Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 1065–1070, February 1996
Cell Biology

Translation initiation of ornithine decarboxylase and nucleocytoplasmic transport of cyclin D1 mRNA are increased in cells overexpressing eukaryotic initiation factor 4E

(transformation/RNA transport)

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Communicated by Irving M. London, Massachusetts Institute of Technology, Cambridge, MA, October 23, 1995

ABSTRACT **The structure m^7GpppN (where N is any nucleotide), termed cap, is present at the 5′ end of all eukaryotic cellular mRNAs (except organellar). The eukaryotic initiation factor 4E (eIF-4E) binds to the cap and facilitates the formation of translation initiation complexes. eIF-4E is implicated in control of cell growth, as its overexpression causes malignant transformation of rodent cells and deregulates HeLa cell growth. It was suggested that overexpression of eIF-4E results in the enhanced translation of poorly translated mRNAs that encode growth-promoting proteins. Indeed, enhanced expression of several proteins, including cyclin D1 and ornithine decarboxylase (ODC), was documented in eIF-4E-overexpressing NIH 3T3 cells. However, the mechanism underlying this increase has not been elucidated. Here, we studied the mode by which eIF-4E increases the expression of cyclin D1 and ODC. We show that the increase in the amount of cyclin D1 and ODC is directly proportional to the degree of eIF-4E overexpression. Two mechanisms, which are not mutually exclusive, are responsible for the increase. In eIF-4E-overexpressing cells the rate of translation initiation of ODC mRNA was increased inasmuch as the mRNA sedimented with heavier polysomes. For cyclin D1 mRNA, translation initiation was not increased, but rather its amount in the cytoplasm increased, without a significant increase in total mRNA. Whereas, in the parental NIH 3T3 cell line, a large proportion of the cyclin D1 mRNA was confined to the nucleus, in eIF-4E-overexpressing cells the vast majority of the mRNA was present in the cytoplasm. These results indicate that eIF-4E affects directly or indirectly mRNA nucleocytoplasmic transport, in addition to its role in translation initiation.**

All eukaryotic cellular mRNAs possess a 5′ cap structure that facilitates binding to ribosomes (1, 2). The cap structure is also important for other cellular processes such as mRNA nucleocytoplasmic transport, splicing, and mRNA stability (3–5). A three-subunit initiation factor, eukaryotic initiation factor (eIF) 4F, mediates the translational function of the cap (6). eIF-4F consists of eIF-4E (the cap binding protein subunit), eIF-4A (an RNA helicase), and a high molecular weight subunit termed p220 (whose precise function has not been determined). eIF-4F exhibits RNA helicase activity in conjunction with initiation factor eIF-4B (7, 8). It is thought that the helicase activity is required to melt mRNA 5′ secondary structure to facilitate ribosome binding (for reviews, see refs. 2 and 9). Because of its limiting amount in the cell, eIF-4E has been hypothesized to play a central role in regulation of translation initiation and control of cell growth (9). This is consistent with the findings that treatment of cells with insulin and growth factors inactivates an inhibitor of eIF-4E (10). In addition, phosphorylation of eIF-4E, which increases its affinity to the cap (11), occurs after stimulation of cells by diverse extracellular stimuli that enhance cell growth (for review, see ref. 12).

The regulation of cell growth by eIF-4E has been studied through its overexpression or down-regulation in several cell lines. Overexpression of eIF-4E in NIH 3T3 cells causes tumorigenic transformation (13). In HeLa cells, eIF-4E overexpression results in accelerated proliferation and morphological changes (14). Furthermore, eIF-4E is mitogenic, as its microinjection into serum-starved NIH 3T3 cells activates DNA synthesis (15). These biological activities of eIF-4E are abolished by a mutation of Ser-53 to Ala (13–15) or to either Asp or Glu (A. Lazaris-Karatzas and N.S., unpublished observations). Consistent with these results, inhibition of eIF-4E expression using antisense strategy decreased protein synthesis and partially reverted a ras-transformed phenotype (16). To explain these effects, it has been postulated that overexpression of eIF-4E promotes the translation of mRNAs that are poorly translated because of their long and structured 5′-untranslated region (UTR; refs. 12, 13, and 17). These regions are generally G+C rich and have the potential to fold into stable secondary structures that inhibit ribosome binding (18). Because eIF-4E is a subunit in the eIF-4F complex, and this complex exhibits RNA helicase activity (7), it was argued that increasing the amount of eIF-4E would relieve the translational inhibition of these mRNAs (12). A large proportion of proteins that are involved in regulation of cell growth, differentiation, and development, such as growth factors, growth factor receptors, transcription factors, and homeotic proteins, are encoded by mRNAs with structured 5′-UTRs. Translation of at least some of these mRNAs is expected to be increased in eIF-4E-overexpressing cells.

