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Overexpression of eukaryotic protein synthesis initiation factor 4E in HeLa cells results in aberrant growth and morphology

(cap binding protein/cell growth control/syncytium/episomal vector)

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ABSTRACT **Eukaryotic protein synthesis initiation factor 4E (eIF-4E) is a 25-kDa polypeptide that binds to the 7-methylguanosine-containing cap of mRNA and participates in the transfer of mRNA to the 40S ribosomal subunit, a step that is rate-limiting for protein synthesis under most cellular conditions. eIF-4E is the least abundant of the initiation factors, is present at ≈10% of molar concentration of mRNA, and thus may serve as a site of regulation for the recruitment of mRNA into polysomes. Previous studies have indicated that phosphorylation of eIF-4E at Ser-53 is correlated with an increased rate of protein synthesis in a variety of systems *in vivo* and is required for eIF-4E to become bound to the 48S initiation complex. In this study we show that overexpression of eIF-4E in HeLa cells using an episomally replicating, BK virus-based vector leads to an unusual phenotype: cells grow rapidly, forming densely packed, multilayered foci. They progressively form syncytia, some containing as many as six nuclei, and ultimately lyse 1 month after transfection. Some of these properties are reminiscent of oncogenically transformed cells. Cells transfected with the identical vector expressing a variant of eIF-4E, which contains alanine at position 53 and thus cannot be phosphorylated at the major *in vivo* site, grow normally. Estimations using the Ala-53 variant or a bacterial chloramphenicol acetyltransferase reporter gene in the same vector indicate that the degree of eIF-4E overexpression is 3- to 9-fold more than the endogenous level. These results suggest that eIF-4E may play a key role in cell cycle progression.**

The initiation of translation in eukaryotes can be regulated at the level of 43S complex formation (binding of initiator Met-tRNA$_i^{met}$ to the 40S ribosomal subunit) and at the level of 48S complex formation (binding of mRNA to the 43S complex). The former occurs during virus infection, after interferon treatment, and in other severe and stressful circumstances (1). Under more normal cellular conditions, the second step, formation of the 48S complex, is rate limiting (2), and regulation by mitogens, growth factors, and serum or during mitosis appears to occur at this step (ref. 3 and references therein). mRNA binding to 43S complexes is catalyzed by the eIF-4 group factors, which collectively recognize the 7-methylguanosine-containing cap, melt secondary structure beginning from the 5′ end, and facilitate the scanning of the mRNA sequence for the initiation codon by the 40S subunit (4, 5).

How mRNA recruitment into 48S initiation complexes is regulated is not completely understood, but a factor that is likely to be involved is eIF-4E, a 25-kDa polypeptide that binds to the cap (presumably the first step in mRNA recruitment) and accompanies mRNA transfer to the 48S complex (6). Whether eIF-4E acts initially as a free polypeptide or in a complex with other polypeptides remains to be established.

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eIF-4E is the least abundant of the initiation factors and is present at ≈10% of the molar concentration of mRNA (6) and ribosomes (7, 8). It is a phosphoprotein, the major site of *in vivo* phosphorylation being Ser-53 (9). Phosphorylation of eIF-4E is correlated with elevated protein synthesis in reticulocytes treated with phorbol esters (10), fibroblasts treated with serum (3), B lymphocytes activated with phorbol esters and ionomycin or lipopolysaccharide (11), and 3T3-L1 fibroblasts treated with insulin (12). Conversely, dephosphorylation of eIF-4E is correlated with the inhibition of protein synthesis in HeLa cells after heat shock (8) or during mitosis (13). Furthermore, a variant of eIF-4E in which Ser-53 is replaced with Ala-53 ([Ala^{53}]eIF-4E) cannot be phosphorylated at the major *in vivo* site and is not found on the 48S initiation complex (14), suggesting that eIF-4E cannot participate in the transfer of mRNA to the 48S complex unless it is phosphorylated.

