**Nuclear Eukaryotic Initiation Factor 4E (eIF4E) Colocalizes with Splicing Factors in Speckles**

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**Abstract.** The eukaryotic initiation factor 4E (eIF4E) plays a pivotal role in the control of protein synthesis. eIF4E binds to the mRNA 5' cap structure, m7GppN (where N is any nucleotide) and promotes ribosome binding to the mRNA. It was previously shown that a fraction of eIF4E localizes to the nucleus (Lejbkowicz, F., C. Goyer, A. D'arveau, S. Neron, R. Lemieux, and N. Sonenberg. 1992. Proc. Natl. Acad. Sci. USA. 89: 9612–9616). Here, we show that the nuclear eIF4E is present throughout the nucleoplasm, but is concentrated in speckled regions. Double label immunofluorescence confocal microscopy shows that eIF4E colocalizes with Sm and U1snRNP. We also demonstrate that eIF4E is specifically released from the speckles by the cap analogue m7GpppG in a cell permeabilization assay. However, eIF4E is not released from the speckles by RNase A treatment, suggesting that retention of eIF4E in the speckles is not RNA-mediated. 5,6-dichloro-1-b-D-ribofuranosylbenzimidazole (DRB) treatment of cells causes the condensation of eIF4E nuclear speckles. In addition, overexpression of the dual specificity kinase, Clk/Sty, but not of the catalytically inactive form, results in the dispersion of eIF4E nuclear speckles.

**Key words:** nuclear proteins • peptide initiation factor • RNA caps • RNA splicing • cap-binding protein

**Introduction**

In eukaryotes, all RNA polymerase II transcripts acquire cotranscriptionally the 5'-terminal structure, m7G(5')ppp(5')N termed cap (Salditt-Goregieff et al., 1980). The cap consists of a 7-methylguanosine residue, linked via a 5'-5' triphosphate bridge to the first transcribed nucleotide (Shatkin, 1976). The cap is involved in numerous aspects of mRNA metabolism, including RNA stability (Furuiuchi et al., 1977; Shimotohno et al., 1977; Murthy et al., 1991), 3' end mRNA processing (Hart et al., 1985), pre-mRNA splicing (Konarska et al., 1984; Edery and Sonenberg, 1985), nuclear export (Hamm and Mattaj, 1990; Jarmolowski et al., 1994), and translation initiation (Shatkin, 1985). Mammalian eukaryotic initiation factor 4E (eIF4E) is a 24-kD cap-binding protein (Sonenberg et al., 1978, 1979) that can be purified as a monomer or as a subunit in a complex called eIF4F. eIF4F is a heterotrimeric complex composed of eIF4E, eIF4A, and eIF4G. eIF4A is an ATP-dependent RNA helicase and eIF4G is a modular scaffolding protein that binds to both eIF4E and eIF4A and other proteins (Imataka and Sonenberg, 1997; for review see Gingras et al., 1999). eIF4E plays an important role in the control of gene expression. It is the limiting factor for translation initiation (Duncan et al., 1987) and its overexpression in NIH3T3 cells causes malignant transformation (Lazaris-Karatzas et al., 1993). Conversely, in vivo depletion of eIF4E by antisense RNA partially reverses the transformed phenotype of ras-overexpressing cells (Rinker-Schaeffer et al., 1993).

Nuclear cap-binding proteins that are likely to mediate the various nuclear functions of the cap have been detected and some identified (Patzelt et al., 1983; Rozen and Sonenberg, 1987; Izaurralde et al., 1992). For example, the nuclear cap-binding complex (CBC), composed of CBP20 and CBP80, is involved in mRNA splicing (Izaurralde et al., 1994; Lewis et al., 1996). CBC also stimulates nucleocytoplasmic export of U snRNA (Izaurralde et al., 1995). A study on the Balbiani ring mRNA export of Chironomus tentans demonstrates that CBC binds cotranscriptionally to the cap and accompanies the ribonucleoprotein particle during nuclear export (V isa et al., 1996). A additionally, CBC stimulates mRNA 3' end processing (Flaherty et al., 1997).
Cellular fractionation and immunofluorescence analysis demonstrated that a sizeable fraction (12-33%) of total eIF4E is localized to the nucleus of mammalian cells (Lejbkowicz et al., 1992). Electron microscope studies showed that eIF4E is also present in the nucleus of Saccharomyces cerevisiae (Lang et al., 1994). These results raise the possibility that eIF4E may also play a nuclear role in mRNA metabolism, such as splicing or transport.