Two proteins whose expression is elevated in NIH 3T3 cells overexpressing eIF-4E are cyclin D1 and ornithine decarboxylase (ODC; refs. 19 and 20). An increase in cyclin D1 and ODC proteins occurs without a corresponding increase in their mRNA levels (19, 20). These proteins behave as protooncogene products, since their overexpression in rodent cells leads to transformation (21, 22). Also, ODC mRNA contains a highly structured 5′-UTR, which inhibits translation (23). To investigate the mechanisms by which ODC and cyclin D1 protein synthesis is increased posttranscriptionally by eIF-4E, we examined the polysome distribution of these mRNAs in control cells and cells overexpressing eIF-4E. We report that the expression of ODC and cyclin D1 correlates with the eIF-4E overexpression level. The results presented in this

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Abbreviations: eIF, eukaryotic initiation factor; UTR, untranslated region; ODC, ornithine decarboxylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; snRNA, small nuclear RNA.

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paper indicate that increased translation is directly correlated with both translational initiation and mRNA export from the nucleus.

MATERIALS AND METHODS

Cell Culture. NIH 3T3 fibroblast cells were infected with a pMV7/eIF-4E retrovirus, pMV7/eIF-4E Ala 53, or pMV7 vector alone as described (13). Cells were grown in Dulbecco's modified essential medium supplemented with 10% fetal bovine serum. NIH 3T3/pMV7 and NIH 3T3/pMV7/eIF-4E cell lines were established by selection for neomycin resistance in active G418 (Sigma) at 500 μg/ml. Selection was removed 1 week prior to the experiments.

Analysis of Anchorage-Independent Growth. Cells (10^4 cells) were grown in soft agar as described (13). Cloning efficiency was measured after 20 days by counting clones (>10–12 cells) in duplicate plates.

Protein Extraction and Analysis. For Western blotting experiments, cells were rinsed twice with ice-cold PBS and scraped using a rubber policeman. Cells were resuspended in lysis buffer [10 mM Tris·HCl (pH 7.4), 1 mM EDTA, 1% Nonidet P-40, leupeptin (10 μg/ml), aprotinin (25 μg/ml), pepstatin (10 μg/ml)] and lysed by two freeze–thaw cycles. Cell debris was pelleted at 12,000 × *g* for 20 min, and the supernatant was recovered for Western analysis. Fifty micrograms of protein [as determined by a Bradford analysis in duplicate (Bio-Rad)] was resolved on an SDS/15% polyacrylamide gel and transferred onto Immobilon membranes (Millipore). Immunodetection was performed at room temperature in TBS buffer [10 mM Tris·HCl (pH 8.0), 150 mM NaCl] containing 0.5% gelatin and 0.1% Tween 20. eIF-4E, ODC, and cyclin D1 were detected with rabbit polyclonal antibodies against the respective proteins. eIF-4A was detected using an anti-eIF-4A monoclonal antibody (a kind gift from H. Trachsel, Bern, Switzerland). Primary antibodies were detected with either ^{125}I-labeled protein A (Amersham) or ^{125}I-labeled anti-mouse IgG. Membranes were exposed to x-ray film and signals were quantified using a Fuji BAS2000 phosphor imaging system.

Polyribosome Analysis. For preparation of cytoplasmic extracts, cells from three 15-cm tissue culture plates (25–30% confluent) were treated with cycloheximide (100 μg/ml; Sigma) for 5 min at 37°C, washed with PBS containing cycloheximide, and harvested by trypsinization. Polyribosomes were prepared as described (24) with slight modifications. The cells were pelleted by centrifugation, swollen for 2 min in 375 μl of low salt buffer [LSB; 20 mM Tris (pH 7.5), 10 mM NaCl, and 3 mM $MgCl_2$] containing 1 mM dithiothreitol and 50 units of recombinant RNasin (Promega), and lysed by addition of 125 μl of lysis buffer [1× LSB/0.2 M sucrose/1.2% Triton N-101 (Sigma)] followed by 10 strokes with a Dounce homogenizer. The nuclei were pelleted by centrifugation in a microcentrifuge at top speed for 30 sec. The supernatant (cytoplasmic extract ≈ 500 μl) was poured into a new tube containing 50 μl of heparin (10 mg/ml; Sigma), 15 μl of 5 M NaCl, and 1 mM dithiothreitol. Equal optical density units (260 nm) of cytoplasmic extracts from 4E-overexpressing or wild-type NIH 3T3 cells (≈200 μl) were layered over 0.5–1.5 M linear sucrose gradients (in LSB) and centrifuged at 45,000 rpm in a Beckman SW50 rotor for 90 min at 4°C. Gradients were fractionated using an ISCO density gradient fractionator equipped with an absorbance monitor (254 nm) and collected into 1/10th volume of 10% SDS. The RNA from each fraction was extracted and analyzed by Northern blot as described (24).