In the present study we have tested the ideas that eIF-4E is rate-limiting for protein synthesis and that phosphorylation at Ser-53 is essential for its function by overexpressing eIF-4E in HeLa cells with an episomally replicating vector. Overexpression of the wild type (wt) containing Ser-53 resulted in accelerated cell growth and division, but unexpectedly, cells grew to form dense multilayered foci and later become multinucleated. Analogous expression of the [Ala^{53}] eIF-4E variant did not produce these effects, confirming the importance of phosphorylation at Ser-53 and demonstrating that the vector alone was not responsible for this phenotype. These results indicate that overproduction of functional eIF-4E causes the rate of mitosis to exceed that of cytokinesis and suggest that eIF-4E phosphorylation (or dephosphorylation) may play an essential role in cell cycle progression.†

MATERIALS AND METHODS

Expression vectors. The RDB vector is a composite that includes most of the BK papovavirus (18); sequences from pSV2-neo (19) including the simian virus 40 promoter and polyadenylylation region and the aminoglycoside phosphotransferase gene, which confers resistance to the antibiotic G418; a mouse mammary tumor virus promoter driven by a cytochrome P-450 enhancer that can be induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (20); and pGem-7Zf(+) (hereafter pGem-7; Promega Biotec) sequences for bacterial origin of replication and multiple cloning sites. The vector is summarized in Fig. 1. RDB-0 is the vector with no insert in the

Abbreviations: eIF-4E, eukaryotic initiation factor 4E; wt, wild type; [Ala^{53}]eIF-4E, variant polypeptide in which Ser-53 is replaced with Ala-53, expressed from a cDNA; CAT, chloramphenicol acetyltransferase.

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†These results have been presented in preliminary form (15, 16). After submission of this manuscript to the sponsor, similar observations with NIH 3T3 and Rat 2 fibroblasts were published (17).

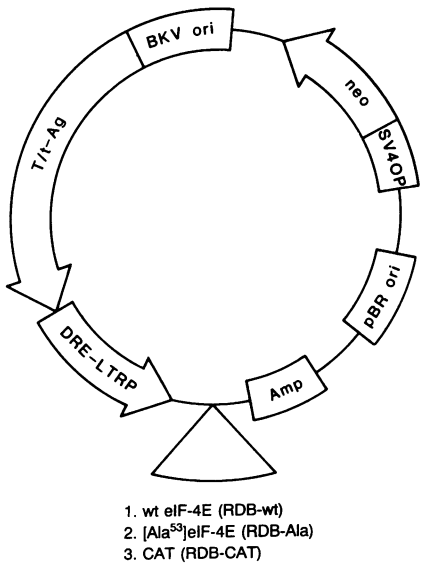

FIG. 1. An outline of the vectors RDB-wt, RDB-Ala, and RDB-CAT. Arrows indicate the direction of transcription. The thick arc following the murine mammary tumor virus (MMTV) promoter indicates the genes inserted to form the various vectors. BKV ori, BK viral origin of replication; T/t Ag, large and small tumor antigens; DRE-LTRP, dioxin-responsive enhancer and the promoter in the long terminal repeat of MMTV; Amp, ampicillin-resistance gene; pBR ori, bacterial origin of replication; SV40P, simian virus 40 early promoter; neo, neomycin-resistance gene (encoding aminoglycoside phosphotransferase).

multiple cloning site. Details of its construction and properties will be published elsewhere.

The cDNA encoding wt Ser-53-containing eIF-4E is from plasmid pTEEC (6), whereas the cDNA for [Ala^{53}]eIF-4E is in the plasmid pTCALA (14). pTCEEC was cut with *Cla* I and *Hin*dIII, and the wt eIF-4E-encoding fragment was inserted in the corresponding sites of the polylinker of pGem-7. pTCALA was cut with *Pvu* II and *Bam*HI. After the addition of a *Hin*dIII linker to the blunt *Pvu* II-cut end, the [Ala^{53}]eIF-4E encoding fragment was cloned in the corresponding sites of the polylinker of pGem-7. Finally, the 3′-terminal noncoding regions of the two inserts were rendered identical by removal of the sequence from 1080 to 1269 (using the numbering system of ref. 21) of the eIF-4E inserts, in the form of a *Nsi* I fragment extending from position 1080 to the *Nsi* I site of pGem-7. The gene for chloramphenicol acetyltransferase (CAT) was isolated by cutting plasmid pMcat4.1 (20) with *Hin*dIII and *Bam*HI and was inserted into the pGem-7 polylinker. In the case of all three pGem-7 constructions (wt eIF-4E, [Ala^{53}]eIF-4E, and CAT), the modified BK virus was cut with *Eco*RI and *Sal* I and inserted between the *Eco*RI and *Xho* I sites of the polylinker.

