2004; Sonenberg and Gingras, 1998). In S. cerevisiae, even 100 fold overexpression of eIF4E had only a minor effect on growth rates (Lang et al., 1994). In Xenopus (Wakiyama et al., 2001) and S. pombe (Hashemzadeh-Bonehi et al., 2003), overexpression of either of the two eIF4Es modestly increases translation in oocytes or had not affected cell growth, respectively. Here we observed that the overexpression of eIF4E-1 transgene produces phenotypic defects in early embryos (a time when endogenous eIF4E-1 is expressed most strongly) or when it was expressed in more than one copy in the developing eye. As we have recently demonstrated for Drosophila elf4B (Hernández et al., 2004c), here we provide in vivo evidence for phenotypic defects produced by changes in eIF4E levels in a non-oncogenic or cell cycle-sensitized genetic background, both in embryo and in the developing eye. These defects are produced in a dose-dependent manner. Altogether, these data suggest that in a wild type genetic background only a very high level of overexpression of eIF4E-1 might result in phenotypic effects. This would explain why no phenotypic defects were obtained by overexpression of Drosophila elf4E-1 in previous studies (Lachance et al., 2002; Zhang et al., 2000). It seems likely that those cells having an excess of eIF4E over eIF4G, like in rabbit reticulocyte lysates (Rau et al., 1996), yeast (von der Haar and McCarthy, 2002) and Drosophila embryos (Hernández and Rivera-Pomar, unpublished), are less sensitive to a further increase in the amount of free eIF4E. This implies that eIF4G, not eIF4E, is the limiting factor in the formation of eIF4F during the initiation of translation.

4. Experimental procedures

4.1. Identification and sequence analysis of eIF4E isoforms

Drosophila elf4E-1 sequence was taken from Hernández and Sierra (1995) and that for elf4E-2 from Hernández et al. (1997). Sequences for Drosophila annotated genes CG8023
(eIF4E-3), CG10124 (eIF4E-4), CG8277 (eIF4E-5), CG1442 (eIF4E-6), CG32859 (eIF4E-7) and CG33100 (eIF4E-8) were taken from Lasko (2000) and from the Ensembl Project (Birney, 2004), program version number 21.3a.1, from the Wellcome Trust Sanger Institute (Cambridge, UK; web site: http://www.ensembl.org/Drosophila_melanogaster). Sequences lineup were performed using the CLUSTAL W algorithm (Thompson et al., 1994) in the Megalign program of the DNA Star software package and optimized by eye. Sequence identity values were obtained using the programs BESTFIT and GAP, of the Genetics Computer Group Sequence Analysis Software, Wisconsin, USA.

4.2. Plasmids construction

The open reading frames (ORF) of all eight eIF4E were PCR-amplified using sequence-specific primers (Metabion). For eIF4E-1, eIF4E-3 and eIF4E-4, pBS-eIF4E-l (Hernández and Sierra, 1995) and ESTs LD034967 and GH01027 from the Berkeley Drosophila Genome Project were used as templates, respectively. eIF4E-2, eIF4E-5, eIF4E-6, eIF4E-7 and eIF4E-8 were amplified from a cDNA library constructed from pupae poly A+-RNA. After PCR amplification, cDNA fragments were cloned onto pCR2.1 vector (Invitrogen) and both strands fully sequenced, thus creating the constructs 4Es-pCR2.1. All Drosophila eIF4Es and Drosophila eIF4G (Hernández et al., 1998) ORFs were further subcloned onto the vectors p301-TRP1/GAL1 and p301-HIS3/GAL1, which allow for expression of cDNAs upon induction on galactose-containing media (Altmann et al., 1993), to create the respective plasmids 4Es-TRP1, 4Es-HIS3 or 4G-TRP1. Full-length eIF4E-1 cDNA (Hernández and Sierra, 1995) was subcloned onto the vector pUAST (Brand and Perrimon, 1993) to create the plasmid pUAS-4E1. Drosophila eIF4Es, eIF4G and 4E-BP (Bernal and Kimbrell, 2000) ORFs were cloned onto the vectors pGB79 or pGAD424 (Clontech), respectively, to build the bait constructs 4Es-BD (Binding Domain) or the prey construct 4EBP-AD (Activator Domain), respectively.

4.3. Northern blot and quantitative real-time RT-PCR

Total RNA of staged wild type Drosophila (Oregon R) was isolated using the RNaseasy Mini Kit (Qiagen), digested with RNase-free DNase I (Qiagen) and quantified by spectrophotometry. Northern Blot was performed as described (Hernández and Sierra, 1995) using 100 ng of [32P]-labelled DNA probes and hybridized at 50 °C. 100 ng of total RNA per reaction was used as template for quantitative real-time RT-PCR using the QuantiTect SYBR green RT-PCR kit (Qiagen) in an Engine Opticon System (M.J. Research Inc.). Primers were sequence-specific 25-mer oligonucleotides (Metabion) designed to amplify 100 bp-long fragments in each case.