Cellular Fractionation. Preparation of cytoplasm-free nuclei was done according to described procedures (25, 26). Cells were trypsinized, rinsed twice in ice-cold PBS, and resuspended with slow pipetting in lysis buffer B [10 mM Tris (pH 8.4), 140 mM NaCl, 1.5 mM $MgCl_2$, 0.5% Nonidet P-40, 1 mM dithiothreitol, and RNasin (100 units/ml)] with slow pipetting. Nuclear suspensions were centrifuged at 1000 × *g* for 3 min, and the supernatant was saved as the cytoplasmic fraction. Nuclear pellets were rinsed and resuspended in lysis buffer B. One-tenth volume of detergent [3.3% (wt/wt) sodium deoxycholate and 6.6% (vol/vol) Tween 40] was added under slow vortexing, and the nuclear suspension was incubated on ice for 5 min. Nuclei were pelleted by centrifugation at 1000 × *g* for 3 min, and the supernatant (termed postnuclear fraction) was saved. Nuclei were rinsed once in lysis buffer B. This protocol yielded intact nuclei as determined by light microscopy with no significant cytoplasmic material as determined from lysine tRNA content (see Fig. 4).

RNA Extraction and Analysis. Cell fractions were precipitated with 2.5 volumes of ethanol, and RNA was purified with Trizol (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. RNA from the nuclear fractions was extracted directly with Trizol and treated with RNase-free DNase I. Purified RNA was quantitated by spectrophotometry. For Northern analysis, 25 μg of RNA was analyzed on 1.1% agarose/formaldehyde gels. RNA was transferred onto Hybond C membrane (Amersham), and membranes were baked for 2 h at 80°C. Hybridization was performed with ^{32}P-labeled mouse cDNA probes (Ready-To-Go DNA labeling kit; Pharmacia) or with a 5′ ^{32}P-labeled DNA 18-mer complementary to the 3′ end of two lysine tRNA species (1 and 2) at a concentration of 10^6 cpm/ml. Membranes were washed and exposed to x-ray film.

RESULTS

Cyclin D1 and ODC Expression Is Directly Proportional to eIF-4E Levels and Transformation Efficiency. Cyclin D1 and ODC are two proteins whose expression is enhanced in the NIH 3T3 cell line (P2) that overexpresses eIF-4E (19, 20). To determine the generality of this correlation, and its importance for transformation, we examined the effects of eIF-4E overexpression on the transformation phenotype (ability to grow in soft agar) and the increase in cyclin D1 and ODC levels in several independently derived cell lines. Using retrovirus infection, we overexpressed the wild-type eIF-4E and a nonfunctional eIF-4E protein containing a mutation of Ser-53 to an Ala in NIH 3T3 cells. In addition, for control cell lines, NIH 3T3 cells were infected with a retrovirus containing the vector alone. G418-resistant cell lines were established (13), and the levels of eIF-4E, ODC, and cyclin D1 in the cell lines were determined by Western blot analysis. Transformation was analyzed by a soft agar assay. Fig. 1*A* shows the correlation between the extent of eIF-4E overexpression and transformation as determined by cloning efficiency in soft agar. This was performed in 16 independent G418-resistant cell lines that were derived from the pMV7/4E infection and two cell lines derived from infection with virus (pMV7) alone. There is a direct correlation between the amount of eIF-4E and the ability of the cells to grow in soft agar (correlation coefficient, $r = 0.89$). Expression of the Ala-53 mutant did not result in the transformation of NIH 3T3 cells, confirming earlier reports (13, 27), and even eliminated the low background level of growth in soft agar of the parental NIH 3T3 cells. Expression of cyclin D1 and ODC proteins was analyzed in several clones (Fig. 1*B*). Cyclin D1 and ODC levels are increased proportionally to the eIF-4E (wild type) overexpression (correlation coefficients = 0.97 and 0.76, respectively) and are found to be significantly increased (>2.5-fold) in most cell lines in which wild-type eIF-4E is overexpressed (Fig. 1*B*). No change in cyclin D1 and ODC protein levels was detected in NIH 3T3 cells overexpressing the mutant eIF-4E (Ala-53). Fig. 1*C* shows a representative Western blot of two cell lines overexpressing ≈2.5-fold more eIF-4E (4E-20) and mutant eIF-4E (Ala-3) relative to the parental cell line. The amount of ODC and cyclin D1 protein is increased ≈3-fold in wild-type eIF-4E-

