eIF4E promotes nuclear export of cyclin D1 mRNAs via an element in the 3′UTR

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The eukaryotic translation initiation factor eIF4E is a critical modulator of cellular growth with functions in the nucleus and cytoplasm. In the cytoplasm, recognition of the 5′ m^{7}G cap moiety on all mRNAs is sufficient for their functional interaction with eIF4E. In contrast, we have shown that in the nucleus eIF4E associates and promotes the nuclear export of cyclin D1, but not GAPDH or actin mRNAs. We determined that the basis of this discriminatory interaction is an ~100-nt sequence in the 3′ untranslated region (UTR) of cyclin D1 mRNA, we refer to as an eIF4E sensitivity element (4E-SE). We found that cyclin D1 mRNA is enriched at eIF4E nuclear bodies, suggesting these are functional sites for organization of specific ribonucleoproteins. The 4E-SE is required for eIF4E to efficiently transform cells, thereby linking recognition of this element to eIF4E mediated oncogenic transformation. Our studies demonstrate previously uncharacterized fundamental differences in eIF4E-mRNA recognition between the nuclear and cytoplasmic compartments and further a novel level of regulation of cellular proliferation.

Introduction

The eukaryotic translation initiation factor eIF4E is involved in modulation of cellular growth. Moderate overexpression of eIF4E leads to dysregulated growth and malignant transformation (Lazaris-Karatzas et al., 1990). The levels of eIF4E are elevated in several human malignancies including a subset of myeloid leukemias and breast cancer (Nathan et al., 1997; Topisirovic et al., 2003b). Importantly, both the nuclear and cytoplasmic functions of eIF4E contribute to its ability to transform cells (Sonenberg and Gingras, 1998; Strudwick and Borden, 2002). In the cytoplasm, eIF4E is required for cap-dependent translation, a process highly conserved from yeast to humans (Sonenberg and Gingras, 1998). Here, eIF4E binds the methyl 7-guanosine (m^{7}G) cap moiety present on the 5′ end of mRNAs and subsequently recruits the given mRNA to the ribosome (Sonenberg and Gingras, 1998).

In the nucleus, eIF4E functions to promote export from the nucleus to the cytoplasm of at least two reported mRNAs, cyclin D1 and ornithine decarboxylase (ODC), but does not alter GAPDH or actin mRNA export (Rousseau et al., 1996; Lai and Borden, 2000; Cohen et al., 2001; Topisirovic et al., 2002, 2003a). Since the first report of the nuclear localization of eIF4E 12 yr ago (Lejbkowicz et al., 1992), studies showed that up to 68% of cellular eIF4E is in the nucleus (Iborra et al., 2001), where it associates with nuclear bodies in a wide variety of organisms including yeast (Lang et al., 1994), *Drosophila* (Cohen et al., 2001), *Xenopus* (Strudwick and Borden, 2002), and humans (Cohen et al., 2001; Iborra et al., 2001; Topisirovic et al., 2003b). These bodies are found in all cell types reported including nearly 30 cell lines and primary cells from diverse lineages such as NIH3T3, HEK293T, U2OS, K562, and U937 (this paper; Lejbkowicz et al., 1992; Lai and Borden, 2000; Cohen et al., 2001; Strudwick and Borden, 2002; Topisirovic et al., 2002, 2003a). In mammalian cells, a large subset of eIF4E nuclear bodies coincides with those associated with the promyelocytic leukemia protein PML (Lai and Borden, 2000; Cohen et al., 2001; Topisirovic et al., 2003a,b). PML was the first identified regulator of eIF4E-dependent mRNA export (Cohen et al., 2001). The RING domain of PML directly binds the dorsal surface of eIF4E, reducing its affinity for the m^{7}G cap by >100-fold (Cohen et al., 2001; Kentsis et al., 2001). This loss of cap-binding activity correlates with a loss of the mRNA export function and loss of transformation activity of (Cohen et al., 2001; Topisirovic et al., 2002, 2003a).

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Abbreviations used in this paper: CBC, cap-binding complex; ODC, ornithine decarboxylase; SNAAP, specific nucleic acids associated with protein; UTR, untranslated region.

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There is evidence that the mRNA export function of eIF4E is linked to its oncogenic transformation activity. In a subset of primary human myeloid leukemia specimens, eIF4E-dependent cyclin D1 mRNA export is substantially up-regulated (Topisirovic et al., 2003b). Additionally, a mutant form of eIF4E, W73A, enters the nucleus colocalizing with endogenous eIF4E nuclear bodies, enhances the transport of cyclin D1 mRNAs to the cytoplasm and subsequently transforms immortalized cells (see Fig. 3, A and E; this paper; Cohen et al., 2001; Topisirovic et al., 2003a). This occurs despite the fact that W73A eIF4E cannot bind eIF4G and thus cannot act in translation (Sonenberg and Gingras, 1998).

Observations made by our group and the Sonenberg laboratory that eIF4E functionally discriminates between cyclin D1 and GAPDH mRNAs are surprising because the traditional view is that eIF4E binds the m^7G cap found on all mRNAs regardless of other sequence specific features. Thus, this functional discrimination presents a conundrum in terms of our understanding of eIF4E mRNA recognition in the nucleus. We explore the possibility that in the nucleus, eIF4E recognition of mRNA is fundamentally different than in the cytoplasm. Here, we identify a 100-nt sequence from the cyclin D1 3′UTR which sensitizes this mRNA to eIF4E in the nucleus and is involved in eIF4E mediated cell transformation.

Results

eIF4E physically associates with cyclin D1 mRNAs in the nucleus

To understand the underlying basis for the specificity of eIF4E's effects on promotion of mRNA export, we examined the novel possibility that eIF4E physically associated only with specific mRNAs in the nucleus. In this way, eIF4E-dependent promotion of export of cyclin D1 mRNAs could arise through a specific physical interaction of this mRNA with eIF4E in the nucleus. First, we examined whether eIF4E immunoprecipitates with cyclin D1 or housekeeping genes like GAPDH and actin mRNAs in total cell lysates and subsequently in nuclear and cytoplasmic fractions in a variety of cell lines including U2OS, NIH3T3, K562, U937, and HEK293T cells. Results were the same across cell lines so only representative results are shown here (Fig. 1). Note that both the mRNAs, and the eIF4E examined here, are endogenous. RNAs were detected for each experiment independently using multiple PCR strategies including quantitative RT-PCR and semi-quantitative PCR. Consistent results were always obtained using these different methodologies.

Immunoprecipitation studies indicated that in total cell lysates, eIF4E bound both cyclin D1 and GAPDH mRNAs, as expected because these mRNAs are capped (Fig. 1 A). In the nuclear fraction, eIF4E physically associates with a readily detectable fraction of cyclin D1 mRNA (Fig. 1 B). Yet, no detectable association between eIF4E and GAPDH mRNA or actin mRNA is observed in the nuclear fraction in contrast to total cell lysates or the cytoplasmic fractions (Fig. 1 A and not depicted). These results are confirmed by our semi-quantitative and independently RT-PCR analysis (Fig. 1, B, D, and E). Also, eIF4E associates only with processed cyclin D1 mRNAs in the nucleus, as observed using specific primers and RT-PCR (not depicted).

Importantly, the ability of eIF4E to associate with GAPDH or cyclin D1 mRNAs was monitored using material from the same eIF4E immunoprecipitations. Thus, differences in binding affinity between GAPDH and cyclin D1 are not a result of differences in immunoprecipitation efficiency or in the quality of the fractionation between experiments. Controls for the quality of these immunoprecipitations and fractionations are given below and discussed in the Materials and methods.

The above findings suggested that eIF4E-mRNA recognition in the nucleus could be substantially different to that in the cytoplasm. In particular, it was critical to establish the importance of cap binding for eIF4E-mRNA recognition in the nucleus. Thus, we examined which features of eIF4E were required for interaction with cyclin D1 mRNA in the nuclear fraction using the GST pull-down approach referred to as specific nucleic acids associated with protein (SNAAP; Trifillis et al., 1999). Here nuclear lysates were incubated with glutathione sepharose-bound wild-type or mutant forms of eIF4E-GST or GST (Fig. 1 C). Consistent with the immunoprecipitation findings, wild-type eIF4E associates with cyclin D1 but not GAPDH mRNAs. No association is observed with GST (Fig. 1 C) or an unrelated mRNA-binding protein αCP1-GST (not depicted) for either mRNA. The W56A eIF4E mutant, which does not bind the cap, does not bind cyclin D1 indicating that eIF4E still requires its cap-binding activity to associate with mRNAs in the nuclear fraction (Fig. 1 C). We extended these studies to test whether the dorsal surface mutant, W73A, can still associate with cyclin D1 mRNA in the nucleus, because this mutant readily enhances transport of cyclin D1 when expressed (Topisirovic et al., 2002, 2003a). Importantly, W73A mutant is deficient in translation but not transport (Sonenberg and Gingras, 1998; Cohen et al., 2001; Topisirovic et al., 2002, 2003a). This mutation does not detectably reduce binding to cyclin D1 mRNA as compared with wild type (Fig. 1 C). Note that previous biophysical studies indicate that W56A and W73A mutants have structures indistinguishable from wild-type eIF4E (Kentsis et al., 2001). Thus, there appears to be a correlation between the ability of eIF4E to physically associate with cyclin D1 mRNA in the nucleus and the ability of eIF4E to enhance transport of these mRNAs.

