Multiple Isoforms of Eukaryotic Protein Synthesis Initiation Factor 4E in Caenorhabditis elegans Can Distinguish between Mono- and Trimethylated mRNA Cap Structures*

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The rate-limiting step for cap-dependent translation initiation in eukaryotes is recruitment of mRNA to the ribosome. An early event in this process is recognition of the m7GTP-containing cap structure at the 5'-end of the mRNA by initiation factor eIF4E. In the nematode Caenorhabditis elegans, mRNAs from 70% of the genes contain a different cap structure, m3,2,2,7GTP. This cap structure is poorly recognized by mammalian eIF4E, suggesting that C. elegans may possess a specialized form of eIF4E that can recognize m3,2,2,7GTP. Analysis of the C. elegans genomic sequence data base revealed the presence of three eIF4E-like genes, here named ife-1, ife-2, and ife-3. cDNAs for these three eIF4E isoforms were cloned and sequenced. Isoform-specific antibodies were prepared from synthetic peptides based on nonhomologous regions of the three proteins. All three eIF4E isoforms were detected in extracts of C. elegans and were retained on m7GTP-Sepharose. One eIF4E isoform, IFE-1, was also retained on m3,2,2,7GTP-Sepharose. Furthermore, binding of IFE-1 and IFE-2 to m7GTP-Sepharose was inhibited by m3,2,2,7GTP. These results suggest that IFE-1 and IFE-2 bind both m7GTP- and m3,2,2,7GTP-containing mRNA cap structures, although with different affinities. In conjunction with IFE-3, these eIF4E isoforms would permit cap-dependent recruitment of all C. elegans mRNAs to the ribosome.

All eukaryotic cytosolic mRNAs and many eukaryotic viral mRNAs contain a 5'-terminal capping group (1). The most commonly occurring cap structures contain 7-methylguanosine in a 5'-to-5' triphosphate linkage to the first transcribed nucleotide residue, which is often 2'-O-methylated as well. The presence of a cap on mRNA stimulates translation as well as stabilizes the mRNA against degradation. The former of these effects is thought to be mediated by the binding of a 25-kDa initiation factor, eIF4E (eukaryotic initiation factor 4E), to the cap (2). eIF4E is a member of the eIF4E class of initiation factors, which also includes eIF4A, eIF4B, and eIF4G; collectively these factors recruit mRNA to the 43 S initiation complex and melt mRNA secondary structure (3). The primary structure of eIF4E has been deduced from cDNA in a variety of species (4–9), and the tertiary structure has recently been solved in the case of mouse (10) and yeast (11). In plants, there are at least two eIF4E isoforms, termed eIF4E and eIF(iso)4E (12, 13); the former is expressed in most tissues, whereas the latter is expressed only in floral organs and developing tissues (9).

eIF4E is regulated by at least three processes. First, the phosphorylation of eIF4E correlates positively with the rate of translation in a large number of systems (4) and increases the affinity of the protein for cap analogues 3–4-fold (14). Second, eIF4E availability is regulated by eIF4E-binding proteins, the phosphorylation of which, in response to insulin and other mitogens, releases them from eIF4E and permits eIF4E binding to eIF4G (15). Third, eIF4E levels are regulated at the transcriptional level (16). Changes in the intracellular levels of eIF4E have a profound effect on cellular growth control. Ectopic overexpression of eIF4E leads to accelerated cell growth, transformation in culture and tumorigenesis in nude mice, prevention of apoptosis in growth factor-restricted fibroblasts, and elevated intracellular levels of growth-regulated proteins such as cyclin D1, c-Myc, ornithine decarboxylase, ornithine aminotransferase, P23, vascular endothelial growth factor, and fibroblast growth factor-2 (reviewed in Ref. 17). Reduction in intracellular eIF4E levels by expression of antisense RNA results in phenotypic reversal of ras-transformed fibroblasts (18). eIF4E mRNA levels are also elevated in a variety of cells that have been oncogenically transformed by in vivo transfection, viral infection, or chemical mutagenesis (19), and naturally occurring breast and head-and-neck tumors express elevated levels of eIF4E (Ref. 20 and references therein).

The structural requirements for recognition of the cap by mammalian and plant eIF4E have been determined by inhibition of in vitro translation by cap analogs, quenching of tryptophan fluorescence in eIF4E by cap analogs, and translation of mRNAs synthesized with modified cap structures (reviewed in Ref. 2). Binding requires the presence of the 7-methyl group, but changing the substituent at N7 to an aromatic group increases binding affinity. Addition of one methyl group at the N2 position of guanine has little effect on binding, but addition of a second methyl group (m2,2,7GTP; Scheme 1) drastically decreases it (21–23), presumably due to the loss of the H-bond between the N2 of m7G and Glu-103 of eIF4E (10).