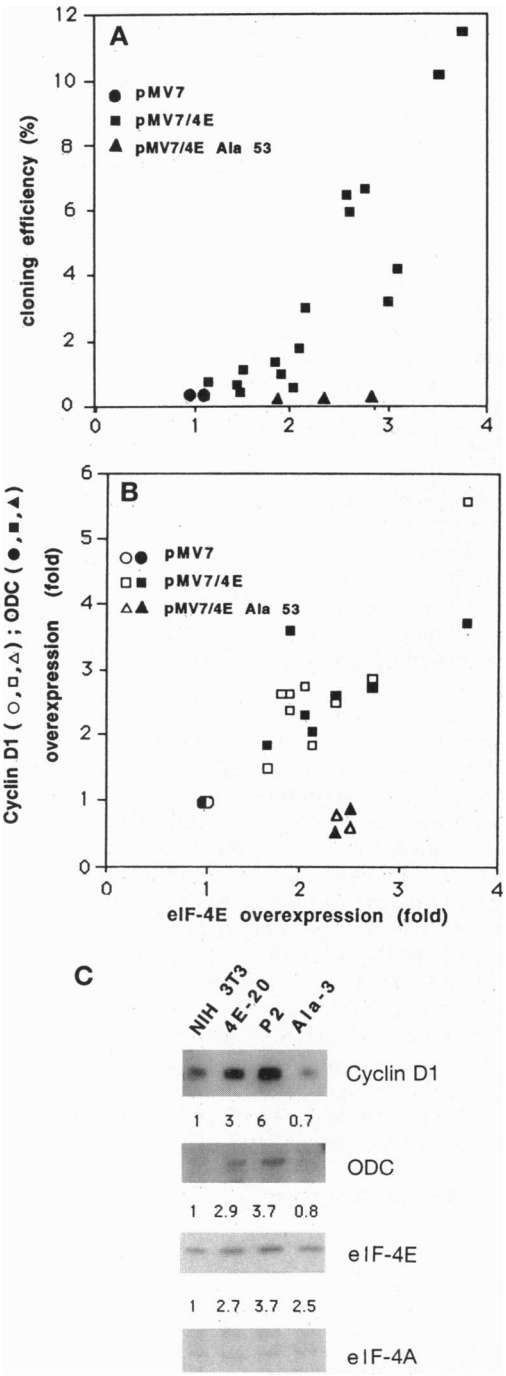

FIG. 1. Correlation of eIF-4E overexpression with growth in soft agar and overexpression of cyclin D1 and ODC. (*A*) NIH 3T3 cell lines derived from retrovirus infection with pMV7 alone (●), pMV7/eIF-4E (■), or pMV7/eIF-4E Ala 53 (▲) were analyzed in parallel for their eIF-4E levels by Western blotting and for their cloning efficiency in a soft agar assay. (*B*) Cell clones derived from retrovirus infection with pMV7 alone (○, ●), pMV7/eIF-4E (□, ■), or pMV7/eIF-4E Ala 53 (△, ▲) were analyzed in parallel for eIF-4E, cyclin D1, and ODC levels by Western blotting. ODC expression is indicated by closed symbols and cyclin D1 is indicated by open symbols. (*C*) Immunodetection of cyclin D1, ODC, eIF-4E, and eIF-4A was performed in representative cell lines overexpressing wild-type eIF-4E (4E-20 and P2) or the eIF-4E Ala 53 mutant (Ala-3) or containing the vector alone (NIH 3T3). eIF-4A protein levels in the various cells were constant, and the numbers below the lanes represent the fold increase in protein expression relative to eIF-4A protein.

overexpressing cells and is diminished by 30% in cells overexpressing mutant eIF-4E.

