MOLECULAR AND CELLULAR BIOLOGY, Mar. 1992, p. 1234–1238
0270-7306/92/031234-05$02.00/0

Vol. 12, No. 3

The mRNA 5′ Cap-Binding Protein, eIF-4E, Cooperates with v-*myc* or E1A in the Transformation of Primary Rodent Fibroblasts

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Received 9 July 1991/Accepted 8 December 1991

We present evidence that eIF-4E, the mRNA 5′ cap-binding protein, cooperates with two immortalizing oncogenes, v-*myc* and E1A, to cause transformation of rat embryo fibroblasts. eIF-4E alone can transform rat embryo fibroblasts when selection is applied. The pattern of transformation by eIF-4E is similar to that of p21 Ras, raising the possibility that eIF-4E shares a common signal transduction pathway with p21 Ras.

Translational control is an important mechanism of regulation of gene expression and likely plays a critical role in cell growth, development, and differentiation. Most of the translational control is exerted at the level of initiation (12). The overall rate-limiting step in translation initiation is the binding of the ribosome to the mRNA (12). Several features of the 5′ region of eukaryotic mRNAs, including the 5′ cap structure and the 5′ untranslated region, are important for translational control. The mRNA 5′ cap structure, present on all cellular eukaryotic mRNAs, facilitates ribosome binding to mRNA (for reviews, see references 27 and 34).

A 24-kDa phosphoprotein, termed eIF-4E, binds specifically to the mRNA cap structure. This protein is a component of a three-subunit complex, eIF-4F, that is involved in ATP-dependent unwinding of 5′ mRNA secondary structure (27, 28, 34). eIF-4E is the limiting component among the initiation factors (5, 9). Consequently, eIF-4E and the complex, eIF-4F, likely play an important role in regulation of translation. There are numerous lines of evidence to support such a role. The mechanism by which eIF-4E regulates the rate of translation initiation is not entirely understood, but phosphorylation on a single serine residue (Ser-53 [31]) apparently enhances eIF-4E activity (13, 22). Consistent with this, the phosphorylation state of eIF-4E in a cell is proportional to its proliferative state. Stimulation of cell growth by serum, tumor necrosis factor α, platelet-derived growth factor, and insulin results in enhanced phosphorylation of eIF-4E (7, 8, 19, 23). Similarly, cells transformed by oncogenes contain a higher proportion of phosphorylated eIF-4E than do normal cells (7). Furthermore, under circumstances whereby cellular protein synthesis is reduced (e.g., mitosis, heat shock, or adenovirus infection), phosphorylation of eIF-4E is decreased (2, 5, 10). The importance of phosphorylation of Ser-53 for control of cell growth was further demonstrated by the finding that wild-type eIF-4E acts as a proto-oncogene, whereas a mutated form of eIF-4E, in which Ser-53 was changed to alanine [4E(Ala)], had no oncogenic activity (16). Also, overexpression of wild-type eIF-4E, but not 4E(Ala), deregulated HeLa cell growth (4). In other experiments, it was demonstrated that eIF-4E is mitogenic, inasmuch as injection of recombinant eIF-4E into serum-starved NIH 3T3 cells triggered the onset of DNA synthesis and also caused morphologic transformation of the cells (33). Again, the mutant form, 4E(Ala), did not display such activities (33). Together, these results emphasize the role of eIF-4E in growth signal transduction and cell proliferation.

Transformation of nonestablished rodent cells, as opposed to established cell lines, by single viral or cellular oncogenes is generally inefficient and requires special selection conditions (14, 15, 29, 35–37). Numerous reports documented the requirement for complementation between members of two distinct groups of oncogenes to transform primary cells (for recent reviews, see references 11 and 37). One group consists of nuclear genes, such as *myc*, E1A, and polyomavirus large T, that are primarily involved in transcriptional control and are capable of immortalizing nonestablished primary cells (1, 15, 25, 29). The second group consists of cytoplasmic oncogenes, such as activated p21 *ras* or middle T, that are generally capable of transforming established cell lines and are involved in cytoplasmic signal transduction pathways (11, 15, 36, 37).

The mechanism by which eIF-4E transforms cells is not known. An understanding of this mechanism should yield important insights into the transduction pathways that interdigitate between growth signals and the translation machinery. As a first step in these studies, we examined whether eIF-4E can transform primary cells and whether it requires a complementing oncogene for transformation.

