Functional Characterization of Five eIF4E Isoforms in Caenorhabditis elegans*

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Recognition of the 5’-cap structure of mRNA by eIF4E is a critical step in the recruitment of most mRNAs to the ribosome. In Caenorhabditis elegans, ~70% of mRNAs contain an unusual 2,2,7-trimethylguanosine cap structure as a result of trans-splicing onto the 5’ end of the pre-mRNA. The characterization of three eIF4E isoforms in C. elegans (IFE-1, IFE-2, and IFE-3) was reported previously. The present study describes two more eIF4E isoforms expressed in C. elegans, IFE-4 and IFE-5. We analyzed the requirement of each isoform for viability by RNA interference. IFE-3, the most closely related to mammalian eIF4E-1, binds only 7-methylguanosine caps and is essential for viability. In contrast, three closely related isoforms (IFE-1, IFE-2, and IFE-5) bind 2,2,7-trimethylguanosine caps and are partially redundant, but at least one functional isoform is required for viability. IFE-4, which binds only 7-methylguanosine caps, is most closely related to an unusual eIF4E isoform found in plants (nCBP) and mammals (4E-HP) and is not essential for viability in any combination of IFE knock-out. ife-2, ife-3, ife-4, and ife-5 mRNAs are themselves trans-spliced to SL1 spliced leaders. ife-1 mRNA is trans-spliced to an SL2 leader, indicating that its gene resides in a downstream position of an operon.

Eukaryotic mRNAs and small nuclear RNAs synthesized by RNA polymerase II are posttranscriptionally modified to form a 5’-5’ GpppN linkage (1). The 5’-terminal G is methylated at N7 while still in the nucleus to yield an MMG cap. The cap of small nuclear RNAs is then further methylated at N2 in the cytoplasm to yield a TMG cap (2). Methylation of small nuclear RNAs is dependent upon the binding of Sm proteins to form small nuclear ribonucleoproteins. Formation of the TMG cap is the targeting signal for import of small nuclear ribonucleoproteins back into the nucleus to take part in pre-mRNA splicing (3, 4). mRNAs, on the other hand, which possess only the MMG cap, remain in the cytoplasm.

In some primitive eukaryotes, including Caenorhabditis elegans, mRNAs acquire a TMG cap through the process of trans-splicing (5). Primary transcripts from approximately 70% of protein-coding genes are trans-spliced to 22-nt SL sequences, such that the original MMG caps are replaced with the TMG caps from the SL small nuclear RNAs (6, 7). Also common in C. elegans is the organization of genes into operons that are transcribed from a single promoter into a polycistronic RNA (8). trans-Splicing results in the processing of these primary transcripts into monocistronic mRNAs. Generally, the mRNA from the first cistron is trans-spliced to SL1, whereas mRNAs from downstream cistrons are trans-spliced to SL2 or SL2 variants (8). mRNAs that are not trans-spliced retain the original MMG cap. Thus, both MMG- and TMG-capped mRNAs are found in the cytoplasm of C. elegans. Both types of mRNA enter polyribosomes and are translated, indicating that they are competent to interact with the translational machinery (9).

The recruitment of mRNAs to ribosomes is catalyzed by the eIF4E group of translation initiation factors (reviewed in Refs. 10 and 11). The mRNA cap is specifically recognized by eIF4E. At least two isoforms of eIF4E exist in humans, eIF4E-1 (12) and 4E-HP (13), which are quite divergent in primary sequence. In plants, three isoforms have been described, eIF4E (14, 15), eIF(iso)4E (16), and nCBP (17). All eIF4E proteins characterized from higher eukaryotes are highly selective for MMG caps. Cap analogs containing TMG are 17-fold less inhibitory than the corresponding MMG-containing cap analogs in a rabbit reticulocyte cell-free translation system (18). Substitution of a TMG cap for the MMG cap on β-globin mRNA reduces its translational efficiency by 75% in the same system (19).