Analysis of ODC and Cyclin D1 mRNA Polysome Profiles in eIF-4E-Overexpressing Cells. The posttranscriptional mode by which the amounts of ODC and cyclin D1 are increased in eIF-4E-overexpressing cells is not understood. To examine whether translation of cyclin D1 and ODC mRNAs *per se* is increased in eIF-4E-overexpressing cells, we first analyzed the polysome distributions of ODC mRNA in the eIF-4E-overexpressing cells (P2) and in control NIH 3T3 cells. Cytoplasmic RNA was analyzed by centrifugation on sucrose gradients followed by Northern blotting using a mouse cDNA ODC probe (Fig. 2). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was used as a control for the amount of mRNA loaded. In the parental NIH 3T3 cells, most of ODC mRNA sedimented with small polysomes (containing two or three ribosomes), consistent with the poor translation of the ODC mRNA (Fig. 2*C*). However, in cells overexpressing eIF-4E, there was a significant increase (5-fold) in the amount of ODC mRNA in heavier polysomes (>5 ribosomes; Fig. 2*B*). These results demonstrate that translation initiation on ODC mRNA in eIF-4E-overexpressing cells is stimulated. It is also noteworthy that the amount of cytoplasmic ODC mRNA was increased (2-fold after normalization against GAPDH mRNA) in cells overexpressing eIF-4E. Such an increase has not been previously observed when total cellular ODC mRNA was analyzed (20). The explanation for the increase in the amount of ODC mRNA is addressed below.

A polysome distribution analysis similar to that done for ODC mRNA was performed for cyclin D1 mRNA. Surprisingly, and in marked contrast to the polysome profile of ODC, cyclin D1 polysome distribution was qualitatively unaffected by eIF-4E overexpression, but rather the total amount of polysomal cyclin D1 mRNA was increased (Fig. 3). In both control and eIF-4E-overexpressing cells, cyclin D1 mRNA sedimented with large polysomes, indicating efficient translation initiation, but the total amount of cytoplasmic cyclin D1 mRNA was significantly increased (6-fold after normalization against GAPDH mRNA) in eIF-4E-overexpressing cells. The amount of cyclin A mRNA did not change significantly, but the sedimentation profile shifted by one fraction toward larger polysomes in the 4E-overexpressing cells. The level of c-myc mRNA decreased, and a shift similar to the cyclin A1 profile was noted for 4E-overexpressing cells. The apparent decrease in c-myc mRNA levels may be compensated by increased translation initiation, because recent results have shown that c-myc expression is elevated in CHO cells overexpressing eIF-4E (28). Actin mRNA levels increased slightly in the 4E-overexpressing cells while sedimenting in large polysomes. Experiments were also performed with cells overexpressing a mutant form of eIF-4E (Ser-53 to Ala), and no significant changes in the amount and distribution of polysomal cyclin D1 RNA relative to control cells were observed (data not shown).

Increased Level of Cyclin D1 and ODC Cytoplasmic mRNAs Is Due to Increased Nucleocytoplasmic Transport. The finding that the amount of cytoplasmic cyclin D1 was increased in eIF-4E-overexpressing cells was unexpected inasmuch as it was reported earlier that the levels of total cyclin D1 mRNA in eIF-4E-overexpressing cells were not changed substantially relative to the parental cells (19). One possible explanation for the apparent discrepancy between the earlier results (19) and those described here is the sources of the mRNA. Rosenwald *et al.* (19) analyzed total RNA, whereas the distribution of cytoplasmic mRNA in polysomes was analyzed in this study. Thus, it is possible that cyclin D1, and to a lesser extent ODC, mRNA is differentially compartmentalized between the nucleus and cytoplasm in parental versus eIF-4E-overexpressing NIH 3T3 cells.

To address this possibility, we performed Northern blot analysis of cyclin D1 and ODC mRNAs in nuclear and cytoplasmic fractions. To prepare nuclei that are free of trace cytoplasmic contaminants, we used a protocol whereby cyto-

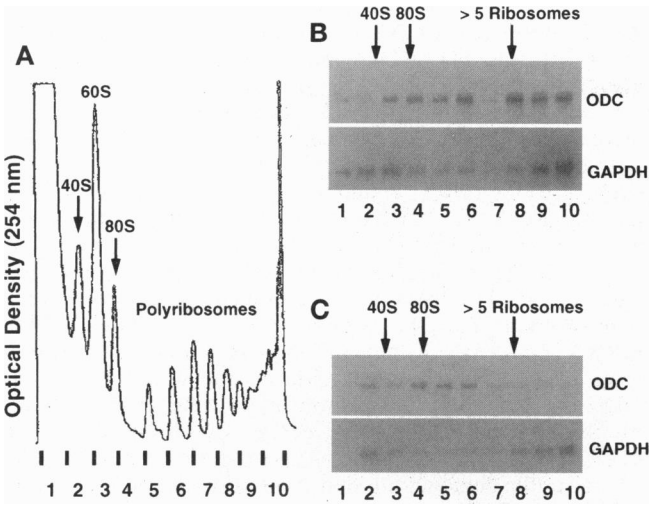

FIG. 2. Polysome distribution of ODC mRNA in eIF-4E-overexpressing cells. (*A*) Polysomes from NIH 3T3 cells or eIF-4E-overexpressing (P2) cells were prepared and resolved by sedimentation on sucrose gradients as described in *Materials and Methods*. The distribution of ODC and GAPDH mRNAs across the gradients was analyzed by Northern blotting with labeled cDNA probes. (*B*) eIF-4E-overexpressing NIH 3T3 cells. (*C*) NIH 3T3 cells.