We extended these findings to further demonstrate the requirement for the m^7G cap for association of mRNA with eIF4E in the nucleus (Fig. 1, D and E). We monitored the ability of m^7G cap analogue (m^7GpppG) to compete for mRNA binding using semi-quantitative PCR and independently, quantitative RT-PCR methods. Consistent with the above results using the W56A mutant, the cap analogue successfully disrupts the association of cyclin D1 mRNAs with eIF4E. In contrast, GpppG, which does not bind eIF4E, does not disrupt its association with cyclin D1. In either case eIF4E does not associate with GAPDH mRNA. Together, these findings indicate that eIF4E requires the m^7G cap in order to associate with specific mRNAs in the nucleus. Note that treatment with m^7GpppG or GpppG did not alter the amount of eIF4E immunoprecipitated by eIF4E antibody in these reactions (unpublished data).

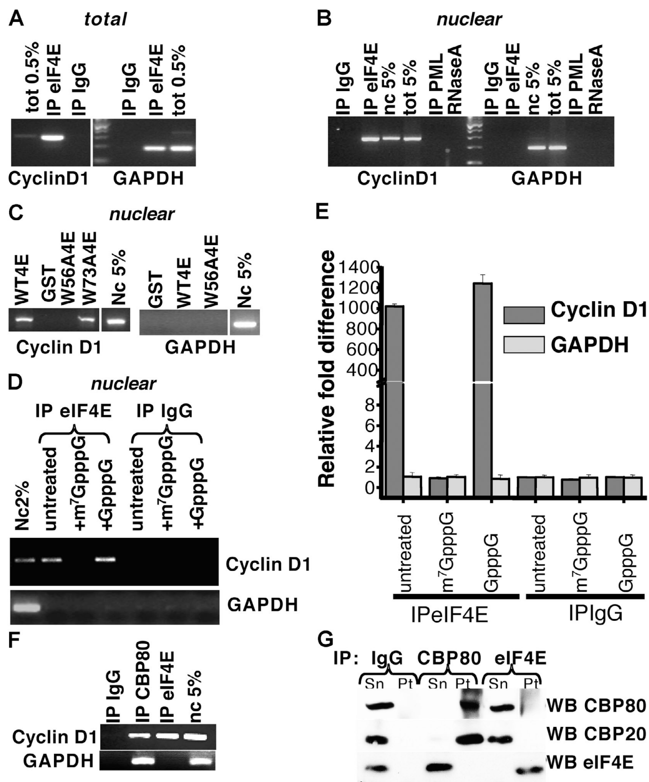

Figure 1. **eIF4E associates with cyclin D1 but not GAPDH mRNA in the nuclear fraction of U2OS or HEK293T cells.** (A) U2OS total cell lysates were immunoprecipitated (IP) with either an eIF4E antibody or mouse immunoglobulin (IgG) as a control. RNAs were detected by RT-PCR as indicated. Tot represents 0.5% of input RNA. (B) U2OS nuclear lysates were immunoprecipitated using antibodies to eIF4E (mAb eIF4E), PML (mAb PG-M3), or mouse IgG. RNase A indicates treatment before IP as a negative control. Total and nuclear (nc) represent 5% of RNA input. (C) U2OS nuclear lysates were subjected to SNAAP analysis with eIF4EWT- GST and mutant (W56A and W73A) fusion proteins. GST only was used as a negative control. Bound RNAs were detected by RT-PCR. Nc represents the percentage of input as indicated. RT-PCRs for A–C were detected by ethidium bromide staining. (D) As a control for cap dependence, the ability to compete for eIF4E binding by addition of 50 μM m^7GpppG cap analogue or 50 μM GpppG negative control was tested in the nuclear fraction of HEK293T cells. The ability of cyclin D1 and GAPDH mRNA to immunoprecipitate with eIF4E after treatments as indicated was monitored by semi-quantitative PCR. (E) Parallel RT-PCR methods to the experiments in A–C confirm the above results indicating eIF4E selectively binds cyclin D1 in a cap-dependent manner. Relative fold values were as calculated as described in the Materials and methods for both cyclin D1 and GAPDH mRNAs. (F) K562 nuclear lysates were immunoprecipitated using antibodies to eIF4E (mAb eIF4E), CBC (pAb CBP80), or mouse IgG; nc represent 5% of RNA input. (G) Proteins from immunoprecipitations by eIF4E and CBP antibodies or mouse IgG were analyzed by Western blot (WB). White line indicates that intervening lanes have been spliced out.

Interestingly, when the cytoplasmic fractions of cells were incubated with eIF4E-GST, all mRNAs bound, similar to the results we observed for immunoprecipitation experiments using the total lysates or cytoplasmic fraction (unpublished data). It is of interest that even when nuclear lysates are incubated with recombinant eIF4E in the SNAAP assay, we do not observe an association with GAPDH mRNA (Fig. 1 C). This raises the possibility that eIF4E-mRNA recognition is restricted in nuclear lysates by other regulatory elements that are not present in the cytoplasm, where cap binding is sufficient to mediate these interactions.

As a positive control, we extended our experiments to determine whether both GAPDH and cyclin D1 mRNA bound to the other nuclear cap-binding proteins, CBP 80 and CBP 20 which together form the cap-binding complex (CBC). In general, CBC associates with all transcripts cotranscriptionally (Visa et al., 1996). Immunoprecipitations were performed using an antibody to CBP 80. Results were monitored by semi-quantitative and independently by RT-PCR methods. Parallel experiments were performed with eIF4E antibodies using the same nuclear fractions. As expected, CBC associates with both cyclin D1 and GAPDH transcripts, whereas eIF4E associates only with cyclin D1 mRNA (Fig. 1 F). We further determined whether the CBC associates with eIF4E. Using immunoprecipitation (Fig. 1 G) and separately immunofluorescence (not depicted), we observed no association between the CBC and eIF4E. These findings are consistent with previous reports showing no coimmunoprecipitation between CBC and eIF4E (Ishigaki et al., 2001; Lejeune et al., 2002). However, we cannot rule out the possibility of a transient interaction between the CBC and eIF4E that we cannot detect by these methods. Together, these data suggest that eIF4E–cyclin D1 mRNA and CBC–cyclin D1 mRNA complexes are distinct complexes in the nucleus.

We cannot rule out the possibility that, in the nucleus, eIF4E binds a low level of GAPDH mRNA, which is beyond the detection limits of our RT-PCR methods. Even if this is the case, we readily detect an enrichment of up to 1,000-fold for cyclin D1 relative to GAPDH mRNAs despite the relative differences in abundance, with GAPDH being the much more abundant mRNA in both fractions (Fig. 1 C and see Fig. 3 B). Thus, using two independent methods, immunoprecipitation and SNAAP, we demonstrate that eIF4E physically associates with specific mRNAs in the nuclear fraction. Furthermore, eIF4E requires its cap-binding activity for this association but not W73 on the dorsal surface.

Recent findings by another laboratory suggest that eIF4E associates with all mRNAs in the nuclear fraction (Lejeune et al., 2002), whereas the data we present here clearly indicate that eIF4E binds cyclin D1 but not GAPDH or actin mRNAs in the nuclear fraction. The most likely reason for this discrepancy is differences in experimental approach. One major difference is that we monitor association of eIF4E with endogenous, not overexpressed, mRNAs (Fig. 1). Overexpression could lead to the formation of RNPs that are different from endogenous RNPs. Thus, we initiated our studies with endogenous eIF4E as well as endogenous mRNAs. Detection of bound mRNAs in immunoprecipitated fractions is also critical for optimal interpretation of these experiments. We confirmed our results with quantitative RT-PCR methods in order to ensure that background binding of mRNAs was not mistaken for real binding. Furthermore, we obtain the same results using different eIF4E antibodies or reconstituting the complexes with eIF4E-GST. Clearly, our specificity correlates well with previous observations by our group and the Sonenberg group that eIF4E overexpression up-regulates cyclin D1 but not GAPDH or actin mRNA transport and correspondingly up-regulates cyclin D1 but not GAPDH and actin protein levels.