Many, but not all, splicing factors are concentrated in sub-nuclear structures termed “speckles”. The speckles (20-50 speckles per nucleus) are irregular shaped bodies. Although the precise function of the speckles remains controversial, there is evidence that the speckles are sites of posttranscriptional splicing (Xing et al., 1993, 1995) and of splicing component storage and/or assembly (Puvion and Puvion-Dutilleul, 1996; Spector, 1996). Here, we show that the nuclear fraction of eIF4E colocalizes with splicing factors in the speckles. We demonstrate that the nuclear distribution of eIF4E is sensitive to RNA polymerase II transcription inhibitors and the availability of cap structures, but not to RNase treatment. Similar to serine/arginine-rich (SR) splicing factors, the localization of eIF4E is regulated by the dual specificity kinase, Clk/Sty.

Materials and Methods

Plasmids and Antibodies

Plasmids encoding myc-Clk/Sty wt and myc-Clk/StyK190R were kindly provided by J.C. Bell (University of Ottawa, Ottawa, Canada). Human anti-Sm sera and human anti-U1 snRNP sera were provided by the Center for Disease Control (Atlanta, GA). mAb 5C3 was a kind gift from X.D. Fu and T. Maniatis (Harvard University, Cambridge, MA). 10C6 is an anti-mouse eIF4E mAb (Lejbkowicz et al., 1992), Texas red- and fluorescein-conjugated secondary antibodies were purchased from Molecular Probes, Inc.

Immunofluorescence Assay

CV-1 monkey kidney cells and HeLa cells were plated at 2 × 10⁴ per chamber on Lab-Tek chamber slides (Nunc) and grown to subconfluence in DME supplemented with 10% FBS. Cells were fixed and processed for immunofluorescence 24 h after transfection. The immunofluorescence shows that nuclear eIF4E is excluded from the nucleoli and concentrated in speckles that are characteristic of those observed to contain splicing factors (Fig. 1). To determine whether eIF4E colocalizes with splicing factors, antibodies against three different components of the splicing machinery were used: anti-SC35 (an SR protein; Fu and Maniatis, 1990); anti-Sm; and anti-U1 snRNP. To ascertain that incubation with the three antibodies results in the characteristic nuclear pattern reported earlier, double label immunofluorescence was performed (Lerner et al., 1981; Fu and Maniatis, 1990). Staining with anti-SC35 (Fig. 2 A, a and d), anti-Sm (Fig. 2 A, b), and anti-U1 snRNP (Fig. 2 A, e) shows a typical speckled pattern. In addition, SC35 colocalizes with Sm (Fig. 2 A, c) and U1 snRNP (Fig. 2 A, f), as visualized in the merged pictures and as previously described (Fu and Maniatis, 1990).

Cell Permeabilization Assay

The assay was done as described previously, except for a few modifications (Adam et al., 1990). In brief, HeLa cells were plated at low density on coverslips, grown in DME/10% FBS for at least 24 h, and the media was changed 2-4 h before the experiment. Coverslips were briefly rinsed in transport buffer (20 mM Hapes/KOH, pH 7.3, 110 mM potassium acetate, 2 mM sodium acetate, 5 mM magnesium acetate, 1 mM EGTA, 2 mM dithiothreitol, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin) and incubated for 4 min at RT in transport buffer containing 40 μg/ml digitonin. Coverslips were gently rinsed and inverted on a paraffin sheet over a drop of transport buffer containing 2 μg/ml BSA and 50 mM mGppG or GpppG. The reaction was done at 30°C for 25 min. Cells were rinsed, fixed in transport buffer containing 3% formaldehyde, and processed for immunofluorescence as described above. This experiment was performed three times with the same results.

RNase Digestion

RNase treatment was performed as previously described (Spector et al., 1991). In brief, cells (CV-1) were fixed in methanol for 2 min at −20°C, rinsed in PBS, and incubated with RNase A (100 μg/ml) for 2 h at RT. Cells were washed several times and processed for immunofluorescence as described.

Transient Transfections

HeLa cells were plated at low density on 60-mm dishes in DME/10% FBS. At 50% confluency, cells were transfected with 10 μg of plasmid DNA by the calcium phosphate transfection method (Graham and Van der Eb, 1973). Cells were fixed and processed for immunofluorescence 24 h after transfection.