Cell Transfection and Selection. HeLa cells (ATCC-CCL2) were obtained from American Type Culture Collection and cultured in Dulbecco's modified Eagle's medium with 10% (vol/vol) fetal bovine serum in 100×20 mm dishes. The cells were washed twice with medium lacking serum before addition of a 2-ml suspension containing 20–30 μg of plasmid DNA in Hepes-buffered saline (20 mM Hepes/0.75 mM Na_2HPO_4/140 mM NaCl, pH 7.05) containing 0.08 M $CaCl_2$. After 20 min, 5 ml of medium (containing serum) was added for 5 hr. After recovery for 24 hr in fresh medium, the cells were plated in 75-cm^2 flasks. The next day, 0.2 mg of the antibiotic G418 was introduced per ml of medium to kill cells that did not contain the vector-borne aminoglycoside phosphotransferase gene. The medium was changed daily for 4 days and thereafter once a week. The cells were grown as mass cultures.

Microscopic Observations and Photographs. Cells were viewed through a Nikon N4004 microscope at ×20 with a green/amber filter. For photography, a Nikon 2020 camera was used with Kodak 400 T-max film.

Isolation of eIF-4E. eIF-4E from control HeLa cells and RDB-Ala-transformed cells was isolated from cytoplasmic cell extracts on a m^{7}GTP-Sepharose column (Pharmacia P-L Biochemicals) following the procedure of Webb *et al.* (22). Approximately 3 × 10^7 cells (one confluent culture in a 225-cm^2 flask) were lysed in 2 volumes of 20 mM Hepes (pH 7.6)/20 mM KOAc/0.1 mM EDTA/0.2 mM ATP/0.2 mM GTP/1% Triton X-100. The protein bound to the column was eluted with 1 ml of 0.1 M m^{7}GTP and precipitated by the addition of 0.1 ml of 100% trichloroacetic acid. After extensive washing with acetone, the protein pellet was dried and dissolved in sample buffer for NaDodSO$_4$/PAGE analysis (23).

RESULTS

A bacterial–mammalian cell shuttle vector that replicates episomally in mammalian cells was constructed to express eIF-4E in HeLa cells (Fig. 1). Three separate plasmids were made containing the cDNAs for either unmodified (Ser-53-containing) eIF-4E (RDB-wt), the variant [Ala^{53}]eIF-4E (RDB-Ala), or bacterial CAT (RDB-CAT).

HeLa cells transformed with the RDB-wt and RDB-Ala vectors were followed over time. After 4 days of G418 section, untransformed cells had died, and small colonies of resistant cells formed in the case of both vectors. Eleven days after selection, RDB-Ala-transfected cells had grown to small colonies morphologically indistinguishable from untransfected HeLa cells—i.e., flat, spindle-shaped, and with only a small proportion of round, dividing cells (Fig. 2 *Left*). In contrast, most of the RDB-wt-transfected cells were refractile, suggesting they were rounded-up and undergoing cell division (Fig. 2 *Right*). They also grew in foci that were larger than the RDB-Ala colonies and were many cells thick. To obtain an estimate of the difference in growth rate, cells in individual 11-day colonies were photographed and counted. RDB-Ala-transformed colonies contained 71 ± 24 cells (mean ± SD) whereas RDB-wt-transformed colonies contained 207 ± 71 cells. From these values one can calculate that the mean generation time of RDB-wt-transfected cells was shortened by 20%.

After one month, the RDB-Ala-transfected cells had formed a confluent monolayer, but most of the RDB-wt-transformed cells had lysed. Fig. 2 shows the remains of foci in which most of the cells had died and those remaining were of unusual morphology. Closer examination of RDB-wt-transfected cells 2 days earlier reveals large, strangely shaped cells, many of which contain multiple nuclei (Fig. 3). In some cells, as many as six nuclei can be distinguished (e.g., center of Fig. 3 *Lower*).

This experiment has been performed a total of nine times with identical results. In each instance, untransfected HeLa and RDB-Ala-transfected cells exhibited indistinguishable growth and morphological characteristics. RDB-wt transfection, on the other hand, consistently resulted in rapidly growing foci that degenerated into syncytia, most of which died after 1 month. Attempts to preserve these cells by varying the selection protocol have prolonged their survival but never resulted in a stable cell line.