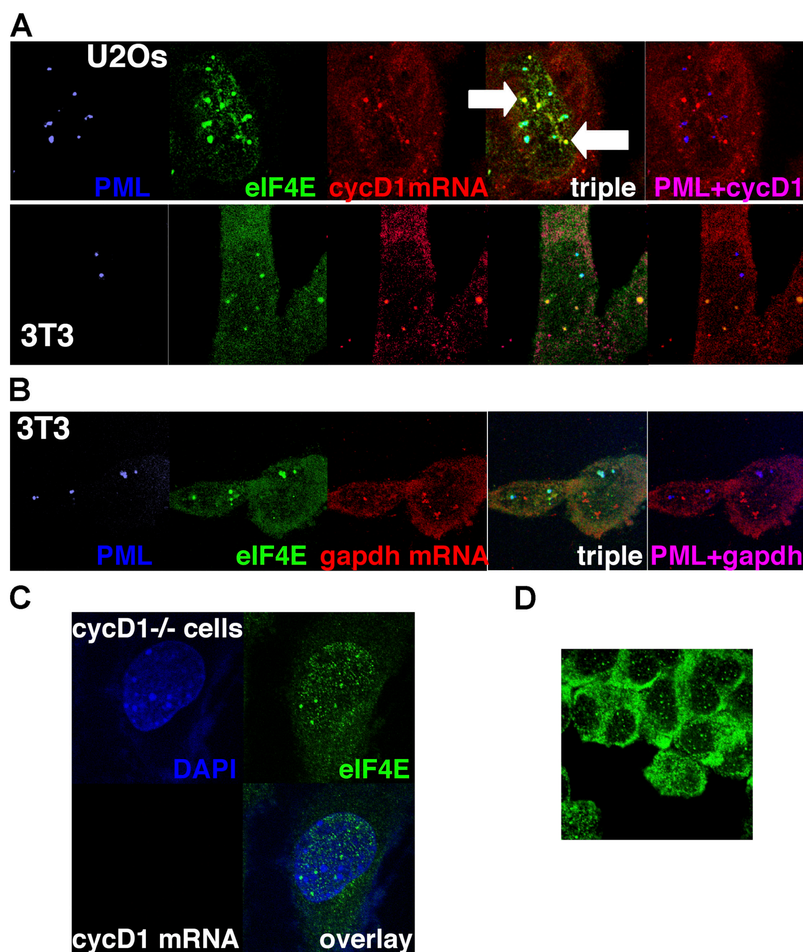

Figure 2. **Cyclin D1 but not GAPDH mRNAs colocalize with a subset of eIF4E nuclear bodies.** (A) Co-localization of cyclin D1 mRNA with PML and eIF4E proteins was analyzed in U2OS or NIH3T3 cells. Cyclin D1 mRNA was detected using in situ hybridization with a digoxigenin labeled nick-translated probe to cyclin D1 (red). Cells were then immunostained using an eIF4E mAb conjugated directly to FITC (green) and PML mAb 5E10 (blue). (B) The same as in A, except digoxigenin nick-translated probes to GAPDH were used for in situ hybridization. Within these panels, different combinations of overlays of the same micrographs are shown to highlight the localization of cyclin D1 mRNAs with eIF4E nuclear bodies (see arrows). (C) In situ hybridization for cyclin D1 mRNAs and immunostaining for eIF4E protein in cyclin $D1^{-/-}$ cells was performed as described above. (D) HEK293T cells contain eIF4E bodies similar in size, number and morphology observed for other cell types. Cells were stained with a pAb to eIF4E (Morley and Pain, 1995). Staining with mAb eIF4E gave identical results (not depicted). For all panels, confocal micrographs represent a single optical section through the plane of the cell.

Cyclin D1 mRNAs are localized to a subset of eIF4E nuclear bodies

Because eIF4E specifically associates with cyclin D1 mRNA in the nucleus, we examined whether cyclin D1 mRNA specifically associates with eIF4E nuclear bodies. In this way, eIF4E nuclear bodies could be sites of assembly of specific RNPs or functional storage sites. Studies were performed in U2OS and NIH3T3 cells. The localization of cyclin D1 or GAPDH mRNAs was determined using in situ hybridization and the localization of eIF4E and another component of the nuclear body, PML, through immunofluorescence. The results were monitored using confocal microscopy. Similar results are observed in both U2OS and NIH3T3 cells (Fig. 2, A and B).

These studies reveal that cyclin D1 mRNAs (red) are found throughout the cytoplasm and nucleoplasm but are additionally enriched in bodies in the nucleus. These local sites of enrichment colocalize with a subset of eIF4E nuclear bodies (green). Sites of colocalization of eIF4E nuclear bodies and cyclin D1 mRNAs are shown in yellow with two of several such sites marked with arrows (Fig. 2). Note that the objective for all experiments in Fig. 2 was 100× with further magnifications as follows: twofold for A–C; and 1.5-fold for D. The current resolution of these studies does not enable us to distinguish whether cyclin D1 mRNAs are found on the surface or within the eIF4E bodies. Consistent with previous studies (Lai and Borden, 2000; Cohen et al., 2001), there are two populations of eIF4E nuclear bodies, those, which contain PML, and those, which do not. The majority of eIF4E (green) and PML (blue) colocalize to the same nuclear bodies (light blue) and, as observed previously for many cells, there are additional eIF4E bodies (Fig. 2 A, green; Lai and Borden, 2000; Cohen et al., 2001). Importantly, mRNAs were never observed to colocalize with PML nuclear bodies consistent with previous studies showing RNA did not localize with PML nuclear bodies (Boisvert et al., 2000). Thus, cyclin D1 mRNAs localize to the subset of eIF4E nuclear bodies that do not contain PML. As expected, GADPH mRNAs do not localize with either PML or eIF4E nuclear bodies (Fig. 2 B). These results are consistent with the observation that nuclear GAPDH mRNAs do not physically associate with eIF4E and do not have their export modulated by eIF4E overexpression (Topisirovic et al., 2002, 2003a). As a negative control, probes for cyclin D1 in situ hybridization in cyclin $D1^{-/-}$ cells revealed no signal indicating that these probes are specific for cyclin D1 (Fig. 2 C). Furthermore, RNase treatment completely abolishes signals (not depicted).

As expected given the above results, immunoprecipitation studies with a PML antibody reveal no association with either cyclin D1 or GAPDH mRNAs (Fig. 1 B). These data are consistent with our previous findings that PML reduces the affinity of eIF4E for the m^7G cap by >100-fold (Kentsis et al., 2001), thus disabling RNA binding. Because eIF4E requires its

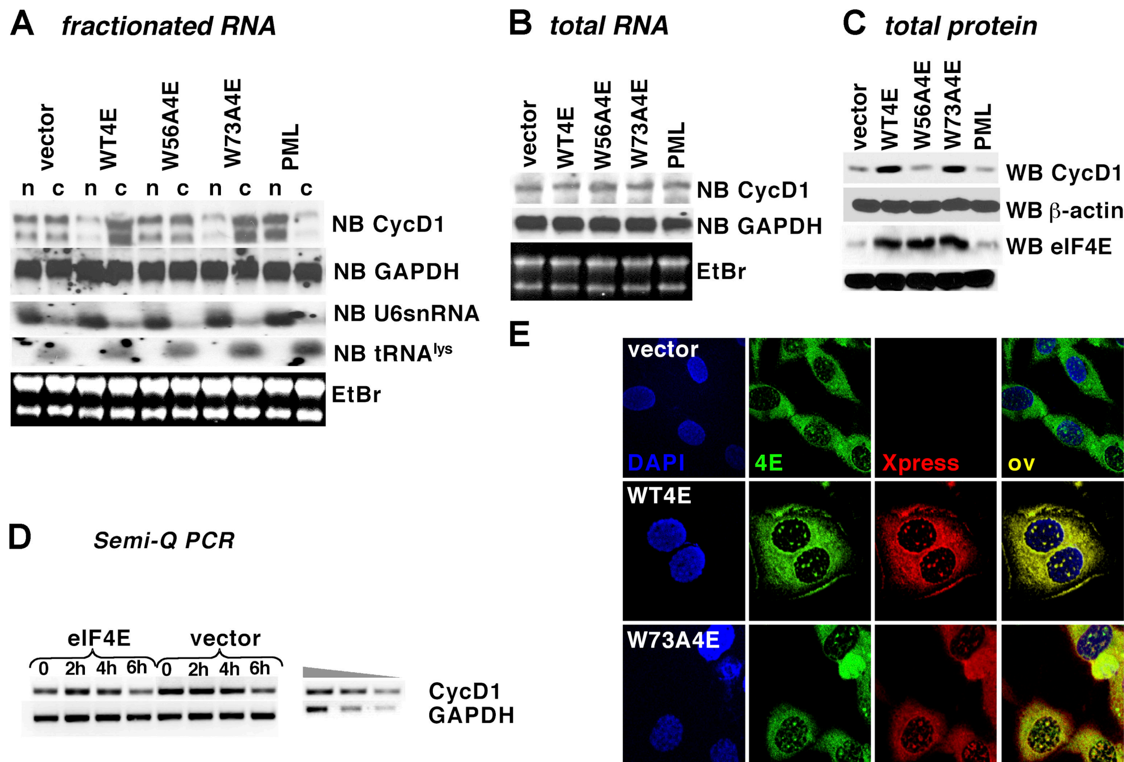

Figure 3. **eIF4E enhanced nucleocytoplasmic transport of cyclin D1 RNA.** (A) Nuclear (n) and cytoplasmic (c) fractions were isolated from NIH3T3 cells stably transfected with eIF4E WT, eIF4E mutants (W56A and W73A) or PML and RNAs were detected by Northern blot (NB) as indicated. U6snRNA (nuclear) and tRNALys (cytoplasmic) were used as markers for the quality of the fractionation. (B) Northern blot analysis of total RNAs isolated from NIH3T3 cells transfected as described in A. Ethidium bromide stained gels in A and B demonstrate the quality of the isolated RNA. (C) eIF4E enhanced mRNA transport leads to up-regulated protein levels of corresponding mRNAs. Total cell lysates from NIH3T3 cells transfected as indicated were analyzed for protein content by Western blot (WB). (D) Semi-quantitative PCR indicates that eIF4E overexpression does not alter cyclin D1 mRNA stability. Act D indicates hours of actinomycin treatment. Right panel represents decreasing amounts of RNA used in RT-PCR showing that conditions are semi-quantitative. (E) Mutant and wild-type eIF4E proteins still form nuclear bodies. NIH3T3 cells overexpressing Xpress-tagged eIF4E wild-type or W73A mutant were immunostained with anti-Xpress antibody to detect exogenous eIF4E (red) and or mAb eIF4E directly conjugated to FITC (green) to detect both endogenous and exogenous protein. The confocal micrograph represents a single optical section through the cell.

cap-binding activity for interaction with cyclin D1 (Fig. 1, D and E), it is consistent that cyclin D1 mRNAs are not found at PML containing eIF4E nuclear bodies.