plasmic components that are tightly bound to the nucleus are removed by detergent treatment (25, 26). Using this method, we obtained highly purified nuclei, as assessed by light microscopy of nuclei. We analyzed the RNA distribution in three cellular fractions: cytoplasm (supernatant of first lysis), postnuclear fraction (supernatant of detergent-washed nuclei), and nucleus. The distribution of total RNA in the different fractions from both NIH 3T3 and P2 cells was as follows: cytoplasm, 35%; postnuclear fraction, 50%; and nuclear fraction, 15% (Table 1). An equal amount of RNA from each fraction was analyzed on gels. Consequently, the intensity of the signals in Fig. 4 does not reflect the relative subcellular distribution of the mRNAs in the cell (see Table 1). To test for nuclear integrity, we examined the distribution of nuclear U6 small nuclear (snRNA) in the different subcellular fractions. This RNA participates in pre-mRNA splicing and is not known to have a cytoplasmic phase (29). A large fraction (≈50% in P2 cells and ≈30% in NIH 3T3 cells) of the U6 RNA was confined to the nuclear fraction (Fig. 4 and Table 1). It is possible that because of its small size (108 nucleotides) this RNA leaks out more readily than mRNAs. Consistent with this possibility, larger RNA species, such as the putative unspliced forms of actin and GAPDH mRNAs (indicated by dots in the right margin) were confined to the nucleus (Fig. 4). We also analyzed the distribution of lysine tRNA, which serves as a cytoplasmic marker, and showed that it was almost completely excluded from the nucleus, indicating that the nuclei were not contaminated with cytoplasm.

Table 1. RNA distribution in cytoplasmic, postnuclear, and nuclear fractions of P2 and parental NIH 3T3 cells

	% of RNA in subcellular fractions					
	Cytoplasmic		Postnuclear		Nuclear	
RNA	3T3	P2	3T3	P2	3T3	P2
Cyclin D1	50	85	12	10	38	5
ODC	30	60	40	35	30	5
Actin	35	30	50	47	15	13
GAPDH	30	30	50	55	20	15
Lys tRNA	30	30	65	60	5	10
U6 snRNA	50	40	20	10	30	50

Equal amounts of RNA were analyzed on blots and quantified from Fig. 4 by phosphor imaging and normalized for the RNA distribution in the cell. The total amount of RNA in the different subcellular fractions was as follows: cytoplasmic, 35%; postnuclear, 50%; and nuclear, 15%.

The total amount of cyclin D1 and ODC mRNAs is not significantly altered (1.5- to 2-fold) in eIF-4E-overexpressing cells relative to parental cells when adding up the signals in nuclear, postnuclear, and cytoplasmic fractions and normalizing for the relative distribution of RNA in the different fractions (Fig. 4). However, there is a striking difference in the ratio of cytoplasmic to nuclear cyclin D1 mRNA between P2 cells and parental NIH 3T3 cells. In NIH 3T3 cells, a large fraction of cyclin D1 mRNA (38% after normalization to represent the relative amount in intact cells) is confined to the nucleus, but this nuclear fraction decreased dramatically to ≈5% in P2 cells (Table 1). For ODC mRNA, the change in cellular distribution between NIH 3T3 and P2 cells was similar