In these experiments, cells first appeared in G418-resistant colonies, and with time these developed into densely packed foci. It was of interest to test whether the foci would develop without G418 selection to rule out the possibility that they were artifacts of the transfection and selection protocol. Cells were transfected with RDB-wt and then allowed to grow without G418. Foci were observed again and at the same

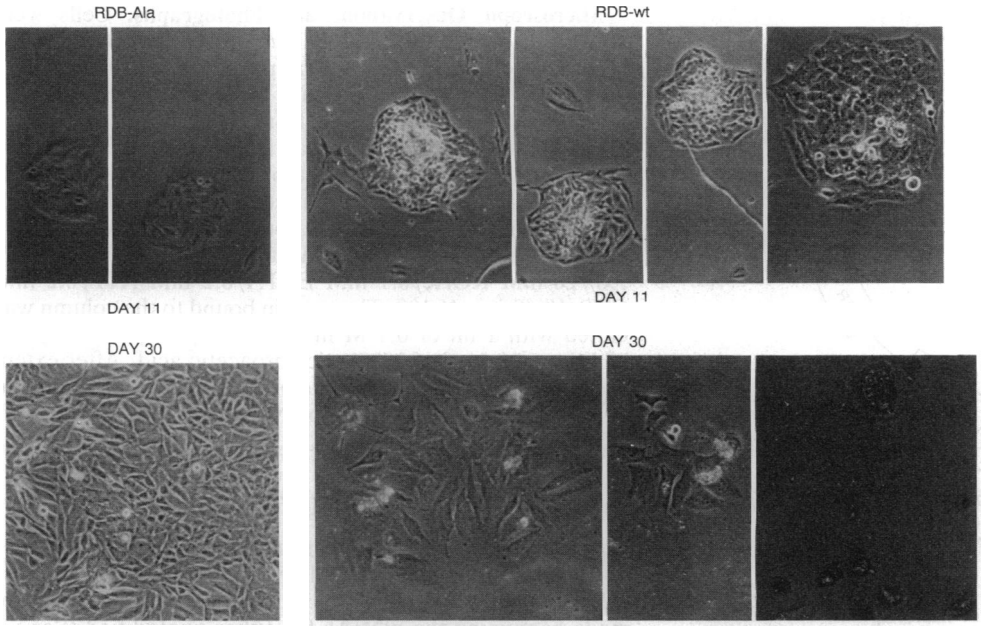

FIG. 2. Growth progression of G418-resistant cells transformed with RDB-Ala and RDB-wt. Transfection protocols are described in *Materials and Methods*. (*Left*) RDB-Ala-transfected cells 11 (*Upper*) and 30 (*Lower*) days after the beginning of G418 selection. (*Right*) The same for RDB-wt-transfected cells. All of the frames were photographed at the same magnification.

frequency as in the experiments where G418 selection was imposed. Fig. 4 shows the growth of an individual focus of cells at 7, 10, and 15 days in the absence of selection. The contrast between refractile, rounded-up cells in the focus and the lawn of normal cells in monolayer is apparent. When G418 was added at 20 days, the lawn of cells died, but those in the focus were completely resistant (Fig. 4, day 23). Many of these cells also appeared to be multinucleated. All of the cells in the focus died by day 30, as observed in the previous experiments. These results indicate that the foci do not arise spontaneously; all of the normally growing cells in the lawn are untransfected, whereas all of the rapidly growing cells in the focus are transformed with RDB-wt. The formation of the multilayered foci amidst a monolayer of untransformed cells indicates that the cells in the foci are not contact-inhibited.

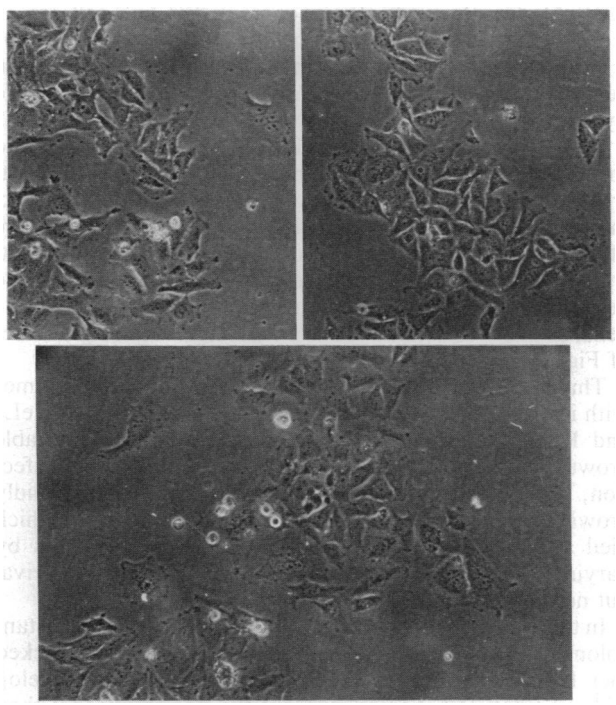

FIG. 3. Multinucleated cells transformed with RDB-wt shown in greater details after 27 days of selection in G418. Magnification is higher than in Fig. 2.

