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Interaction of three *Caenorhabditis elegans* isoforms of translation initiation factor eIF4E with mono- and trimethylated mRNA 5′ cap analogues★✪

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Translation initiation factor eIF4E binds the m^7G cap of eukaryotic mRNAs and mediates recruitment of mRNA to the ribosome during cap-dependent translation initiation. This event is the rate-limiting step of translation and a major target for translational control. In the nematode *Caenorhabditis elegans*, about 70% of genes express mRNAs with an unusual cap structure containing $m_3^{2,2,7}G$, which is poorly recognized by mammalian eIF4E. *C. elegans* expresses five isoforms of eIF4E (IFE-1,

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Abbreviations: m^7G, 7-methylguanosine; m^7GMP, 7-methyl-GMP; m^7GDP, 7-methyl-GDP; m^7GTP, 7-methyl-GTP; $m_3^{2,2,7}GTP$, 2,2,7-trimethyl-GTP; m^7GpppG, P^1-7-methylguanosine-P^3-guanosine-5′,5′-triphosphate; $m_3^{2,2,7}GpppG$, P^1-2,2,7-trimethylguanosine-P^3-guanosine-5′,5′-triphosphate; m^7G cap, m^7GpppN, where N is any nucleoside; $m_3^{2,2,7}G$ cap, $m_3^{2,2,7}GpppN$, where N is any nucleoside; eIF, eukaryotic initiation factor; IFE, *C. elegans* initiation factor 4E; K_{as}, equilibrium association constant.

IFE-2, etc.). Three of these (IFE-3, IFE-4 and IFE-5) were investigated by means of spectroscopy and structural modelling based on mouse eIF4E bound to m^7GDP. Intrinsic fluorescence quenching of Trp residues in the IFEs by iodide ions indicated structural differences between the apo and m^7G cap bound proteins. Fluorescence quenching by selected cap analogues showed that only IFE-5 forms specific complexes with both m^7G- and $m_3^{2,2,7}$G-containing caps (K_{as} 2×10^6 M^{-1} to 7×10^6 M^{-1}) whereas IFE-3 and IFE-4 discriminated strongly in favor of m^7G-containing caps. These spectroscopic results quantitatively confirm earlier qualitative data derived from affinity chromatography. The dependence of K_{as} on pH indicated optimal cap binding of IFE-3, IFE-4 and IFE-5 at pH 7.2, lower by 0.4 pH units than that of eIF4E from human erythrocytes. These results provide insight into the molecular mechanism of recognition of structurally different caps by the highly homologous IFEs.

The cap structure m^7GpppN (where N is any nucleoside, Fig. 1) is present at the 5′ ends of RNA polymerase II transcripts, including primary transcripts of all eukaryotic mRNAs and most U-type snRNAs (uridine-rich small nuclear RNAs) (Furuichi & Shatkin, 2000). The cap is added enzymatically to RNA precursors in the nucleus during the initial phases of transcription and acts as one of the signals for nuclear export (Hamm & Mattaj, 1990). After export, mRNAs remain in the cytoplasm where they serve as templates for protein synthesis. The cap both stimulates translation and protects against nucleolytic degradation (Rhoads, 1985). By contrast, snRNAs bind Sm proteins in the cytoplasm, their m^7G cap is hypermethylated to $m_3^{2,2,7}$GpppN (Fig. 1), and then they are imported back to the nucleus where they play a catalytic role in the splicing of pre-mRNAs (Mattaj, 1986). The $m_3^{2,2,7}$G cap and Sm proteins constitute a bipartite nuclear targeting signal (Hamm *et al.*, 1990).

The m^7G cap in mRNA is specifically recognized by a 25-kDa protein – termed eIF4E. Binding of eIF4E to the m^7G cap occurs during formation of the 48S initiation complex, which is rate-limiting for translation initiation under normal (e.g., non-stressed) conditions (Raught *et al.*, 2000). eIF4E is in turn bound to the 180-kDa initiation factor eIF4G, which also binds eIF4A, a 46-kDa ATP-dependent RNA helicase, eIF4B, a 70-kDa protein that stimulates eIF4A helicase activity, the 550-kDa complex eIF3, and poly(A)-binding protein (Raught *et al.*, 2000). This complex

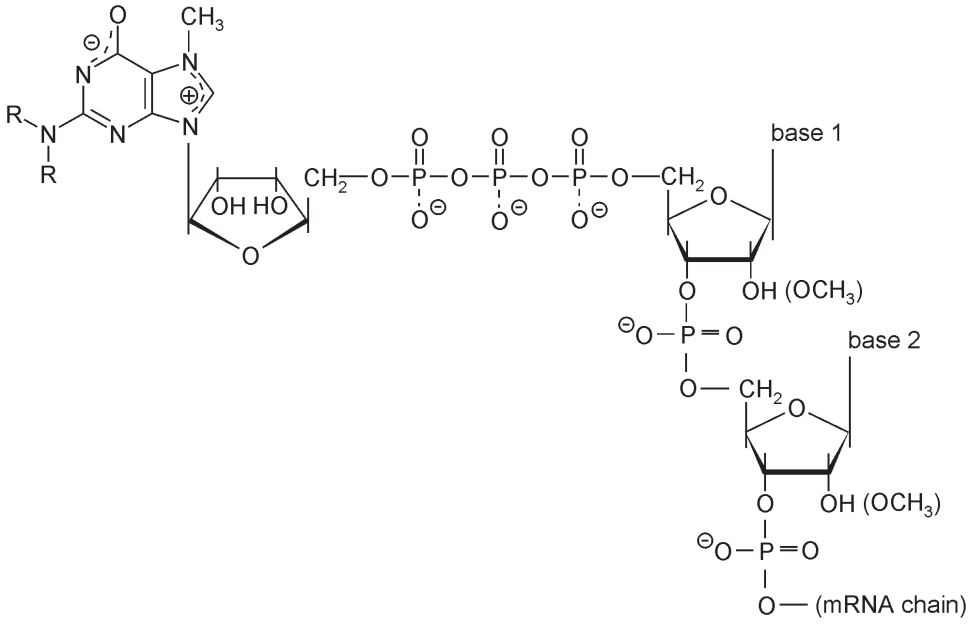

Figure 1. Structures of mono- (R = H) and trimethylated (R = CH_3) mRNA 5′ caps.

