Eukaryotic Translation Initiation Factor 4E Regulates Expression of Cyclin D1 at Transcriptional and Post-transcriptional Levels*

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Regulation of the cell cycle is orchestrated by cyclins and cyclin-dependent kinases. We have demonstrated previously that overexpression of eukaryotic translation initiation factor 4E (eIF-4E) in NIH 3T3 cells growing in 10% fetal calf serum leads to highly elevated levels of cyclin D1 protein without significant increase in cyclin D1 mRNA levels, suggesting that a post-transcriptional mechanism is involved. (Rosenwald, I. B., Lazaris-Karatzas, A., Sonenberg, N., and Schmidt, E. V. (1993) Mol. Cell. Biol. 13, 7358–7363). In the present research, we did not find any significant effect of eIF-4E on polysomal distribution of cyclin D1 mRNA. However, the total amount of cyclin D1 mRNA associated with polysomes was significantly increased by eIF-4E overexpression. Further, we determined that the levels of both cyclin D1 protein and mRNA are increased in serum-deprived cells overexpressing eIF-4E. Nuclear run-on experiments demonstrated that the rate of the cyclin D1 transcription is not down-regulated in serum-deprived cells overexpressing eIF-4E. Thus, elevated levels of eIF-4E may lead to increased transcription of the cyclin D1 gene, and this effect becomes visible when serum deprivation down-regulates the rate of cyclin D1 mRNA synthesis in control cells. However, artificial overexpression of cyclin D1 mRNA in serum-deprived cells in the absence of eIF-4E overexpression did not cause the elevation of cyclin D1 protein, and this overexpressed cyclin D1 mRNA accumulated in the nucleus, suggesting that one post-transcriptional role of eIF-4E is to transport cyclin D1 mRNA from the nucleus to cytoplasmic polysomes.

Mitogenic stimulation leads to increased rates of protein synthesis, which is required for entry of resting cells into the cell cycle (2–5). The increase in net protein synthesis after mitogenic stimulation of resting cells is connected with mitogen-induced expression of genes coding for translation initiation factors (6–10). In addition to the total increase in protein synthesis, it is reasonable to expect that there should be selective increases in the synthesis of growth-promoting proteins. One of the translation initiation factors whose levels are increased after mitogenic stimulation of resting cells is the rate-limiting mRNA cap-binding protein eukaryotic translation initiation factor 4E (eIF-4E), which may be involved in unwinding of mRNA 5' secondary structures, mRNA splicing, mRNA 3' processing, and mRNA nucleocytoplasmic transport (11–13). An important role for eIF-4E in cell growth has been demonstrated in experiments in which microinjection of this translation initiation factor into quiescent NIH 3T3 cells induced them to enter the S phase (14). Furthermore, overexpression of eIF-4E transforms both established and primary cells (15–17). It has also been demonstrated that the c-myc oncogene increases the expression of eIF-4E by a transcriptional mechanism (6), while ras and src oncoproteins increase the function of eIF-4E by increasing its phosphorylation (34, 35).

We have examined previously the role of eIF-4E in the expression of cyclin D1/PRAD1/bcl-1 (1). One of the functions of cyclin D1 is to bind the tumor suppressor protein, pRB, which allows hyperphosphorylation and inactivation of pRB by a cyclin D1-dependent kinase that is necessary for G1/S transition (18, 19). Cyclin D1 is expressed late in G1, and its induction by growth factors requires new protein synthesis (20, 21). In addition, cyclin D1 is identical to the PRAD1 oncogene that is overexpressed in parathyroid and breast tumors and is located near the bcl-1 breakpoint in some lymphomas (21–23). We have found that the levels of cyclin D1 protein, but not pRB, are specifically and very strongly increased in continuously growing NIH 3T3 cells overexpressing eIF-4E (1). We did not observe a proportional increase in cyclin D1 mRNA levels, a finding which suggests that the effect of eIF-4E on cyclin D1 expression involves a post-transcriptional mechanism. In the present report we describe our progress on identifying the nature of regulation of cyclin D1 gene expression by translation initiation factor 4E.