Untransfected HeLa cells were strongly contact-inhibited and became arrested at confluence (data not shown).

The simplest interpretation of these results is that the abnormal growth phenotype is caused by the overexpression of a functional eIF-4E protein by the RDB-wt vector. The G418-resistant cells obtained with the RDB-0 vector (no inserted cDNA), RDB-CAT, or RDB-Ala did not exhibit these effects, ruling out the possibility that the phenotype could be caused by the vector alone. The [Ala^{53}]eIF-4E protein expressed by RDB-Ala differs by only one amino acid residue from the wt protein produced by RDB-wt. This amino acid substitution (Ser-53 → Ala) does not affect cap binding but renders the protein incapable of binding to the 48S initiation complex in an *in vitro* assay (14). It is interesting that overexpression of the [Ala^{53}]eIF-4E protein does not appear to interfere with mRNA translation by competition with the endogenous protein for cap binding. Perhaps it is the availability of phosphorylated eIF-4E, rather than eIF-4E itself, which is rate-limiting for protein synthesis.

Further understanding of this phenomenon would be facilitated by knowledge of the extent of overexpression. However, estimation of the eIF-4E level in RDB-wt-transformed cells is problematical because the cells die after 30 days; the number of cells in the foci from even several flasks is insufficient to perform accurate eIF-4E quantitations. Two approaches were taken to circumvent this problem. In the first, CAT activity was measured in RDB-CAT-transformed cells selected in an identical fashion to the RDB-wt-transformed cells (Table 1). Comparison with purified enzyme standards indicated that the cellular concentration of CAT was approximately 27 nM. Previously, we estimated by immunological methods that the concentration of eIF-4E in rabbit reticulocytes was 8 nM (7), Thus, if one assumes that (*i*) RDB vector-encoded CAT and eIF-4E, both of which are 25 kDa, accumulate to the same level, (*ii*) the endogenous eIF-4E concentration in HeLa cells and rabbit reticulocytes is the same, and (*iii*) the CAT standard used in Table 1 was fully active, then the degree of overexpression was 3-fold. In separate experiments with RDB-CAT-transformed cells, we have found that the level of CAT estimated by enzymatic activity and by Coomassie blue staining after $NaDodSO_4$/PAGE was similar (data not shown).

The second approach was to measure the [Ala^{53}]eIF-4E content of the RDB-Ala-transformed cells, which grow continuously and can yield sufficient material for analysis. Cy-