Most nematode mRNAs have a 22-nucleotide trans-spliced leader sequence at their 5'-ends (24, 25). In Caenorhabditis elegans, a number of different spliced leaders have been described (26), and mRNAs from ~70% of the genes contain a spliced leader (27). Trans-splicing results in an mRNA with an
The effects of the trimethyl cap on translational efficiency in *C. elegans* has not been studied, trans-splicing enhances the translational efficiency of mRNAs in the parasitic nematode *Ascaris* (25). This increase reflects synergistic effects of both the spliced leader and the m<sup>2,2,7</sup>GTP-containing cap.

The inability of eIF4E from the species studied to date to bind m<sup>2,2,7</sup>GTP-containing caps contrasts with the fact that most mRNAs of *C. elegans* contain such caps, suggesting that eIF4E from *C. elegans* may differ qualitatively from that of other species. We therefore set out to isolate eIF4E from *C. elegans* to determine its properties. Surprisingly, we found multiple eIF4E isoforms. Moreover, the different types varied in their abilities to recognize m<sup>7</sup>GTP and m<sup>3,2,7</sup>GTP.

**EXPERIMENTAL PROCEDURES**

**Materials**—Carbenicillin, leupeptin, pepstatin, TAME,<sup>1</sup> and aproctin were purchased from Sigma. Maleimide-activated keyhole limpet hemocyanin and bovine serum albumin were purchased from Pierce. Ni<sup>2+</sup>-nitrilotriacetic acid-agarose (from Qiagen, Chatsworth, CA). The protease inhibitor E-64 and Complete<sup>2</sup> Protease Inhibitor tablets (without EDTA) were obtained from Boehringer Mannheim. Affi-Gel 501 resin was obtained from Bio-Rad. The following antibodies were purified on columns of Affi-Gel 501 to which each synthesized protein was linked: anti-peptide antibodies were prepared against the peptide ASSLHSSDAPVAEKS, CKHAIYAVEPREEK, and TTAGACGGCGGATTTCTCG, respectively: CGAACCGGATCCATGACTGAAACGGA, GAGGGACGCAAGC- and -3, respectively: CLSLHSSDAPVAEKS, CKHAIYAVEPREEK, and TCCGAGGATCCAGTCGCAGCTCC, TCCGTAGCGGAA, TGGTGAGATCTCGAGAATATGCTTAAGGAG, and phosphatase conjugate were purchased from Novagen (Madison, WI).

**Preparation of C. elegans Extracts**—*C. elegans nematode growth medium agar and *C. elegans* wild type strain N2 were grown on plates supplemented with chicken egg yolk (35). Animals were harvested, cleaned by one or more washes with *C. elegans* nematode growth medium agar and stored at 4 °C. Cells were thawed in the presence of buffer B (25 mM Tris·HCl, pH 7.5, 300 mM NaCl, 5 mM β-mercaptoethanol, and one Complete<sup>2</sup> Protease Inhibitor tablet/25 ml) and lysed by sonication (3–6 bursts of 10 s each). The lysate was spun at 15,000 × g for 15 min at 4 °C. The supernatant was used as the source of the purified proteins.

**Synthesis of Affinity Chromatography—**C. elegans extracts (10–20 ml) were applied to 0.2-ml columns of m<sup>7</sup>GTP-Sepharose or m<sup>3,2,7</sup>GTP-Sepharose equilibrated in buffer A (20 mM MOPS, pH 7.5, 1 mM EDTA, 100 mM KCl, 10% (v/v) glycerol, and 0.5 mM dithiothreitol), and the flow-through fraction was collected. Columns were washed with 10 ml of buffer A followed by 10 ml of buffer B containing 100 μM GTP. Proteins were eluted with 2 ml of buffer B containing either 100 mM ATP or m<sup>3,2,7</sup>GTP (depending on the column matrix), and 0.2-ml fractions were collected.

**Preparation of anti-peptide antibodies**—The cDNA constructs expressed eIF4E isoforms containing an N-terminal addition consisting of thioredoxin, a 5-peptide sequence, and a His<sub>6</sub>-tag. *C. elegans* eIF4E cDNAs were also kindly provided by the Yugi Kahara laboratory (Japan) as Lac clones corresponding to expressed sequence tags for IFE-1 (yk364a1), IFE-2 (yk452e8), and IFE-3 (yk81f11). All cDNA constructs were sequenced and compared with genomic sequences to determine intron/exon boundary structures. Two discrepancies were observed. First, a 9-nucleotide insertion was present in the IFE-3 coding region of yk81f11 but not IFE-3, resulting in the addition of Gln and Arg. Second, a single nucleotide change was observed in IFE-3 compared with yk452e8 and genomic DNA, resulting in Pro-114 instead of Leu-114. This likely represents a PCR-induced mutation.