Results

Nuclear eIF4E Colocalizes with Splicing Factors

An mAb (10C6) that recognizes eIF4E in the nucleus (Lejbkowicz et al., 1992) was used for immunostaining of HeLa cells. The immunofluorescence shows that nuclear eIF4E is excluded from the nucleoli and concentrated in speckles that are characteristic of those observed to contain splicing factors (Fig. 1). To determine whether eIF4E colocalizes with splicing factors, antibodies against three different components of the splicing machinery were used: anti-SC35 (an SR protein; Fu and Maniatis, 1990); anti-Sm; and anti-U1 snRNP. To ascertain that incubation with the three antibodies results in the characteristic nuclear pattern reported earlier, double label immunofluorescence was performed (Lerner et al., 1981; Fu and Maniatis, 1990). Staining with anti-SC35 (Fig. 2 A, a and d), anti-Sm (Fig. 2 A, b), and anti-U1 snRNP (Fig. 2 A, e) shows a typical speckled pattern. In addition, SC35 colocalizes with Sm (Fig. 2 A, c) and U1 snRNP (Fig. 2 A, f), as visualized in the merged pictures and as previously described (Fu and Maniatis, 1990).

Inhibition of RNA polymerase II transcription by the nucleoside D-Rib was shown to cause the rounding up of splicing factor speckles (Spector et al., 1993). To determine whether eIF4E is also sensitive to RNA pol II transcription inhibition, HeLa cells were treated with D-Rib alone or in the presence of cycloheximide to inhibit protein synthesis. A addition of D-Rib in the presence or absence of cycloheximide resulted in the rounding up of eIF4E (Fig. 3, c and d), SC35 (Fig. 3, g and h), and Sm (Fig. 3, k and l) speckles. Similar results were
also obtained when cells were treated with α-amanitin (data not shown). Incubation with cycloheximide alone had no effect on the distribution of either protein (Fig. 3, b, f, and g). These results show that similar to splicing components, the nuclear distribution of eIF4E is sensitive to RNA pol II transcription and is independent of translation.

eIF4E Is Released from the Nucleus by the Monomethylated Cap Analogue, but Not by RNase Treatment

Because eIF4E mediates the mRNA cap structure function during translation initiation, it was of interest to determine the effect of cap analogues on eIF4E localization. HeLa cells were permeabilized with digitonin, rinsed to remove soluble proteins, and incubated with m7GpppG or GpppG. Following treatment, cells were rinsed to wash away proteins that were released from the nucleus upon treatment. Incubation with buffer alone did not alter the speckled pattern of eIF4E or SC35 (Fig. 4 A, left; compare with Fig. 2 A). However, incubation of cells with m7GpppG specifically released eIF4E from the nucleus (Fig. 4 A, middle), whereas GpppG had no significant effect (Fig. 4 A, right). The signal intensity for SC35 was not significantly changed by m7GpppG or GpppG. These results might suggest that the nuclear eIF4E is associated with the cap structure of mRNA in the speckles. Alternatively, it is possible that eIF4E in the speckles is not RNA-bound, but is released from the speckles as a consequence of its interaction with the vast excess of exogenous cap analogue in the nucleoplasm, which competes for the binding of eIF4E with speckled elements.

One of the elements in the speckles that binds eIF4E could be capped RNA. To determine whether eIF4E is retained in the nucleus in an RNA-dependent manner, CV-1 cells were treated with RNase A and the localization of eIF4E was examined by immunofluorescence (Fig. 4 B). RNase A treatment had no effect on the localization of eIF4E (Fig. 4 B, a and d). As previously reported, the Sm (Fig. 4 B, c and f), but not SC35 (Fig. 4 B, b and e), distribution is dramatically changed after this treatment.
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whereby it becomes diffuse in the nucleoplasm (Spector et al., 1992). These results suggest that the localization of eIF4E in the speckles does not appear to require its association with RNA. However, this negative result needs to be interpreted with caution. RNase treatment might not be expected to remove eIF4E from the speckles, if when bound to RNA, eIF4E interacts with both RNA and other insoluble proteins or structural elements in the speckles.

The Nuclear Distribution of eIF4E Is Regulated by the Dual Specificity Kinase Clk/Sty

Splicing factors of the SR family are substrates for the dual specificity kinase, Clk/Sty (Colwill et al., 1996). Expression of mammalian Clk/Sty wt in COS-1 cells causes the release of SR proteins from the speckles, whereas a catalytically inactive Clk/Sty does not (Colwill et al., 1996; Caceres et al., 1998). To examine whether Clk/Sty also affects the localization of eIF4E, HeLa cells were transiently transfected with myc-Clk/Sty wt or the catalytically inactive myc-Clk/Sty K190R, and the localization of eIF4E was determined by immunofluorescence. Expression of Clk/Sty wt, but not Clk/Sty K190R, resulted in the disruption of the eIF4E speckles (Fig. 5, A and B, top). As previously reported, in transfected cells using anti-SC35 or the anti-SR mAb 104, which is specific for phosphorylated SR proteins and recognizes all SR family members (Colwell et al., 1996; Caceres et al., 1998), Clk/Sty wt, but not the catalytically inactive kinase, released the SR splicing factors from the speckles (Fig. 5, A and B, middle and bottom; Colwell et al., 1996). Taken together, these results indicate that the nuclear localization of eIF4E is regulated by the dual specificity kinase, Clk/Sty.