In summary, cyclin D1 mRNAs localize to a subset of eIF4E nuclear bodies. Localization of mRNAs to the bodies is specific and is likely to be functionally important for their subsequent transport to the cytoplasm. In this way, eIF4E nuclear bodies may be assembly sites for specific eIF4E-RNPs, which enable promotion of export to the cytoplasm. Furthermore, it appears that, in the nucleus, there must be features particular to the bound mRNAs that impart the observed specificity of eIF4E.

Physical association of eIF4E with mRNAs is correlated with enhanced mRNA transport

Above, we demonstrate that both wild-type eIF4E and the W73A mutant physically associate with cyclin D1 mRNA in the nuclear fraction but that the W56A mutant, which is deficient in cap binding, does not (Fig. 1 C). To determine whether there is a correlation between binding and mRNA transport, we assessed the ability of these mutants to promote transport of cyclin D1 mRNA. Stably transfected NIH3T3 cells expressing mutant or wild-type proteins were fractionated and mRNAs monitored by Northern analysis (Fig. 3 A and Table I) as described previously (Topisirovic et al., 2002). U6snRNA and tRNALys serve as fractionation controls. Note that GAPDH is not altered in any case, as expected. Furthermore, the mutant proteins are expressed to similar levels (Fig. 3 C) and total levels of cyclin D1 mRNA are not altered by any of the constructs (Fig. 3 B). Furthermore, the stability of the cyclin D1 transcript is not affected by eIF4E (Fig. 3 D and Table II).

Importantly, eIF4E and the W73A mutant promote cyclin D1 mRNA transport where more cyclin D1 transcripts are clearly visible in the cytoplasmic fractions versus vector controls. Importantly, the W56A mutant does not alter the subcellular distribution of cyclin D1 mRNA transcripts (Fig. 3 A and

Table I. **Relative Nc/Cyt ratio of cyclin D1 mRNA in cells transfected as indicated (densitometry analysis of Northern blot experiments)**[a]

Vector	1.110 ± 0.490
WT4E	0.171 ± 0.0828
W56A4E	1.194 ± 0.365
W73A4E	0.216 ± 0.102
PML	4.552 ± 0.632

[a]Cyclin D1 mRNA levels were normalized to GAPDH mRNA; ± value represents SD from three independent experiments.

Table II. **Relative cyclin D1 mRNA level after actinomycin D treatment of cells transfected with eIF4EWT or vector control (measured by RT-PCR)[a]**

Time	4EWT	Vector
0	1 ± 0.3422	1 ± 0.5820
2 h	0.1654 ± 0.04	0.1314 ± 0.04600
4 h	0.0718 ± 0.0120	0.0567 ± 0.0108
6 h	0.0199 ± 0.0028	0.0218 ± 0.0084

[a]Cyclin D1 mRNA levels were normalized to GAPDH mRNA; ± value represents SD from three independent experiments.

Table I). One of the consequences of eIF4E-dependent mRNA transport is increased protein levels due to higher concentrations of these mRNAs in the cytoplasm and thus increased availability of these mRNAs to the translational machinery. Consistent with the above fractionation studies, cyclin D1 protein levels are elevated in wild-type and W73A mutant experiments but there is no increase when the W56A mutant is overexpressed. Thus, the physical association of cyclin D1 mRNA with the nuclear fraction of eIF4E is strongly correlated with the enhanced transport of cyclin D1 mRNA from the nucleus to the cytoplasm.

PML overexpression leads to the nuclear retention of cyclin D1 but not GAPDH mRNAs (Fig. 3 A), as well as reduced cyclin D1 but not GAPDH or actin protein levels (Fig. 3 C). This is consistent with the results from immunoprecipitation and in situ studies, where PML inhibits formation of eIF4E–cyclin D1 mRNA complexes (Fig. 1 B and Fig. 2 A). Once again it links the ability of eIF4E to physically interact with RNAs to the ability to promote mRNA transport.

Previous studies demonstrated that eIF4E could enter the nucleus by interaction with the 4E transporter protein (4ET; Dostie et al., 2000). Here, mutation of the dorsal surface (W73A) impaired association with the 4ET and thus impaired nuclear entry (Dostie et al., 2000). Thus, we performed experiments to ensure that the W73A mutant still entered the nucleus and formed nuclear bodies (Fig. 3 E). Using confocal microscopy, we examined the subcellular distribution of overexpressed eIF4E or the W73A mutant using the Xpress epitope tag and additionally an antibody to eIF4E, which recognizes both endogenous and overexpressed protein. It is clear from the confocal micrographs that the W73A mutant is readily detectable in the nucleus and associates with endogenous eIF4E nuclear bodies (Fig. 3 E). Thus, it appears that when the W73A mutant is overexpressed it uses an alternate route or can overcome the weaker binding to 4ET, gets transported into the nucleus and associate with nuclear bodies (Fig. 3 E). Similar studies with the W56A mutant indicated no alteration in subcellular distribution as compared with wild type (not depicted). In addition, wild-type and mutant forms of eIF4E are expressed to similar levels (Fig. 3 C). eIF4E levels are expressed to similar levels (Fig. 3 C). Note that the objective was 100× for these micrographs with a further 1.5-fold magnification.

Identification of an RNA structural element that mediates eIF4E sensitivity in the nuclear compartment

To determine if the association of mRNAs with eIF4E in the nucleus and eIF4E-dependent mRNA transport are mediated through some specific mRNA sequence, we analyzed 3′ and 5′ UTRs from our model mRNA cyclin D1. A series of chimeric

