Alternatively Spliced Transcripts from the Drosophila eIF4E Gene Produce Two Different Cap-binding Proteins*

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Eukaryotic initiation factor 4E (eIF4E) is the subunit of eIF4F that 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-polyacrylamide gel electrophoresis 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.

Translation of eukaryotic mRNAs is a complex process that involves numerous components and is regulated at many steps (Merrick and Hersh, 1996). A critical point in the initiation of translation is the binding of the mRNA to the 43 S pre-initiation complex, which requires the initiation factor eIF4F.1 In mammals eIF4F consists of three subunits, eIF4E, eIF4A, and eIF4G (Grifo et al., 1983; Edery et al., 1983). The eIF4E subunit binds the cap structure, m7G(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 (Hiremath et al., 1985; Duncan et al., 1987), 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, 1989; Metz et al., 1992; Rychlik et al., 1987; Hernández and Sierra, 1995). 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 multi-copy 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 (Takahara et al., 1981; Edery et al., 1983; Grifo et al., 1983), but the yeast and Drosophila eIF4F proteins lack the eIF4A polypeptide (Goyer et al., 1989, 1993; 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 et al., 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 been purified previously 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 (Hernández 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**

Lysis of Drosophila Adult Tissues—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 of buffer A (50 mM HEPES, pH 7.6, 70 mM KCl, 2 mM dithiothreitol, 10% glycerol, 0.1 mM EDTA, 5 mM magnesium acetate, 40 mg/ml phenylmethylsulfonyl fluoride, 50 mg/ml TLCK, 0.5 mg/ml aprotinin). The unlysed material was pelleted for 15 min at 5000 × 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 × g for 25–30 min in a Beckman 45Ti rotor. Drosophila eIF4E has been shown previously 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 (Mates et al., 1987; Webster et al., 1991). Briefly, the
supernatant was spun for 2 h at 260,000 × g in a Beckman 70Ti rotor. A 0–70% ammonium sulfate fraction of the post-ribosomal supernatant was then dialyzed against buffer B (20 mM HEPES, pH 7.6, 120 mM KCl, 1 mM dithiothreitol, 3% glycerol, 0.1 mM EDTA, 40 mg/ml phenylmethylsulfonyl fluoride, 50 mg/ml TLCK, 0.5 mg/ml aprotinin). All steps were performed at 4 °C.

Cap Column Chromatography—Cap column chromatography was carried out on post-ribosomal supernatants as in Maroto and Sierra (1989) using m7GTP-Sepharose (Pharmacia Biotech Inc.) and the cap analogue mGDP (Sigma). A total of 22 mg of protein from the post-ribosomal supernatant was added to 0.5 ml of m7GTP-Sepharose and incubated for 2.5 h at 4 °C. The beads and protein were then poured onto a disposable column (Bio-Rad) 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 mGDP in buffer B.

Analysis of Proteins and Preparation for Microsequencing—Proteins (10 μl) were analyzed 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 polyvinylidene difluoride 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 analyzed 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'-AAACCCCGCTGCTGAAA-3') and antisense (5'-CAGTGGTTGACCATCCT-3') primers; the primer sequences were obtained from the previously published eIF4E sequence (Hernández and Sierra, 1995). PCR buffer (Life Technologies, Inc.) was supplemented with 1.5 mM MgCl2, 0.4 mM each dNTP, and 2.5 units of Taq DNA polymerase (Life Technologies, Inc.). Thermocycling was performed in a Perkin-Elmer-Cetus instrument using the following conditions: 2 cycles of 95 °C for 2 min, 46 °C for 2 min, and 72 °C for 4 min followed by 20 cycles of 95 °C for 40 s, 46 °C for 1 min, and 72 °C for 50 s. Reactions were then supplemented with 4 mM EDTA and precipitated in 1 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 3/4th 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-32P]dCTP by random priming (Oligolabeling Kit, Pharmacia) and used to screen 150,000 individual plaques of a 0–2-h embryo cDNA library constructed in λZAP. 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 λFIX1I.2 To screen the genomic library, a 1.4-kb fragment from the Drosophila eIF4E gene was amplified by PCR using sense (5'-TGAGGAGACGGAGAG-3') and antisense (5'-GCCGCCGTG-CTCTCT-3') primers and labeled with [α-32P]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-h embryo cDNA library (Brown and Kafatos, 1988). Amplification primers were 5'-CGATTAGTGGACATCATTAG-3' (SP6 primer, sense) and 5'-GGCGGTGTGGTGTGATAG-3' (primer A, antisense). A second round of PCR using the same primers was done using 3/4th of the ammonium-precipitated material from round one. To specifically amplify the 5′ end of the 2.0-kb eIF4E transcript, a PCR primer that was used as template was 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-base pair product was amplified. Further amplification reactions were done using this product as template with primers 5'-TTGTTGGAGACGGAGAG-3'.