serves to recruit mRNA to the 43S initiation complex and unwind secondary structure in the mRNA 5′ untranslated region. The stimulatory effect of eIF4E on translation is regulated through the interactions with the 4E-binding proteins 4E-BP1, 4E-BP2 and 4E-BP3, as well as through phosphorylation of those proteins (Gingras *et al.*, 1999; 2001) and eIF4E itself (Rhoads, 1993; Sonenberg, 1996).

eIF4E levels in cells are also regulated at the transcriptional level (Rosenwald *et al.*, 1993). Abnormal overexpression of eIF4E has a profound effect on cellular growth and tumorigenesis (Rhoads, 1991; Sonenberg, 1996; De Benedetti & Harris, 1999). eIF4E overexpression activates the *ras* oncogene whereas overexpression of GAP, a negative regulator of Ras, reverses the transformed phenotype (Lazaris-Karatzas *et al.*, 1992). The aggressive growth phenotype of transformed cells is also suppressed by eIF4E antisense RNA (De Benedetti *et al.*, 1991) or by overexpressing 4E-BP1 and 4E-BP2 (Rousseau *et al.*, 1996). eIF4E is involved also in regulating apoptosis. Elevated eIF4E levels prevent apoptosis of NIH-3T3 cells subjected to serum deprivation (Polunovsky *et al.*, 1996). Overexpression of 4E-BP1 or treatment with rapamycin of *ras*-transformed fibroblasts – both factors suppressing the activity of eIF4E – increases their susceptibility to apoptosis (Tan *et al.*, 2000).

The primary structure of eIF4E is known for a wide variety of plant, animal and fungal organisms (McKendrick *et al.*, 1999). Interestingly, multiple isoforms of eIF4E differing in primary structure have been found in a single species, e.g., two isoforms in humans, eIF4E-1 (Rychlik *et al.*, 1987) and 4E-HP (Rom *et al.*, 1998), and three isoforms in plants (Metz *et al.*, 1992; Allen *et al.*, 1992; Rodriguez *et al.*, 1998; Ruud *et al.*, 1998). The amino-acid sequences in all these proteins exhibit conservation of a central core domain, suggesting a similar tertiary structure of the protein. The cocrystal structure of mouse eIF4E bound to m^7GDP (Marcotrigiano *et al.*, 1997) and NMR structure of yeast eIF4E bound to m^7GDP (Matsuo *et al.*, 1997) showed that the purine moiety is sandwiched between the indole rings of two Trp residues (Trp-56 and Trp-102 in mouse, Trp-58 and Trp-104 in yeast). Glu-103 in mouse eIF4E (equivalent to Glu-105 in yeast eIF4E) interacts with the N1 and N2 protons of m^7G by hydrogen bonding, whereas the N7 methyl group makes a van der Waals contact with the side chain of Trp-166.

Although the mRNAs of most eukaryotic organisms contain exclusively m^7G caps, those of the nematode *Caenorhabditis elegans* contain both $m_3^{2,2,7}$G caps (transcribed from about 70% of the genes) and m^7G caps (transcribed from about 30% of the genes) (van Doren & Hirsh, 1990; Zorio *et al.*, 1994). The $m_3^{2,2,7}$G cap is acquired along with a conserved 22-nucleotide RNA sequence during *trans*-splicing of pre-mRNA (Blumenthal, 1998). Both types of *C. elegans* mRNA (m^7G-capped and $m_3^{2,2,7}$G -capped) are capable of associating with polysomes and being translated (Liou & Blumenthal, 1990).

All eIF4E proteins from higher eukaryotes characterized to date strongly prefer the m^7G over the $m_3^{2,2,7}$G cap. This is based on several types of experiments: inhibition of *in vitro* translation by cap analogues (Cai *et al.*, 1999), translation of mRNAs capped with modified structures (Darżynkiewicz *et al.*, 1988), and quenching of intrinsic eIF4E fluorescence by titration with cap analogues (Carberry *et al.*, 1990; Wieczorek *et al.*, 1999). The fact that *C. elegans* expresses and translates $m_3^{2,2,7}$-capped mRNAs suggests that eIF4E of this organism may be different. Recently five isoforms of eIF4E were identified in *C. elegans* (Jankowska-Anyszka *et al.*, 1998; Keiper *et al.*, 2000). Experiments employing affinity chromatography on m^7GTP- and $m_3^{2,2,7}$GTP-Sepharose provided qualitative evidence that the eIF4E isoforms differed in the ability to discriminate between m^7G- and $m_3^{2,2,7}$G-containing caps. The aim of the present study was to obtain quantitative information on the cap binding properties of *C. elegans* eIF4E isoforms by fluorescence quenching.

MATERIALS AND METHODS

Materials. Recombinant *C. elegans* eIF4E isoforms (IFE-3, IFE-4 and IFE-5) were expressed in *E. coli* as described previously (Jankowska-Anyszka *et al.*, 1998; Keiper *et al.*, 2000). After final purification by affinity chromatography on m^7GTP-Sepharose, the proteins were subjected to dialysis in 20 mM Hepes, pH 7.5, 1 mM EDTA, 600 mM KCl, 5% (v/v) glycerol to remove bound m^7GTP. Total concentrations of eIF4E isoforms were determined from absorption, using molar extinction coefficients calculated on the base of number of tryptophan and tyrosine residues in the proteins (McCubbin *et al.*, 1988). m^7GTP (Darżynkiewicz *et al.*, 1985), $m_3^{2,2,7}$GTP (Jankowska *et al.*, 1993), m^7GpppG and $m_3^{2,2,7}$GpppG (Stępiński *et al.*, 1995) were synthesized as described previously.