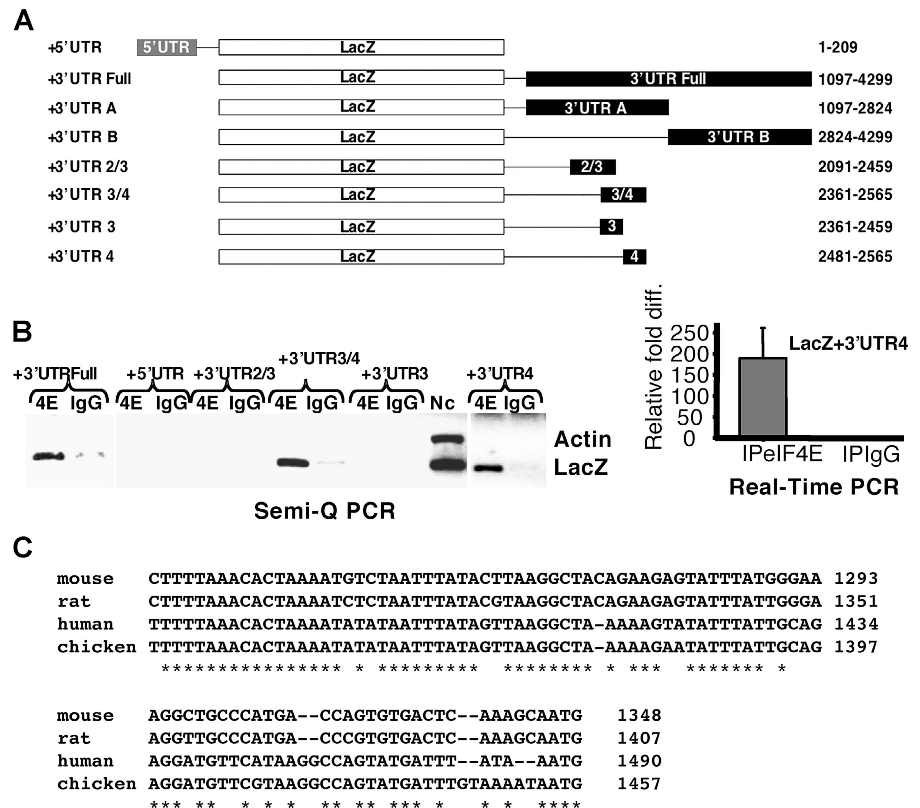

Figure 4. **eIF4E specifically associates with 4E-SE from the 3′UTR of cyclin D1.** (A) Schematic representation of chimeric constructs used in this study. Full 5′ and 3′UTR and different parts of 3′UTR of human cyclin D1 mRNA were cloned up- or downstream of LacZ, respectively. Numbers represent position of UTR fragments in cyclin D1 mRNA. (B) NIH3T3 cells were transiently transfected with chimeric LacZ constructs containing UTR-LacZ, LacZ-3′UTR, or LacZ constructs different parts of cyclin D1 3′UTR. The nuclear fractions of the transfected cells were immunoprecipitated with mAb eIF4E or mouse IgG for a control. LacZ and β-actin were detected by semi-quantitative RT-PCR and ethidium bromide staining (left). Nc indicates the nuclear fraction before IP and is 5% input of nuclear mRNA. For the RT-PCR method (right), relative fold enrichment is shown for the IP eIF4E fraction versus the IP IgG fraction indicating the enrichment of LacZ+3′UTR4 in the IP eIF4E. (C) Sequence alignment of cyclin D1 4E-SE from ClustalW (Thompson et al., 1994). GenBank/EMBL/DDBJ accession numbers are: human gi: 16950654, mouse gi: 6680867 and rat gi: 31377522. GenBank/EMBL/DDBJ accession no. for chicken is from the Ensembl database is gallus Gallus|5.14792937-14795000 and gi: U40844.

constructs were made fusing the coding region of LacZ to the 5′ or 3′UTRs of cyclin D1 (Fig. 4 A). We assessed whether these sequences were necessary and sufficient to enable chimeric mRNAs to associate with endogenous eIF4E in the nucleus and subsequently have their export modulated. Experiments were performed in NIH3T3 and HEK293T cells, which gave identical results. Note that HEK293T cells form nuclear bodies similar in size and morphology to those observed for NIH3T3 cells (Fig. 2 D). Initial semi-quantitative PCR results were confirmed by quantitative RT-PCR methods using the standard curves method (Fig. 4 B).

We monitored the ability of the nuclear fraction of eIF4E to associate with these mRNAs using immunoprecipitation in conjunction with PCR (Fig. 4 B). Importantly, eIF4E does not immunoprecipitate with LacZ mRNA, does not immunoprecipitate with Lac Z-cyclin D1 5′UTR chimeric mRNA, but does associate with chimeric LacZ mRNA that contains the entire 3′ UTR of cyclin D1. We made additional chimeric LacZ constructs with two different parts of the 3′UTR using an EcoRI site positioned approximately in the center of 3′UTR of cyclin D1 cDNA, and showed that chimeric RNA that contains first part of cyclin D1 3′UTR (+3′UTRA) immunoprecipitates with nuclear eIF4E whereas the second part (+3′UTRB) does not. Analysis of additional chimeric constructs containing different elements from the first part of cyclin D1 3′UTR revealed that the 100-bp sequence from the 3′UTR of cyclin D1 (located 2,471–2,565 bp in human cyclin D1 cDNA) is necessary and sufficient for association with eIF4E, so we refer to it as an eIF4E-*s*ensitive element (4E-SE). Importantly, this element is the highly conserved between human, mouse, rat, and chicken sequences (Fig. 4 C). In fact, the 4E-SEs between humans and chicken are nearly identical with 94% conservation versus 59% similarity over the rest of the 3′UTR. The presence of the 4E-SE in mammals and birds suggests that it is evolutionarily conserved.

To assess if the interaction of chimeric mRNAs with eIF4E was functional, we examined the effects of eIF4E overexpression on their export (Fig. 5). mRNA export was monitored using subcellular fractionation in conjunction with semi-quantitative RT PCR (Fig. 5 A), northern methods (Fig. 5, B–D) or quantitative RT-PCR (Table III). eIF4E does not modulate the transport of LacZ or LacZ chimeras that do not contain the 4E-SE (Fig. 5 and Table III), which is consistent with the observation that eIF4E does not bind these mRNAs (Fig. 4 B). Note that total mRNA levels determined from the same transfected cells indicated that LacZ mRNAs levels were not modulated (Fig. 6 B) nor were their stability (Fig. 6 C). Thus, there is a strong correlation with the ability of eIF4E to associate (directly or indirectly) with the 3′UTRs of these mRNAs and promote their transport. Increased export of LacZ mRNA, and thus the higher levels of cytoplasmic mRNAs when the 4E-SE is present, is correlated with higher levels of LacZ protein (Fig. 6 A). Consistent with our earlier observations, overexpression of the W56A mutant does not alter transport of either LacZ or LacZ-4E-SE as compared with wild-type eIF4E nor did the W56A mutant alter protein production of either LacZ construct (Fig. 5 D and Fig. 6 A; Table III). Thus, the mRNAs retain their cap dependence. Furthermore, all of the chimeric constructs had similar levels of total mRNA indicating that differences observed at the protein level were posttranscriptional and that differences in association with eIF4E and transport were not due to differences in expression of the constructs (Fig. 6 B). Importantly, LacZ-4E-SE transport is negatively regulated by PML (Fig. 6 A), as we observed for endogenous cyclin D1 mRNA (Fig. 3, A–C). Together, these results indicate that both the 4E-SE and the m^7G cap are required for eIF4E to enhance transport of these mRNAs.

The 4E-SE contributes to eIF4E mediated oncogenic transformation

We extended these studies to establish whether the 4E-SE contributed to the physiological activities of eIF4E and thereby to assess the functional significance of this RNA element (Fig. 7). Our previous studies correlated eIF4E-dependent promotion of cyclin D1 mRNA export with the transformation activities of eIF4E so we examined the contribution of the 4E-SE to this activity. Transformation activity was assessed by monitoring the number of foci formed upon eIF4E overexpression in a cyclin $D1^{-/-}$ fibroblast cell line. Note that the distribution of eIF4E nuclear bodies is not altered in cyclin $D1^{-/-}$ as compared with other cell types (Fig. 2 C). First, we determined that eIF4E

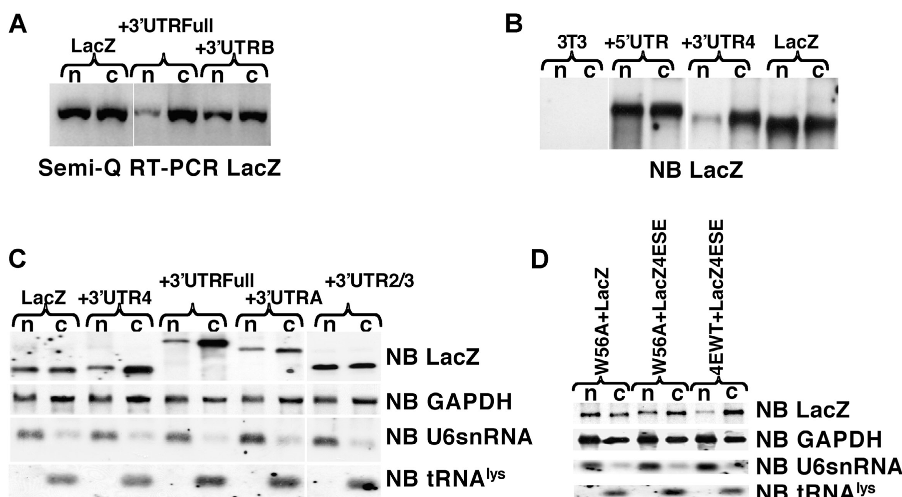

Figure 5. **4E-SE is sufficient for eIF4E-mediated mRNA transport.** PolyA RNA purified from nuclear (n) and cytoplasmic (c) fractions of NIH3T3 cells, cotransfected with eIF4E-2Flag and chimeric LacZ constructs (as indicated), were analyzed by (A) semi-quantitative RT-PCR and ethidium bromide staining (left) or (B) Northern blot (NB). (C and D) Northern blot analysis of polyA RNA purified from nuclear (n) and cytoplasmic (c) fractions of HEK293T cells cotransfected with eIF4E or W56A eIF4E and chimeric LacZ constructs (as indicated). Corresponding aliquots taken before polyA RNA purification indicate quality of the fractionations.

