Activated eIF4E-binding Protein Slows G₁ Progression and Blocks Transformation by c-myc without Inhibiting Cell Growth*

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Translation initiation is poised between global regulation of cell growth and specific regulation of cell division. The mRNA cap-binding protein (eIF4E) is a critical integrator of cell growth and division because it is rate-limiting for translation initiation and is also rate-limiting for G₁ progression. Translation initiation factor eIF4E is also oncogenic and a candidate target of c-myc. Recently, an activated inhibitory 4E-binding protein (4EBP) that blocks eIF4E was used to study its regulation of Drosophila growth. We adopted this approach in mammalian cells after identifying an autosensing mechanism that protects against increased levels of 4EBP1. Increased 4EBP1 induced a quantitative increase in the inactivated phosphorylated form of 4EBP1 in vitro and in vivo. To overcome this protective mechanism, we introduced alanine substitutions at four phosphorylation/inactivation sites in 4EBP1 to constitutively activate a 4EBP₁ to block eIF4E. Overexpression of activated 4EBP₁ inhibited cell proliferation and completely blocked transformation by both eIF4E and c-myc, although it did not block all tested oncogenes. Surprisingly, expression of the activated 4EBP₁ increased cell size and protein content. Activated 4EBP₁ blocked both cell proliferation and c-myc transformation by inhibiting G₁ progression and increasing apoptosis, without decreasing protein synthesis. Our results identify mammalian eIF4E as rate-limiting for cell cycle progression before it regulates cell growth. It further identifies G₁ control by translation initiation factors as an essential genetic target of c-myc that is necessary for its ability to transform cells.

Cell proliferation is the product of cell growth, cell cycle progression, apoptosis, and terminal differentiation (1). Whereas cell division requires cell growth (2, 3), mechanisms by which cell growth or growth regulators might control cell division remain less clear. The process of translation initiation can integrate cell growth and division because it is both rate-limiting for the first step in protein synthesis and has specific effects on cell cycle regulation (4, 5). Translation initiation factor eIF4E, which binds the m'GpppN mRNA cap structure, is the central molecule in translation initiation because it is the least abundant factor regulating translation in mammalian cells (6). Moreover, eIF4E is a key step in translation initiation because it induces malignant transformation when overexpressed in mammalian cells (7).

Genetic models of eIF4E function illustrate its importance in both growth and cell division. Mutations in translation initiation factor eIF4E in budding yeast (cdc33) cause both a G₁ arrest phenotype (8) and limit protein synthesis (9). Unlike yeast, however, loss of Drosophila eIF4E limits growth with only downstream effects on cell cycle progression (10). Furthermore, overexpression of eIF4E produces no discernible phenotype in Drosophila (10, 11). Studies of mammalian eIF4E have been largely confined to cell culture systems. Studies using HeLa cells showed that DNA synthesis was altered in response to both overexpression and antisense inhibition of eIF4E (12, 13) but did not simultaneously assess effects on cell growth. These results, together with its transforming functions, strongly suggest that limiting amounts of eIF4E likely affect mammalian cell division before regulating cell growth (7).

The c-myc oncogene is a paradigm for a gene that exerts profound effects on both cell growth and cell division (14). Nevertheless, which of its many functions is essential to the role for myc in human cancer remains a critical question. A remarkably diverse set of myc target genes has emerged (15). Broadly, c-myc targets genes that regulate cell cycle progression and genes that regulate cell growth (14). Growth functions of c-myc were first identified in cells overexpressing c-myc and confirmed in c-myc null cells (16, 17). Increased protein synthesis precedes S phase entry after activation of chimeric proteins that fuse c-myc with the estradiol receptor (17, 18). c-myc-induced proliferation correlates with an increase in protein synthesis in the B cells of transgenic mice overexpressing c-myc (19). Delayed cell division rates in c-myc null fibroblasts match their decreased protein synthesis rates (20).

Genetic analyses of dMyc first confirmed that its primary function in Drosophila is growth regulation (21–23). These genetic studies seemed poised to simplify our understanding of the proto-oncogenic functions of myc until an interesting discrepancy between fly myc and mammalian myc emerged. Although Drosophila genetics placed dMyc in a group of cell growth regulators, mouse genetics proved c-myc to primarily regulate cell division (24). We are therefore particularly interested in evaluating both the cell division control and cell growth control functions of eIF4E as candidate mediators of the effects of myc (17).

Our identification of the mRNA cap-binding protein (translation initiation factor 4E, eIF4E) as a candidate myc target gene first led us to propose that one of the primary functions of myc might be to regulate cell growth (18). The presence of an essential myc-binding site in the eIF4E promoter demonstrated
a potentially direct role for c-myc as an activator of eIF4E transcription (25). Recent differential expression screens have identified both additional translation initiation factors and ribosomal proteins as candidate c-myc target genes (26–30). The lack of apparent correlation between myc-induced genes, genes down-regulated in c-myc null cells, and genes required for transformation by c-myc remains puzzling (31). For example, carbonyl-phosphate synthetase-aspartase transcarbamylase-dihydro-orotase gene expression clearly responds to loss of c-myc, but carbonyl-phosphate synthetase-aspartase transcarbamylase-dihydro-orotase apparently plays little role in tumors induced by c-myc (32). Recently, down-regulation of the expression of the H-ferritin gene was shown to be required for cell transformation by c-myc (33). Two additional myc targets are also critical to its transforming functions, PRDX3 and eIF4E (44). Because translation initiation factor eIF4E is a particularly interesting c-myc target gene, we chose this factor as a target gene, we transformed B21 strain of Escherichia coli, and GST fusion proteins were made using standard methods (49). RNA Synthesis and in Vitro Translation—pBEF5′UTR-eIF4E was linearized and used as a template to synthesize capped mRNA in vitro using a kit from Ambion. T7 RNA polymerase (New England Biolabs) was used to synthesize 4EBP mRNA in vitro. 0.2 μg of RNA was used in a rabbit reticulocyte lysate in vitro translation reaction. Purified 4EBP proteins were incubated with the lysates for 10 min at room temperature prior to RNA template addition. 5 μl of the translation products were analyzed by 12% SDS-PAGE.

### EXPERIMENTAL PROCEDURES

#### Plasmids and Expression Constructs—The plasmid pBEF5′ was a gift from Dr. John Lawrence. The eIF4E coding sequence was from pMVeIF4E from Dr. Nahum Sonenberg. pDOL-erbB2 was from Dr. D. Stern (47, 48). pCEPMyc was the kind donation of Dr. Evelyn Guillot. Commercial vectors used for the described constructs include pGEX6p-1 and pCEP4. Additional plasmids used are outlined in Table I.