MATERIALS AND METHODS

Plasmids and cell lines. eIF-4E constructs in the retroviral expression vector pMV7 (13a) were as previously described (16). A v-*myc*-containing plasmid (pSVv-*myc*) and an EJ *ras* plasmid (pEJ6.6) were provided by R. A. Weinberg (Massachusetts Institute of Technology). pSVv-*myc* consists of a 5.5-kb insert encoding the entire avian p110 *gag-myc* gene with long terminal repeats at each end subcloned into the *Eco*RI site of pSV2gpt, replacing the *gpt* coding sequence (15). pEJ6.6 contains a 6.6-kb genomic fragment of the transforming allele of Ha-c-*ras* (32). An E1A-containing plasmid was provided by G. Matlashewski (McGill University) and consists of the E1A region of adenovirus type 5 subcloned into pJ10DC (21).

Rat embryo fibroblast (REF) cultures were provided by M. Bastin (University of Sherbrooke). REFs were prepared as previously described (1), by mincing and dissecting 14-day-old Fisher rat embryos with trypsin. The suspension was passed through a gauze mesh to isolate single cells, collected with fetal calf serum, and centrifuged at 1,800 rpm.

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TABLE 1. Colony- or focus-forming ability of REFs cotransfected with eIF-4E and v-*myc* or E1A

Plasmid(s)	No. (avg) of:		No. with long-term growth[b]		Transformation[c] phenotype
	Foci[a]	Colonies (with G418)	−G418	+G418	
1. pMV7	0, 0, 0, 0 (0)	2, 7, 4, 5 (4)	0	0/5	−
2. pMV7 + E1A[d]	0, 0 (0)	6, 2 (4)	0	4/8	−
3. pMV7 + v-*myc*	6, 4 (5)	3, 3 (3)	0	0/4	−
4. v-*myc* + Ha-*ras*	15, 10, 24 (16)	ND[e]	6/6	ND	+
5. E1A + Ha-*ras*	18, 25 (22)	ND	3/3	ND	+
6. v-*myc*	6, 5, 10 (10)	ND	0	ND	−
7. Ha-*ras*	0, 0, 0 (0)	ND	0	ND	−
8. pMV7/4E(wt[f])	0, 0, 0, 2, 0 (0)	50, 86, 30, 66, 85 (63)	0	6/6	+
9. pMV7 + Ha-*ras*	0, 3 (1)	83, 50 (66)	0	3/3	+
10. pMV7/4E(wt) + v-*myc*	121, 85, 95, 100 (100)	223, 121, 95, 108 (136)	6/6	6/6	+
11. pMV7/4E(wt)+ E1A[d]	31, 23, 11, 22 (22)	65, 50, 68, 58 (60)	5/5	5/5	+
12. pMV7/4E(Ala) + v-*myc*	0, 0 (0)	0, 0 (0)	0	0	−
13. pMV7/4E(Ala) + E1A[d]	8, 5 (6)	3, 2 (2)	0	0/3	−
14. pMV7/4E(Ala)	0, 0, 0 (0)	0, 0, 0 (0)	0	0	−
15. pMV7/4E(wt) + Ha-*ras*	2, 0, 0 (1)	ND	0	ND	−

[a] A focus is defined as an outgrowth on a monolayer of cells. Some of the observed foci were not transformed (see last column) and could not be established as cell lines.
[b] Number of cell lines capable of more than 10 passages per total number of cell lines tested.
[c] Transformation was defined according to phenotype. Further criteria for transformation are shown in Table 2.
[d] Cotransfection was performed with 0.5 μg of E1A plasmid.
[e] ND, not done.
[f] wt, wild type.

The cell pellet was subsequently plated in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. DNA transfections into passage 2 REFs were performed as described previously (1). Briefly, REFs were plated at 5×10^5 cells per 100-mm dish 24 h before transfection. The cells were transfected with 1.25 μg of plasmid DNA, unless otherwise indicated, and carrier DNA was added to a final concentration of 15 μg, using the calcium phosphate method (38). The precipitate was applied to the cells for 24 h before selection with G418 (500 μg/ml). Plates were refed every 2 to 3 days with Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Individual foci or colonies were picked and expanded after 3 to 4 weeks.