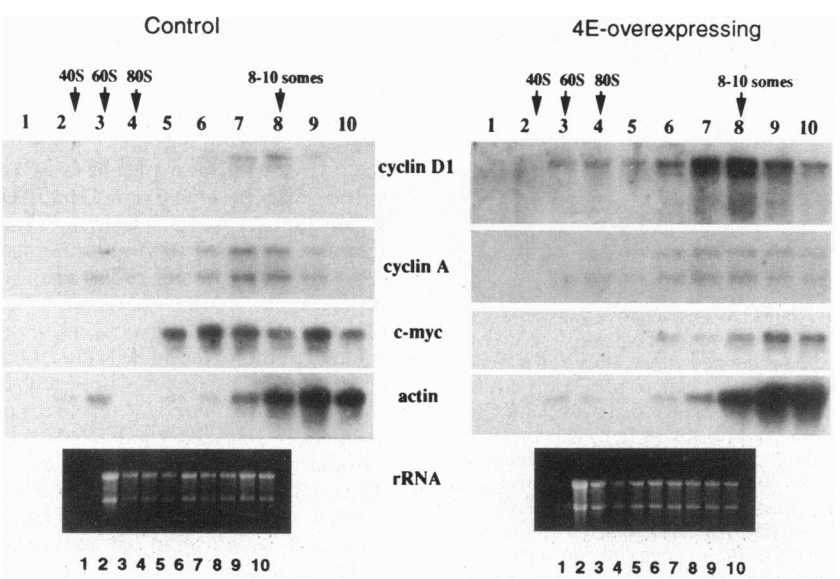

FIG. 3. Polysome distribution of cyclin D1 mRNAs in eIF-4E-overexpressing cells. Polysomes from NIH 3T3 cells (*Left*) or eIF-4E-overexpressing (P2) cells (*Right*) were prepared and resolved by sedimentation on sucrose gradients as described in *Materials and Methods*. The amount of cyclin D1, cyclin A, c-myc, and actin mRNAs was determined by Northern blot analysis. Ethidium bromide staining of the gel is shown at the bottom.

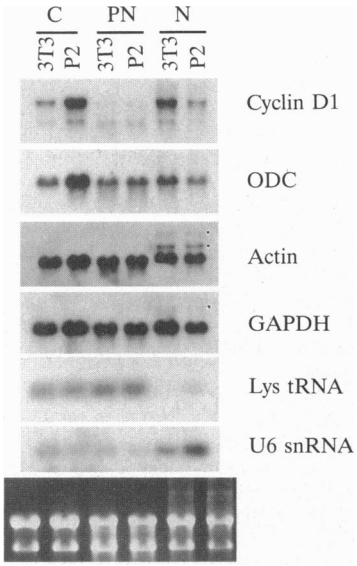

FIG. 4. Subcellular localization of cyclin D1 and ODC mRNAs. NIH 3T3 control and P2 cells were fractionated into three subcellular compartments as described in *Materials and Methods*. Total RNA was purified and an equal amount of RNA (30 μg) from cytoplasmic (C), postnuclear (PN), and nuclear (N) fractions was resolved on gels followed by Northern blotting as described in *Materials and Methods*. Cyclin D1, ODC, β-actin, GAPDH, and U6 snRNA cDNAs were used as probes for the corresponding RNAs. An oligonucleotide probe was used for detection of lysine tRNA. Ethidium bromide staining of the gel is shown at the bottom. This experiment was repeated twice, and the values obtained did not vary by more than 15%.

if slightly less dramatic. The amount of ODC mRNA in the nucleus decreased from 30% in the parental cell line to 5% in P2 cells. In sharp contrast to cyclin D1 and ODC, no significant changes occurred in the nuclear to cytoplasmic ratio for actin and GAPDH mRNAs in P2 cells. For these mRNAs, only $\approx$15% is confined to the nucleus in both NIH 3T3 and P2 cells. For most of the mRNAs tested, there was a large fraction present in the postnuclear wash compartment in both NIH 3T3 and P2 cell lines, except for cyclin D1 mRNA. The significance of this observation is not immediately clear, and we have not pursued this further. In conclusion, these results indicate that the increase of cyclin D1 protein in cells overexpressing eIF-4E results from the increased export of the mRNA to the cytoplasm.

DISCUSSION

The findings in this paper strongly support the hypothesis that overexpression of eIF-4E enhances translation of certain mRNAs (e.g., ODC) at the level of translation initiation. Importantly, these findings also demonstrate that overexpression of eIF-4E could cause an unpredicted increase in the ratio of cytoplasmic to nuclear mRNA.

We have tested in this study several different mRNAs including those that encode cyclin D1 and ODC. The expression of ODC is enhanced at the level of translational initiation, as expected according to our current model for the function of eIF-4E in translation. The model is based on the activity of the eIF-4F complex (of which eIF-4E is a component) as an RNA helicase. It is thought that the RNA helicase activity is required for melting the 5′-UTR mRNA secondary structure to facilitate ribosome binding. Since eIF-4E is the most limiting factor among all initiation factors, the unwinding of the 5′ secondary structure in the mRNA is expected to be dependent on eIF-4E. Consequently, mRNAs that contain extensive secondary structure in their 5′-UTR are expected to be poorly translated. Indeed, excess secondary structure in the mRNA 5′-UTR inhibits translation initiation (18, 30). ODC mRNA contains a highly structured 5′-UTR of 284 nucleotides, which is inhibitory to translation (23). Furthermore, treatment of cells with growth factors or insulin stimulated ODC mRNA translation without a corresponding increase in mRNA levels (31, 32). These treatments lead to an increase in eIF-4E phosphorylation and its ability to bind better to the cap structure (11). The 5′-UTR of cyclin D1 is also longer than average (170 nucleotides), but it is predicted to be less structured than ODC mRNA.