UV absorption and fluorescence measurements. UV absorption spectra were recorded on a Cary 300 spectrophotometer and fluorescence spectra on a Perkin Elmer LS 50B luminescence spectrometer, applying the conventional right-angle geometry of detection in a 0.4 cm × 1.0 cm cell. The temperature of the cell-housing block was adjusted to 20 ± 0.1°C. For all fluorescence measurements, the excitation wavelength of 280 nm, corresponding to the absorption maximum of eIF4E, was used along with the excitation bandwidth of 5 nm and the emission bandwidth of 8 nm. The intensity of the excitation beam was reduced 50 fold with a special filter to protect the protein during titration. Fluorescence quenching was performed by adding 1.0 to 60 μl aliquots of the cap analogue at 150 μM concentration to 1200 μl of IFE solution (concentration 0.2 to 0.6 μM) in 20 mM Hepes (pH range 6.6 to 8.4) containing 100 mM KCl, 0.2 mM EDTA and 1 mM dithiothreitol. Iodide titrations were performed the same way by adding 3 M KI to IFE solution in a pH 7.2 buffer.

Due to partial overlap of the IFE and cap fluorescence spectra, the fluorescence emission due solely to the protein was obtained by subtracting the ligand contribution using least-square fitting. The spectra were subsequently corrected for dilution of the sample during the course of titration and for absorption of the cap analog (inner-filter effect) as described previously (Wieczorek *et al.*, 1993). Fluorescence intensities were obtained by integrating the whole, corrected fluorescence spectrum. The equilibrium association constant, K_{as}, of the IFE-cap complex was determined by non-linear least-square fitting of the theoretical curve to the experimental data points, according to the equation.

$$\Delta F = \frac{\Delta F_{\max}}{2C_{P,tot}}\left\{C_{L,tot} + C_{P,tot} + \frac{1}{K_{as}} - \sqrt{\left(C_{L,tot} + C_{P,tot} + \frac{1}{K_{as}}\right)^2 - 4C_{L,tot}C_{P,tot}}\right\}$$

where $C_{L,tot}$ and $C_{P,tot}$ are the total concentration of the cap analogue and IFE protein, respectively; $\Delta F = F_0 - F$ is the difference between the initial IFE fluorescence intensity (F_0) and that for the $C_{L,tot}$ concentration of the cap analogue (F); and $\Delta F_{max} = F_0 - F_{eq}$ is the difference between the initial and the final (F_{eq}) fluorescence intensities, after saturation of the protein with the cap analogue. Data analysis and calculations were performed by means of the programs Prism 2 (GraphPad Software, Inc.) and Origin 6 (Microcal Software, Inc.).

NMR spectroscopy. ^{1}H NMR spectra of m^7GMP were recorded on a Varian UNITYplus 500 MHz spectrometer at 25°C at 3 mM concentration in aqueous phosphate buffer (10% of ^2H_2O for signal locking), and at pH values of

7.2, 7.7, 7.9, or 8.2. The strong water signal was suppressed by presaturation.

Modelling of the IFE tertiary structures by homology. Three-dimensional molecular models of IFE-3, IFE-4 and IFE-5 were built with the MODELLER program (Sali & Blundell, 1993) using the crystal structure of the mouse eIF4E–m^7GDP complex (PDB code: 1EJ1) (Marcotrigiano *et al.*, 1997) as a template. Initially, the CLUSTAL W program (Thompson *et al.*, 1994) was used to generate multiple sequence alignments for the proteins. Taking into account the general requirements of the eIF4E fold, some corrections were performed manually to preserve conservation of specific residues and to adjust the exact positions of inserted and deleted amino-acid sequences. In some cases, 3D evaluation procedure (Venclovas *et al.*, 1999) was used to obtain the final structure-based sequence alignment. The final models were subjected to visual inspection to remove improper packing of residues, cavities and steric clashes.

The water- and iodide-accessible surfaces of the proteins were determined by means of the program NACCESS (S.J. Hubbard, J.M. Thornton, Department of Biochemistry and Molecular Biology, University College, London, GB, 1993), using van der Waals radii of 1.4 Å and 2.15 Å, respectively.

RESULTS AND DISCUSSION

The five isoforms of eIF4E existing in *C. elegans* may be divided into three classes (Keiper *et al.*, 2000) on the basis of their primary sequences, cap-binding specificities, and requirements for embryonic viability (Jankowska-Anyszka *et al.*, 1998; Keiper *et al.*, 2000). Class A contains IFE-3, the isoform most similar to human eIF4E and the only isoform that is alone required for viability. IFE-3, like mammalian eIF4E, is retained on m^7GTP- but not $m_3^{2,2,7}$GTP-Sepharose. Class B consists of IFE-1, IFE-2 and IFE-5, proteins with high primary sequence identity, which are retained on both m^7GTP- and $m_3^{2,2,7}$GTP-Sepharose. None of these three proteins alone are required for viability, but the absence of all of them (caused by interfering RNA) causes 99% embryonic lethality. Class C contains IFE-4, the form most different in primary structure from the others; it is not essential for viability and is retained by m^7GTP- but not $m_3^{2,2,7}$GTP-Sepharose.