Soft agar and tumorigenesis assays. Analysis in soft agar was performed as previously described (16). Briefly, 2×10^4 cells were plated in duplicate in 30-mm dishes in 0.33% soft agar. Colonies were scored after 21 days, and only colonies containing 10 or more cells were counted.

Tumorigenesis was assessed by injection of cells into nude mice as previously described (16). Cells were washed and resuspended in phosphate-buffered saline, and 10^6 cells were injected subcutaneously into the hind legs of 4- to 8-week-old *nu/nu* mice. The mice were examined at 2-day intervals.

Western immunoblot analysis. Western blot analysis was performed as previously described (16). Total cell lysate was resolved by electrophoresis on a sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gel and blotted onto a nitrocellulose membrane. The blot was incubated with polyclonal rabbit anti-eIF-4E serum and monoclonal mouse antiactin serum and then incubated with ^{125}I-protein A to detect eIF-4E and with ^{125}I-goat anti-mouse antiserum (NEN) to detect β-actin. Autoradiography was for 2 days.

RESULTS

To characterize the transformation and tumorigenic properties of eIF-4E, we examined its capacity to act as an oncogene in an REF transformation assay (15). We first wanted to establish that our REFs could be immortalized by bona fide immortalizing genes and could be transformed by combinations of immortalizing and transforming genes. To this end, we examined whether REFs could be immortalized by the nuclear immortalizing gene v-*myc* or E1A. Independent G418-resistant cells, exhibiting normal morphology, were obtained in low frequency (about four colonies per dish) by transfection of the E1A gene together with the neomycin resistance gene in a retroviral vector (pMV7 [13a]). Half of the colonies could be established as untransformed cell lines (Table 1, line 2; Fig. 1C). Thus, our REFs could be immortalized by a bona fide immortalizing gene. However, we were not able to establish permanent cell lines by v-*myc* transfection, despite indirect evidence that it functions as an immortalizing gene (11) (we observed a total of six G418-resistant colonies in two different experiments, but these cells could not be passaged more than five times [Table 1, line 3]). REFs became transformed when transfected by a combination of an activated Ha-c-*ras* with either the v-*myc* or E1A gene, as had been previously reported for rodent primary cells (14, 15, 17, 29) (Table 1, lines 4 and 5; Fig. 1, E and F compared with Fig. 1A). All of the transformed clones that were examined were capable of long-term growth in culture (Table 1, lines 4 and 5). Transfection of the activated Ha-c-*ras* and v-*myc* by themselves did not result in transformation (Table 1, lines 6 and 7).

We next examined the ability of eIF-4E by itself to immortalize or transform REFs. When transfected in the absence of selection, eIF-4E did not cause either transformation or immortalization of REFs (Table 1, line 8). However, in the presence of G418 as a selection agent, transformed colonies were generated, and these were efficiently cloned into permanent cell lines (Table 1, line 8; Fig. 1H). These cell lines were capable of anchorage-independent growth (average cloning efficiency of 30%) and formation of tumors of unlimited growth in nude mice (Table 2, line 5). Hence, eIF-4E behaves similarly to the *ras* oncogene in that it cannot transform REFs under standard growth conditions but is capable of transforming REFs when selection is