#### Protein Purification—Plasmids pGEX6p-1, pGEF4EBP, and pGEF4EBP-eIF4E were transformed into the B21 strain of Escherichia coli, and GST fusion proteins were made using standard methods (49).

#### Cell Transfection, Selection, and Cell Growth Assay—Rat1a cells were from Dr. Chi Dang at The Johns Hopkins University. TGR cells were from Dr. John Sediwy at Brown University (44). Both cell types were initially transfected with pMV7, pMVHA4EBP, pMV4EBP, and pMV4EBP, respectively, together with either pCEP4 or pCEPMyc. Colonies selected in the presence of 500 μg/ml geneticin and 200 μg/ml hygromycin were stained and photographed to assess effects of constructs on cell survival. Individual colonies were initially isolated using trypsin-impregnated cloning filters. Because all colonies expressed the transfected proteins due to the double selection applied to all transfections, pooled transfected cells were used in all subsequent studies. Proliferation curves were determined by counting viable cells harvested from triplicate 10-cm plates over a 4-day period (before confluence). All cell counts are presented together with least square trend lines. Soft agar cloning was performed as described (46).

Subconfluent Rat1a and TGR cells were labeled with 10 μM bromodeoxyuridine-cell labeling reagent (Amer sham Biosciences) for 30 min and harvested for cell cycle analysis. Cells were fixed in 80% ethanol for at least 1 h, incubated in anti-bromodeoxyuridine antibody (BD Biosciences) for 30 min, and exposed to anti-mouse fluorescein secondary antibody (Vector Laboratories) for 30 min. Cells were resuspended in propidium iodide (70 μg/ml) supplemented with RNase A (25 μg/ml) and analyzed. DNA content was measured using a FACScan cytemeter (BD Biosciences). Apoptosis score by quantifying sub-genomic fragmented DNA was confirmed by staining cells with 4′,6-diamidino-2-phenylindole (DAPI) (Roche Applied Science).

### Rat1a and NIH3T3 Transformation Assays—Clonogenicity of the Rat1a transfec tants in soft agar was performed as described (46). NIH3T3 cells were cotransfected with 1.5 μg of pMV7, pMVeIF4E, pDOL-erbB2, or pSV40T antigen together with an additional 1.5 μg of pMV7, pMVHA4EBP, pMV4EBP, or pMV4EBP using LipofectAMINE (Invitrogen). Transformed foci were identified using 1% methylene blue staining 3–4 weeks after plating. Mean ± S.D. of transformed foci per plate were evaluated using six 60-mm plates per assay condition.

### Table I

| Plasmid          | Parent | Cloning | Insert Description                  | Changed|
|------------------|--------|---------|------------------------------------|---------|
| p4EBP5′UTR4EBP   | p4EBP1 | In pGEM4 | 4EBP1 5′UTR and 4EBP CDS           | Gift from Dr. J. Lawrence |
| pGEX4EBP        | pGEX6p-1 | BamHI, NotI | 4EBP PCR CDS                     | Leu<sup>−</sup>−Met<sup>−</sup>− GA |
| pGEF4EBP<sub>μ, μ</sub> | BamHI, NotI | EcoRI, HindIII | 4EBP PCR CDS                  | Thr<sup>35</sup> → Ala, Thr<sup>45</sup> → Ala, Ser<sup>64</sup> → Ala, Thr<sup>99</sup> → Ala, Thr<sup>45</sup> → Ala, Ser<sup>64</sup> → Ala, Thr<sup>99</sup> → Ala, Leu<sup>−</sup>−Met<sup>−</sup>− GA |
| pMVE4EBP        | pMV7   | EcoRI, HindIII | Activated 4EBP plus HA tag         | |
| pMV4EBP<sub>μ</sub> | pMV7   | EcoRI, HindIII | Activated 4EBP with inactivation of eIF4E binding domain plus HA tag | |
| pCEP4EBP        | pCEP4  | EcoRI, HindIII | 4EBP PCR CDS                     | |
| pCEP4EBP<sub>μ</sub> | pCEP4  | EcoRI, HindIII | Activated 4EBP plus HA tag         | |
| pCEP4eIF4E      | pCEP4  | Nhel, Sall | eIF4E CDS                         | |

1 The abbreviations used are: 4EBP, 4E-binding protein; PHAS, phosphorylated heat and acid-stable; FC5, fetal calf serum; FBS, fetal bovine serum; HA, hemagglutinin; GST, glutathione S-transferase; DAPI, 4′,6-diamidino-2-phenylindole; DODC, ornithine decarboxylase; TOP, target of rapamycin; mTOR, mammalian target of rapamycin; FAC5, fluorescence-activated cell sorter. (Rat1a) that can be transformed by myc and eIF4E in a single step (45, 46).
Activated 4EBP1 Blocks G1 Progression

Immunoblot and Protein Synthesis Analysis—For Western analyses, cells were lysed in Laemmli loading buffer. 10 μg of protein sample was subjected to 12% SDS-PAGE and transferred to polyvinylidene difluoride membrane. Membranes were hybridized with anti-actin (N-350; Roche Applied Science), a mouse anti-HA antibody (12CA5; Roche Applied Science), or rabbit anti-4EBP1 antibodies (gift from Dr. John Lawrence) as indicated and detected by ECL (Amer sham Biosciences) as indicated in the figures.

Protein content per cell was determined by lysis of a known number of cells in ELB lysis buffer. Measurement of the protein content of an aliquot of these lysates was performed by using a kit from Bio-Rad.

Protein synthesis rates were determined by adding 20 μCi of [35S]methionine (PerkinElmer Life Sciences) to each plate for 1–3 h as indicated. Labeled cells were lysed, and incorporated counts were determined by trichloroacetic acid precipitation as described previously (13). [35S] incorporated at the indicated time points was plotted, and a line was drawn with the least squares trend line is shown.

Polysonal Profile Analysis and RNA Blots—One 100-mm diameter plate containing the indicated cells was harvested for each polysomal analysis. Confluent cells were harvested and lysed in 300 μl of RSB (10 mM NaCl, 10 mM Tris-HCl (pH 7.4), 15 mM MgCl₂) containing 100 μg/ml heparin, 1.2% deoxycholate (50), 51). Nuclei were pelleted for 3 min in a microcentrifuge at 4 °C. The 300-μl extract was layered over 11.5 ml of a 15–45% (w/w) sucrose gradient with a 0.5-ml cushion of 45% sucrose. The gradients were centrifuged at 37,000 rpm for 2.5 h in a SW41 (Beckman) rotor at 4 °C. After centrifugation the A₅₀₀ was continuously monitored and recorded across the gradient. The gradients were divided into polysonal (containing disomes and greater) and monosomal fractions (containing monosomes, ribosomal subunits, and mRNA ribonucleoproteins). For RNA blots of the polysonal and sub-polysonal fractions, RNA was extracted from the polysonal and sub-polysonal fractions using Trizol reagent, and 50% of the harvested RNA was analyzed using standard RNA blotting techniques (49).