MATERIALS AND METHODS

Cell Cultures—Parental NIH 3T3 cells and cells overexpressing wild-type eIF-4E (4E[P2]) or mutated (not active) eIF-4E (4E[Ala]) have been described previously (1, 16). NIH 3T3 cells overexpressing cyclin D1 mRNA following transfection with cyclin D1 gene under the control of the mouse mammary tumor virus (MMTV) promoter have also been described (1). Cells were grown in Dulbecco's modified Eagle's medium (containing glutamine, penicillin/streptomycin) supplemented with 1 The abbreviations used are: eIF-4E, eukaryotic translation initiation factor 4E; MMTV, mouse mammary tumor virus; FCS, fetal calf serum.
Northern Blot Analysis—Cells were lysed by guanidine thiocy-
ate, and RNA was pelleted by centrifugation in a CsCl gradient. Total cellular RNA (10 μg/lanne) was size fractionated on formaldehyde-agarose gels, transferred to Hybond-N nylon membranes, and cross-linked by UV light. The membranes were hybridized overnight at 42 °C with cyclin D1, c-myc, EF-1α, actin, or rRNA cDNA fragments of wild-

Nuclear Run-on Assay—Run-on experiments were performed on pu-
rified nuclei as described (36) with the following modifications. For each cell sample 5 × 10^7 nuclei were purified and used for the assay. Total transcription was measured after purification of RNA using Trizol (Life Technologies Inc.) and scintillation counting of incorporated[^32P]UTP. Labeled RNA at a concentration of 10^5 counts/min/μl was hybridized to cDNA immobilized on nitrocellulose membrane for 48 h. 200 ng of each DNA probe (Fig. 4) were used for dot-blot after alkaline denaturation (37). After hybridization filters were rinsed and treated with 10 μg/ml RNase H for 5 min at 42 °C in 2 × SSC, and membranes were exposed against a Phosphor Image screen (37).

Western Blot Protein Analysis—Western blot analysis was performed as described previously (1) except that the cells were lysed in a modified buffer containing 0.5% Nonidet P-40, 420 mM NaCl, 20 mM Tris, pH 7.5, 2 mM phenylmethylsulfonyl fluoride, 0.02 mM leupeptin. Each cell suspension was passed 15 times through a 20G1 gauge needle to lyse the nuclei following which the lysates were kept on ice for 15 min, centri-

FIG. 1. Expression of cyclin D1 protein in serum-deprived eIF-
4E-overexpressing cells. Cells were plated in 15-cm culture dishes in 10% FCS and grown to three-quarters confluence, cultured for 3 days in 0.5% serum, and treated with fresh 10% FCS added directly to the culture media for the indicated periods before protein extraction. For Western blot analysis 40 μg of protein/lane were analyzed for each sample.

by serum; this is followed by an increase in cyclin D1 protein. The overexpression of mutated (non-functional) eIF-4E does not increase expression of cyclin D1. In contrast, constitutively high expression of functional eIF-4E leads to an increase in cyclin D1 protein levels. Interestingly, the level of cyclin D1 protein in serum-deprived eIF-4E overexpressing cells (0 time point in [4E(P2)] group) is significantly higher than in control cells stimulated with 10% FCS for 12 h.