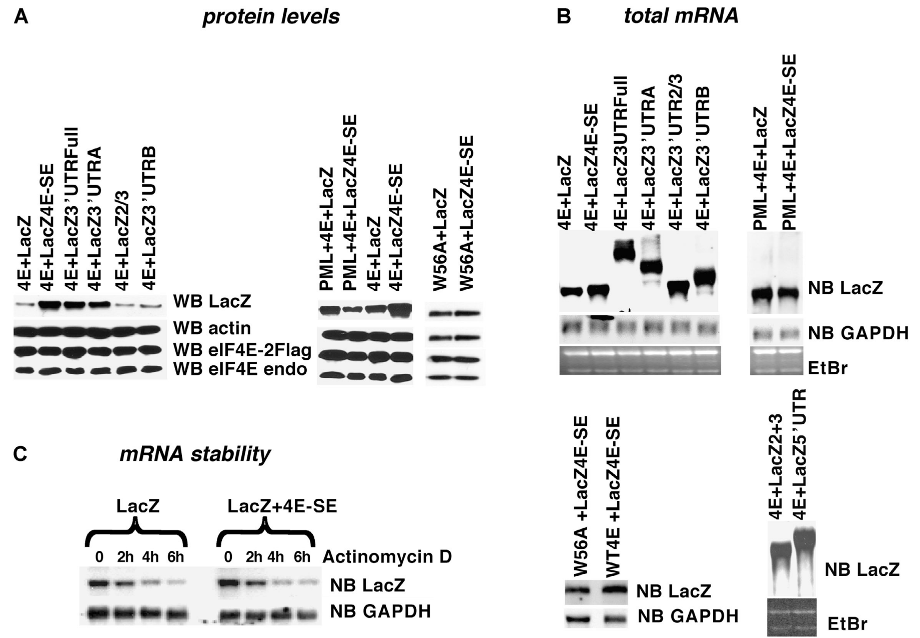

Figure 6. **The presence of the 4E-SE correlates with increased LacZ protein levels.** (A) Protein levels were analyzed by Western blot (WB) of total cell lysates from HEK293T cells transiently cotransfected with eIF4E-2Flag constructs and indicated chimeric LacZ constructs or PML, eIF4E-2Flag, and chimeric LacZ constructs. Note that HEK293T cells have eIF4E nuclear bodies (Fig. 3 C). (B) Northern blot (NB) analysis of total RNA from HEK293T cells cotransfected as indicated. Note that none of the total RNA levels are altered by any of the transfections. (C) Semi-Q PCR analysis indicates that the presence of the 4E-SE does not detectably alter LacZ mRNA stability. Act D indicates hours treated with actinomycin D. GAPDH is shown as a loading control.

transformed cyclin D1$^{-/-}$ cells relative to vector controls. Reintroduction of cyclin D1 constructs containing the full-length 3′UTR (cycFull) led to substantially more foci than cells transfected with eIF4E alone (Fig. 7 A). However, eIF4E's transformation activity was not augmented by introduction of cyclin D1 with no 3′UTR (cycTrunc) being the same as eIF4E overexpressing cells alone. Importantly, introduction of eIF4E and cyclin D1, with only the 100 nt 4E-SE (cyc4E-SE), transformed cells as well as constructs containing the full-length 3′UTR. Thus, in the context of cyclin D1$^{-/-}$ cells, the transformation activity of eIF4E is only increased by reintroduction of cyclin D1 when the 4E-SE is present.

Consistently, only those cells transfected with cyclin D1-3′UTR (cycFull) or cyclin D1-4E-SE (cyc4E-SE) showed increased cyclin D1 protein levels in contrast to vector controls or cells transfected with cyclin D1 with truncated 3′UTR (cycTrunc; Fig. 7 B). Thus, the presence of the 4E-SE is tightly tied to eIF4E's ability to export cyclin D1 and subsequently to efficiently transform cells. These effects can be extended to endogenous eIF4E. Cells expressing cycFull or cyc4E-SE, even in the absence of overexpressed eIF4E, produce more cyclin D1 protein than those cells expressing the truncated version of cyclin D1 (Fig. 7 B). We confirm this is occurring at the mRNA transport level by fractionation and RT-PCR methods (Fig. 7 C). We demonstrate that the ratio of nuclear to cytoplasmic cyclin D1 mRNA is ∼250 times greater in those cyclin D1$^{-/-}$ cells expressing the cycTrunc construct than those expressing the cycFull or cyc4E-SE constructs. Thus, the cycTrunc is not as efficiently transported to the cytoplasm as cycFull and cyc4E-SE constructs are. Importantly, the distribution of GAPDH was not altered by any of these constructs (unpublished data). Northern analysis confirmed these findings and indicated that fractionations were clean (unpublished data). Thus, the presence of the 4E-SE allows more efficient export of cyclin D1 mRNA using either endogenous or exogenous eIF4E.

Table III. **Relative ratio of cytoplasmic versus nuclear LacZ mRNA of cells transfected as indicated (measured by RT-PCR)[a]**

Construct	Relative C:N LacZ mRNA ratio
4EWT ± LacZ	1 ± 0.082
4EWT ± 3′UTR4	462.496 ± 38.114
4EWT ± 3′UTR Full	373.934 ± 30.195
4EWT ± 3′UTR2/3	0.823 ± 0.069
4EWT ± 3′UTRB	1.187 ± 0.119
W56A ± lacZ	1.159 ± 0.124
W56A ± 3′UTR4	1.918 ± 0.286

[a]LacZ mRNA levels were normalized to GAPDH mRNA; ± value represents SD from three independent experiments.

Discussion

These studies reveal that eIF4E associates with and regulates nuclear mRNAs in a fundamentally different manner than cytoplasmic mRNAs. Unlike the cytoplasmic fraction of eIF4E where cap binding is sufficient for its functional interaction with mRNAs, in the nucleus eIF4E appears to associate with regulatory factors that restrict its association with mRNA lacking 4E-SEs. Because eIF4E binds the m^{7}G cap, we hypothesize that other factors directly bind the 4E-SE in the 3′UTR and through physical association with eIF4E increase its affinity for this subset of mRNAs (Fig. 8). An mRNA looping model is another possibility, where eIF4E cap binding is stabilized by direct contact with the 4E-SE, through an unknown mechanism (Fig. 8). It seems likely that not only cyclin D1 but also many other mRNAs could be regulated in this way (unpublished data), especially given that ODC also has its transport regulated in this manner (Rousseau et al., 1996).

Our studies and recent reports indicate that eIF4E does not associate with the CBC nor does it associate with unspliced mRNA (Ishigaki et al., 2001; Lejeune et al., 2002). These studies

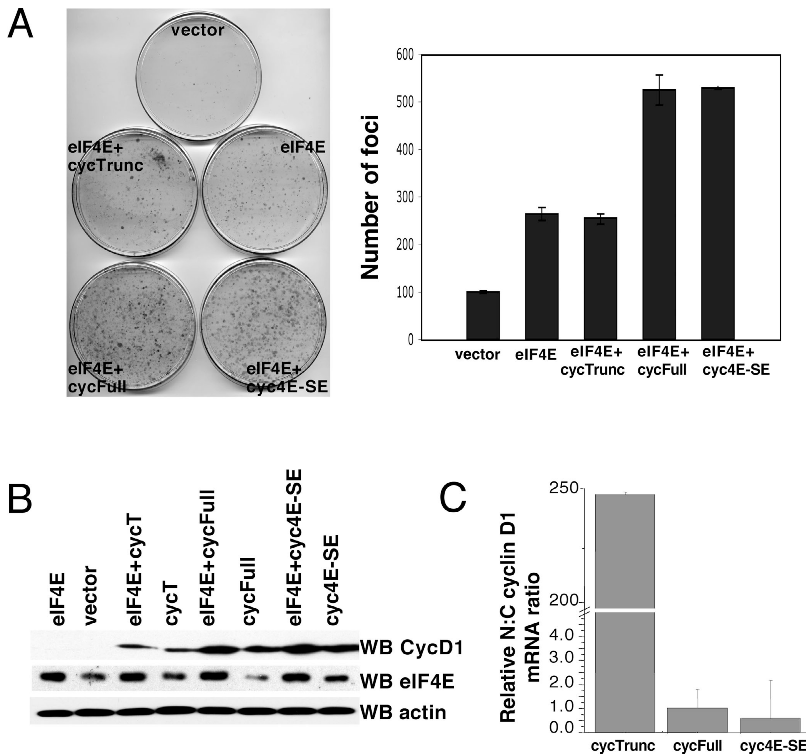

Figure 7. **The 4E-SE contributes to eIF4E mediated oncogenic transformation.** (A) Cyclin D1$^{-/-}$ cells were stably transfected with eIF4E or cotransfected with either the coding region of cyclin D1 constructs without the 3′UTR (cycTrunc), the coding region of cyclin D1 with the full-length 3′UTR (cycFull), and the coding region of cyclin D1 with only the 100 nt 4E-SE (cyc4E-SE), and analyzed for anchorage-dependent foci formation assays. Three independent experiments were preformed in triplicate and error bars indicate ±SD. Number of foci are relative to vector control, which was set to 100%. (B) Western blot analysis (WB) of total cell lysates from cyclin D1$^{-/-}$ cells stably transfected as indicated, showing increased cyclin D1 protein level in cells transfected with constructs containing full-length 3′UTR or 4E-SE compared with the truncated form lacking the 3′UTR sequence. (C) Results of quantitative RT-PCR experiments using endogenous eIF4E in cyclin D1$^{-/-}$ cells. The relative ratios of nuclear (N)/cytoplasmic (C) cyclin D1 mRNA was determined using RT-PCR with the relative standard curves method. Values were normalized to CycFull by setting its ratio arbitrarily to 1. Standard methods were used to propagate SDs from these experiments.