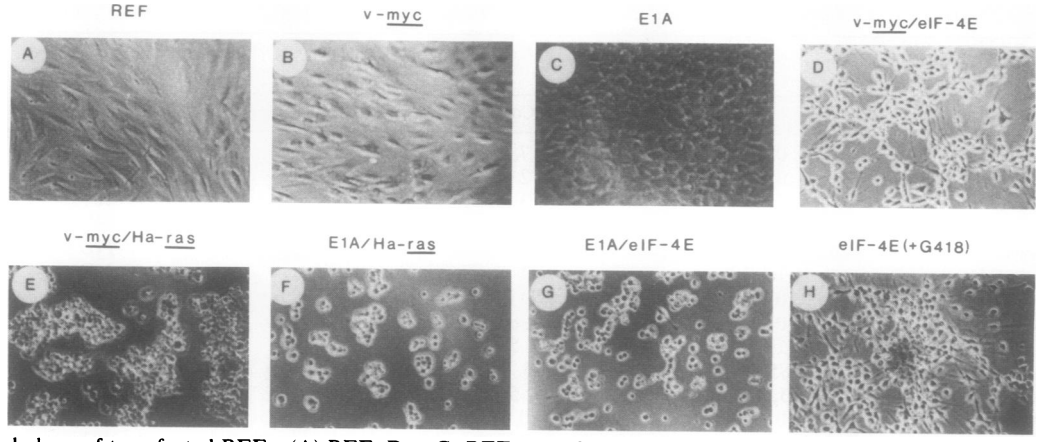

FIG. 1. Morphology of transfected REFs. (A) REF; B to G, REFs transfected with v-*myc* (cell lines could not be established, see text) (B), E1A (C), pMV7/4E and v-*myc* (D), v-*myc* and Ha-c-*ras* (E), E1A and Ha-c-*ras* (F), pMV7/4E and E1A (G), and pMV7/4E (H) in the presence of G418. Cells were photographed (magnification, ×160) after reaching confluency.

applied (Table 1, line 9) (14, 15, 35). It might be significant that the transformation efficiencies of eIF-4E and Ras are similar in the presence of G418, consistent with a similar mechanism of transformation (see below). A possible explanation for the ability of eIF-4E to transform cells only under selective pressure, as previously suggested for *ras*, will be addressed in Discussion.

To further examine the analogy between eIF-4E and p21 Ras, we studied the ability of eIF-4E to transform REFs in cooperation with the immortalizing, nuclear oncogenes v-*myc* and E1A. Cotransfection of pMV7/4E with either v-*myc* or E1A resulted in an average of 100 or 22 transformed foci, respectively (Table 1, lines 10 and 11). When selection with G418 was applied after transfection of pMV7/4E with either v-*myc* or E1A, the number of transformed foci was increased by 1.5- to 3-fold (Table 1, lines 10 and 11). All of the foci that were subsequently cloned were capable of continuous growth upon multiple (>20) passages (Table 1, lines 10 and 11; Fig. 1D and G). As expected, the transformed clones obtained by cotransfection of pMV7/4E and v-*myc* were capable of anchorage-independent growth and were tumorigenic in nude mice (Table 2, line 6). The tumors produced by eIF-4E-transformed REFs grew incessantly. This differs from what has been observed when REFs were transformed by a combination of v-*myc* and Ha-c-*ras* (15), in which case tumors grew to a definite size. The abrupt cessation of growth of tumors harboring activated Ha-c-*ras* and v-*myc* genes has prompted the suggestion that an additional gene(s) is necessary for the generation of fully malignant neoplasia (37). Our results suggest either that eIF-4E may be activating more pathways than does p21 Ras or, alternatively, that eIF-4E is more potent than p21 Ras in activating a downstream target required for tumor progression.

TABLE 2. Anchorage-independent growth and tumorigenicity of REFs cotransfected with the combination of eIF-4E and v-*myc* or E1A

Cells	Growth in soft agar[a] (no. positive/ no. tested)	Tumorigenicity in nude mice		
		No. of tumors/mice injected	Avg tumor size (cm)	Latency[b] (days)
1. REF	ND[c]	0/2		
2. v-*myc* + Ha-*ras*	2/2	4/4[d]	2.0	10–15[e]
3. E1A + Ha-*ras*	2/2	ND		
4. pMV7 + E1A	0/3	ND		
5. pMV7/4E(wt[f])	4/4	6/6[d]	3.5	10–12[g]
6. pMV7/4E(wt) + v-*myc*	4/4	8/8[d]	3.5	10–12[g]

[a] Colonies were examined 21 days after plating. Positives are defined as clones with >25% cloning efficiency in soft agar.
[b] Defined as the number of days after injection when a palpable tumor was observed.
[c] ND, not determined.
[d] Values represent the sum of injections from two independent clones.
[e] Tumor size was recorded until growth ceased, ~30 days after injection. Mice were maintained for an additional 40 days and sacrificed.
[f] wt, wild type.
[g] Tumors displayed unlimited growth. Tumor size was recorded before sacrifice, at 60 days after injection.

We show that transformation requires a wild-type eIF-4E, containing Ser-53. Mutation of this serine to alanine [(pMV7/4E(Ala)], which was previously shown to abrogate transformation activity in the NIH 3T3 assay, also abolished the transformation activity in REFs (Table 1, lines 12 to 14). As mentioned above, transfection of REFs with E1A (pMV7 + E1A) in the presence of neomycin selection gave rise to immortalized cell lines (Table 1, line 2). However, when E1A was cotransfected with pMV7/4E(Ala), immortalized cells were not obtained (Table 1, line 13; fewer colonies [five in two different experiments] were generated in the presence of G418, but the cells could not be propagated into established cell lines). The reason for this is not immediately clear but might reflect a *trans*-dominant negative effect of eIF-4E(Ala), as has been previously suggested from experiments with established cell lines (16).