Because naturally occurring TMG-capped mRNAs are abundant in C. elegans, the organism must possess a mechanism to recognize these mRNAs and initiate their translation. We previously reported the characterization of MMG- and TMG-binding proteins from C. elegans and the cloning of three cDNAs (ife-1, ife-2 and ife-3) encoding eIF4E isoforms (20). Among these isoforms, IFE-3 has a cap specificity similar to mammalian eIF4E-1, binding only MMG. IFE-1 and IFE-2, on the other hand, bind either MMG or TMG, although apparently with different affinities. All three isoforms can be purified from worm extracts. We now report the cloning of two additional eIF4E cDNAs from C. elegans, ife-4 and ife-5, as well as the...
characterization of their encoded proteins and the requirement for each of the five proteins for viability.

EXPERIMENTAL PROCEDURES

Materials—Leupeptin, pepstatin, and Nα-p-tosyl-L-arginine methyl ester were purchased from Sigma. m7GTP and m2,2,7GTP-Sepharose were obtained from Amersham Pharmacia Biotech. E64 and CompleteTM Protease Inhibitor tablets were purchased from Roche Molecular Biochemicals. m2,2,7GTP and m2,2,7GTP-Sepharose were synthesized as described previously (20). Oligodeoxynucleotides were purchased from Life Technologies, Inc.

Sequence Analysis of C. elegans eIF4E cDNAs—The predicted protein sequences of IFE-4 and IFE-5 were identified on cosmid C05D9 and from Life Technologies, Inc. obtained from Amersham Pharmacia Biotech. E64 and CompleteTM Protease Inhibitor tablets/25 ml were used. Affinity purification of mGTP-binding proteins from C. elegans strain N2 was performed as described previously (20).

Cap Binding Specificity Assay—Purified recombinant IFE-4 and IFE-5 (25 µg) were diluted into 0.1 ml of buffer A (50 mM HEPES-KOH, pH 7.6, 50 mM KCl, 2 mM EDTA, 1 mM dithiothreitol, 5% (v/v) glycerol) and applied to a 0.1-ml m7GTP-Sepharose or a m2,2,7GTP-Sepharose column. The columns were washed with 0.2 ml of buffer A, and the proteins were eluted with 0.1 ml of 100 mM mGTP or m2,2,7GTP mixed m7GTP-Sepharose or a m2,2,7GTP-Sepharose as described for recombinant human eIF4E-1 (26) except that 2 mM EDTA in the sequence of IFE-5. Preparation of anti-peptide antibodies and immunoblotting were performed as described previously (27). Antibodies against IFE-4 were purified on columns of Affi-Gel 501 (Bio-Rad) to which the immunogenic peptide was linked via the Cys residue (28). Serum against IFE-5 was used directly at a dilution of 1:500. Affinity-purified antibodies against IFE-1, IFE-2, and IFE-3 were previously described (20).

Spliced Leader Assay—Poly(A) RNA purified from C. elegans total RNA by a single round of oligo(dT)-cellulose chromatography (29). N2 poly(A)- RNA (40 ng) was reverse transcribed with rTth polymerase (Perkin-Elmer) at 60 °C for 20 min with specific ife primers (primers 1.2–5.2 for ife-1 through ife-5, respectively). Either primer SL1 or SL2, corresponding to the spliced leaders SL1 or SL2 (30), was added, and DNA was amplified for 35 cycles with the same polymerase according to the following regimen: 95 °C for 1 min, 59 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 7 min. The resulting products were digested with NdeI and Xhol and subcloned into pET21b (Novagen) to yield pETife1 and pETife2. Inserts were removed from these plasmids with Xhol and subcloned into the same sites in pBluescript SK (Stratagene) to form pSKife1, pSKife2, and pSKife3 as excised from the library in the presence of E. coli. Digestion with the restriction enzyme SgrAI generated a 450-bp insert, which was subcloned into pBluescript SK (Stratagene). The resulting construct served as a template for the PCR amplification of a 500-bp fragment that was subcloned into pET21b (Novagen). This construct contained the entire 450-bp insert, as well as a small flanking region at the 5′ end. The 5′ end of the insert contained a HindIII site, and the 3′ end contained a BamHI site.