Discussion

Nuclear eIF4E is shown here to be distributed throughout the nucleoplasm, but is concentrated in speckles. Electron microscope studies identified the splicing factor-enriched speckles as clusters of interchromatin granules surrounded by perichromatin fibrils (Spector, 1993). The fluorescence from the speckles, as detected with antibodies against various splicing factors, such as SC35, corresponds to interchromatin granules and some perichromatin fibrils, whereas the more dispersed nucleoplasmic signal represents perichromatin fibrils, which are mostly localized throughout the nucleus (Spector, 1996). Although numer-

Figure 3. Effect of transcription inhibition on the localization of eIF4E. HeLa cells were incubated with buffer alone (a, e, and i), cycloheximide (b, f, and j), DRB (c, g, and k), or in the presence of cycloheximide and DRB (d, h, and l) for 3 h. Cells were fixed and the localization of eIF4E, SC35, and Sm was determined by indirect immunofluorescence with the anti-eIF4E mAb 10C6 (top), anti-mouse SC35 mAb (middle), and with a human anti-Sm antisera (bottom). Primary antibodies were detected with Texas red-conjugated secondary antibodies and analyzed with a Kontron IBAS confocal imaging system. Images were taken from a 63× objective of a Zeiss LSM 410 microscope. Bar, 10 μm.

Figure 4. Nuclear eIF4E is released from the speckles by the monomethylated cap structure, but not by RNase treatment. A, HeLa cells were permeabilized with digitonin and incubated with buffer alone (left), m7GpppG (middle), or GpppG (right) for 25 min. The cells were rinsed and fixed as described in Materials and Methods. The localization of eIF4E (top) and SC35 (bottom) was determined by indirect immunofluorescence with the anti-eIF4E mAb 10C6 and monoclonal anti-SC35, respectively. Primary antibodies were detected with Texas red-conjugated mouse secondary antibodies and analyzed with a Kontron IBAS confocal imaging system. Images were taken from a 63× objective of a Zeiss LSM 410 microscope. Bar, 10 μm. B, CV-1 cells were fixed with methanol and incubated with PBS (a–c) or 100 μg/ml RNase A. The localization of eIF4E (a and d), SC35 (b and e), and Sm (c and f) was determined by indirect immunofluorescence with the anti-eIF4E mAb 10C6 and monoclonal anti-SC35, respectively. Primary antibodies were detected with Texas red-conjugated mouse secondary antibodies. Images were taken from a 63× objective of a Bio-Rad MRC-600 confocal imaging system mounted on a Nikon Diaphot-TMD microscope as previously described (Lejbkowicz et al., 1992). Bar, 10 μm.
ous studies have provided abundant information about the composition of the speckles, the precise function of these structures remains unclear. One model suggests that the speckles are splicing component storage sites, from which they are recruited to nascent pre-mRNA transcription sites (Singer and Green, 1997). This is consistent with the observations that most pre-mRNAs are spliced cotranscriptionally (Beyer and Osheim, 1988; Spector, 1996; Slee- man and L amond, 1999), and that nascent RNA detected by short pulses of $[^{3}H]$uridine primarily localize to the perichromatin fibrils and not to the interchromatin granules (Spector, 1996). The detection by in situ hybridization of several pre-mRNAs and corresponding spliced mRNAs within the speckles suggests that some splicing may also occur within these structures (Xing et al., 1993, 1995).

In a cell permeabilization assay, eIF4E was specifically released from the nucleus by m7GpppG (Fig. 4 A). One interpretation of this result could be that eIF4E is associated with pre-mRNA in the speckles, and that this association is disrupted by m7GpppG. This model is consistent with previous electron microscope studies demonstrating that the speckles contain poly A RNA (Carter et al., 1991). Alternatively, it is possible that the speckled eIF4E is not associated with mRNA and that eIF4E in the speckles is released to the nucleoplasm as a result of its interaction with the excess cap analogue. This is consistent with the resistance of eIF4E in the speckles to RNase treatment. However, as indicated earlier, the resistance of eIF4E to RNase treatment is not definitive proof for the lack of association of eIF4E with mRNA. Taken together, our results suggest that eIF4E is retained in the speckles primarily through its association with proteins and not RNA.