We chose IFE-3, IFE-4 and IFE-5 as representatives of the three classes for further physicochemical characterization and quantitative determination of cap binding specificity. The molecular structures of eIF4E isoforms from *C. elegans* have not yet been experimentally determined. In order to have a preliminary basis to interpret the spectroscopic data, the structures of IFE-3, -4 and -5 were modelled by sequence homology as described in Materials and Methods, taking advantage of the high similarity of the IFE primary structures (50% for IFE-3, 25% for IFE-4 and 42% for IFE-5) to that of mouse eIF4E. The known crystallographic structure of the mouse eIF4E-m^7GDP complex was used as template (Marcotrigiano *et al.*, 1997). According to expectations, the modelled structures of all three isoforms (not shown) appeared to have very similar tertiary folds, and were similar to that of the template eIF4E. The model structures gave no clear explanation for the observed differences in affinities of mono- and trimetyloguanosine caps for the eIF4E isoforms.

Fluorescence spectra of IFEs

The values of emission maximum and quantum yield of Trp residues in proteins can vary greatly due to variations in protein structure (Lakowicz, 1999). Fluorescence intensities of equimolar solutions of the IFE proteins (Fig. 2A) reflect to some extent the number of Trp residues in each protein: ten in IFE-5, nine in IFE-3, and five in IFE-4 (Jankowska-Anyszka *et al.*, 1998; Keiper *et al.*, 2000). The proportionality between fluorescence emis-

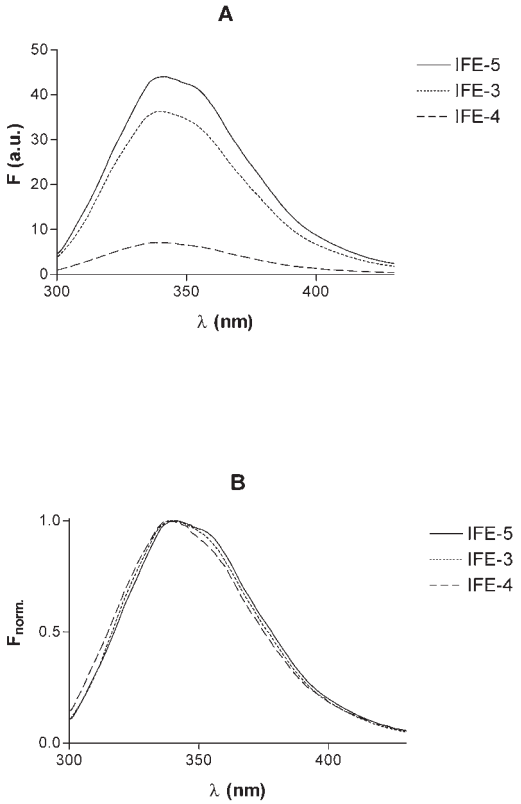

Figure 2. Fluorescence emission spectra of IFE isoforms.

Fluorescence spectra (excitation wavelength 280 nm) in 20 mM Hepes, pH 7.2, 100 mM KCl, 0.2 mM EDTA, 1 mM DTT, at 1 μM protein concentration (A) and after normalization to unity at the wavelengths corresponding to the maximal fluorescence intensities (B).

sion and Trp content holds for IFE-5 and IFE-3, but a large deviation is observed for IFE-4; one would have expected about 50% signal intensity for IFE-4 cf. IFE-5 if each Trp contributed equally to the total emission. The data suggest that more of the Trp residues in IFE-4 are buried inside the protein, leading to fluorescence quenching by charged or aromatic amino-acid side chains. Our computer modelling of the three IFE structures bound to m^7GDP showed that the fluorescence of the buried Trp residues is likely to be quenched by Arg, Lys, or Phe residues situated 3–5 Å from the indole rings. The fraction of unquenched, solvent-accessible Trp residues in the model structure of IFE-4 (Table 1) is smaller than in IFE-3 and IFE-5, thus leading to a substantially lower fluorescence emission of IFE-4. Iodide quenching experiments (Fig. 3) yielded

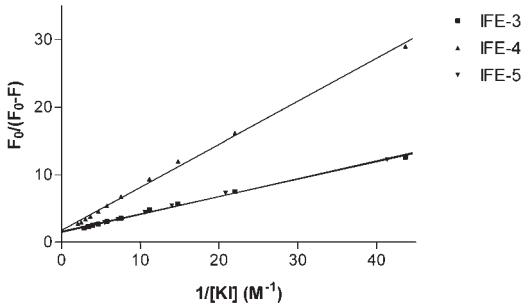

Figure 3. Modified Stern-Volmer plot of IFE fluorescence quenching by potassium iodide.

the fraction of Trp fluorescence accessible to quenching: 65% for IFE-3, 57% for IFE-4, and 68% for IFE-5 (Table 1). These values independently confirm the lower contribution of buried Trp residues to the total fluorescence for IFE-4. In the normalized spectra of the IFE proteins (Fig. 2B), the positions of the emission maxima are in good agreement with the iodide quenching data. The IFE-3 spectrum is slightly blue-shifted comparing to that of IFE-5, while the IFE-4 emission maximum is blue-shifted even more (Table 1).

The number of solvent-exposed Trp residues and their solvent-accesible surfaces, as calculated from the modelled structures, roughly agree with the corresponding fractions of total fluorescence quenched by the iodide ions (Table 1). However, the area of solvent-accessible Trp surface is greater in IFE-3 than in IFE-5, whereas the proportion of fluorescence quenched by iodide was in the reverse order. The discrepancies could be ascribed, at least partially, to differences between the structures of the apo and ligand-bound proteins, due to a probable conformational rearrangement upon binding of the ligand.