suggest that the transfer of capped cyclin D1 mRNA transcripts from the CBC to eIF4E happens after splicing and before cyclin D1 mRNA gets exported from the nucleus. Because eIF4E and CBC do not coimmunoprecipitate or colocalize, this interaction is likely transient one. We cannot rule out the possibility of a completely novel mechanism by which the cap of cyclin D1 mRNA is protected by some unknown means between leaving the CBC RNP and associating with eIF4E. This is an area of future investigation.

mRNAs that get exported in an eIF4E-dependent fashion may undergo some alternative, eIF4E-dependent type of mRNA quality surveillance. Previous studies suggested that the nuclear fraction of eIF4E might be involved in low level nuclear translation as part of mRNA quality surveillance (Iborra et al., 2001). However, our studies with the W73A mutant indicate that nuclear translation is not required for the observed transport function because this mutant is active in transport but not translation, because it cannot bind eIF4G (Sonenberg and Gingras, 1998; Gingras et al., 1999). Specialized pathways for transport of growth-promoting mRNAs such is cyclin D1, and control of this process by factors such as PML, may have evolved in order to coordinate gene expression with cellular proliferation.

eIF4E nuclear bodies must be intact in order to act in mRNA export because their disruption is correlated with a loss of export activity (Topisirovic et al., 2003a; Kentsis et al., 2004). Our data suggest that assembly of eIF4E transport RNPs happens in or around eIF4E bodies. The colocalization of cyclin D1 mRNAs with PML-negative eIF4E nuclear bodies suggests that these sites are areas for assembly of specific subtypes

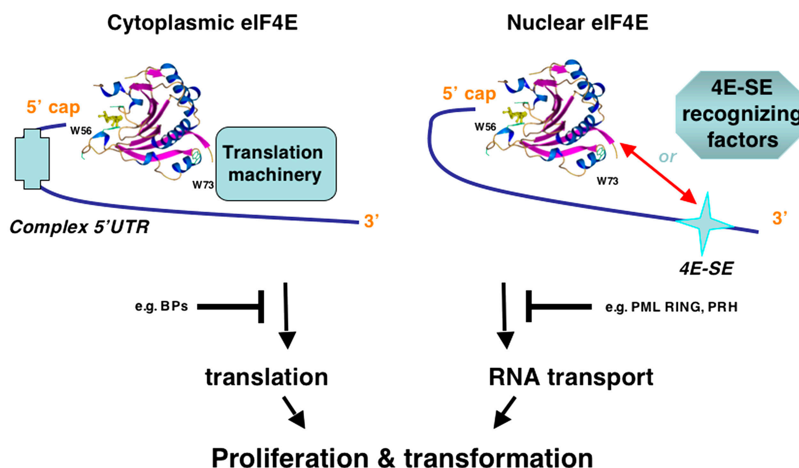

Figure 8. **A model for specific mRNA recognition by eIF4E in the nucleus.** The three dimensional structure of eIF4E is shown (1ej1; Marcotrigiano et al., 1997). Arrows indicate β-sheets; coils indicate α-helices. The m^{7}GDP cap analogue is shown in yellow and the structure is rendered in PREPI (S. Islam and M. Sternberg, Imperial College). We propose two possibilities for 4E-SE recognition in the nucleus, one that eIF4E by itself binds both, cap and 4E-SE, and the second one where some other factors mediate 4E-SE sensitivity in the nucleus, as discussed in the text. For eIF4E found in the cytoplasm, the translation machinery denotes the remaining portion of eIF4F and associated factors, for example, PABP, where this general terminology was used for simplification. The BPs indicates eIF4E-binding proteins that inhibit translation. PML and PRH are factors which inhibit eIF4E mediated transport. Importantly, mRNAs that are sensitive to eIF4E in one compartment may or may not be sensitive in the other compartment, as discussed in the text.

of RNPs which permit more efficient export of this restricted subset of mRNAs to the cytoplasm. In this way, expression of these targeted mRNAs could be modulated quite quickly. It seems likely that nuclear eIF4E RNPs involved in promotion of mRNA export are different from those functioning in translation, because the W73A mutant is still active in transport (Cohen et al., 2001; Topisirovic et al., 2003a). Consistently, eIF4E does not appear to bind eIF4G in the nucleus (McKendrick et al., 2001) but eIF4G is an integral part of the eIF4E RNP in the cytoplasm (Sonenberg and Gingras, 1998). Clearly these results suggest major differences in functionalities of the corresponding nuclear and cytoplasmic eIF4E RNPs.

eIF4E-dependent promotion of mRNA export could provide an immediate response system by which the cell responds to stress and/or growth conditions before transcriptional reprogramming. We speculate that this process is not limited just to cyclin D1 mRNA but that other mRNAs involved in growth regulation could be regulated this way, including ODC (Rousseau et al., 1996) and many others (unpublished data). The ability of eIF4E to promote the export of growth promoting mRNAs such as cyclin D1 allows it to turn on a cellular growth promoting program thereby positioning eIF4E as a critical node in the growth regulatory network. eIF4E regulating proteins, such as PML (this paper) and nuclear homeodomain proteins such as PRH, which directly bind eIF4E (Topisirovic et al., 2003a) are well positioned to act upstream of eIF4E. Although, this network also includes important regulation of translation by the eIF4E-binding proteins (4EBPs; Sonenberg and Gingras, 1998), our findings suggest that these transport and translation networks may not completely overlap. For instance, cyclin D1 mRNA is sensitive to eIF4E at the transport level, but not at the translation level (Rousseau et al., 1996). In contrast, ODC mRNA is sensitive to eIF4E at both levels (Rousseau et al., 1996). ODC mRNA, like cyclin D1 mRNA, contains a 4E-SE element (unpublished data). PML appears to be a critical negative regulator of this nuclear network, thereby shutting down production of a wide variety of growth promoting proteins simultaneously and thus, inhibiting eIF4E-mediated growth and transformation. These activities rely on eIF4E RNA recognition through both the m^7G cap and the 4E-SE. eIF4E promotion of export of specific mRNAs represents an exciting new point of growth regulation in the cell and a novel regulatory pathway which when dysregulated could contribute to human cancers.

Materials and methods

Constructs

All UTR-LacZ fusion constructs were made in pcDNA3.1LacZ vector (Invitrogen) and positioned 5′ or 3′ of the coding region of LacZ as appropriate. For cloning of cyclin D1 3′UTR, the NotI restriction site was created in pD1-1 construct (human cyclin D1 gene in pGEM7Zf; Motokura and Arnold, 1993), 150 bp upstream of stop codon by in vitro mutagenesis (Quickchange kit; Stratagene) and full-length 3′UTR was cloned using NotI and XbaI downstream of LacZ (referred here as LacZ-3′UTRFull). Fragments containing the first part of cyclin D1 3′UTR were generated using NotI and EcoRI, and second part of cyclin D1 3′UTR using EcoRI and XbaI (note that there is EcoRI site at the position 2,824 bp in human cyclin D1 cDNA) and cloned under NotI–XbaI and EcoI–XbaI, downstream of LacZ (LacZ 3′UTRA and LacZ 3′UTRB). Individual sequences were amplified using specific primers containing NotI or XbaI restriction sites at their 5′ ends. LacZ+3′UTR2/3 contains segment 2,091–2,459 bp from cyclin D1 mRNA, LacZ+3′UTR 3 contains sequence 2,361–2,459 bp, LacZ3/4 contains segment 2,361–2,565 and LacZ+3′UTR4 contains sequence 2,481–2,565 bp from human cDNA. The 5′UTR was amplified from cyclin D1 cDNA (ATCC MGC-2316) and cloned using the HindIII site, upstream of AUG codon for Xpress tag. pcDNA2Flag-eIF4E construct was made by inserting of eIF4E cDNA into the EcoRI–NotI sites (pcDNA2F vector was gift from Z. Ronai, Burnham Institute, La Jolla, CA). eIF4E mutants in pcDNA2Flag were made by in vitro mutagenesis (Quickchange kit; Stratagene). pMV vector, pMV-eIF4E wild type (a gift from N. Sonenberg, McGill University, Montreal, Quebec, Canada) or mutants, pLINKSV40-PML and bacterial expression constructs were described previously (Lazaris-Karatzas and Sonenberg, 1992; Cohen et al., 2001). Human cyclin D1 cDNA without the full-length 3′UTR (ATCC MGC-2316) was cloned in pMV vector using EcoRI and HindIII (cyclin D1 truncated). Cyclin D1 full construct was made by using HindIII–XbaI fragment from pCDNALacZ-3′UTR that was blunt ended and cloned under HindIII in pMV-cyclin D1Trunc (note that there is HindIII site in human cyclin D1 cDNA at position 1,206 bp). 4E-SE-4 from cyclin D1 3′UTR was PCR amplified, blunt ended and cloned under HindIII in pMV-cyclin D1Trunc (cycD14E-SE).