Polysomal Distribution of Cyclin D1 mRNA in Growth-ar-
rested and Stimulated Cells—To find out whether eIF-4E af-
facts cytosolic distribution of cyclin D1 mRNA we performed a polysome analysis in mutated (Ala) eIF-4E and wild-type eIF-
4E-overexpressing cells. Both Ala and 4E(P2) cells were ini-
tially deprived of serum growth factors for 3 days (designated by R in Fig. 2) and then stimulated (activated) by serum for 4 h. As can be seen in the Fig. 2A, cyclin D1 mRNA is detectable in the polysomes of Ala cells when they are activated (design-
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Increase in Cyclin D1 Protein Correlates with the Level of eIF-4E in Serum-deprived and -stimulated Cells—We have pre-
viously reported that the steady-state levels of cyclin D1 protein, normalized to total cellular protein, are dramatically in-
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tional eIF-4E. As shown in Fig. 1, the level of eIF-4E is very low in serum-deprived (resting) NIH 3T3 cells. The increase in eIF-4E is observed after resting NIH 3T3 cells are stimulated

RESULTS

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tion of resting NIH 3T3 cells (EF-1α) by serum, but there are higher levels of cyclin D1 mRNA in polysomes of both serum-deprived and stimulated 4E(P2) cells. To verify that such a significant in-
crease in the amount of cyclin D1 mRNA found in polysomes of 4E(P2) cells is not due to overloading the RNA-preparing gel, we extracted RNA from polysomal and subpolyosomal fractions of resting and stimulated Ala and 4E(P2) cells and loaded approximately equal amounts of RNA on the gel. The amount of cyclin D1 mRNA is much higher in polysomes of wild-type eIF-4E overexpressing cells than in polysomes of Ala cells under conditions of serum deprivation (R) and stimulation (A), see Fig. 2B. The effect of eIF-4E is specific, since its overexpression had no effect on the amounts of c-myc and actin mRNAs asso-
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Thus, cyclin D1 mRNA was found predominantly associated with polysomes in resting cells and no change in the distribu-
tion of cyclin D1 mRNA was seen following mitogenic activation. Another important finding (Fig. 2A) is that polysomal distribution of translationally regulated mRNA for elongation factor 1α (EF-1α) is not affected by eIF-4E at all. As demonstrated previously (25, 26), we have found that EF-1α mRNA is localized in subpolyosomal fractions of resting cells, but is shifted to heavy polysomes upon mitogenic stimulation. How-
ever, overexpression of functional eIF-4E has no effect on polysomal distribution of EF-1α mRNA in both resting or serum-
activated cells (Fig. 2A). These findings suggest that translational regulation of EF-1α is independent of eIF-4E.

The Total Levels of Cyclin D1 mRNA Are Increased by eIF-4E Overexpression in Serum-deprived Cells—To understand the reason for the observed increase in the amount of cyclin D1 mRNA in polysomes of eIF-4E overexpressing cells, we determined the levels of cyclin D1 mRNA in serum-deprived and

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The Total Levels of Cyclin D1 mRNA Are Increased by eIF-4E Overexpression in Serum-deprived Cells—To understand the reason for the observed increase in the amount of cyclin D1 mRNA in polysomes of eIF-4E overexpressing cells, we determined the levels of cyclin D1 mRNA in serum-deprived and
stimulated parental NIH 3T3 cells and in eIF-4E-overexpressing cells by Northern blot. As can be seen in the Fig. 3, the levels of cyclin D1 mRNA are very low in serum-deprived NIH 3T3 cells and nonfunctional eIF-4E-overexpressing cells (Ala). The levels of cyclin D1 mRNA increase after resting cells are stimulated with 10% FCS. In contrast, the level of cyclin D1 mRNA remains high in serum-deprived eIF-4E-overexpressing cells, and it does not increase significantly after serum stimulation. The effect of eIF-4E on cyclin D1 mRNA levels is specific as there is no difference in the expression of cyclin A and c-myc in 4E(P2) and control cells. Thus, the increased amount of cyclin D1 mRNA in polysomes of serum-deprived eIF-4E-overexpressing cells (Fig. 2) can be explained at least in part by an increase in total amount of cyclin D1 mRNA in these cells.