Ribosomal Protein Synthesis and Immunoprecipitation Analysis of Ornithine Decarboxylase Synthesis—Cell fractionation for preparation of ribosomes was performed as described by Greco and Madjar (52). 20 μl of [35S]methionine (PerkinElmer Life Sciences) was added to media for incorporation into confluent monolayers of Rat1a cells transfected either with the empty vector or 4EBP1-expressing vector in 10 cm diameter dishes. Labeling continued for 3 h before the cells were harvested in buffer A (0.25 M sucrose, 25 mM KCl, 5 mM MgCl₂, and 50 mM Tris-HCl (pH 7.4)). Nuclei were removed by centrifugation at 750 g for 10 min after addition of 0.7% Nonidet P-40. Mitochondria were then incubated for 30 min with the lysate containing the antibody (Affiniti Research Products, PW8880) was added and incubated for another 10 min. The post-mitochondrial supernatant was then adjusted to 0.5 M KCl and for 10 min after addition of 0.7% Nonidet P-40. Mitochondria were washed three times with the lysis buffer before 50 μl of protein gel loading buffer was added and run into standard 10% polyacrylamide gels. Radioactive incorporation into the immunoprecipitated ODC was assessed by overnight autoradiography of the gel.

RESULTS

Translation initiation factor eIF4E is a particularly interesting mediator of cell growth and division because it transforms cells (7). We therefore chose to evaluate a molecular inhibitor of eIF4E, the eIF4E-binding protein (4EBP1; PHAS1) (53) as a potential inhibitor of eIF4E function. To evaluate possible mechanisms that might attenuate 4EBP1 effects in vitro (40), we first tested the effect of the addition of 4EBP1 on translation rates of its own mRNA in vitro. We generated 4EBP1 mRNA in vitro, which was then translated in rabbit reticulocyte lysates in the presence of additional purified GST or GST-4EBP1 protein (Fig. 1A). 4EBP1 synthesis rates were determined by comparing radioactive [35S]methionine incorporation as demonstrated by autoradiography on standard protein gels. Purified 4EBP1 caused a minimal decrease in its own translation rate (Fig. 1B, lanes 2 and 3 versus lanes 4 and 5). On closer analysis, a slower migrating form of 4EBP1 appeared when purified GST-4EBP1 was added (lanes 4 and 5 in Fig. 1B). Treatment with λ phosphatase confirmed that the slower migrating form of 4EBP1 was phosphorylated. Our results suggested that addition of 4EBP1 results in a direct or indirect induction of 4EBP kinase activity present in the reticulocyte lysate. Autoradiography of the translational environment by 4EBP1 was reported previously (54, 55) after inducible up-regulation of eIF4E and has been attributed to mTOR. We determined that this effect depends on a direct interaction between 4EBP1 and eIF4E because the translational inhibition and phosphorylation did not appear when a Leu⁵⁶→Met⁵⁹ → GA mutation in the interaction domain of 4EBP1 with eIF4E was added (Fig. 1C).

To prevent inactivation of 4EBP by this autoregulatory mechanism, we simultaneously mutated its threonines 35, 45, and 69 and serine 64 to alanines. We sought to develop a constitutively active 4EBP that might provide a better reagent to study the role of translation initiation in growth control. We used pM7V to develop mammalian expression constructs that contained no insert, 4EBP1 itself, or the constitutively active form of 4EBP1 (4EBPµ). We further activated the alanine-substituted mutant by an additional mutation in the interaction domain of 4EBP1 to prevent its interactions with eIF4E (4EBPµ). (Fig. 1D). We initially studied Rat1a cells because eIF4E transformed them in a single step, as reported previously (46) for c-myc. Rat1a cells were stably transfected using combinations of the constructs shown in Fig. 1D together with the control vector pCEP4 or pCEP-eIF4E. The resulting plates were stained, and minimal changes in colony viability, number, and size were observed in response to the activated 4EBPµ (not shown).

Single clones expressing the 4EBP constructs, but without eIF4E, were initially selected and tested for expression of 4EBP1 or 4EBPµ in Western assays (Fig. 1E). As seen in vitro, 4EBP1 migrated as doublets containing additional phosphorylated 4EBP1 whether it was the exogenous HA-tagged 4EBP1 or the endogenous 4EBP1 in the transfected cells (Fig. 1E, lane 2 versus lanes 3–7). No doublets were observed in cell lines transfected with the mutant HA-tagged 4EBPµ (Fig. 1F). Endogenous 4EBP1 showed a clear shift toward a preponderance of the phosphorylated upper band of the doublet in the presence of the constitutively activated mutant (Fig. 1F, lane 2 versus lanes 3–6). To explore the role of translation initiation in cell division, we evaluated the effects of the expression of the 4EBPµ on cell proliferation. Because the dual selection applied to our transfections produced uniform expression of the transfected plasmids in all tested clones, we evaluated pooled transfectants of the various plasmid combinations in subsequent studies. The activated 4EBPµ inhibited cell proliferation during logarithmic growth in high serum concentrations better than the phosphorylatable normal 4EBP (Fig. 2, A and B). Inhibition by 4EBPµ required interactions with eIF4E because an additional loss of the eIF4E interaction domain by the Leu⁵⁶→Met⁵⁹ → GA mutation (4EBPµ) abrogated the inhibitory effects of 4EBPµ. This construct was effectively expressed at roughly comparable levels as the 4EBPµ (Fig. 2C). The 4EBPµ was sufficient to block the transforming function of eIF4E in Rat1a cells (Fig.
The HA-tagged 4EBP\(_\mu\) effectively bound to eIF4E because it was readily found in protein lysates purified by 7\(^{th}\)G chromatography and detected using an anti-HA antibody (Fig. 2E). We observed little HA-tagged non-mutant 4EBP1 bound to the eIF4E that adhered to the 7\(^{th}\)G column (lane 5, Fig. 2E). This is likely due to the absence of unphosphorylated forms of HA-4EBP in the transfected cells (Fig. 2F). Taken together, these results demonstrate that a constitutively active 4EBP\(_\mu\) blocks cell proliferation by blocking eIF4E.