Plasmids pETife1, pETife2, and pETife3 were excised as phagemids from EST clones YK3641A, YK4526E, and YK811F in a Zap (generously supplied by Yuji Kohara, National Institute of Genetics, Mishima, Japan) using the Rapid Excision Kit (Stratagene). cDNAs for ife-1, ife-2, and ife-3 were amplified from pSKife1, pSKife2, and pSKife3 with primers 1.1, 2.1, and 3.1, respectively, together with primer T7. The PCR products for ife-1 and ife-2 were digested with NdeI and XhoI and subcloned into plasmid pET21a (Novagen) to form pETife1 and pETife2, respectively. The product for ife-3 was digested with NcoI and XhoI and subcloned into pET21d (Novagen) to form pETife3. PCT constructs were used to express IFE proteins (except IFE-2) in Escherichia coli, and pSK constructs were used for in vitro transcription (see below).

Purification of Recombinant and Natural eIF4E Isomers—Recombinant C. elegans eIF4E isoforms (rIFE-1, rTthIFE-2, rIFE-3, etc.) were obtained in E. coli strain BL21(DE3)pLysS from plasmids pETife1, pETife2, pETife3, pETife4, and pETife5. rTthIFE-2, encoded by pTSife2, consists of a fusion protein of thioredoxin, the S peptide of ribonuclease A, and a Hisa tag; it was isolated on Ni2+-nitritotriacetic acid-agarose (20). rIFE-1, rIFE-3, rIFE-4, and rIFE-5 were isolated as described for recombinant human eIF4E-1 (26) except that 2 mM EDTA and one CompleteTM Protease Inhibitor tablet/25 ml were used. Affinity purification of mGTP-binding proteins from C. elegans strain N2 was performed as described previously (20).

Comparison of C. elegans eIF4E cDNAs—The predicted protein sequences of IFE-4 and IFE-5 were identified on cosmid C05D9 and from Life Technologies, Inc. obtained from Amersham Pharmacia Biotech. E64 and CompleteTM Protease Inhibitor tablets/25 ml were used. Affinity purification of mGTP-binding proteins from C. elegans strain N2 was performed as described previously (20).

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produce plasmids p2a (SL2-ife-1), p41a (SL1-ife-4), p51xc (SL1-ife-5) and p62xc (SL2-ife-5). The 5’ end of each insert was sequenced (>200 bp).

**RNAi Assays**—General procedures for handling and maintaining the *C. elegans* N2 strain have been described (32). Double-stranded RNAs were prepared by transcribing individual *ife* cDNAs in plasmids pSKife1 through pSKife5 with T3 and T7 RNA polymerases (Epicenter Technologies, Madison, WI). The sense and antisense strands were mixed together at 100 ng/μl each and injected into either the intestine or gonads of young adult hermaphrodites (33, 34). 6–8 h after injection, each animal was transferred onto a fresh plate. Eggs were counted at 24-h intervals for 3–4 days and monitored for embryonic lethality. For every set of RNAi injections, a 200-ng/μl solution of double-stranded RNA for the 64-kDa subunit of Cleavage Stimulatory Factor, which produces 100% embryonic lethality (Williams and Blumenthal, unpublished), was injected as a positive control. The negative control was buffer TE.

**RESULTS**

Cloning of *ife*-4 and *ife*-5 cDNAs—Homology searches using the human eIF4E-1 (12) and 4E-HP (13) sequences revealed five genes encoding *C. elegans* eIF4E isoforms. These were termed *ife*-1, *ife*-2, etc., and their encoded proteins, IFE-1, IFE-2, etc. Characterization of *ife*-1, *ife*-2, and *ife*-3 and identification of corresponding ESTs were reported earlier (20). No ESTs corresponding to *ife*-4 or *ife*-5 have yet been identified in the *C. elegans* data base of the DNA Data Bank of Japan. cDNAs containing the complete open reading frame sequences of the *ife*-4 and *ife*-5 genes were amplified by RT-PCR from *C. elegans* RNA. Analysis of trans-splicing (see below) verified that the predicted initiation codons for *ife*-4 and *ife*-5 were the 5’-most ATG codons in each mRNA.