The Effect of eIF-4E Overexpression on the Transcription Rate of Cyclin D1 Gene—To determine whether overexpression of eIF-4E leads to increased cyclin D1 gene transcription under serum starvation conditions, we performed a nuclear run-on assay. Nuclei were prepared from Ala and 4E(P2) cells continuously growing in 10% FCS or serum-deprived by culturing in 0.5% FCS for 3 days (Fig. 4). As shown in Fig. 4, there is no apparent increase in cyclin D1 transcription in the 4E(P2) cells overexpressing wild-type eIF-4E when grown in 10% FCS, which agrees well with an absence of significant increase in cyclin D1 mRNA in 4E(P2) cells grown in 10% FCS (1). Furthermore, the general transcription rate (data not shown), as well as transcription of both actin and GAPDH decreases in Ala and 4E(P2) cells when they are serum-deprived. In contrast, the rate of transcription of cyclin D1 gene remains the same when 4E(P2) cells are serum-deprived, while in control Ala cells it is significantly down-regulated. The rate of transcription of cyclin D1 gene is 9-fold higher in serum-deprived 4E(P2) cells than in serum-deprived Ala cells when normalized to the general transcription rate. These findings indicate that the high levels of cyclin D1 mRNA observed when 4E(P2) cells are serum-deprived (Fig. 3) are due to constitutively high rates of cyclin D1 gene transcription.

Increased Cyclin D1 mRNA Levels Are Not Sufficient to Provide for the Increase in Cyclin D1 Protein Levels in Serum-deprived Cells—To find out whether increased concentration of cyclin D1 mRNA can account for the observed increase of cyclin D1 at the protein level in serum-deprived eIF-4E-overexpressing cells (Fig. 1), we determined the levels of cyclin D1 mRNA and proteins in NIH 3T3 cells transfected with a cyclin D1 expression vector in which cyclin D1 transcription is controlled by a dexamethasone-inducible MMTV promoter (CD-10 cells, Ref. 1). Fig. 5 (left) shows that the levels of cyclin D1 mRNA are much higher in serum-deprived CD-10 cells than in parental NIH 3T3 cells and are further increased by treatment with dexamethasone. However, Western blot analysis (Fig. 5, right) revealed that there is no increase in cyclin D1 protein in serum-deprived CD-10 cells whether treated or untreated with dexamethasone. Thus, increased levels of cyclin D1 mRNA per se are not able to provide for elevated levels of cyclin D1 protein.

Increased Amounts of Exogenously Overexpressed Cyclin D1 mRNA Accumulate in the Nucleus—In order to account for the observed discrepancy in cyclin D1 mRNA and protein levels, we...
analyzed the distribution of cyclin D1 mRNA between the nucleus and the cytoplasm in cyclin D1 mRNA-overexpressing cells. We found that cyclin D1 mRNA is concentrated in the nuclei of CD-10 cells and is not transported efficiently to cytoplasm (Fig. 6). Furthermore, we have found that nucleocytoplasmic transport of cyclin D1 mRNA is more efficient in functional eIF-4E-overexpressing cells than in control cells.

Increased Expression of Cyclin D1 Protein in the Cells Overexpressing eIF-4E Is Not Sufficient for Their Proliferation in the Media with Low Serum Concentration—Since cyclin D1 protein levels are much higher in cells overexpressing eIF-4E than in control cells under conditions of serum deprivation (Fig. 1), we addressed the question whether elevation of cyclin D1 protein levels correlates with the ability of eIF-4E-overexpressing cells to proliferate when they are deprived of serum growth factors. In order to do so, cells initially plated in 10% FCS were cultured in 0.5% FCS, and cell cycle distribution was analyzed (Fig. 7). The data suggest that increased expression of cyclin D1 is not sufficient for the cells to proliferate in low serum, as wild-type eIF-4E-overexpressing cells withdraw from the cell cycle almost as efficiently as control cells during the initial 6 days of serum deprivation. However, overexpression of eIF-4E appears to allow the cells to re-enter the cell cycle at a later period (Fig. 7), while the control NIH 3T3 and Ala cells die out with G0/G1 DNA content (data not shown). One possible explanation is that the synthesis of autocrine growth factors is increased by eIF-4E, and these factors gradually accumulate in the media reaching a mitogenic concentration after several days of culturing.