We next tested the ability of the activated 4EBP\(_\mu\) to block Rat1a transformation by c-myc. We first cotransfected Rat1a cells with four G418-selectable constructs (MV7 control, 4EBP1, 4EBP\(_\mu\), or 4EBP\(_\mu\)) in the absence and presence of a hygromycin-selectable c-myc expression plasmid. Neither colony viability, number, nor colony sizes were changed by 4EBP\(_\mu\) expression (not shown). We assessed the proliferation rate of cells expressing 4EBP\(_\mu\), c-myc, and the combination of 4EBP\(_\mu\)/c-myc (Fig. 3A). c-myc increased proliferation of cells expressing the 4EBP\(_\mu\), which proliferated more slowly than control cells. Cells expressing both c-myc and 4EBP\(_\mu\) proliferated at the same rate as control cells.

We next sought to determine whether the 4EBP\(_\mu\) could block tumorigenesis by c-myc (Fig. 3, B and C). 4EBP1- and 4EBP\(_\mu\)-expressing pooled transfectants were assayed in both soft agar colony formation assays and nude mice. myc-mediated transformation was moderately suppressed by 4EBP1 (Fig. 3B). In contrast, 4EBP\(_\mu\) had a significant effect on the transforming capability of c-myc, reducing the number of colonies to below control levels. We determined that this effect depends on a direct interaction between 4EBP1 and eIF4E because the inhibition of soft agar colony formation did not appear when a Leu\(^{58}\)–Met\(^{59}\)→GA mutation that removes a portion of the interaction domain of 4EBP1 with the eIF4E was inserted. The activated four alanine-substituted 4EBP\(_\mu\) was then rendered incapable of interacting with eIF4E by an additional Leu\(^{58}\)–Met\(^{59}\)→GA mutation to make 4EBP\(_\mu\)-\(\mu\). 4EBP1- and 4EBP\(_\mu\)-mediated transfor-

mation was confirmed in vivo (Fig. 3C).

We demonstrated previously (25) a direct effect of c-myc on the eIF4E promoter, suggesting that c-myc can directly regulate eIF4E expression. In this report, we wanted to assess the biological importance of that regulation in the transformation function of myc. Given the central role of eIF4E in translation, blocking its function might also have a general effect on transformation by other oncogenes. We therefore tested the 4EBP\(_\mu\) construct (4EBP\(_\mu\)) was cotransfected with c-myc (last bar, Fig. 3B). The effect of the 4EBP\(_\mu\) on myc-mediated transformation was confirmed in vivo (Fig. 3C).

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To confirm the effects of the 4EBP$_\mu$ in more normal diploid fibroblasts, we transfected rat TGR cells (44). Surprisingly, although a putative growth inhibitor like 4EBP1 should decrease cell size, expression of the 4EBP$_\mu$ caused an obvious phenotypic enlargement of TGR cells (Fig. 4A). We confirmed this effect by using standard FACS-based forward scatter analysis (Fig. 4B). We then evaluated the effects of the 4EBP$_\mu$ on protein concentration per cell to evaluate one possible basis for the change in cell size. Consistent with the cell size data, 4EBP$_\mu$ increased the protein content per cell of TGR cells, although these changes were less significant than the other measures (Fig. 4C).

The large cell phenotype and the increased protein per cell in cells transfected with 4EBP$_\mu$ suggested that the 4EBP$_\mu$ might preferentially exert its anti-proliferative effects on regulation of cell division. To evaluate this possibility, cell cycle staging was performed. The 4EBP$_\mu$ decreased S phase cells 24 h after growth induction of transfected TGR pools expressing the 4EBP$_\mu$ expression vector (Fig. 4D). The 4EBP$_\mu$ expression particularly increased the proportion of cells in G$_1$. Given the large cell phenotype in the G$_1$-delayed 4EBP$_\mu$-expressing cells, it seemed possible that protein synthesis rates might not be decreased by the 4EBP$_\mu$ construct. Although translation initiation is thought to be the rate-limiting step in protein synthesis, few in vivo experimental models have directly tested this proposition (57). We therefore evaluated protein synthesis rates in control transfected TGR cells or TGR cells expressing 4EBP$_\mu$ (Fig. 4, E and F). We measured $[\text{35S}]$methionine incorporation after growth arrest (Fig. 4E) and growth induction (Fig. 4F), and we found that stable expression of the 4EBP$_\mu$ had minimal effects on this measure of protein synthesis in TGR cells (Fig. 4, E and F).

We next sought to confirm the protein synthesis results in Rat1a cells expressing 4EBP or 4EBP$_\mu$, and to further evaluate the effect of the 4EBP$_\mu$ on protein synthesis in cells expressing c-myc (Fig. 5). We measured $[\text{35S}]$methionine incorporation af-