The cDNA sequences matched the *ife*-4 and *ife*-5 genes absent the intron sequences (data not shown). 5’ and 3’ splice sites (including trans-splicing acceptor sites; see below) in both genes conformed closely to the consensus sequences for *C. elegans* (5). The *ife*-4 gene contains three introns of 292, 483, and 419 bp following, respectively, codons 21 (Met), 81 (Thr), and 161 (Arg). This gene structure is unique among the *ife* genes. *ife*-5 contains a single intron of 54 nt after codon 152 (Gly). This Gly residue is conserved in *ife*-1, *ife*-2, *ife*-3, and *ife*-5 (see below), but an intron is found at this position only in the genes encoding IFE-1, IFE-2, and IFE-5 (data not shown).

Alignment of all five IFE amino acid sequences shows that they are more similar throughout the central core and more divergent in the NH2- and COOH-terminal regions (Fig. 1A). The sequence of *ife*-4 encodes a putative protein of 212 amino acids with a molecular mass of 24,583 Da. Surprisingly, IFE-4 is more similar to human 4E-HP (48% identity) than to the other *C. elegans* eIF4E isoforms (27–35% identity; Fig. 1B). Despite its sequence divergence from the other IFEs, however, IFE-4 contains the five Trp residues in its central core that are characteristic of eIF4E proteins (12, 35).

The sequence of *ife*-5 encodes a putative protein of 201 amino relatedness (e.g. IFE-1 is most closely related to IFE-5). Residues identical in three of the five sequence are shaded. B, the percentage of amino acid identity was calculated from pairwise alignments with the GAP algorithm of the Wisconsin Software Package using a gap penalty of 2.0 and a gap extension penalty of 0.05. Similar alignments to human eIF4E-1 (GenBank™ accession number M15353) and human 4E-HP (AF047695) were performed. The ability of each protein to bind caps was determined by gel mobility shift assay. C. The sequence of *ife*-5 encodes a putative protein of 201 amino relatedness (e.g. IFE-1 is most closely related to IFE-5). Residues identical in three of the five sequence are shaded. B, the percentage of amino acid identity was calculated from pairwise alignments with the GAP algorithm of the Wisconsin Software Package using a gap penalty of 2.0 and a gap extension penalty of 0.05. 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Targeted Inactivation of ife Genes—We interfered with expression of each of the ife genes using RNAi (36) to determine which of the IFEs had essential functions in C. elegans. In this technique, expression of a specific gene is inhibited by the injection of the corresponding double-stranded RNA into young adult worms, and then phenotypes are assayed in their progeny. Phenotypes obtained through the RNAi technique have been shown to be gene-specific and are essentially equivalent to phenotypes observed for null mutations (34, 37).

Various ife genes were inactivated individually and in combination by RNAi (Table I). Inactivation of ife-3, which is the most similar to the human eIF4E-1 gene, resulted in 100% embryonic lethality (Set 1), whereas inactivation of ife-1, ife-2, ife-4, or ife-5 alone showed no embryonic lethality whatsoever. RNAi against the combination of ife-1 and ife-2 was lethal to 75% of the embryos (Set 2). Similarly, inactivation of ife-2 and ife-5 caused 89% embryonic lethality. Simultaneous inactivation of ife-1, ife-2, and ife-5 resulted in 99% embryonic lethality. On the other hand, none of the combinations in which ife-4 was inactivated was deleterious to the worms, including simultaneous inactivation of ife-4, ife-1, and ife-5 (Set 3).
trans-Splicing of ife mRNAs—The fact that some eIF4E isoforms recognize TMG caps suggests that they are required to translate trans-spliced mRNAs. We next determined whether the mRNAs encoding the IFEs were themselves trans-spliced. Antisense oligonucleotides to each of the ife cDNAs were used in conjunction with sense primers to either SL1 or SL2 to amplify products by RT-PCR from poly(A)⁺ RNA. At least one trans-spliced mRNA was detected for each ife gene.