**DISCUSSION**

We have found previously that increased expression of cyclin D1 protein in continuously growing eIF-4E-overexpressing cells cannot be explained simply by elevated cyclin D1 mRNA levels, suggesting the involvement of post-transcriptional regulation (1). In this paper we provide data suggesting the complex involvement of eIF-4E in the regulation of cyclin D1 gene expression. First, the levels of eIF-4E protein become critical in determining the amount of total cyclin D1 mRNA when cells are deprived of growth factors (Fig. 3). Further, the levels of total cyclin D1 mRNA (Fig. 3) and the amount of cyclin D1 mRNA in polysomes of serum-deprived and stimulated eIF-4E-overexpressing cells are greatly increased (Fig. 2). These increases correlate with the elevated levels of cyclin D1 protein in these cells (Fig. 1). Furthermore, the transcription rate of cyclin D1 gene remains high when 4E(P2) cells are deprived of growth factors, but it is readily down-regulated in control Ala cells (Fig. 4). It has been demonstrated recently that eIF-4E increases the levels of transcription factors responsible for increased synthesis of autocrine growth factors is increased by eIF-4E, and these factors gradually accumulate in the media reaching a mitogenic concentration after several days of culturing.

**Fig. 5. Increased levels of cyclin D1 mRNA in serum-deprived cells are not sufficient for elevation of cyclin D1 protein.** NIH 3T3 cells transfected with MMTV-driven cyclin D1 expression vector (CD-10, Ref. 1) and control parental cells (NIH) were serum deprived as described above. For the last 24 h of serum deprivation, cells were treated (+) or not treated (−) with dexamethasone to induce MMTV promoter. Left, Northern blot analysis of total cyclin D1 mRNA level. Right, Western blot analysis of cyclin D1 protein level.

**Fig. 6. The overexpressed cyclin D1 mRNA is accumulated in the nuclei of CD-10 cells.** Cells were made quiescent by serum deprivation as described above, and total (T), nuclear (N), and cytoplasmic (C) RNA were extracted and analyzed by Northern blot.

**Fig. 7. Cell cycle analysis of eIF-4E-overexpressing cells during serum deprivation.** Cells were plated at 200 × 10^3/well in 6-well culture plates in 10% FCS. The following day they were washed once with serum-free Dulbecco’s modified Eagle’s medium and further cultured in Dulbecco’s modified Eagle’s medium containing 0.5% FCS. Cells were harvested by trypsinization at indicated days, stained with propidium iodide, and cell cycle distribution was determined by flow cytometry.

terleukin-2 gene expression in T-lymphocytes (40). It is likely that eIF-4E facilitates translation of some transcription factor which is rate limiting for cyclin D1 mRNA synthesis under conditions of serum deprivation.

However, the failure of increased cyclin D1 mRNA levels to provide for elevated cyclin D1 protein levels (when mRNA is overexpressed by exogenous cyclin D1 expression vector) in serum-deprived or continuously growing cells, Fig. 5 and Ref. 1) suggests that the post-transcriptional step is also involved in the control of cyclin D1 expression. The role of post-transcriptional events is also suggested by findings that strong overexpression of cyclin D1 mRNA in transgenic mice from the same construct as in our NIH 3T3 cells provides for much less pronounced (although significant) increase in cyclin D1 protein in the mammary glands of those mice (38). Also, dramatic increase in cyclin D1 protein levels (Ref. 1) and the lack of changes in both the transcription rate of cyclin D1 gene (Fig. 4) and its mRNA levels (Ref. 1) in 4E(P2) cells continuously growing in 10% FCS, suggest the important role for post-transcriptional control. The molecular interactions of eIF-4E protein and cyclin D1 mRNA in post-transcriptional regulation are not understood yet. However, high GC content in the 5′-untranslated region and possible secondary structures in the 5′-untranslated region probably do not play a crucial role in this regulation because most of the 5′-untranslated region is de-
lated in the MMTV cyclin D1 construct providing high levels of cyclin D1 mRNA (cd+10 cells, Figs 5 and 6) without corresponding increase in cyclin D1 protein levels.

