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′ m7G 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 a 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 (m7G) 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 m7G 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).
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'G 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. mRNAs 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 eCP1-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'G cap for association of mRNA with eIF4E in the nucleus (Fig. 1, D and E). We monitored the ability of m'G cap analogue (m'GpppG) 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'G cap in order to associate with specific mRNAs in the nucleus. Note that treatment with m'GpppG or GpppG did not alter the amount of eIF4E immunoprecipitated by eIF4E antibody in these reactions (unpublished data).
where cap binding is sufficient to mediate these interactions. Other regulatory elements that are not present in the cytoplasm, that eIF4E-mRNA recognition is restricted in nuclear lysates by incubation with GAPDH mRNA (Fig. 1 C). This raises the possibility that even when nuclear lysates are incubated with recombinant eIF4E in the SNAAP assay, we do not observe an association with GAPDH mRNA. 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 m7GpppG 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 associate with all mRNAs in the nuclear fraction (Lejeune et al., 2002), whereas the data we present here clearly indicate that eIF4E associates with both cyclin D1 mRNA 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 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.

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 recombiant 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.
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 (Topicirovic 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 m7G cap by >100-fold (Kentsis et al., 2001), thus disabling RNA binding. Because eIF4E requires its
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).

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

| Construct | Nc/Cyt Ratio |
|-----------|--------------|
| 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 |

*Cyclin D1 mRNA levels were normalized to GAPDH mRNA; ± value represents SD from three independent experiments.*
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. 3A), as well as reduced cyclin D1 but not GAPDH or actin protein levels (Fig. 3C). This is consistent with the results from immunoprecipitation and in situ studies, where PML inhibits formation of eIF4E–cyclin D1 mRNA complexes (Fig. 1B and Fig. 2A). Once again it links the ability of eIF4E to physically interact with RNAs to the ability to promote mRNA transport.

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 constructs containing UTRs were cloned up- or downstream of LacZ, respectively. Numbers represent position of UTR fragments in cyclin D1 mRNA. NIH3T3 cells were transiently transfected with chimeric LacZ constructs containing UTR-LacZ, LacZ-3'UTR, or LacZ constructs containing 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'UTR in the IP eIF4E. Sequence alignment of cyclin D1 4ESE 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 (c.1.14,792,997-14,795,000) and gi: U40844.

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. 3E). 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. 3E). 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. 3C). eIF4E levels are expressed to similar levels (Fig. 3C). Note that the objective was 100X for these micrographs with a further 1.5-fold magnification.

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

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

*Cyclin D1 mRNA levels were normalized to GAPDH mRNA; ± value represents SD from three independent experiments.*
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 elf4E 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 elf4E to associate with these mRNAs using immunoprecipitation in conjunction with PCR (Fig. 4 B). Importantly, elf4E does not immunoprecipitate with LacZ mRNA, does not immunoprecipitate with LacZ-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 elf4E 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 elf4E, so we refer to it as an elf4E-sensitive 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 elf4E was functional, we examined the effects of elf4E 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). elf4E 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 elf4E 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 elf4E 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 elf4E 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 elf4E 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′G cap are required for elf4E to enhance transport of these mRNAs.

The 4E-SE contributes to elf4E mediated oncogenic transformation

We extended these studies to establish whether the 4E-SE contributed to the physiological activities of elf4E and thereby to assess the functional significance of this RNA element (Fig. 7). Our previous studies correlated elf4E-dependent promotion of cyclin D1 mRNA export with the transformation activities of elf4E so we examined the contribution of the 4E-SE to this activity. Transformation activity was assessed by monitoring the number of foci formed upon elf4E overexpression in a cyclin D1+/- fibroblast cell line. Note that the distribution of elf4E nuclear bodies is not altered in cyclin D1-/- as compared with other cell types (Fig. 2 C). First, we determined that elf4E...
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 fractions 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.

### 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 m1G 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

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

| Construct | Relative C:N LacZ mRNA ratio |
|-----------|-----------------------------|
| 4EWT ± LacZ | 1 ± 0.082 |
| 4EWT ± 3′UTR | 462.496 ± 38.114 |
| 4EWT ± 3′UTR Full | 373.934 ± 30.195 |
| 4EWT ± 3′UTR/2/3 | 0.823 ± 0.069 |
| 4EWT ± 3′UTR | 1.178 ± 0.119 |
| W56A ± LacZ | 1.159 ± 0.124 |
| W56A ± 3′UTR | 1.918 ± 0.286 |