A single amplified product for ife-1 mRNA indicated that it is trans-spliced to SL2 (Fig. 4A). The presence of the SL2 leader suggests that the ife-1 gene is downstream of a cluster of genes that form an operon (8). Consistent with that prediction, ife-1 is the third of five genes clustered within a 14-kilobase region of C. elegans chromosome III (Fig. 4C) (38). Intergenic distances in this putative operon range from 122 to 449 bp. The sequence of the SL2-ife-1 PCR product indicated that the spliced leader was added 2 nt upstream of the initiation codon of the open reading frame reported for ife-1 (20), similar to the spacing observed for most trans-spliced mRNAs (5). Three of the 11 known ife-1 ESTs contain more sequence upstream of this ATG (yk364a1, 11 bp; yk385e3, 31 bp; yk275b11; 92 bp). They are colinear and correspond to part of a putative exon 161 nt upstream of (and spliced to) the same 3′ splice site used by SL2. The longer mRNAs was detected by RT-PCR, suggesting that they are not trans-spliced. Only clone yk275b11 contains another ATG upstream, and it is in frame with the reported ife-1 open reading frame (20).

Three products were observed with SL1 and primer 2.2 (Fig. 4A); however, sequencing of the two larger products indicated that they were not related to ife-2 (data not shown). The smallest SL1-ife-2 product was further amplified by a second round of PCR using the same primers (Fig. 4B). The length of the ife-2-derived product was consistent with existing ESTs for ife-2, one of which contains part of the SL1 sequence spliced 6 nt upstream of the ATG (yk469e7).

Major RT-PCR products corresponding to ife-3 and ife-4 mRNAs were produced using primer SL1 but not primer SL2 (Fig. 4A). The length of the ife-3-derived product was consistent with existing ESTs for ife-3. EST clone yk81f11 encodes part of the SL1 sequence 4 nt upstream of the initiation codon of ife-3. The sequence of the SL1-ife-4 PCR product indicated that the mRNA was trans-spliced 2 nt upstream of the ATG that begins the predicted open reading frame of the ife-4 gene. The SL1-ife-4 PCR product therefore represents an mRNA that encodes the complete IFE-4 protein.

No discrete products of the expected size were observed for ife-5 mRNA with either the SL1 or the SL2 primer in the primary RT-PCR reaction (Fig. 4A), indicating either that it is not trans-spliced or that the abundance of ife-5 mRNA is low. Secondary PCR using a nested ife-5 primer, primer 5.3, produced both SL1- and SL2-containing products of different sizes (Fig. 4B). The sequence of the SL1-ife-5 secondary PCR product indicated an mRNA that is trans-spliced 3 nt upstream of the putative initiation codon. The sequence of the SL2-ife-5 secondary PCR product indicated that the corresponding mRNA contained an additional exon of 120 nt that was likewise spliced (but in cis) 3 nt upstream of the predicted ATG (data not shown). This putative exon matched sequences 2.5 kilobases upstream of the ife-5 gene on the C. elegans genomic clone, Y57A10. It contained a 93-nucleotide open reading frame followed by a termination codon and then by two out-of-frame ATGs. Although this product may correspond to an authentic bicistronic mRNA, it more likely represents an incompletely spliced intermediate or an aberrant splicing product. Overall, these data suggest that ife-1 mRNAs are trans-spliced to SL2 and that ife-2, ife-3, ife-4, and probably ife-5 mRNAs are trans-spliced to SL1.

**DISCUSSION**

Previously we identified three eIF4E isoforms in *C. elegans*, IFE-1, IFE-2, and IFE-3 (20). The present study identifies two more isoforms, IFE-4 and IFE-5. Because the sequencing of the