How is the effect of eIF-4E overexpression on cyclin D1 and ODC mRNA nucleocytoplasmic distribution explained? One possibility is that overexpression of eIF-4E stimulates the translation of an mRNA that encodes a protein which is important for mRNA export from the nucleus. According to this model, the effect of eIF-4E on cyclin D1 expression would be indirect. A second possibility is that nucleocytoplasmic transport of mRNAs is coupled to translation initiation and both are dependent on eIF-4E. This possibility is intriguing in light of the finding that a significant fraction ($\approx$30%) of eIF-4E is confined to the nucleus (33). Also, the cap structure has been shown to facilitate the nucleocytoplasmic transport of mRNAs and snRNAs (3). There are several nuclear cap binding proteins (34–37) that might mediate mRNA transport. One of these proteins is a complex of two polypeptides of 20 and 80 kDa, termed cap binding protein complex (38). This protein was shown to be required for splicing (38) and was proposed to play an essential role in export of U RNAs but only a minor part in the export of mRNAs (34). Thus, it is possible that cap binding protein complex binds to the cap structure of snRNAs, while eIF-4E binds to the cap structure of mRNAs to facilitate their export to the cytoplasm. This differential binding would then serve to direct mRNAs to the translation machinery and prevent association of ribosomes with snRNAs.

In addition, our findings might be related to the phenomenon of nonsense codon-mediated nuclear degradation of mRNA (26, 39–41). In mammalian cells, nonsense codons cause mRNA degradation in the nucleus. Because nonsense codons are in all likelihood recognized only by the cytoplasmic translation machinery, it was proposed that translation occurs concurrently with the mRNA transport to the cytoplasm (39, 40). Thus, ribosomes that encounter a nonsense codon relay a signal in *cis* to the nucleus that leads to mRNA destabilization. This model was initially termed "translation-translocation" and implied that export and translation are concomitant with splicing (39). Subsequently, studies have demonstrated that nonsense codons decreased the rate of splicing (42, 43). However, other studies argue against coupling of translation and splicing, and the model was called "co-translational export" (ref. 40; for a recent review, see ref. 41).

How could the mRNA specificity of the eIF-4E effect on transport be explained? One hypothesis is based on the differences among 5′ untranslated leader sequences and structures, as suggested above for the effects on translation. Actin and GAPDH possess 5′-UTRs of average length (50–100 nucleotides), G+C content, and predicted stable structure, whereas the 5′-UTRs of ODC and cyclin D1 are longer and have a high G+C content ($\approx$70%). It is thus conceivable that eIF-4E-facilitated transport is inhibited by secondary structure in the same way that stable secondary structure impairs translation. Because the major effect of eIF-4E overexpression on cyclin D1 is at the level of transport, it is possible that nucleocytoplasmic transport is even more sensitive to secondary structure in the 5′-UTR than translation.

It is noteworthy that infection of cells with adenovirus and influenza virus causes inhibition of mRNA transport from the nucleus to the cytoplasm (44), but not all mRNAs are prevented from exiting the nucleus in adenovirus infection (45). The mechanism for the selective inhibition is not known. In addition, both viruses selectively inhibit host protein synthesis

(46, 47). Several lines of evidence suggest that inactivation of eIF-4E by dephosphorylation is responsible for the shut-off of host protein synthesis in adenovirus (48) and possibly in part in influenza virus (49). It would be of interest to determine whether eIF-4E inactivation plays any role in the inhibition of mRNA transport in virus-infected cells.

We thank Vinita Adkar for excellent technical assistance and L. Maquat and M. Wilkinson for helpful comments on the manuscript. We are grateful to P. Coffino for ODC antibodies and ODC cDNA, L. Kleinman for the lysine tRNA oligomer probe, C. J. Sherr for cyclin D1 cDNA, and I. W. Mattaj for the U6 snRNA clone. This work was supported by grants from the Medical Research Council and National Cancer Institute of Canada to N.S. and by an award from CIBA–Geigy to L.G.; I.R. was supported by an award from the U.S. Public Health Service to Jane-Jane Chen and Irving London and by a grant from the Johnson and Johnson Company to Philippe Leboulch and Irving London. D.R. was supported by Institut National de la Santé et de la Recherche Médicale (CS/AMM/2943) and European Molecular Biology Organization (ALTF2-1993) fellowships.

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