*LacZ mRNA levels were normalized to GAPDH mRNA; ± value represents SD from three independent 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 as 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.
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 transit (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 (4E-BPs; 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 m7G 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 pcDNA-LacZ (human cyclin D1 gene in pGEM7zf; Motokura and Aris-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 pMVCyclin 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 (cyd14E-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 Translaction Laboratory), 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-CBP pAb [a gift from L. Maqiat, University of Rochester, Rochester, NY; Ishigaki et al., 2001] anti-GFP Ab [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 D11+); Perez-Roger et al., 1999] cells were maintained in 5% CO2 at 37°C in DME ( Gibco BRL, Life Technologies), supple-mented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin. eIF4E and PML stably transfected NIH3T3 were made as described previ-ously (Topisirovic et al., 2002, 2003a). Transient transfection of NIH3T3 was performed using either GeneJummer Transfection Reagent (Stratagene) or Lipofectamine Plus reagent (Invitrogen) according to the manufac-turer’s instructions. Transient transfections of NIH3T3 were performed using Calcium Phosphate Transfection kit (Invitrogen). Stable transfections of cyclin D11−/− 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 × 106 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. 1/20 were split in three aliquots, two of which, when indicated in the text, were incubated with 50 µM mGpppG 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 Sensi-Script Reverse Transcription kit (QIAGEN). RT-PCR was performed in triplicate with the Quantitect SYBR green RT-PCR Kit (QIAGEN) in an Opticon thermal cycler (MJR). Obtained RT-PCR data was analyzed with Option software (MJR). Primers used for cyclin D1 RT-PCR were cyf5′-caggagcaggagctgcgc-3′ and cyr5′-acaaggggtcgccgcagcgc-3′; and for GAPDH amplification GAPDHf5′-acccaatcgtacccatgc-3′ and GAPDHr5′-caggagcaggagctgcgc-3′.
DHR, 5′-ttcaacaccagcctgtc-3′. 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: cyclHMF, 5′-ctctctctcctatatgcca-3′; cyclHMR, 5′-ctggcagcaggctacct-3′; Actf, 5′-atgctgccctcccagagctg-3′; and Actf, 5′-catgtcactctggctgatactgc-3′.

Controls for quality of immunoprecipitation and fractions
Several steps were taken to ensure that variability between experiments did not lead to false positive or false negative results. The immunoprecipitated sample which 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 merase 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 tRNALys (cytoplasmic) as we reported previously. Cell extracts were added to 50 μl of RBB buffer containing 0.5% NP-40, and after incubation of 30 min at 4°C, 500 μg of yeast RNA 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 from fractionated RNA using Oligotex mRNA Mini Kit (Qiagen). Probes for cyclin D1, GAPDH, U6, and tRNALys were hybridized with BglII fragments (cyclin D1 specific 5SA, 5′-cttgaacaccagcctcggagg-3′ and 5′-gactgtaaaccagcctgag-3′ and labeled with brightiang Psoralen-Biotin kit (Ambion). Immunofluorescence, in situ hybridization, and laser scanning confocal microscopy
Immunofluorescence experiments were 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 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). Immunofluorescence experiments were performed as described previously (Topisirovic 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 of RBB buffer containing 0.5% NP-40, and after incubation of 30 min at 4°C, 500 μg of yeast RNA 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.

References
Boisvert, F.M., M.J. Hendzel, and D.P. Bazett-Jones. 2000. Promyelocytic leukemia (PML) nuclear bodies are protein structures that do not accumulate RNA. J. Cell Biol. 148:283–292.

Borden, K.L.B., M.N. Boddy, J. Sally, N.J. O’Reilly, S. Martin, K. Howe, E. Solomon, and P.S. Freemont. 1995. The structure of the RING finger domain from the acute promyelocytic leukaemia proto-oncoprotein PML. EMBO J. 14:1531–1542.

Cohen, N., M. Sharma, A. Kentsis, J.M. Perez, S. Strudwick, and K.L. Borden. 2001. PML RING suppresses oncogenic transformation by reducing the affinity of eIF4E for mRNA. EMBO J. 20:4547–4559.

Dostie, J., M. Ferraiuolo, A. Pause, S.A. Adam, and N. Sonenberg. 2000. A novel shuttling protein, 4E-T, mediates the nuclear import of the mRNA cap-binding protein, eIF4E. EMBO J. 19:3142–3156.

Gingras, A.C., B. Raught, and N. Sonenberg. 1999. eIF4E initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu. Rev. Biochem. 68:913–963.

Iborra, F.I., D.A. Jackson, and P.R. Cook. 2001. Coupled transcription and translation within nuclei of mammalian cells. Science. 293:1139–1142.

Ishigaki, Y., X. Li, G. Serin, and L.E. Maquat. 2001. Evidence for a pioneer round of mRNA translation: mRNAs subject to nonsense-mediated decay in mammalian cells are bound by CBP80 and CBP20. Cell. 106:607–617.

Izaurralde, E., J. Lewis, C. Gamberi, A. Jarmolowski, C. McGuigan, and I.W. Mattaj. 1995. A cap-binding protein complex mediating U snRNA export. Nature. 376:709–712.

Kentsis, A., E.C. Dwyer, J.M. Perez, M. Sharma, A. Chen, Z.Q. Pan, and K.L. Borden. 2001. The RING domains of the promyelocytic leukemia protein PML and the arenaviral protein z repress translation by directly inhibiting translation initiation factor eIF4E. J. Mol. Biol. 312:609–623.