Affinity of mono- and trimethylated cap analogues for the IFEs

Association of eIF4E with a cap analogue results in quenching of intrinsic Trp fluores-

Table 1. Iodide quenching of apo IFEs fluorescence *vs.* Trp accessibility to solvent in the modelled IFE structures

Protein	Total fluorescence quenching by iodide [%][a]	Number of exposed Trp residues[b]	Number of buried Trp residues[b]	Total surface of solvent-accesible Trp residues [Å^2][b]	Emission maximum λmax [nm][c]
IFE-3	65	4	5	435	333.5
IFE-4	57	1	4	158	337.5
IFE-5	68	4	6	400	341.0
EIF4E$_d$		3	5	247	

[a]Data from Fig. 3; [b]data from molecular modelling (see Materials and Methods); [c]data from Fig. 2; [d]data from crystallography (Marcotrigiano *et al.*, 1997).

cence in the protein (Wieczorek *et al.*, 1999; McCubbin *et al.*, 1988; Carberry *et al.*, 1989; Ueda *et al.*, 1991), presumably due to stacking of m^{7}G between two Trp residues (Marcotrigiano *et al.*, 1997; Matsuo *et al.*, 1997). The equilibrium association constant, K_{as}, can be derived from titration of eIF4E with increasing amounts of a cap analogue. High K_{as} values of 10^6 M^{-1} to 10^7 M^{-1} reflect the tight, specific binding of the ligand to the protein. A 30% decrease of the fluorescence intensity was observed in the case of IFE-5 (Fig. 4A) as well as IFE-3 and -4 (not shown) on binding of the monomethylated cap analogues m^{7}GTP and m^{7}GpppG. Similar fluorescence quenching was observed for IFE-5, when titrated with the trimethylated caps m$_3^{2,2,7}$GTP (Fig. 4B) and m$_3^{2,2,7}$GpppG (not shown). The spectra shown in Figs. 4A and 4B are uncorrected for the emission of the free ligand. The decrease in fluorescence intensity due to the ligand quenching was extracted from the spectra and corrected for sample dilution and inner-filter effect to yield the resulting ΔF. The theoretical curve was used to fit numerically the ΔF values (Fig. 4A and B, insets) as described in Materials and Methods, providing the equilibrium association constants K_{as} of the IFE–cap analogue complexes (Table 2). The titration experiments were performed at pH 7.2, optimal for the specific binding of the cap analogues to the IFE proteins (see below). The K_{as} values for the specific binding of the cap analogues to

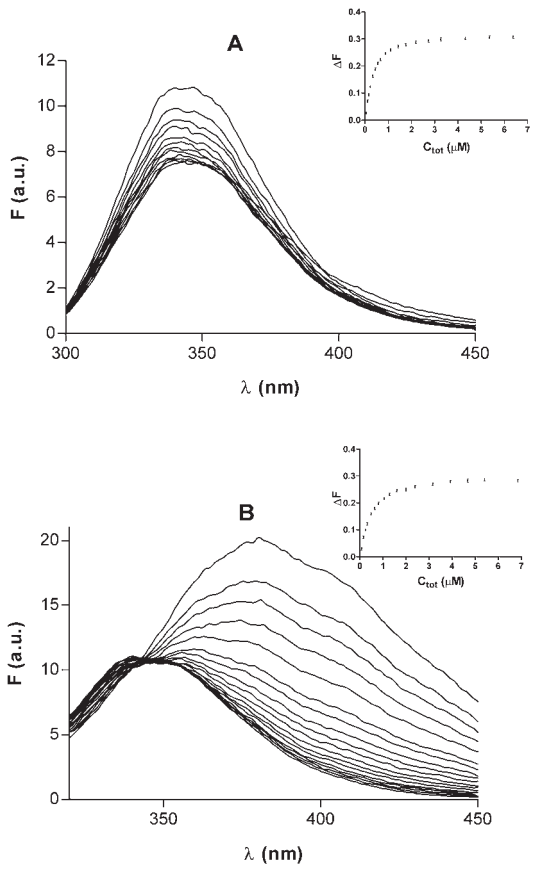

Figure 4. Typical IFE-5 fluorescence quenching spectra upon increasing concentration.

(A) m^{7}GTP, (B) m$_3^{2,2,7}$GTP. The corresponding titration curves are shown as insets.

the IFE proteins are in the range of 2×10^6 M^{-1} to 7×10^6 M^{-1}. Nonspecific IFE–cap interactions are characterized by K_{as} values

more than two orders of magnitude lower. In the latter case, only an upper limit of about $0.06 \times 10^6\ M^{-1}$ can be estimated from such titrations.

In chromatographic experiments (Jankowska-Anyszka *et al.*, 1998; Keiper *et al.*, 2000), the proteins retained on and eluted from either m^7GTP-Sepharose or $m_3^{2,2,7}$GTP-Sepharose were subsequently subjected to SDS/polyacrylamide gel electrophoresis and stained with Coomassie blue. All the isoforms considered (IFE-3, -4 and -5) were retained on the former column whereas only IFE-5 was captured on the latter. Although the intensity of staining need not always reflect the amount of a protein, the relative intensities of the bands corresponding to IFE-3, IFE-4 and IFE-5 correlate with the amounts of the isoforms bound to m^7GTP or $m_3^{2,2,7}$GTP during the titration experiments. Thus, the quantitative, fluorometrically determined values of K_{as} are in good agreement with the qualitative results of affinity chromatography.