4.4. Proteins labeling and cap binding analysis

Two microgram of each pCR2.1-eIF4E DNA was subjected to transcription/translation in vitro using the TNT-coupled Reticulocyte Lysate System (Promega) in the presence of a [35S]Met and [35S]Cys mixture (14.3 mCi/ml: Amersham), as described by the manufacturer. Labeled proteins were subjected to cap-binding analysis by affinity chromatography on a m7GTP-Sepharose column (Pharmacia) according to (Maroto and Sierra, 1989) as follows. 150 μl of TNT reactions containing the labeled proteins were dialyzed against buffer A (20 mM HEPES pH 7.6, 100 mM KCl, 0.2 mM EDTA, 0.5 mM dithiothretiol, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride). Eight-hundred and fifty microliter of buffer A were added and the total volume (1 ml) was mixed with 10 μl of m7GTP-Sepharose resin (Pharmacia, Inc.) and gently shaken for 4 h. The resin was collected by centrifugation (370×g for 5 min), washed twice with 1 ml buffer A, poured into a column and further extensively washed with 10 ml buffer A. Nonspecific bound proteins were eluted by washing the column with 10 volumes of buffer A containing 0.1 mM GTP. Specific cap-bound proteins were eluted with buffer A containing 100 μM m7GTP, resolved by SDS-PAGE (12.5% acrylamide) and quantified with the help of a phosphorimager. All steps were performed at 4 °C.

4.5. Western blot

Western blotting was performed using anti-eIF4E-1 (1:1000) (Maroto and Sierra, 1989) or anti-eIF4A (1:5000) (Hernández et al., 2004a) polyclonal antibodies, HRP-coupled goat anti-rabbit secondary antibody (1:20,000) (DianoVA), and the ECL detection kit (Amersham Pharmacia Biotech).

4.6. Mass spectroscopy

Proteins were precipitated with 3 volumes ethanol and 0.1 volumes sodium acetate pH 5.2 for 2 h at −20 °C, centrifuged at 14,000 rpm for 30 min at 4 °C, washed with ethanol 80% and further air-dried. Proteins were resolved by SDS-PAGE and visualized by silver staining. Mass spectrometry of the proteins was performed by the thin-layer method and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) as described (Hartmuth et al., 2002) in a Bruker Reflex IV apparatus. Recorded spectra were analyzed using the TofS.1.1 software (Bruker, Bremen, Germany) and the proteins identified in the Nonredundant Database from the National Center for Biotechnology Information by using MASCOT (Matrix Science, London) as a searching tool. The less-abundant proteins that could not be identified by MALDI-TOF were identified by liquid chromatography-coupled tandem mass spectrometry (LC-MSMS) as described (Hartmuth et al., 2002) in an Ultimate HPLC system containing a Switchos II
pre-column switching device and a Famos autosampler (LC Packings, Idstein, Germany) coupled to an orthogonal quadrupole time-of-flight mass spectrometer (Q-Tof 1, Micromass, Manchester, UK). The electrospray was generated with fused-silica 10-µm PicoTip needles (New Objectives, Cambridge, MA), which were operated at 2.8–3.5 kV.

4.7. Yeast two-hybrid assays

Saccharomyces cerevisiae strain Y190 was co-transformed with bait and prey constructs and plated on selective medium (-try, -leu). The colonies were then transferred to selective medium (-try, -leu, -his) containing 25 mM 3-amino-1,2,4-triazole (Sigma), and the positive interacting colonies assayed for β-galactosidase activity on paper filter as described (Bartel et al., 1993).

4.8. In vivo complementation in S. cerevisiae

4Es-TRP1, 4Es-HIS3 or 4G-TRP1 constructs were used to transform the S. cerevisiae strains T93C that carries a conditionally lethal allele of yeast CDC33 (eIF4E) gene (Altmann et al., 1989b), and CBY19 that carries a double knockout of TIF4631 (eIF4GI) and TIF4632 (eIF4GII) genes (Berset et al., 2003). After transformation, yeast cells were replica plated on YPGal (2% galactose) medium and tested at 30 °C for their capacity to complement the lack of endogenous eIF4E or eIF4G as described (Altmann et al., 1989a).

4.9. Fly stocks, transgenic flies preparations and Scanning electron microscopy of eyes

Flies strains were raised as described (Ashburner, 1989). Construct UAS-eIF4E1 was used to generate transgenic flies yw; P[w UAS-eIF4E1] as described in (Rubin and Spradling, 1982) by microinjection in yw embryos. Ectopic overexpression of transgene UAS-eIF4E1 was achieved by crossing flies yw; P[w UAS-eIF4E1] with the early embryonic Gal4 drivers en-Gal4, NGT40-Gal4, the late embryonic driver T80-Gal4, or the eye imaginal disc drivers Gal4 sev-Gal4, ey-Gal4 and GMR-Gal4. Cuticle preparations were done as described in Ashburner (1989). For Scanning electron microscopy (SEM), adult flies were subjected to sequential ethanol dehydration, critical point drying and then coated with a gold-palladium mix in a Cool Spatter Coater (Fisons Instruments, UK). Images were acquired using a Digital Scanning Microscope DSM960 (Zeiss, Germany).

4.10. Rescue of a null mutant for eIF4E

The mutant l(3)67Af rrl eIF4E, Sb1 (Leicht and Bonner, 1988), which is a null mutant for the gene eIF4E-1,2 (Hernández et al., 2004b; Lachance et al., 2002), and the deficiency mutant Df[3L]29A6(66F3;67B1), rrl eIF4E/TM3 (Leicht and Bonner, 1988), were obtained from Bloomington Stock Center (USA). Stocks Gal4-T80/SM6a-TM6b/l(3)67Af were generated and crossed to UAS-eIF4E1/SM6a-TM6b/Df(3L)29A6. Pupae and adults w; Gal4-T80/UAS-eIF4E1; l(3)67Af/Df(3L)29A6 were identified by their lack of the Cy and Tb markers.

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