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**Fig. 2.** Overexpression of a constitutively active 4EBP$_\mu$ slows cell proliferation and inhibits transformation by eIF4E. A, expression of the 4EBP$_\mu$ inhibits cell proliferation in Rat1a cells. Replicates containing pooled transfectants expressing neo selection alone (Rat1a), 4EBP, or a 4EBP$_\mu$ construct that contains both the four alanine mutations, and a mutation in the eIF4E-binding domain that prevents interaction with eIF4E (as in p4EBD in Fig. 1 above) were plated at 1 $\times$ 10$^6$ cells per 10-cm dish, and proliferation was assessed by daily manual counting on the indicated days. Plates were counted in triplicate for each time point, and a linear regression best-fit line is shown (r$^2$ = 0.95 for all plots). B, expression of the 4EBP$_\mu$ inhibits cell proliferation in Rat1a cells. The same data as plotted in A are re-plotted on a log scale, focusing on days 2–4 when the cells were in log phase growth. An F test was performed using the statistical package XLStatistics (56). F(1,14) = 31.2, p < 0.00001. C, the 4EBP$_\mu$ is effectively expressed in the Rat1a transfectants. Protein lysates were probed with the mouse anti-HA tag antibody (HA) and actin (actin). Lane 1, a protein sample from Rat1a cells transfected with the 4EBP$_\mu$; lane 2, an equivalently loaded sample of Rat1a cells transfected with the 4EBP$_\mu$. D, 4EBP$_\mu$ inhibited transformation by the oncogene eIF4E. Rat1a cell lines that expressed a control vector, eIF4E, or eIF4E with 4EBP$_\mu$ were compared in a soft agar tumorigenicity assay. Transfection with eIF4E increased soft agar colony formation compared with control-transfected cells. This number was dramatically reduced in cells transfected with both eIF4E and 4EBP$_\mu$. E, detection of binding between 4EBP$_\mu$ and eIF4E in Rat1a cells expressing wild type and mutant HA-tagged 4EBP$_\mu$ proteins by m7GTP chromatography. 10 $\mu$g of protein lysates harvested from pMV7 control (C), pMV4EBP (4), and pMV4EBP$_\mu$ (5) transfected cells were loaded in lanes 1–3. 100 $\mu$g of protein lysates from the same cell lines was additionally subjected to m7GTP-Sepharose chromatography. After elution these eIF4E-containing fractions were loaded in lanes 4–6. After standard protein gel electrophoresis, the resulting Western blot was developed with an anti-HA antibody. F, we were surprised to find no HA-tagged 4EBP$_\mu$ in the column eluate in lane 5 of B. Therefore, we evaluated more closely the phosphorylation status of the transfected HA-tagged mutant 4EBP$_\mu$ protein by using an anti-HA tag antibody to determine what proportion of unphosphorylated HA-tagged 4EBP$_\mu$ was present in lysates that might be available to bind to the eIF4E adhering to the m7GTP column in E. The protein lysates used in lanes 1F, lanes 2–5, were compared with a protein sample from a λ phosphatase treated in in vitro translation reaction (IVR) using HA-tagged 4EBP$_\mu$ mRNA (1). This blot demonstrates that essentially all of the transfected HA-4EBP$_\mu$ is found in slower migrating phosphorylated forms when compared with the migration of the in vitro generated HA-4EBP. The lower arrow indicates unphosphorylated 4EBP; the upper arrow labeled P identifies the slower migrating phosphorylated 4EBP form.
Fig. 3. The activated 4EBP1 blocks transformation by c-myc. A. c-myc increases the proliferation of cells expressing 4EBP1. Cells transfected with a control vector, 4EBP1, c-myc, or 4EBP1 with c-myc were seeded at 2 × 10⁵ in 100-mm dishes and grown in media supplemented with 10% fetal calf serum. Cell counts were performed at the indicated time points. B. 4EBP1 expression increased the proliferation of cells expressing c-myc in soft agar colony formation assays. Rat1a cells transfected with a control vector, 4EBP1, or 4EBP1 were cotransfected either with a control vector or a c-myc expression construct and compared in soft agar tumorigenicity assays. C. 4EBP1 blocks transformation by c-myc in immunocompromised mice. Rat1a cells transfected with a control vector, 4EBP1, c-myc, or 4EBP1 with c-myc were injected into nude (nu/nu) mice. Tumors were harvested and weighed after 3 weeks of growth. Mean tumor weights of six tumors in each condition are plotted together with error bars. D. Foci appearing in NIH3T3 cells transfected with pMV7, pDOLerbB2, and pSVA4 in the presence of either pMV7, pMV4EBP, pMV4EBP1, or pMV4EBP1 were assessed by methylene blue staining. The number of foci per plate is shown as a mean ± S.E. for six plates per condition.

...ter growth arrest (Fig. 5, A and C) and growth induction (Fig. 5, B and D). Again, the 4EBP1 failed to inhibit protein synthesis in Rat1a cells (Fig. 5, A and B). Importantly, c-myc induced moderate increases in protein synthesis in both conditions as described in other studies (19), but coexpression with 4EBP1 did not return protein synthesis to control levels in myc-expressing cells in either condition (Fig. 5, C and D). Finally, we again evaluated the effects of the 4EBP1 on protein concentration per cell in combination with c-myc in growth-arrested Rat1a cells. Consistent with the TGR cell data, 4EBP1 increased the protein content per cell of Rat1a cells; myc increased protein concentration per cell, and the 4EBP1 restored the myc cells to a more normal protein concentration per cell, although these changes were less statistically significant than the other measures (Fig. 5E).

We also evaluated the effects of 4EBP1 and 4EBP1 on regulation of cell division in the Rat1a cells to further confirm the TGR cell results (Fig. 6). Cell cycle staging was again performed in actively proliferating Rat1a cells (Fig. 6, A and B) and at two time points after serum induction of growth-arrested cells (Fig. 6, C and D). The 4EBP1 decreased S phase cells in Rat1a cells during log phase proliferation whether proliferating slowly in 0.5% fetal calf serum (FCS) (Fig. 6A) or more rapidly in 2% FCS (Fig. 6B). We then evaluated S phase entry 6 and 24 h after contact-inhibited Rat1a pools expressing the 4EBP1 expression vectors were stimulated with 10% FCS (Fig. 6, C and D, respectively). The 4EBP1 expression again increased the proportion of cells in G1 at 6 h. Both the 4EBP1 and the 4EBP1 increased G1 phase cells at 24 h with the constitutively active mutant exhibiting the greater effect. c-myc accelerates the G1-S phase transition (58). It seemed possible that reversion of myc-induced cellular transformation by activated 4EBP1 might be due to a correction of the mycshortened G1. We compared cell cycle distributions of cells expressing c-myc alone and c-myc together with the 4EBP1 after serum deprivation (Fig. 7A). Serum deprivation led to a marked increase in myc-expressing cells containing fragmented DNA, as expected for cells undergoing apoptotic cell death (59). The addition of 4EBP1 significantly increased this cell population when 4EBP1 was coexpressed with c-myc. These findings were confirmed by DAPI staining (Fig. 7B). In addition, cell cycle distributions after growth induction showed that G1 acceleration by c-myc was completely reversed when 4EBP1 was coexpressed with c-myc (Fig. 7C).