The K_{as} values for the association of m^7GTP and m^7GpppG with the full-length human eIF4E, 1.2×10^6 and 5.7×10^5, respectively (Wieczorek *et al.*, 1999), are close to those measured for *C. elegans* eIF4E isoforms with the same analogues. The dinucleotide analogue m^7GpppG is bound to each IFE protein weaker than its mononucleotide counterpart, similarly as in the case of human eIF4E. This effect can be attributed to two factors: 1) disadvantageous entropic contribution to the free energy of binding in the case of the longer molecule, and 2) more negative charge of the m^7GTP phosphate chain in comparison with m^7GpppG, the former being better stabilized by the positively charged Arg and Lys residues in the cap binding center (Marcotrigiano *et al.*, 1997; Matsuo *et al.*, 1997). IFE-3 and IFE-4 discriminate strongly between mono- and trimethylated cap analogues, as does mammalian eIF4E. Double methylation at N^2 of the m^7G ring disrupts at least one hydrogen bond between the cap and eIF4E. This feature may be responsible for the 4-fold decrease in translation activity of β-globin mRNA capped with $m_3^{2,2,7}$G compared with m^7G (Darżynkiewicz *et al.*, 1988), and the 17-fold decrease in the inhibitory activity of $m_3^{2,2,7}$GTP in comparison to m^7GTP in a rabbit reticulocyte translation system (Cai *et al.*, 1999). By contrast, the affinity of mono- and trimethylated cap analogues for IFE-5 is similar (Table 2). Since IFE-5 has a high sequence homology with eIF4E as well as with IFE-3 and IFE-4, this raises a fundamental question concerning the differences in the cap recognition pattern among those closely related isoforms. The basis for the discrimination between the two cap structures by the active centers of these proteins may be subtle, in light of the structural similarities of the isoforms derived from homology modelling.

Table 2. Equilibrium association constants K_{as} (M^{-1}), e.s.d. ± 30%, of IFE-cap complexes at pH 7.2 and temperature 20°C

Cap analogues	IFE-3	IFE-4	IFE-5
m^7GTP	5×10^6	4×10^6	7×10^6
m^7GpppG	3×10^6	2×10^6	3×10^6
$m_3^{2,2,7}$GTP	$<6 \times 10^4$	$<6 \times 10^4$	3×10^6
$m_3^{2,2,7}$GpppG	$<6 \times 10^4$	$<6 \times 10^4$	2×10^6

K_{as} as a function of pH

The spectrophotometrically determined apparent K_{as} values depend on environmental parameters like pH, ionic strength, and types of ions in solution (Record *et al.*, 1991). The

pH dependence of K_{as} for the binding of m^7GTP and $m_3^{2,2,7}GTP$ to IFE-5 (Fig. 5)

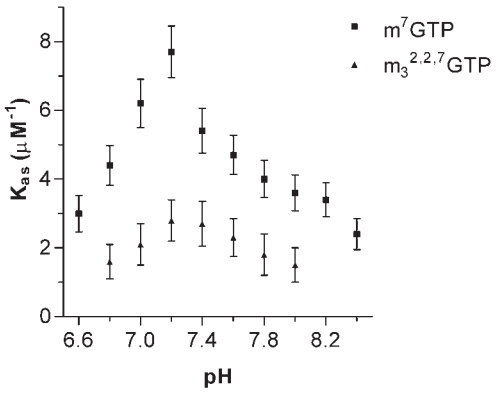

Figure 5. Association equilibrium constant K_{as} of binding of m^7GTP and $m_3^{2,2,7}GTP$ to IFE-5 as a function of pH.

shows that optimal affinity occurs at pH 7.2, lower than pH 7.6 that was observed in the case of full-length eIF4E from human erythrocytes (Carberry *et al.*, 1989). A pH optimum of 7.6 was also found for cell-free translation of globin mRNA in reticulocyte lysate (Rhoads *et al.*, 1983). Similarly, the degree of inhibition of *in vitro* translation by m^7GTP increased from 48% at pH 6.8 up to 82% at pH 7.8 at 200 μM concentration of the cap analog. In this pH range m^7G is a mixture of the positively charged and zwitterionic forms due to dissociation of the N1 proton (pK about 7.2–7.5, Wieczorek *et al.*, 1990). Although preliminary spectroscopic (Carberry *et al.*, 1989) and translation data (Rhoads *et al.*, 1983) pointed to the zwitterionic form of cap that specifically binds eIF4E, only the cationic form of m^7GDP was postulated to be recognized by eIF4E on the basis of the structural, crystallographic studies (Marcotrigiano *et al.*, 1997; Matsuo *et al.*, 1997). The N1 proton is required for hydrogen bond donation to Glu-103. Additionally, the cap analogues at elevated pH in solution undergo opening of the five-membered ring of the 7-substituted guanine, followed by hydrolysis of the glycosidic bond (Darżynkiewicz *et al.*, 1990). The stability of the cap analogues was tested by NMR (not shown). m^7GMP was stable between pH 7.2 and 8.2 but generated breakdown products at higher pH values, where the zwitterionic form dominates.

Our results also suggest that the cationic form of cap analogs is recognized by IFEs. The observed differences in the pH dependence of the IFEs and eIF4E binding can be rather attributed to differences in procedures of the protein sample preparation, e.g. protein isolation and purification.

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REFERENCES

Allen ML, Metz AM, Timmer RT, Rhoads RE, Browning KS. (1992) Isolation and sequence of the cDNA encoding the subunits of the isozyme form of wheat protein synthesis initiation factor 4F. *J Biol Chem.;* **267:** 23232–6.

Blumenthal T. (1998) Gene clusters and polycistronic transcription in eukaryotes. *BioEssays.;* **20:** 480–7.

Cai A, Jankowska-Anyszka M, Centers A, Chlebicka L, Stępiński J, Stolarski R, Darżynkiewicz E, Rhoads RE. (1999) Quantitative assessment of mRNA cap analogues as inhibitors of *in vitro* translation. *Biochemistry.;* **38:** 8538–47.

Carberry SE, Rhoads RE, Goss DJ. (1989) A spectroscopic study of the binding of m^7GTP and m^7GpppG to human protein synthesis

initiation factor 4E. *Biochemistry.;* **28:** 8078–83.

Carberry SE, Darżynkiewicz E, Stępiński J, Tahara SM, Rhoads RE, Goss DJ. (1990) A spectroscopic study of the binding of N-7-substituted cap analogues to human protein synthesis initiation factor 4E. *Biochemistry.;* **29:** 3337–41.

Darżynkiewicz E, Ekiel I, Tahara SM, Seliger LS, Shatkin AJ. (1985) Chemical synthesis and characterization of 7-methylguanosine cap analogues. *Biochemistry.;* **24:** 1701–7.