Kentsis, A., I. Topisirovic, B. Culjkovic, L. Shao, and K.L. Borden. 2004. Ribavirin suppresses eIF4E-mediated oncogenic transformation by physical inactivity of the 7-methyl guanosine mRNA cap. Proc. Natl. Acad. Sci. USA. 101:18105–18110.

Lai, H.K., and K.L. Borden. 2000. The promyelocytic leukemia (PML) protein suppresses cyclin D1 protein production by altering the nuclear cytoplasmic distribution of cyclin D1 mRNA. Oncogene. 19:1623–1634.

Lang, V., N.I. Zanchin, H. Lunsdorf, M. Tuite, and J.E. McCarthy. 1994. Initiation factor eIF-4E of Saccharomyces cerevisiae. Distribution within the cell, binding to mRNA, and consequences of its overproduction. J. Biol. Chem. 269:6117–6123.

Lazaris-Karatzas, A., K.S. Montine, and N. Sonenberg. 1990. Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5′ cap. Nature. 345:544–547.

Lazaris-Karatzas, A., and N. Sonenberg. 1992. The mRNA 5′ cap-binding protein, eIF-4E, cooperates with v-myc or E1A in the transformation of primary rodent fibroblasts. Mol. Cell. Biol. 12:1234–1238.

Ljushkovc, E., C. Goyer, A. Darveau, S. Neron, R. Lemieux, and N. Sonenberg. 1992. A fraction of the mRNA 5′ cap-binding protein, eukaryotic initiation factor 4E, localizes to the nucleus. Proc. Natl. Acad. Sci. USA. 89:9612–9616.
Lejeune, F., Y. Ishigaki, X. Li, and L.E. Maquat. 2002. The exon junction complex is detected on CBP80-bound but not eIF4E-bound mRNA in mammalian cells: dynamics of mRNP remodeling. *EMBO J.* 21:3536–3545.

Marcotrigiano, J., A.C. Gingras, N. Sonenberg, and S.K. Burley. 1997. Cocrystal structure of the messenger RNA 5′ cap-binding protein (eIF4E) bound to 7-methyl-GDP. *Cell.* 89:951–961.

McKendrick, L., E. Thompson, J. Ferreira, S.J. Morley, and J.D. Lewis. 2001. Interaction of eukaryotic translation initiation factor 4G with the nuclear cap-binding complex provides a link between nuclear and cytoplasmic functions of the m(7) guanosine cap. *Mol. Cell. Biol.* 21:3632–3641.

Morley, S.J., and V.M. Pain. 1995. Hormone-induced meiotic maturation in *Xenopus* oocytes occurs independently of p70s6k activation and is associated with enhanced initiation factor (eIF)-4F phosphorylation and complex formation. *J. Cell Sci.* 108:1751–1760.

Motokura, T., and A. Arnold. 1993. PRAD1/cyclin D1 proto-oncogene: genomic organization, 5′ DNA sequence, and sequence of a tumor-specific rearrangement breakpoint. *Genes Chromosomes Cancer.* 7:89–95.

Nathan, C.A., P. Carter, L. Liu, B.D. Li, F. Abreo, A. Tudor, S.G. Zimmer, and A. De Benedetti. 1997. Elevated expression of eIF4E and FGF-2 isoforms during vascularization of breast carcinomas. *Oncogene.* 15:1087–1094.

Perez-Roger, I., S.H. Kim, B. Griffiths, A. Sewing, and H. Land. 1999. Cyclins D1 and D2 mediate myc-induced proliferation via sequestration of p27(Kip1) and p21(Cip1). *EMBO J.* 18:5310–5320.

Rousseau, D., R. Kaspar, I. Rosenwald, L. Gehrke, and N. Sonenberg. 1996. Translation initiation of ornithine decarboxylase and nucleocytoplasmic transport of cyclin D1 mRNA are increased in cells overexpressing eukaryotic initiation factor 4E. *Proc. Natl. Acad. Sci. USA.* 93:1065–1070.

Sonenberg, N., and A.C. Gingras. 1998. The mRNA 5′ cap-binding protein eIF4E and control of cell growth. *Curr. Opin. Cell Biol.* 10:268–275.

Spector, D.L., R.D. Goldman, and L.A. Leinwand. 1998. Cells: A Laboratory Manual. Cold Spring Harbor, Cold Spring Harbor, NY. 116.8–116.16.

Strudwick, S., and K.L. Borden. 2002. The emerging roles of translation factor eIF4E in the nucleus. *Differentiation.* 70:10–22.

Stuurman, N., A. de Graaf, A. Floore, A. Josso, B. Humbel, L. de Jong, and R. van Driel. 1992. A monoclonal antibody recognizing nuclear matrix-associated nuclear bodies. *J. Cell Sci.* 101:773–784.

Visa, N., E. Izaurralde, J. Ferreira, B. Daneholt, and I.W. Mattaj. 1996. A nuclear cap-binding complex binds baltiarni ring pre-mRNA cotranscriptionally and accompanies the ribonucleoprotein particle during nuclear export. *J. Cell Biol.* 133:5–14.