**Expression and Purification of Recombinant Proteins—**Expression of recombinant *C. elegans* eIF4E isoforms was induced with 1 mM isopropyl<sub>1-β-D</sub>-galactopyranoside for 3 h in 1-liter cultures of *E. coli* strain BL21(DE3)pLysS (38) bearing plasmids pTSIFE-1, pTSIFE-2, or pTSIFE-3. Cells were cooled in an ice water bath, pelleted by centrifugation, and stored at −70 °C. Cells were thawed in the presence of buffer B (25 mM Tris·HCl, pH 7.5, 300 mM NaCl, 5 mM β-mercaptoethanol, and one Complete<sup>2</sup> Protease Inhibitor tablet/25 ml) and lysed by sonication (3–6 bursts of 10 s each). The lysate was spun at 15,000 × g for 15 min at 4 °C. The supernatant was below 0.01. The resin was then washed with 10 ml of buffer B containing 40 mM imidazole, and fusion proteins were eluted with 10 ml of buffer B containing 0.2 M NaCl and 0.5 M KCl.

**Immunological Procedures—**Preparation of anti-peptide antibodies and immunoblotting were performed as described previously (39). Antibodies were purified on columns of Affi-Gel 501 to which each synthetic peptide was linked via the Cys residue (40).

**RESULTS AND DISCUSSION**

m<sup>7</sup>GTP- and m<sup>3,2,7</sup>GTP-Sepharose affinity chromatography resins were synthesized to determine whether eIF4E from *C. elegans* recognizes mono- or trimethylated cap structures. Experiments using these affinity chromatography columns as well as those using m<sup>7</sup>GTP-Sepharose and m<sup>3,2,7</sup>GTP-Sepharose indicated the presence of several eIF4E isoforms in *C. elegans* extracts.
tracts from *C. elegans* were prepared under conditions that in other systems preserve cap binding activity and minimize proteolysis and dephosphorylation of proteins. The eluate from the m7GTP-Sepharose column consisted of a complicated pattern of proteins ranging from ;20 to 200 kDa, the most intensely staining of which migrated between 26 and 40 kDa (Fig. 1A, lanes 5–7). These bands represent proteins that were specifically retained on m7GTP-Sepharose, because they were not eluted by the GTP wash (Fig. 1A, lane 4). This complex pattern of proteins is similar to that observed with extracts from higher eukaryotes (Ref. 41 and references therein), for which it has been shown that the major band represents eIF4E, whereas the others represent initiation factors that specifically associate with eIF4E (i.e. eIF4G, eIF4A, eIF3, eIF4B, and eIF4E-binding proteins). Chromatography on m32,2,7GTP-Sepharose produced a simpler collection of retained proteins, with bands at 26 and 37 kDa predominating (Fig. 1B, lanes 5–7). Inclusion of GTP in the extract reduced the amount of the 37-kDa band relative to the 26-kDa band (Fig. 1C), suggesting that the former protein is nonspecifically retained. The 26-kDa band from m7GTP-Sepharose co-migrated with the major protein retained on m32,2,7GTP-Sepharose (data not shown). The slower migrating proteins (Fig. 1, B and C) appear to correspond in molecular mass to a subset of the proteins retained on m7GTP-Sepharose (Fig. 1A) and are presumed to be eIF4E-associated initiation factors. These results indicate, based on molecular mass and retention on affinity columns, that there are several candidates for *C. elegans* eIF4E.

Identification of eIF4E cDNA and Gene Sequences in Genomic Data Bases—Three genes (hereby named ife-1, ife-2, and ife-3) that encode proteins with strong homology to human eIF4E were identified in sequences generated by the *C. elegans* Genome Sequencing Consortium (36). cDNAs corresponding to each of the *C. elegans* ife genes were cloned and sequenced. The calculated molecular masses of the predicted eIF4E proteins, IFE-1, IFE-2, and IFE-3 (Fig. 2A), were 24.3, 25.7, and 27.8 kDa, respectively. Each of the IFE proteins showed strong homology to human eIF4E and to each other (Fig. 2B).

Development of Immunological Reagents—Specific antisera were generated to distinguish between the various eIF4E isoforms. Peptides corresponding to sequences located in the non-homologous C-terminal portion of the proteins (Fig. 2A, boldface) were synthesized and used for generation and purification of antibodies. To test the specificity of the antibodies, the cDNAs for IFE-1, IFE-2, and IFE-3 were cloned into a bacterial vector and expressed as fusion proteins (rIFE-1, -2, and -3).
When the mass of the N-terminal extension is taken into account, these recombinant proteins migrated on SDS-polyacrylamide gel electrophoresis as expected (Fig. 3A). Each anti-peptide antibody recognized only the corresponding isoform of eIF4E when tested against the purified recombinant proteins (Fig. 3, B–D). The protein band migrating below rIFE-2 (arrow) likely represents a truncated form of rIFE-2 because it contains the N-terminal S-peptide (data not shown) but lacks the C-terminal IFE-2 epitope.