ERIC B. Suter, unpublished results.

RESULTS

Two Distinct 35-kDa Cap-Binding Proteins in Drosophila Adults—Using extracts prepared from Drosophila adults, we purified cap-binding proteins by mGTP-Sepharose column chromatography. In accordance with previous reports (Maroto and Sierra, 1989; Zapata et al., 1994; Duncan et al., 1995), the major cap binding activity migrates at approximately 35 kDa on SDS-polyacrylamide gel electrophoresis; 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 NH2-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 (Hernán-
dez 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 an 0–2-h embryonic cDNA library. 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′-UTR than has been recognized previously. 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 NH2-terminal 19 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 NH2-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.

eIF4EI differs from other related proteins by a unique NH2-terminal extension (Hernández and Sierra, 1995). The alternative NH2 terminus found in the eIF4EII sequence more closely resembles those of other eIF-4E proteins (Fig. 3).
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 the single eIF4E gene (Fig. 2). In the five clones that 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, 2F1 (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 16-145 and 1050-1090, Fig. 2), is not apparent on this exposure, but is clearly detected when a fragment of an eIF4E genomic clone containing intron 1 is used as probe (data not shown).
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 probe specific to intron 1 and exon 1A (nucleotides 340–1027) (A), a probe specific to exon 1B (nucleotides 1049–1376) (B), the entire 1.7E1 CDNA and autoradiographed (C). 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.

Three F4E 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 eIF4E1 and which encode eIF4E1I. 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 are similar to those previously reported (Hernández and Sierra, 1995). These results indicate that the 2.0- and 1.7-kb transcripts encode eIF4E1, while the 1.4-kb transcript encodes eIF4E1I. 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 product produced by PCR on a 0–4-h embryonic CDNA library (Brown and Kafatos, 1988). The alternative splicing events that 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 eIF4E1I 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 121 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 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 (Browning et al., 1987; Allen et al., 1992; Metz et al., 1992).

The peptide sequencing data we presented above unambiguously support the existence in vivo of the novel eIF4E1 isoform, but the amino acid sequence we obtained from the faster migrating isoform is not NH2-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 been reported previously as particularly problematic (Duncan et al., 1995). However, the ratio of eIF4E1 to eIF4E1I is relatively constant in numerous extracts we have prepared, with...
eIF4E always the more intense band. Furthermore, any degradation must be specific to eIF4E, as our extraction conditions lead to the recovery of full-length eIF4EII. The difference between the NH2-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 eIF4EII polypeptide, which our NH2-terminal sequencing predicts are in the mature protein, are residues that are not conserved in eIF4E proteins in species other than Drosophila (Fig. 3). It is also possible that the first AUG in the eIF4E open reading frame is not the true initiation codon, as in the eIF4EI sequence there are in-frame initiation 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 (Cavener, 1987; Brown et al., 1994).

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, while 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 and Cavener, 1993; 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 eIF4EII to eIF4EI protein recovered in our Northern blots, and frequently such transcripts are not abundantly expressed (Cavener, 1987; Brown et al., 1994).

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