Darżynkiewicz E, Stępiński J, Ekiel I, Jin Y, Haber D, Sijuwade T, Tahara SM. (1988) β-Globin mRNAs capped with m^7G, $m_2{}^{2,7}G$ or $m_3{}^{2,2,7}G$ differ in intrinsic translation efficiency. *Nucleic Acids Res.;* **16:** 8953–62.

Darżynkiewicz E, Stępiński J, Tahara SM, Stolarski R, Ekiel I, Haber D, Neuvonen K, Lehikoinen P, Labadi I, Lonnberg, H. (1990) Synthesis, conformation and hydrolytic stability of P^1,P^3-dinucleoside triphosphates related to mRNA 5′-cap, and comparative kinetic studies on their nucleoside and nucleoside monophosphate analogs. *Nucleosides & Nucleotides.;* **9:** 599–618.

De Benedetti A, Harris AL. (1999) eIF4E expression in tumors: its possible role in progression of malignancies. *Int J Biochem Cell Biol.;* **31:** 59–72.

De Benedetti A, Joshi-Barve S, Rinker-Schaeffer C, Rhoads RE. (1991) Expression of antisense RNA against initiation factor eIF-4E mRNA in HeLa cells results in lengthened cell division times, diminished translation rates, and reduced levels of both eIF-4E and the p220 component of eIF-4F. *Mol Cell Biol.;* **11:** 5435–45.

Furuichi Y, Shatkin AJ. (2000) Viral and cellular mRNA capping: past and prospects. *Adv Viral Res.;* **55:** 135–84.

Gingras A-C, Raught B, Sonenberg N. (1999) eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu Rev Biochem.;* **68:** 913–63.

Gingras A-C, Raught B, Gygi SP, Niedźwiecka A, Miron M, Burley SK, Polakiewicz RD, Wysłouch-Cieszynska A, Aebersold R, Sonenberg N. (2001) Hierarchical phosphorylation of the translation inhibitor 4E-BP1. *Genes Dev.;* **15:** 2852–64.

Hamm J, Mattaj IW. (1990) Monomethylated cap structures facilitate RNA export from the nucleus. *Cell.;* **63:** 109–18.

Hamm J, Darżynkiwicz E, Tahara SM, Mattaj IW. (1990) The trimethylguanosine cap structure of U1 snRNA is a component of a bipartite nuclear targeting signal. *Cell.;* **62:** 569–77.

Jankowska M, Temeriusz A, Stolarski R, Darżynkiewicz E. (1993) Synthesis of $m_2{}^{2,7}$GTP- and $m_3{}^{2,2,7}$GTP-Sepharose 4B: new affinity resins for isolation of cap binding proteins. *Collect Czech Chem Commun.;* **58:** 132–7.

Jankowska-Anyszka M, Lamphear BJ, Aamodt EJ, Harrington T, Darżynkiewicz E, Stolarski R, Rhoads RE. (1998) Multiple isoforms of eukaryotic protein synthesis initiation factor 4E in *Caenorhabditis elegans* can distinguish between mono- and trimethylated mRNA cap structures. *J Biol Chem.;* **273:** 10538–42.

Keiper BD, Lamphear BJ, Deshpande AM, Jankowska-Anyszka M, Aamodt EJ, Blumenthal T, Rhoads RE. (2000) Functional characterization of five eIF4E isoforms in *Caenorhabditis elegans. J Biol Chem.;* **275:** 10590–6.

Lakowicz JR. (1999) *Principles of fluorescence spectroscopy.* Kluver Academic/Plenum Publishers, New York.

Lazaris-Karatzas A, Smith MR, Frederickson RM, Jaramillo ML, Liu YL, Kung HF, Sonenberg N. (1992) *Ras* mediates translation initiation factor 4E-induced malignant transformation. *Genes Dev.;* **6:** 1631–42.

Liou R-F, Blumenthal T. (1990) *trans*-Spliced *Caenorhabditis elegans* mRNAs retain trimethylguanosine caps. *Mol Cell Biol.;* **10:** 1764–8.

Marcotrigiano J, Gingras A-C, Sonenberg N, Burley SK. (1997) Cocrystal structure of the messenger RNA 5′ cap-binding protein

(eIF4E) bound to 7-methyl-GDP. *Cell.;* **89:** 951–61.

Matsuo H, Li H, McGuire AM, Fletcher CM, Gingras A-C, Sonenberg N, Wagner G. (1997) Structure of translation factor eIF4E bound to m^7GDP and interaction with 4E-binding protein. *Nat Struct Biol.;* **4:** 717–24.

Mattaj IW. (1986) Cap trimethylation of U snRNA is cytoplasmic and dependent on U snRNP protein binding. *Cell.;* **46:** 905–11.

McCubbin WD, Edery I, Altmann M, Sonenberg N, Kay CM. (1988) Circular dichroism and fluorescence studies on protein synthesis initiation factor eIF-4E and two mutant forms from the yeast *Saccharomyces cerevisiae. J Biol Chem.;* **263:** 17663–71.

McKendrick L, Pain VM, Morley SJ. (1999) Translation initiation factor 4E. *Int Biochem Cell Biol.;* **31:** 31–5.

Metz AM, Timmer RT, Browning KS. (1992) Isolation and sequence of a cDNA encoding the cap binding protein of wheat eukaryotic protein synthesis initiation factor 4F. *Nucleic Acids Res.;* **20:** 4096.

Polunovsky V, Rosenwald IB, Tan AT, White J, Chiang L, Sonenberg N, Bitterman PB. (1996) Translational control of programmed cells death: eIF4E blocks apoptosis in growth-factor restricted fibroblasts with physiologically expressed or deregulated Myc. *Mol Cell Biol.;* **16:** 6573–81.