Our findings that exogenously overexpressed cyclin D1 mRNA accumulates in the nucleus (Fig. 6) and the demonstration that the cytoplasmic/nuclear ratio for cyclin D1 mRNA is increased in eIF-4E overexpressing cells3 suggest that expression of cyclin D1 is regulated in part by eIF-4E-facilitated transport of cyclin D1 mRNA from the nucleus to cytoplasm. This is consistent with the localization of eIF-4E in both the nucleus and cytoplasm (33). In addition, our findings that the total level of cyclin D1 mRNA is greatly increased in serum-deprived NIH 3T3 cells when they overexpress wild-type eIF-4E and the lack of down-regulation of cyclin D1 gene transcription in serum-deprived 4E(P2) cells suggest that eIF-4E is involved in the regulation of cyclin D1 gene by increasing its transcription (Figs. 3, 4). Mitogen-inducible regulatory elements in the cyclin D1 promoter have been identified (27) and transcription factors that bind to those elements could be possible candidates for regulation by eIF-4E. In summary, there seem to be at least two distinct steps where eIF-4E regulates the expression of cyclin D1 gene: first is the level of cyclin D1 mRNA and second is recruitment of this mRNA from the nucleus into polyosomes.

The overexpression of cyclin D1 has been shown to accelerate the transition of fibroblasts through the G1 period (28). In addition, microinjection of cyclin D1 antibodies or treatment with cyclin D1 antisense oligonucleotide prevented mitogen-activated NIH 3T3, Rat 2, or human lung fibroblasts from entering the S phase of the cell cycle (28, 29). However, introduction of cyclin D1-overexpressing Rat 6 cells into nude mice did not readily produce tumors because an increase in the number of injected cells from 2 x 10^6 to 1 x 10^7 was required for raising the occurrence of tumors from 25 to 100% of cases (30). Cyclin D1 by itself was not able to transform rat embryo fibroblasts and required cooperation with Ha-ras for transformation (31). Recent findings demonstrate that overexpression of cyclin D1 in mammary glands of transgenic mice leads to tumor formation in mice with a latency of 550 days (38). These data taken together suggest that increased cyclin D1 levels per se may be necessary but not sufficient for cell cycle progression and tumorigenicity, and additional events have to occur to bring about cell proliferation and transformation. Our analysis of the cell cycle in serum-deprived cells demonstrated that marked increase of cyclin D1 protein in cells overexpressing eIF-4E does not prevent them from efficient cessation of their proliferation during first 6 days of serum deprivation (Fig. 7). These data are in agreement with the demonstration that cyclin D1-overexpressing Rat 6 fibroblasts withdraw from the cell cycle almost as efficiently as control cells (30) and with the findings that increased expression of cyclin D1 in response to the treatment of human diploid fibroblast with defined mitogens is not sufficient for their entry into S phase (32).

Remarkably, wild-type eIF-4E-overexpressing cells seem to re-enter the cell cycle after 11 days of culture in 0.5% serum. At this time control A1a and parental NIH 3T3 cells died out (data not shown). One explanation is that eIF-4E-transformed cells secrete growth factor(s) into the media (39), which provide for their survival and re-entry into the cell cycle when a threshold mitogenic concentration is reached. In conclusion, our findings demonstrate that eIF-4E can increase the expression of the important cell cycle regulating protein, cyclin D1, by acting through both transcriptional and post-transcriptional regulatory pathways. Finally, it is most likely that cyclin D1 is only one of many growth-promoting proteins whose expression is directly and/or indirectly regulated by translation initiation factor 4E.

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