Global translation initiation rates are best evaluated by determining the percentage of RNAs present in ribosomal polysomes; polysomal profiles also provide an independent estimate of overall translation initiation rates in cells that may be a more accurate measure of net translation rates in the cell. We therefore compared the effects of the 4EBP1 on translation initiation in Rat1a and TGR cells using polysomal profiles (Fig. 8). An evident decrease in the fraction of ribosomal RNAs contained in polysomes was seen in Rat1a cells expressing the 4EBP1 construct (Fig. 8A) and in TGR cells expressing the
The activated 4EBP$_{\mu}$ increases cell size and protein content in diploid rat fibroblasts by accelerating cell division without inhibiting cell growth. A, photomicrographs of control transfected TGR fibroblasts (left) compared with TGR fibroblasts transfected with the 4EBP$_{\mu}$ construct (right). B, cell size compared by forward scatter in standard FACS analyses demonstrates increased size of TGR cells expressing the 4EBP$_{\mu}$. C, protein concentration per cell increases in TGR cells transfected by the activated 4EBP$_{\mu}$ when proliferating in subconfluent conditions (Growing, $p = 0.81$ by t test) or after growth arrest at confluence (Arrested, $p = 0.32$ by t test). Mean protein concentrations are plotted together with standard deviations. D, the 4EBP$_{\mu}$ inhibits passage through G$_{1}$. TGR cell lines transfected with a control vector or 4EBP$_{\mu}$ were grown to confluence in media supplemented with 10% fetal calf serum; the media were then changed to 0.5% fetal calf serum, and cells were grown for 60 h. These cell lines were then assessed in standard FACS analyses to evaluate entry into S phase. The apoptotic population is seen below in Fig. 7B were not included in this analysis. E and F, the 4EBP$_{\mu}$ has little effect on protein synthesis in TGR cells at growth arrest (E) or after stimulation with 10% FCS (F). Cell lines transfected with a control vector or the construct expressing the 4EBP$_{\mu}$ were grown to confluence in media supplemented with 10% fetal calf serum; the media were then changed to 0.5% fetal calf serum, and cells were grown for 60 h. Two plates per time point were then pulse labeled for 1–3 h with $[^{35}S]$methionine (E), and an additional set of plates had their media changed to 10% fetal calf serum for 24 h (F). After growth induction for 24 h, two plates at each time point were again pulse labeled for 1–3 h with $[^{35}S]$methionine. Counts incorporated during pulse labeling were evaluated by trichloroacetic acid precipitation of cell lysates. Protein synthesis rates were plotted over the linear range of $[^{35}S]$methionine incorporation. The mean at each time point is displayed together with a least squares linear regression trend line for each set of data. $r^2$ was better than 0.95 for all trend lines.

4EBP$_{\mu}$ construct (Fig. 8B), demonstrating global inhibitory effects of our 4EBP$_{\mu}$ construct on translation initiation of many cellular mRNAs in vivo.

We further analyzed the effect of the 4EBP$_{\mu}$ on expression of a 5′-TOP mRNA (ribosomal protein L32-rpl32), actin, and ODC (Fig. 8, C and D). The protein synthetic apparatus, especially ribosomal protein mRNAs, is regulated at the translational level through terminal oligopyrimidine (TOP) sequences present at their 5′ ends (60). Although these sequences have been proposed as regulatory targets of the target of rapamycin (TOR) signaling pathway through effects on 4EBP$_{\mu}$ (61), recent data question this model (51, 62). We therefore evaluated the relative amounts of rpl32 mRNA present in subpolysomal and polysomal fractions in RNA blots of the fractionated RNAs. Surprisingly, the polysomal fractions contained relatively more rpl32 mRNA than the subpolysomal fractions in both Rat1a (Fig. 8C) and TGR (Fig. 8D) cells expressing the 4EBP$_{\mu}$ construct (comparing lanes 3 and 4 to lanes 1 and 2). There were no apparent differences in relative amounts of actin mRNA in these fractions in either cell line. In contrast, ODC is a well characterized target of translational controls. It is also transcriptionally regulated by c-myc, and it can transform cells (63–66). In contrast with the rpl32 mRNA, the ratio of polysomal to subpolysomal ODC mRNA decreased in both Rat1a and TGR cells expressing the 4EBP$_{\mu}$ construct (Fig. 8, C and D), comparing the relative signals in lanes 3 and 4 to lanes 1 and 2.
Fig. 5. The activated 4EBP1 does not inhibit protein synthesis in Rat1a fibroblasts in the absence or presence of c-myc. A and B, the 4EBP1 fails to inhibit protein synthesis in Rat1a cells. Rat1a cells transfected with a control empty vector or the construct expressing the 4EBP1 were grown to confluence, and protein synthesis rates were determined using the same method as in Fig. 4. Protein synthesis rates were plotted over the linear range of [35S]methionine incorporation. The mean at each time point is displayed together with a least squares linear regression trend line for each set of data. r² was better than 0.95 for all trend lines. Transfected and control cells were compared after proliferation arrest (A) or after stimulation with 10% FCS (B). C and D, this analysis was repeated for Rat1a cells transfected with control vector, c-myc, or a combination of c-myc together with 4EBP1 after proliferation arrest (C) or after serum stimulation (D). E, protein concentration per cell increases in Rat1a cells transfected by the activated 4EBP1 after growth arrest at confluence. Protein concentration per cell is plotted as a mean ± S.D. for control Rat1a cells as compared with cells transfected by the control empty vector, the 4EBP1, and the 4EBP1/myc. The protein per cell concentration was further compared between cells simultaneously transfected with myc and the same set of 4EBP constructs. (p = 0.81 for differences between Rat1a and the 4EBP1; p = 0.57 for differences between the 4EBP1 and the 4EBP1/myc; p = 0.086 for differences between myc and myc/4EBP1; and p = 0.11 for differences between the myc/4EBP1 and the 4EBP1/myc).

These results suggest that 4EBP1 may have differential effects on ribosomal protein synthesis, compared with other proteins that are translated from cap-dependent mRNAs. As the most abundant class of proteins, ribosomal proteins compose a significant proportion of all protein content of the cell proteins, and total protein synthesis measurements might reflect the rate of ribosomal protein synthesis. Moreover, the signal transduction pathways known to regulate cell growth exhibit separable effects on ribosomal protein mRNAs and cap-dependent mRNAs (Fig. 9A). Therefore, we measured the rate of incorporation of [35S]methionine into isolated ribosomes in control cells versus those expressing the 4EBP1 (Fig. 9B). As shown, [35S]methionine incorporation into the ribosomal protein fraction of cells was specifically increased in the cells expressing the 4EBP1 construct. We also analyzed the effects of the 4EBP1 on ODC synthesis. We first added 10% fetal bovine serum to the Rat1a controls and 4EBP1 expressors, and we evaluated steady state levels of ODC mRNA and protein. This point was chosen because ODC mRNA levels were minimally increased by serum addition at this time in Rat1a cells (Fig. 9C). Whereas the steady state levels of ODC were increased in the control Rat1a cells, the steady state levels of ODC were not increased in the Rat1a cells expressing the 4EBP1. We then analyzed the synthesis rate of ODC protein and found that the 4EBP1 inhibited [35S]methionine uptake into immunoprecipitable ODC (Fig. 9D).

**DISCUSSION**

Cell proliferation requires that a discrete doubling of DNA be coordinated with continuous growth of all other components of the cell. Cell growth has been hard to define because it is the sum of continuous synthesis of all of the macromolecules of the cell. In contrast, cell division is easier to define because DNA replicates in discrete units. Evolution presumably favors organisms that are best able to adjust their proliferation rate to conditions in the environment. This problem is addressed in yeast in part by regulating cell growth in response to environmental changes and then using cell growth to regulate cell division (1). The realization that mutations in key G1 cyclins cause a small cell phenotype in yeast, and that their mammalian counterparts cause human cancers, suggests that mechanisms coordinating cell growth and division are key cell proliferation controls (2–6).