The localization of eIF4E to the speckles raises the possibility of a role in mRNA processing. This is consistent with the observation that DRB causes the clustering of eIF4E speckles. DRB and α-amanitin inhibit RNA polymerase II transcription at the elongation step (Cochet-Meilhac and Chambon, 1974; Koumenis and Giaccia, 1997; Zhu et al., 1997) and cause the rounding up of splicing factor speckles (Spector et al., 1993; Spector, 1996). It was suggested that the clustering of splicing factors results from the accumulation of splicing components at the storage sites in response to the reduction of pre-mRNA levels (Spector, 1996), and as a consequence, in less splicing. Accordingly, disruption of pre-mRNA splicing in vivo by microinjected oligonucleotides or antibodies against snRNPs also causes the clustering of splicing factors (O’Keefe et al., 1994). This explanation could also apply to eIF4E as a potential component of the splicing machinery.

Since U1 and U2 snRNPs colocalize with snRNPs and SC35 (Huang and Spector, 1992), eIF4E could, in principle, also be associated with the trimethylated cap (m3G) of U snRNAs in the speckles. However, this does not appear likely because U snRNPs can be quantitatively immunoprecipitated from intact particles with an m3G-specific antibody (Bringmann et al., 1983), indicating that this structure is not sequestered by proteins (Kramer et al., 1984). A iso, the affinity of eIF4E for the trimethylated cap structure is tenfold less than that for the monomethylated cap (Wieczorek et al., 1999). Additionally, the m3G cap is a poor inhibitor of cap-dependent translation and substitution of the monomethylated cap with the trimethylated
cap on β-globin mRNA reduced its translation by fourfold (Darzykiewicz et al., 1988).

Expression of the Clk/Sty kinase in COS-1 cells causes the release of the A SF/SF2 SR protein from the speckles (Colwill et al., 1996). The dual specificity Clk/Sty kinase phosphorylates the serine/arginine-rich region (RS domain) of SR splicing factors. Phosphorylation of this domain is required for the activation of SR splicing factors (Mermoud et al., 1994) and has been implicated in protein-protein interactions (Kohtz et al., 1994). Since Clk/Sty interacts with A SF/SF2 through its RS domain, it may also associate and phosphorylate other constituents of the speckles and participate in a more general mechanism of splicing activation. Expression of Clk/Sty wt but not of Clk/StyK399R, dispersed the nuclear eIF4E speckles. These results suggest that the nuclear localization of eIF4E is dependent on the distribution of SR splicing factors or, more generally, on splicing activity. Since eIF4E does not contain an RS motif, the identification of nuclear eIF4E-binding proteins should be helpful in establishing the mechanism by which Clk/Sty releases eIF4E from the speckles.

Since our results raise the possibility that eIF4E is associated with splicing factors, eIF4E could be involved in splicing and/or mRNA export. There is persuasive evidence that the nuclear CCB is important for mRNA splicing (Izaurralde et al., 1994; Lewis et al., 1996) and U snRNA export (Izaurralde et al., 1995). eIF4E perhaps could be required for the processing of a specific subset of snRNAs. eIF4E may be colocalized with splicing components and regulated in a similar fashion to prevent its association with the monomethylated cap structure of snRNAs. A subset of snRNAs, and U1, U2, U4, and U5, are transcribed by RNA polymerase II and acquire a monomethylated cap structure before their export to the cytoplasm for hypermethylation (Huber et al., 1998). These snRNAs are not spliced, targeting of eIF4E to pre-mRNA snRNAs through its interaction with specific splicing factors could provide an effective way to prevent the tethering of snRNAs to the translation machinery.

Previous studies showed that overexpression of eIF4E in NIH 3T3 cells increases cyclin D1 expression by stimulating its snRNA export (Rousseau et al., 1996). However, it is not known whether eIF4E stimulates cyclin D1 snRNA export directly through its association with the cap in the nucleus, or indirectly by increasing the levels of a protein involved in the export of this snRNA. Nevertheless, it seems reasonable that once associated with the cap, eIF4E will accompany the RNA to the cytoplasm. Since eIF4E is retained in rounded up speckles after transcription inhibition and is colocalized with splicing factors, it remains to be determined whether eIF4E binds to the cap early during RNA polymerase II transcription, and whether it could be involved in 3' mRNA processing, splicing, and nucleocytoplasmic transport of mRNA.

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