Antibodies and Western analysis

Antibodies used against PML were described previously (a gift from P. Freemont, Imperial College, London, UK and L. de Jong, University of Amsterdam, Amsterdam, Netherlands; Stuurman et al., 1992; Borden et al., 1995; Topisirovic et al., 2002). Additional antibodies used include mouse monoclonal anti-eIF4E Ab (BD Transduction Laboratories), polyclonal anti-eIF4E Ab (a gift from S. Morley, University of Sussex, Brighton, UK; Morley and Pain, 1995), mouse monoclonal anti–cyclin D1 Ab (BD Biosciences), mouse monoclonal anti-Xpress Ab (Invitrogen), mouse monoclonal anti-GAPDH antibody (MAB374; CHEMICON International, Inc.), anti-CBP80 pAb (a gift from L. Maquat, University of Rochester, Rochester, NY; Ishigaki et al., 2001) anti–CBP-20 (a gift from E. Izaurralde, EMBL, Heidelberg, Heidelberg, Germany; Izaurralde et al., 1995). Western analysis was performed as described previously (Topisirovic et al., 2002, 2003a).

Cell culture and transfection

NIH3T3, U2OS, HEK293T, and Nlog (a gift from H. Land, University of Rochester; cyclin $D1^{-/-}$; Perez-Roger et al., 1999) cells were maintained in 5% CO_2 at 37°C in DME (GIBCO BRL; Life Technologies), supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin. eIF4E and PML stably transfected NIH3T3 were made as described previously (Topisirovic et al., 2002, 2003a). Transient transfection of NIH3T3 was performed using either GeneJammer Transfection Reagent (Stratagene) or Lipofectamine Plus reagent (Invitrogen) according to the manufacturer's instructions. Transient transfections of HEK293T cells were performed using Calcium Phosphate Transfection kit (Invitrogen). Stable transfections of cyclin $D1^{-/-}$ cells were performed using Fugene 6 Transfection Reagent (Roche) according to the manufacturer's instructions. Anchorage-dependent foci formation assays were conducted as described previously (Cohen et al., 2001; Topisirovic et al., 2003a).

Immunopurification of eIF4E, isolation of RNA bound to eIF4E and RT-PCR

Nuclei were isolated from 3×10^9 HEK293T cells aliquoted appropriately, as previously described (Topisirovic et al., 2002), resuspended in ice-cold NET-2 buffer (50 mM Tris-HCL, pH 7.4, 300 mM NaCl, 0.5% [vol/vol] NP-40, 1× complete protease inhibitors [Roche], 200 U/ml SUPERasein [Ambion]) and mechanically disrupted in dounce homogenizer (type B) on ice. Obtained nuclear extracts were cleared by centrifugation at 16,000 *g* for 20 min at 4°C. 1/20 of the supernatant was split in two and used to obtain nuclear RNA and protein, respectively. 19/20 were split in three aliquots, two of which, when indicated in the text, were incubated with 50 μM m^7GpppG and 50 μM GpppG (NEB) in NET-2 buffer for 30 min at 4°C. Each of aforementioned aliquots was split in two and immunoprecipitated as described previously (Ishigaki et al., 2001) with the following modifications: 10 μg of anti-eIF4E mouse mAb (Transduction Laboratories) or 10 μg of mouse IgG (Calbiochem) was used per reaction and after immunoprecipitation, the beads were washed once with NET-2 buffer supplemented with 1 mg/ml of heparin (Sigma-Aldrich). Obtained RNA was treated with RNase free DNase (Promega) according to the manufacturer's instruction. RNA was converted into cDNA using the Sensiscript Reverse Transcription kit (QIAGEN). RT-PCR was performed in triplicate with the QuantiTect SYBR green RT-PCR Kit (QIAGEN) in Opticon thermal cycler (MJR). Obtained RT-PCR data was analyzed with Opticon software (MJR). Primers used for cyclin D1 RT-PCR were cycF, 5′-cagcgagcagcagagtccgc-3′ and cycR, 5′-acaggagctggtgttccatggc-3′; and for GAPDH amplification GAPDHF, 5′-accacagtccatgccatcac-3′ and GAP-

DHR, 5′-tccaccacccgttgctgta. For RT-PCR methods, calculations were done as described by Applied Biosystems. For semi-quantitative PCR, 30 cycles were used, and for RT-PCR, standard methods were used. Primers used for semi-quantitative amplification of GAPDH were the same as for RT-PCR, and for cyclin D1 and actin amplification the following primers were used: cycHMF, 5′-cacttcctctccaaaatgcca-3′; cycHMR, 5′-cctggcgcaggcttgactc-3′; ActF, 5′-atctggcaccacaccttctacaatgagctgcg-3′; and ActR, 5′-cgtcatactcctgcttgctgatccacatctgc-3′.

Controls for quality of immunoprecipitation and fractionations

Several steps were taken to ensure that variability between experiments did not lead to false positive or false negative results. The immunoprecipitated sample was tested to ensure that eIF4E immunoprecipitated itself and that IgG did not bind eIF4E as determined by Western blotting. The specificity of the immunoprecipitation was determined using known positive and negative controls for eIF4E in the nuclear fraction. Thus, the ability of eIF4E antibodies to immunoprecipitate eIF4E but not CBP80 (Fig. 1 G) or RNA Polymerase II was determined (Lai and Borden, 2000). These results are consistent with the findings from the Maquat and our laboratories where it was shown that the nuclear fraction of eIF4E does not associate with these proteins. Furthermore, positive controls for interactions of eIF4E include the ability to associate with the PML protein (Cohen et al., 2001; Topisirovic et al., 2003a,b), as have been reported numerous times. In addition, we demonstrate that the transduction laboratory antibody against eIF4E used here colocalizes with eIF4E antibodies produced in other laboratories (Topisirovic et al., 2004) indicating that the antibody is robust and reliable. Importantly, these experiments ensure that differences in association of various mRNAs with eIF4E are NOT a result of differences in immunoprecipitation efficiency or fractionation quality between experiments.

For fractionation controls, the quality of each nuclear and cytoplasmic fraction was assessed by monitoring the subcellular distribution of U6snRNA (nuclear) and $tRNA^{Lys}$ (cytoplasmic) as we reported previously and show throughout the text. Additional controls performed for each fractionation include Western analysis of the splicing speckles protein which served as a nuclear marker (Sc35) and β-actin, which served as a cytoplasmic marker (Topisirovic et al., 2003a,b). Additional fraction controls were done when sufficient material was available (Topisirovic et al., 2003a,b).

SNAAP protocol was performed as described previously (Trifillis et al., 1999) with the following modifications. Precleared 250 μg of nuclear extracts were added to 50 μg of GST-protein beads in 500 μl RBB buffer containing 0.5% NP-40, and after incubation of 30 min at 4°C, 500 μg of yeast tRNA was added per reaction and incubated overnight at 4°C. All washing of beads was performed in RBB buffer containing 0.25% Triton X-100 and 0.5% NP-40.

Cellular fractionation and Northern analysis

Fractionation and RNA isolation were described previously (Lai and Borden, 2000; Topisirovic et al., 2002). For LacZ, Poly A RNA was purified from fractionated RNA using Oligotex mRNA Mini Kit (QIAGEN). Probes for cyclin D1, GAPDH, U6, and tRNALys for Northern blot analysis were also previously described (Topisirovic et al., 2002). LacZ probe was made by PCR amplification using primers LacZF, 5′-cggtcgctaccattaccagtt-3′ and LacZR, 5′-gacgttgtaaaacgacgggat-3′, and labeled using BrightStar Psoralen-Biotin kit (Ambion).

Immunofluorescence, in situ hybridization, and laser scanning confocal microscopy

Immunofluorescence experiments were as described previously (Cohen et al., 2001; Topisirovic et al., 2002). Fluorescence was observed using 100× optical magnification and 2× digital zoom, unless indicated otherwise, on an inverted laser scanning confocal microscope (model TCS-SP (UV); Leica) exciting at 488, 568, or 351/364 nm (at RT). All channels were detected separately, and no cross talk between the channels was observed. Micrographs represent single sections through the plane of cells with a thickness of ~300 nm. Experiments were repeated three times with more than 500 cells in each sample. In situ hybridization was performed according to Spector et al. (1998), using nick-translated DIG-11-dUTP–labeled (Nick Translation Kit; Roche) cyclin D1 and GAPDH PCR-amplified fragments (cyclin D1 specific 5SA, 5′-catggaacaccagctcctgt-3′ and 3SA, 5′-cgcagccaccacgctccc-3′; and GAPDH specific GAPDHHF, 5′-accacagtccatgccatcac-3′ and GAPDHMR, 5′-tccaccaccctgttgctgggg-3′) and detected using anti-DIG Fab fragments (Roche) followed by donkey anti–sheep Texas red (Jackson ImmunoResearch Laboratories). PML was detected using 5E10 mAb (for U2OS cells) followed by Alexa Fluor 350–conjugated goat anti–mouse Ab (Molecular Probes) or rabbit polyclonal anti-PML Ab (for NIH 3T3 cells) followed Alexa Flour 350–conjugated anti–rabbit Ab (Molecular Probes). eIF4E was detected using FITC-conjugated mouse monoclonal anti-eIF4E Ab (BD Transduction Laboratories). Cells were mounted in Vectashield supplemented with DAPI (Vector Laboratories). Images were obtained using TCS-SP software and displayed using Adobe Photoshop CS 8.0.

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