Four pathways have been genetically shown to coordinate cell growth and cell division in Drosophila: 1) regulatory pathways that impinge on translation initiation; 2) transcriptional
regulation of proliferation by c-myc; 3) nutrient sensing through the TOR pathway; and 4) growth factor signaling through the insulin/phosphatidylinositol 3-kinase pathway (7).

Genetic studies in mammalian cells are obviously more difficult. Interestingly, the translation initiation factor eIF4E and its inhibitor 4EBP lie downstream of all four of the pathways genetically linked to growth control in *Drosophila*. In this report we therefore sought to develop a reagent that would allow us to better define the role of eIF4E as a coordinator of growth control during cell proliferation and to evaluate its role in malignancy.

We focused on the role of translation initiation as a coordinator of cell growth and proliferation because of its central function in regulation of protein synthesis and because loss of translation initiation function delays G1 progression in yeast (8, 9). Intuitively, it seems reasonable to assume that new protein synthesis is at least a first approximation of cell growth because synthesis of all other components of a cell depends on the enzymatic functions of proteins. eIF4E has long been speculated to be the rate-limiting component of the protein synthetic machinery because it is the least abundant of all translation initiation factors and the process of translation initiation is the rate-limiting for protein synthesis in mammalian cells (10).

*Drosophila* again provide genetic support that at least phosphorylation of eIF4E is required for cell growth (11). In contrast, the effects of eIF4E on bulk protein synthesis rates have only rarely been studied in mammalian cells, and when they have been studied eIF4E has not necessarily been the key regulator of protein synthesis (12). Surprisingly, overexpression of eIF4E clearly increases S phase entry in mammalian cells (13, 14) and has specific enough effects on target RNAs to transform cells (15). In this report we sought to develop a non-lethal mechanism to inhibit the function of the eIF4E to characterize its effects on protein synthesis, cell cycle progression, and malignant transformation.

A PHAS target of insulin regulation was first identified as a
strong inhibitor of eIF4E function by virtue of direct binding (16). Although the 4EBP1 inhibitor of the function of eIF4E seemed a likely tool for our studies, its initial potency as an inhibitor of proliferation was not as impressive as hoped (17, 18). As a simple first step to better evaluate the potency of 4EBP1, we added bacterially synthesized 4EBP1 to in vitro extracts producing 4EBP1 (Fig. 1). Where we expected an immediate cessation of translation, 4EBP1 had little effect on its own translation. Instead, the addition of 4EBP1 shifted the newly synthesizing 4EBP1 into its inactive phosphorylated form. This autosensing mechanism is likely to be fundamental in cell growth because it can obviously protect the cell against lethal increases in 4EBP.

Akt, TOR, mitogen-activated protein kinase, S6 kinase, and Cdc2 have all been identified as kinases capable of 4EBP1 phosphorylation (19–22). It seems clear that threonines 35, 45, and 69 together with serine 64 are targets for 4EBP1 inactivation by phosphorylation events (23–25). In mammals, TOR seems to cooperate with phosphatidylinositol-3-kinase-dependent path members to phosphorylate S6 kinase and 4EBP1 (19, 25–27). Importantly, not all phosphorylation events equally block the 4EBP1-eIF4E interaction (24), and several experiments implicate multiple stages of phosphorylation that hierarchically result in the final phosphorylation of all four candidate sites before the 4EBP-eIF4E interaction is completely blocked (67, 68). Furthermore, an increased number of alanine substitutions of these target sites was shown to increase 4EBP1’s inhibition of cap-dependent translation (24). We therefore mutated all four phosphorylation-inactivation target sites together to develop our reagent. Given the large number of changes in 4EBP structure generated by this strategy, we demonstrated a continued interaction between the activated 4EBP1a and eIF4E, and we further confirmed that inactivation of the eIF4E interaction domain killed the activated 4EBP1a (Fig. 2).

The ability of eIF4E to transform cells remains the best demonstration that it regulates specific mRNAs involved in malignant proliferation. Our interest in eIF4E stems from the possibility that some of the transforming function of myc is due to regulation of eIF4E (18, 25). By using a standard cell line that is transformed by c-myc in a single step, we therefore determined that the function of eIF4E is indeed necessary for myc to transform cells (Fig. 3). Our result certainly does not imply that eIF4E is the only target for c-myc during oncogenesis, nor does it demonstrate that eIF4E is only targeted by c-myc. Indeed, eIF4E is a known ras and src effector (40, 69). The spectrum of oncogenes that require alterations in eIF4E function may well be quite large, although evidently not all oncogenes require altered eIF4E function given our inability to block erbB2 transformation (Fig. 3). However, our best evidence for a specific interaction between eIF4E and c-myc remains our demonstration of a myc-binding site in the eIF4E promoter.

We further assessed the activity of the 4EBP1a in more normal diploid rat embryonic cells (Fig. 4). To our surprise, constitutive blockade of eIF4E caused a large cell phenotype in normal diploid cells even though it potently inhibited cell proliferation. We confirmed that this was due to a selective delay in G1, whereas the 4EBP1a failed to decrease cellular protein synthesis. Moreover, protein content of 4EBP1a-expressing cells mildly increased. These data are all consistent with previous studies showing an important cell cycle regulatory role for
eIF4E (7, 13). We have added the important additional information that this effect is a specific one and not due to generalized increases in the synthesis of all proteins in the cell. We further confirmed that the same effects could be seen in Rat1a cells (Figs. 5 and 6).

Surprisingly, whereas both myc and the 4EBP\(\mu\) increased cellular protein content individually, the combined expression of both apparently blocked their respective effects on protein content. We consider that this is likely due to the differential mechanisms underlying the content changes in the two cases. The increased protein in the 4EBP\(\mu\)-expressing cells is likely due to its disproportionate inhibition of proteins regulating cell division. In contrast, the increased protein content of myc-expressing cells is likely due to its disproportionate enhancement of proteins regulating cell growth. When the two are added together, myc presumably enhances the synthesis of growth-promoting elements in the 4EBP\(\mu\) expressers so that cell growth and cell division come back into balance (Fig. 5E, compare Rat1a and myc/4EBP\(\mu\) cells). Interestingly, this is also reflected in the G1 phase of the myc/4EBP\(\mu\) transfectants whose G1 phase is identical to that seen in control Rat1a cells (Fig. 7C).