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**TABLE II**

Viability of F1 embryos following ife gene inactivation by RNAi

| Set  | Gene(s) | Number of worms injected | Embryonic lethality |
|------|---------|--------------------------|--------------------|
| 1    | ife-3  | 64                       | 100                |
| 2    | ife-1  | 75                       | 0                  |
| 3    | ife-2  | 56                       | 0                  |
| 4    | ife-5  | 40                       | 0                  |
| 5    | ife-1 + ife-2 | 12                 | 75                |
| 6    | ife-1 + ife-5 | 9                  | 0                 |
| 7    | ife-2 + ife-5 | 12                 | 89                |
| 8    | ife-1 + ife-2 + ife-5 | 60              | 99                |
| 9    | ife-4  | 73                       | 0                  |
| 10   | ife-4 + ife-1 | 12                | 0                 |
| 11   | ife-4 + ife-2 | 8                  | 0                 |
| 12   | ife-4 + ife-5 | 17                 | 0                 |
| 13   | ife-4 + ife-1 + ife-5 | 14          | 0                 |

* Results are organized according to the group of IFEs (A, B, or C; see "Discussion") targeted for inactivation.
* Young adult hermaphrodites were injected, and F1 progeny were monitored for 4 days.
* Calculated as the number of dead embryos produced divided by the total number of embryos produced × 100%.

**FIG. 4. ife mRNAs are trans-spliced.** Poly(A)⁺ RNA (40 ng) from *C. elegans* was subjected to RT-PCR (35 cycles) using ife-specific primers in conjunction with SL1 or SL2 primers (Table I). A, primary RT-PCR products were resolved by electrophoresis in a 2% agarose gel and stained with ethidium bromide. B, approximately 20 ng of purified primary RT-PCR product was subjected to secondary PCR (20 cycles) using ife-2- or ife-5-specific primers (primers 2.2 and 5.3, respectively) in conjunction with SL1 or SL2 primers, and the products were analyzed as in A. C, organization of the ife-1 operon. Five predicted genes are encoded in the operon: (a) a homolog of the mouse *nud C* gene, (b) a putative CCAAT-binding transcription factor gene, (c) translation initiation factor gene, (d) a putative acetyl-CoA thiolase gene, and (e) a homolog of the mouse *myotubularin/mmt* gene.
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C. elegans genome is essentially complete (21) and because no other obvious candidate genes are found by BLAST sequence searches (22), these five proteins are likely to represent the complete set. Their characterization with respect to primary structure, gene structure, cap binding specificity, and requirement for embryonic viability allows us to group them into three classes.

Class A contains IFE-3, the most similar of the five IFE proteins to human eIF4E-1 (47% identity). Mammalian eIF4E-1 is by far the best characterized translational cap-binding protein (10, 39). It is found free in the cytoplasm, in a complex with PHAS-1 (4E-BP), and in initiation complexes bound to eIF4G, where it brings together the 5’- and 3’-termini of the mRNA, the 40 S ribosomal subunit, and the RNA helicase eIF4A. The rate of protein synthesis and regulation of the cell cycle are highly dependent on intracellular levels of eIF4E-1 (10, 40) and eIF4G-1 (41, 42). Depletion of eIF4E-1 levels by antisense RNA (43) or binding to PHAS-1 (44) results in reduced protein synthesis and slow cell growth; elevation of eIF4E-1 levels by ectopic vector expression or microinjection causes accelerated cell growth (45), malignant transformation (46), and protection against apoptosis (47). eIF4E-1 discriminates against TMG caps in favor of MMC caps (18, 19), a characteristic shared by IFE-3 (20). Of all the IFE proteins, only IFE-3 is absolutely required for embryonic viability (Table II, Set 1). Surprisingly, the other IFE proteins failed to substitute for IFE-3, although all of them bind to MMC caps (Ref. 20 and Fig. 2A). This is not due to level of expression, because IFE-3 and IFE-1 are approximately equally abundant in preparations of cap-binding proteins from C. elegans (20). Therefore, IFE-3 plays some unique role in protein synthesis (e.g. specific interactions with other initiation factors, binding to non-trans-spliced mRNA, tissue distribution, temporal expression, etc.) that makes it essential to the nematode.

Class B includes IFE-1, IFE-2, and IFE-5. These isoforms are the most closely related in primary structure of the five proteins (57–80% identity; Fig. 1B), and they form a cluster on the phylogenetic tree (Fig. 1C). Their genes contain an identically placed intron that is absent in IFE-3 and IFE-4. Finally, all three bind TMG as well as MMC caps, whereas IFE-3 and IFE-4 bind only MMC caps. These similarities in structure and function suggest that Class B IFE genes are descended from a common ancestral gene.