To determine if any of the proteins that were retained on m7GTP- or m3GTP-Sepharose (Fig. 1) included IFE-1, -2, or -3, eluates of affinity columns were subjected to immunoblotting with isoform-specific antibodies. All three isoforms were detected in the elute from m7GTP-Sepharose (Figs. 3, E–H, lanes 1). The major bands at 26 and 31 kDa correspond to IFE-1 and IFE-3, respectively. IFE-2, on the other hand, corresponds to a minor band migrating at 28 kDa; it is often undetectable by silver or Coomassie staining but is readily detectable by immunoblotting. The 26-kDa protein retained on m3GTP-Sepharose (Fig. 3E, lane 2) was recognized by the anti-IFE-1 antibody (Fig. 3F, lane 2), indicating that IFE-1 can bind both m7GTP- and m3GTP-Sepharose. However, neither IFE-2 nor IFE-3 was retained on m3GTP-Sepharose (Fig. 3, G and H, lanes 2). Anti-IFE-1 antibody appeared to recognize a single protein species (Fig. 3F), but anti-IFE-2 and anti-IFE-3 antibodies appeared to recognize closely spaced doublets (Fig. 3, G and H). This may reflect post-translational modification or alternatively spliced forms of IFE-2 and -3.

The cap-binding specificities of the eIF4E isoforms were further characterized with cap analogs to compete for binding to affinity columns (Fig. 4). Extracts were applied to m7GTP- or m3GTP-Sepharose in the presence of m7GTP or m3GTP as competitors. No proteins were retained on m7GTP-Sepharose when m7GTP was used as competitor (Fig. 4, A and C, lanes 2 versus lanes 1), nor were any proteins retained on m3GTP-Sepharose when m3GTP was used as competitor (Fig. 4, B and D, lanes 3 versus lanes 1). Surprisingly, when m3GTP was used as a competitor during m7GTP-Sepharose chromatography, retention of both IFE-1 and IFE-2 was prevented, whereas binding of IFE-3 was unaffected (Fig. 4C, lane 3 versus 1). Similarly, when m7GTP was used as a competitor with a m3GTP-Sepharose column, IFE-1 was not retained (Fig. 4, B and D, lanes 2 versus 1). The fact that binding of IFE-1 to either resin was competed by either cap analog indicates that IFE-1 recognizes both cap structures through the same binding site.

The unexpected finding that IFE-2 apparently recognizes m3GTP (Fig. 4C, lane 3 versus 1) but is not retained on m3GTP-Sepharose (Fig. 3G, lane 2) suggests that it has an intermediate binding affinity: strong enough to allow m3GTP to serve as competitor but too weak to allow retention on an affinity resin. Alternatively, IFE-2 may be hindered in its interaction with immobilized m3GTP but not with the free nucleotide. However, the most likely interpretation at present is that C. elegans eIF4E isoforms have differential affinities for the trimethyl cap structure, the relative order being IFE-1 > IFE-2 ≈ IFE-3. This order of affinity is inversely correlated with the relative homologies to human eIF4E, which recognizes only m7GTP (Fig. 2B). Furthermore, the two C. elegans isoforms that recognize m3GTP are more similar to each other than to IFE-3 (Fig. 2B). Interestingly, the m3GTP-binding isoforms contain an extra amino acid stretch (amino acid 164–170 in IFE-1; see Fig. 2C) as well as an additional Trp residue (amino acids 20 in IFE-1); these may account for the difference in nucleotide-binding specificity.

It is not clear why multiple IFE isoforms are present in C. elegans. It is interesting that in this organism roughly 70% of mature mRNAs contain trimethylated, trans-spliced leaders. This presents a unique situation with respect to recruitment of mRNA for translation. There is the potential for differential selection of mRNAs depending on the nature of the cap: monoversus trimethylated. Furthermore, because the sequence of an mRNA adjacent to the cap can have a profound effect on its association with eIF4E and recruitment to the translational apparatus (42), there is also the potential for recruitment of classes of mRNAs based on a common splice leader sequence (SL1 versus SL2) by altering levels and/or activities of specific eIF4E isoforms. Determination of the temporal and cell-specific
expression of IFE isoforms may provide useful information on the role of cap binding proteins in translation and in the development of the organism.

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Note Added in Proof—The Genome Sequencing Consortium has identified two additional eIF4E-like genes in C. elegans: ife-4, identified as predicted gene C05D9.5, and ife-5, located on YAC Y57A10, suggesting an even greater degree of complexity of regulation of translation and/or development in C. elegans.

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