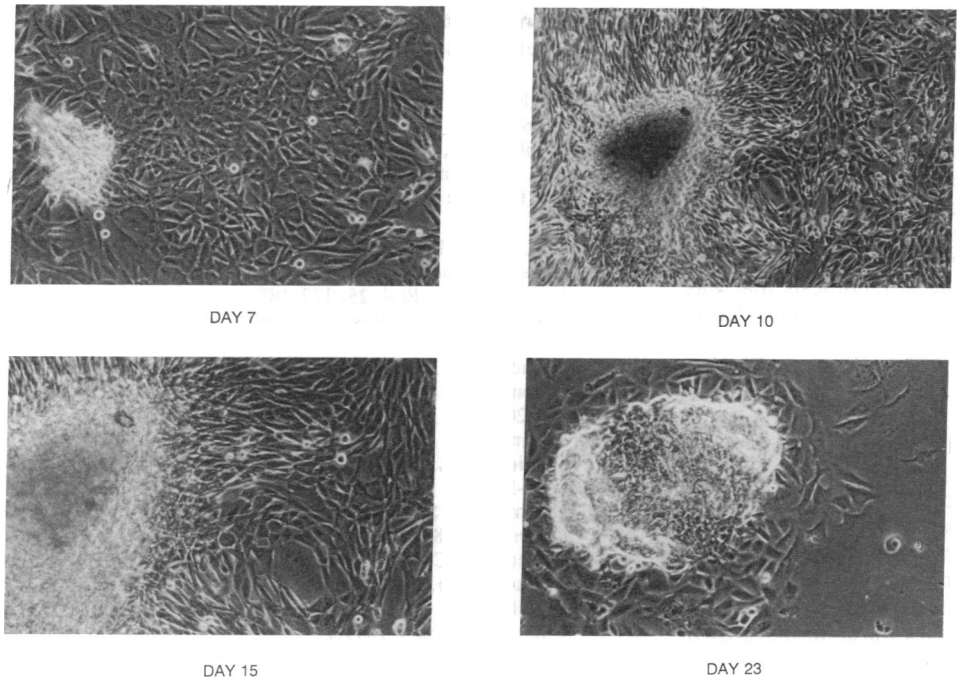

FIG. 4. Growth progression of a single focus of RDB-wt-transformed cells without G418 selection. The same area of the culture was photographed 7, 10, 15, and 23 days after transfection. G418 was added 20 days after transfection. For this experiment, the cells were transfected at subconfluence to prevent overgrowth of the mass culture.

toplasmic extracts were made from untransfected HeLa and RDB-Ala-transformed cells and the eIF-4E was enriched by affinity chromatography on m^7GTP-Sepharose. Bound protein was subjected to $NaDodSO_4$/PAGE and stained with Coomassie blue (Fig. 5). Total protein from the two lines exhibited the same pattern, but affinity-purified protein from RDB-Ala-transformed cells contained both more eIF-4E and more p220, a component of the eIF-4F complex (4, 5). Densitometry of the eIF-4E bands indicated that RDB-Ala-transformed cells contained 8.7-fold more eIF-4E than did untransformed HeLa cells. Considering both methods of quantitation, the level of overexpression is estimated to be 3- to 9-fold.

DISCUSSION

The phenotype of HeLa cells overexpressing wt eIF-4E indicates several things. First, at early stages, they are undergoing cell division more frequently than control cells, based on their numbers and rounded-up appearance, and in fact seem to be continuously dividing (Figs. 2 and 3). Despite the ultimate lethality of wt eIF-4E overexpression, cell division must occur many times before cells die because the foci, which occur infrequently (several per flask), are almost certainly clonally derived. Second, they lose contact inhibition, since they grow many cells thick, even in the midst of confluent untransformed cells (Fig. 4). Third, at latter stages, the rate of mitosis exceeds the rate of cytokinesis, since cells form syncytia. It is possible that cell cycle progression is normal, though accelerated, during the period when eIF-4E levels are building up but becomes abnormal when a critical level is reached.

The experiments presented establish that the abnormal growth phenotype is due to eIF-4E and not to the vector

Table 1. Level of CAT expression in RDB-CAT-transformed cells

Sample	Cm acetylated, %	CAT in assay, ng	Volume of 10^6 cells, μl	CAT in cells, nM
Bacterial CAT	1.3	3		
	4.3	15		
	13	57		
	34	225		
	53	900		
RDB-CAT-transformed cells	5.4	20	30	27

CAT assays were carried out in 0.1-ml reactions with 10 μM [1,2-^{14}C]chloramphenicol (Cm; 10^5 cpm) and 10 mM acetyl-coenzyme A (24). Cell extract from 10^6 RDB-CAT-transformed cells was used in these assays, and reactions were carried out for 1 hr. Standards for CAT activity (purified bacterial enzyme) were purchased from Boehringer Mannheim. Acetylated products, which were separated by thin-layer chromatography and assayed for radioactivity, are expressed as a percentage of input radioactivity.