The structural basis for the difference in cap-binding specificities of the IFE proteins is not known. Two possibilities are: (a) the proteins that bind MMC caps exclusively (IFE-3 and IFE-4) are unable to bind TMG caps because of steric hindrance with the additional methyl groups on N2, but this steric hindrance is absent in the TMG-binding proteins (IFE-1, IFE-2, and IFE-5) and (b) the TMG-binding proteins contain residues that form Van der Waals’ contacts with the N2 methyl groups, but these residues are missing in the MMG-binding proteins. Thus, it is instructive to examine amino acid sequence motifs common to all three TMG-binding proteins that are absent in both MMG-binding proteins. This comparison can be further limited by the three-dimensional structure of mouse eIF4E-1 (48), which indicates that the residues closest to the N2 position of m7GDP are in the “S1-S2 loop” (Lys-49 to Asp-59) and the “S3-S4 loop” (Asp-96 to Arg-109). There are only four such motifs in TMG-versus MMG-binding proteins: Asn-Asp versus Lys-Ala or Met-Arg at positions 24 and 25 (using the numbering system of IFE-1; see Fig. 1); Ser versus Glu or Asp at position 30; Arg versus Cys or Tyr at position 34; and Asp versus Glu or Thr at position 71. These provide initial candidates for site-directed mutagenesis in an attempt to alter cap specificity.

RNA interference experiments also supported the similarity of the TMG-binding isoforms (Class B). Unlike Class A, the knock-out of any one Class B member did not affect viability (Table II, Set 2), suggesting an overlap in their functions. However, knock-out of all combinations of Class B members except ife-1 plus ife-5 produced embryonic lethality, the strongest effect (99% lethality) being the knock-out of all three Class B members. This suggests that the presence of TMG-binding isoforms is necessary for the viability of the developing embryo. TMG-capped mRNAs have been observed to efficiently associate with polyribosomes in vivo (9), and it is presumed that these proteins promote the translation of TMG-containing mRNAs. However, the three Class B members are not equivalent. IFE-2 was sufficient for viability in the absence of both IFE-1 and IFE-5. Neither IFE-1 nor IFE-5 alone was sufficient in the absence of the other Class B proteins. Only IFE-1 plus IFE-5 were sufficient to compensate for the loss of IFE-2. Paradoxically, IFE-2 binds only weakly to TMG caps (20), yet is more important for viability than the strong TMG-binding isoforms. Class B proteins may be unique to organisms such as nematodes and trypanosomes, which possess TMG-capped mRNAs (8).

Recognition of TMG-capped mRNAs by Class B IFEs may also have an autoregulatory function. Each of the five isoforms is encoded by at least one trans-spliced mRNA. trans-Splicing of ife mRNAs also means that they contain TMG caps, yet only Class B IFE proteins are able to bind such mRNAs. It is possible that the translational efficiency of TMG-capped mRNAs, including all five ife mRNAs, is regulated by the level of Class B proteins. The fact that the ife-1 gene resides in an operon suggests that it may also be coordinately regulated with the other four genes in that operon at the level of transcription. However, the identity of the genes clustered with ife-1 (Fig. 4C) do not immediately suggest a rationale for such coordinate regulation.

Class C consists of IFE-4, which is the most unusual of the C. elegans eIF4E isoforms. It is more similar to Arabidopsis nCBP (17) and mammalian 4E-HP (13) than to any other IFE protein (Fig. 1C). Also, its gene has an intron/exon structure that is unique among the ife genes. RNAi experiments also suggest that the requirement for Class C proteins also differs from that for Class A and Class B proteins. IFE-4 was completely dispensable in the worms; no lethality was produced by knock-out of ife-4 either singly or in combination with other ifes (Table II, Set 3). Either IFE-4 is functionally redundant in the worm or its function is restricted to a small population of cells that have no apparent role in animal development. It is curious that a nonessential eIF4E isoform would be conserved in widely separated species across two eukaryotic kingdoms.

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