Raught B, Gingras A-C, Sonenberg N. (2000) Regulation of ribosomal recruitment in Eukaryotes. In *Translational control of gene expression.* Sonenberg N, Hershey JWB, Mathews MB. eds, pp 245–93. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Record MT Jr, Ha JH, Fisher MA. (1991) Analysis of equilibrium and kinetic measurements to determine thermodynamic origins of stability and specificity and mechanism of formation of site-specific complexes between proteins and helical DNA. *Methods Enzymol.;* **208:** 291–343.

Rhoads RE. (1985) The cap structure of eukaryotic messenger RNA and its interaction with cap-binding protein. *Prog Mol Subcell Biol.;* **9:** 104–55.

Rhoads RE. (1991) Protein synthesis, cell growth and oncogenesis. *Curr Opin Cell Biol.;* **3:** 1019–24.

Rhoads RE. (1993) Regulation of eukaryotic protein synthesis by initiation factors. *J Biol Chem.;* **268:** 3017–20.

Rhoads RE, Hellmann GM, Remy P, Ebel JP. (1983) Translational recognition of messenger ribonucleic acid caps as a function of pH. *Biochemistry.;* **22:** 6084–8.

Rom E, Kim HC, Gingras A-C, Marcotrigiano J, Favre D, Olsen H, Burley SK, Sonenberg N. (1998) Cloning and characterization of 4EHP, a novel mammalian eIF4E-related cap-binding protein. *J Biol Chem.;* **273:** 13104–9.

Rodriguez CM, Freire MA, Camillere C, Robaglia C. (1998) The *Arabidopsis thaliana* cDNA coding for eIF4E and eIF(iso)4E are not functionally equivalent for yeast complementation and are differentially expressed during plant development. *Plant J.;* **13:** 465–73.

Rosenwald IB, Rhoads DB, Callanan LD, Isselbacher KJ, Schmidt EV. (1993) Increased expression of eukaryotic translation initiation factors eIF-4E and eIF-2 alpha in response to growth induction by c-myc. *Proc Natl Acad Sci U S A.;* **90:** 6175–8.

Rousseau DR, Gingras A-C, Pause A, Sonenberg N. (1996) The eIF4E-binding proteins 1 and 2 are negative regulators of cell growth. *Oncogene.;* **13:** 2415–20.

Ruud KA, Kuhlow C, Goss DJ, Browning KS. (1998) Identification and characterization of a novel cap-binding protein from *Arabidopsis thaliana*. *J Biol Chem.;* **273:** 10325–30.

Rychlik W, Domier LL, Gardner PR, Hellmann GM, Rhoads RE. (1987) Amino acid sequence of the mRNA cap-binding protein from human tissues. *Proc Natl Acad Sci U S A.;* **84:** 945–9.

Sali A, Blundell TL. (1993) Comparative protein modeling by satisfaction of spatial restraints. *J Mol Biol.;* **234:** 779–815.

Sonenberg N. (1996) mRNA 5′ cap-binding protein eIF4E and control of cell growth. In *Transcriptional control.* Hershey JWB, Mathews MB, Sonenberg N. eds, pp 245–69. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Stępiński J, Bretner M, Jankowska M, Felczak K, Stolarski R, Wieczorek Z, Cai A, Rhoads RE, Temeriusz A, Haber D, Darżynkiewicz E. (1995) Synthesis and properties of P^1,P^2-, P^1,P^3- and P^1,P^4-dinucleoside di-, tri- and tetraphosphate mRNA 5′-cap analogues. *Nucleosides & Nucleotides.;* **14:** 717–21.

Tan AT, Bitterman PB, Sonenberg N, Peterson M, Polunovsky V. (2000) Inhibition of Myc-dependent apoptosis by eukaryotic translation initiation factor 4E requires cyclin D1. *Oncogene.;* **19:** 1437–47.

Thompson JD, Higgins DG, Gibson TJ. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.;* **22:** 4673–80.

Ueda H, Maruyama H, Doi M, Inoue T, Morioka H, Tanaka T, Nishikawa S, Uesugi S. (1991) Expression of a synthetic gene for human cap binding protein (human IF-4E) in *Escherichia coli* and fluorescence studies on interaction with mRNA cap structure analogues. *J Biochem* (Tokyo).; **109:** 882–9.

van Doren K, Hirsh D. (1990) mRNAs that mature through *trans*-splicing in *Caenorhabditis elegans* have a trimethylguanosine cap at their 5′ termini. *Mol Cell Biol.;* **10:** 1769–72.

Venclovas C, Ginalski K, Fidelis K. (1999) Addressing the issue of sequence-to-structure alignments in comparative modeling of CASP3 target proteins. *Proteins*.; **37:** (Suppl 3) 73–80.

Wieczorek Z, Darżynkiewicz E, Stępiński J, Lonnberg H. (1990) Fluorescence studies on P^1,P^3-dinucleoside triphosphates related to mRNA cap: acidity and intramolecular stacking. *Collect Czech Chem Commun.;* **55:** 2765–8.

Wieczorek Z, Stępiński J, Darżynkiewicz E, Lonnberg H. (1993) Association of nucleosides and their 5′-monophosphates with a tryptophan containing tripeptide, Trp-Leu-Glu: the source of an overestimation by fluorescence spectroscopy. *Biophys Chem.;* **47:** 233–40.

Wieczorek Z, Niedźwiecka-Kornaś A, Chlebicka L, Jankowska M, Kiraga K, Stępiński J, Dadlez M, Drabent R, Darżynkiewicz E, Stolarski R. (1999) Fluorescence studies on association of human translation initiation factor eIF4E with mRNA cap-analogues. *Z Naturforsch.;* **54c:** 278–84.

Zorio DAR, Cheng NN, Blumenthal T, Spieth J. (1994) Operons as a common form of chromosomal organization in *C. elegans*. *Nature.;* **372:** 270–2.