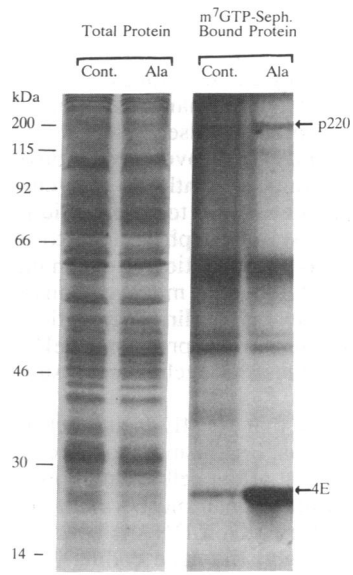

FIG. 5. Comparison of the amount of eIF-4E isolated from control (Cont.) HeLa and RDB-Ala-transformed cells. Cell extracts were prepared as described. For visualization of total protein, 50 μl were subjected to $NaDodSO_4$/PAGE and stained with Coomassie blue. For eIF-4E quantitation, 1 ml was applied to a 2-ml m^7GTP-Sepharose column, and the entire protein sample eluted from the column was applied to the gel. The gel was scanned and digitized with a BioImage Visage 2000 laser densitometer. The area corresponding to the eIF-4E band was quantified with a peak-area integrator. The arrows indicate the positions of eIF-4E and the p220 component of eIF-4F.

alone, since RDB-0, RDB-CAT, and RDB-Ala all caused cells to be transformed to G418 resistance but did not alter the growth phenotype. They do not rule out, however, a cooperative effect of eIF-4E with DNA sequences in the vector or with another vector-encoded product—e.g., tumor (T) antigen. Expression of eIF-4E from an unrelated vector will be necessary to eliminate this possibility. The experiments further establish that only eIF-4E capable of being phosphorylated at Ser-53 produces the unusual growth phenotype. This provides *in vivo* confirmation for the necessity of this phosphorylation for eIF-4E function, a hypothesis that was previously suggested by the correlation of phosphorylation with an increased rate of protein synthesis and by the *in vitro* ribosome transfer experiment (see the Introduction). Finally, the quantitation of eIF-4E, though indirect, suggests that derangement of cell growth occurs at relatively low levels of eIF-4E overexpression (3- to 9-fold). It is curious that the as yet unidentified kinase and phosphatase, which together determine the degree of eIF-4E phosphorylation, apparently do not compensate for the increased amount of eIF-4E by reducing the degree of phosphorylation. Perhaps the kinase has the catalytic capacity to accommodate higher amounts of eIF-4E, but the phosphatase does not.

One possibility that could account for the abnormal growth phenotype is that an excess of eIF-4E relieves competition for some rare mRNA encoding a protein that is important for cell cycle progression. For example, the protooncogene c-*myc* is thought to play a central role in controlling growth and division (25). Abnormalities of this gene and its pattern of expression have been implicated in the genesis of a variety of human tumors. Both c-myc mRNA and c-myc protein are very short lived, and their accumulation is precisely limited to that moment of the cell cycle, known as "competence," in which a decision is made to leave G_0 or G_1 stages and enter S phase. The murine and human c-myc mRNAs are poorly translated in quiescent cells, primarily due to sequences in the 5′ untranslated region encoded by exon 1 (26, 27). As eIF-4E is part of the cellular machinery that unwinds mRNA 5′ secondary structure and is implicated in the recruitment of at least one mRNA from the untranslated mRNP pool to polysomes (3), it is logical to assume that an increase in active eIF-4E would selectively enhance the translation of mRNAs like c-myc that contain high secondary structure.

Another possibility is that overexpression of eIF-4E causes premature or continuous synthesis of cyclins. The level of cyclins is maximal at the G_2 to M transition and then drops abruptly at the onset of anaphase, due to their proteolysis (28). Upon selective degradation of cyclin mRNA, activated *Xenopus* eggs fail to enter mitosis. Conversely, protease inhibitors that prevent cyclin degradation block starfish oocytes in M phase. Overexpression of eIF-4E could result in continuous synthesis of cyclins and result in unscheduled entry into mitosis.

It is also possible that eIF-4E itself functions as a signal for cell division. In most organisms, growth controls operate (*i*) during the G_1 period of the cell cycle to prevent entry into S phase if certain nutritional and hormonal requirements are not met and (*ii*) during G_2 if DNA is not correctly replicated (29–34). Interestingly, the gene coding for eIF-4E in yeast was recently found to be the product of the cell division cycle gene *cdc-33* (35). The phenotype of *cdc-33* mutants resembles cells arrested at the G_0/G_1 boundary during amino acid starvation. If active (phosphorylated) eIF-4E is part of the signal to begin S phase, then it may be critical to dephosphorylate eIF-4E at the conclusion of S phase to prevent another round of DNA synthesis. In fact, as noted above, Bonneau and Sonenberg (13) found that eIF-4E is dephosphorylated during mitosis. The overexpression of eIF-4E may override this control mechanism and allow S phase to begin prematurely, before cytokinesis had occurred.

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