The proliferative effects of c-myc overexpression are normally balanced by increases in myc-induced apoptosis (59). Survival and overproliferation of myc-overexpressing cells therefore requires over-riding signals mediated by the Akt-survival pathway (74). Importantly, Akt functions in part by phosphorylating and inactivating 4EBP\(\mu\) (75). Thus, introduction of our non-phosphorylatable 4EBP\(\mu\) (expressing cells and to enhance the rate of cell division (Fig. 7). The combined effects of the 4EBP\(\mu\) to delay the accelerated G1-S transition in myc-expressing cells and to enhance the rate of apoptosis in myc-expressing cells probably combine to lower the tumorigenic potential of myc-transformed cells (Fig. 3).

This observation may be useful in future considerations of therapeutic approaches to treatment of cancers caused by c-myc.

We last sought to evaluate the effect of the 4EBP\(\mu\) on translation initiation rates to examine its effects on regulators of growth or cell division. The relative amount of RNA found in the polysomal fraction of ribosomes indicates the relative ini-

Fig. 9. Differential effects of 4EBP\(\mu\) on ribosomal protein synthesis and ornithine decarboxylase. A, regulation of translation initiation by signal transduction pathways. Indicated are the ras-raf pathways (left column) and the phosphoinositide 3-kinase (PI3K) pathways (right column). The ras-raf pathway controls activation of the 5’ mRNA cap-binding protein (eIF4E) as mediated by the mitogen-activated protein kinase signal integrating kinase (Mnk). A separate pathway signals through the mammalian target of rapamycin (mTOR). This pathway may regulate cap-dependent translation through its regulation of the eIF4E-binding protein (4EBP\(\mu\)) that inactivates eIF4E. The 4EBP\(\mu\) are hyperphosphorylated to release eIF4E so that it can interact with the 5’ cap and assemble the eIF4F initiation complex. mRNAs involved in ribosomal biogenesis contain a terminal oligopyrimidine sequence (TOP). The TOR pathway also regulates these mRNAs through its regulation of the ribosomal protein subunit S6 kinase (S6K). These pathways show that regulation of ribosomal protein synthesis is under fundamentally different controls than regulation of those mRNAs that are dependent on eIF4E for translation. B, [\(35\)S]methionine incorporation into ribosomes of Rat1a cells expressing the empty vector control or the 4EBP\(\mu\) constructs. Incorporation into the isolated ribosomal fraction was performed as described under “Experimental Procedures,” and total counts/min incorporated per 10\(^6\) cells is plotted as a mean ± S.D. for three plates growing in 0.5% FBS or 10% FBS as indicated. The proliferation induction was performed as described in Fig. 5, A and B, except that we only examined the 3-h time point of incorporation. C, confluent control (C) and 4EBP\(\mu\) (\(\mu\)) cells were growth-arrested in 0.5% FBS for 72 h and then stimulated for the indicated times (in hours) by growth in medium containing 10% FBS. RNA and protein were harvested and analyzed by standard Northern and Western blots as described under “Experimental Procedures” for ornithine decarboxylase and actin protein (top two rows) and mRNA (bottom two rows). D, synthesis rates of ODC protein were further analyzed by immunoprecipitation studies performed as described under “Experimental Procedures.” The immunoprecipitations were performed after a 3-h [\(35\)S]methionine incubation in methionine-free medium in the presence of 0.5% (serum .5) or 10% FBS (serum 10) that followed 72 h of growth arrest in 0.5% FBS. Cells containing the empty vector control plasmid (C) were compared with cells transfected by the 4EBP\(\mu\) construct (\(\mu\)).
tuation rates of all mRNAs taken together (50). Consistent with the known functions of the 4EBP, our 4EBP1 had a general inhibitory effect on translation initiation (Fig. 9, A and B). Here we also show that despite its general inhibitory effect on the sum of all mRNAs, the 4EBP1 has differing specific effects on various mRNAs. Surprisingly, a ribosomal protein mRNA shifts into the polysomal fraction as a result of the 4EBP1 expression. This finding is confirmed by our comparison of net ribosomal protein synthesis to DCC synthesis in Rat1a cells expressing the 4EBP1 construct (Fig. 9).

Explanations for the apparent difference between total [35S]methionine incorporation that showed little response to the 4EBP1 versus its inhibitory effect on polysomal profiles are not obvious from our data. This difference can be partially explained by over-representation of the more abundant ribosomal proteins in [35S]methionine incorporation studies versus polysomal profiles. However, additional factors must also be involved. We did not directly test rates of translation elongation in these experiments that may differ among the various classes of mRNAs examined. Furthermore, whereas translational elongation is not generally viewed as an important control element of protein synthesis, direct or indirect effects of 4EBP1 may be differentially affecting translational elongation of the ribosomal proteins versus other more cap-dependent mRNAs. We also did not evaluate either translation termination or ribosomal recycling, which will both affect net protein synthesis. In addition, the 4EBP1 could alter the rates of labeling of different classes of mRNAs due to shifts in the specific activity of the [35S]methionine pools available to the various mRNAs being translated in these experiments. These complications do not fundamentally change our conclusion that the 4EBP1 construct has differential effects on those mRNAs that regulate the G1-S transition, however.

It was shown previously (78) that overexpression of eIF4E does not relieve translational repression of TOP mRNAs, so it should not be surprising that an agent that blocks eIF4E should have little effect on the TOP mRNAs. This result is also anticipated by the findings of Meyuhas and co-workers (51, 79) who found that the TOR pathway that regulates 4EBP is not a major regulator of TOP mRNAs. We speculate that the actual shift into the polysomal fraction may be reactive to the effects of the 4EBP1, rather than the result of a direct interaction between 4EBP1 and eIF4E. However, to address this issue will require additional experimentation. The protein or proteins that bind to the TOP remain unknown. In contrast, we find that translation initiation of ODC, a strong promoter of cell proliferation that is known to be translationally regulated by eIF4E, is indeed inhibited by our 4EBP1. Our results provide strong confirmation of the view that eIF4E regulates translational initiation of subsets of mRNAs critical to cell division. The relative stringency of loss of translation initiation obviously varies over a spectrum, as illustrated by prt-1/cdc63 mutants (4). In the extreme case, loss of growth capacity leads to cell death. A milder loss of translation capacity arrests cells in G1 during the most growth-sensitive phase. This mechanism must provide a selective advantage to cells because it stops cell division before protein synthesis rates fall below a critical threshold. Our work emphasizes the importance of further identification of critical cell cycle targets of the translational machinery (5).

c-myc is a relatively weak activator of a broad array of cellular functions (26). This result fits previous demonstrations of its weak activation potential in reporter gene experiments (80). The limited set of genes whose expression is lost in myc null cells also fits this model (31), although loss of target gene expression must be interpreted cautiously in knockout experi-

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