One interpretation of these results is that eIF-4E exerts its transforming activity through a signalling pathway that is shared or converges on that of p21 Ras. To further explore this possibility, we transfected REFs with a combination of an activated Ha-c-*ras* and pMV7/4E. Only two foci have been obtained in three different experiments, and these could not be established as permanent cell lines (Table 1, line 15). Thus, eIF-4E and Ras cannot cooperate in transformation of REFs.

To ascertain that the transformation of REFs correlated with increased expression of eIF-4E, Western blot analysis was performed. We examined 10 different cell lines for

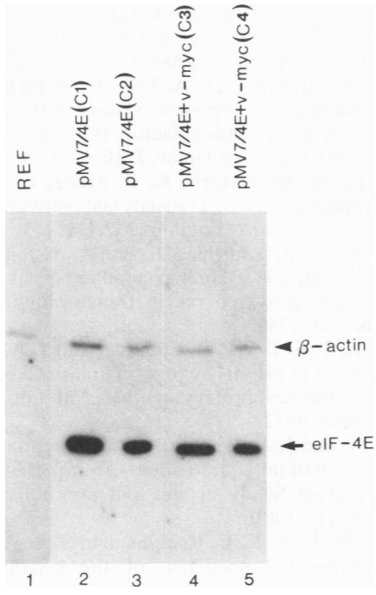

FIG. 2. Western blot analysis. Total cell extract (40 μg) was subjected to electrophoresis on an SDS–12.5% polyacrylamide gel, and Western blotting was done as described in Materials and Methods. Lanes: 1, extracts from REFs; 2, REFs transfected with pMV7/4E (C1) followed by selection with G418; 3, REFs transfected with pMV7/4E (C2) followed by selection with G418; 4, REFs cotransfected with pMV7/4E and v-*myc* in the absence of selection (C3); 5, REFs cotransfected with pMV7/4E and v-*myc* in the absence of selection (C4). Positions of β-actin (arrowhead) and eIF-4E (arrow) are indicated.

protein levels following transfection with pMV7/4E and selection for G418 resistance and found that the amount of eIF-4E protein was increased (approximately 5- to 15-fold) compared with levels in REFs. The results from two of these cell lines are shown in Fig. 2 (compare lanes 2 and 3 with lane 1). REFs cotransfected with pMV7/4E and v-*myc* demonstrated a lower increase in the levels of eIF-4E protein regardless of the presence or absence of selection (approximately eightfold; compare lanes 4 and 5 with lane 1). Similar levels of expression were observed when eIF-4E was cotransfected with E1A (data not shown). It is noteworthy that similarly to these findings, higher levels of Ras were also observed when REFs were transfected with Ha-c-*ras* alone compared with the levels observed when it is cotransfected with v-*myc* (15).

DISCUSSION

Several conclusions can be drawn from our results. First, eIF-4E is oncogenic in primary, nonestablished REFs, thus substantiating our earlier conclusions, based on transformation of established cell lines (NIH 3T3 and Rat-2), that eIF-4E is a proto-oncogene. It is also noteworthy that in the present study, eIF-4E was introduced into REFs by DNA transfection, whereas infection had been used previously for transformation of established cell lines (16). Second, oncogenic transformation of primary cells by eIF-4E abides by one of the cardinal principles of tumorigenesis in animals, namely, that conversion of normal cells to tumor cells is a multistage process that involves several genes. Furthermore, transformation of primary cells in culture requires the collaboration of at least two oncogenes (11, 36, 37). Because eIF-4E collaborates with nuclear oncogenes in transformation, it is possible that it acts in a *ras*-dependent signal transduction pathway. The finding that eIF-4E does not cooperate with an activated Ha-c-*ras* to transform primary cells conforms to the tenet (although there are exceptions; for examples, see references 26 and 30) that two cytoplasmic oncogenes are not capable of cooperating in transformation of REFs. A simple interpretation of this observation is that most cytoplasmic oncogenes activate the same or convergent pathways or that one pathway inhibits the other (11, 37). This again is consistent with the possibility that eIF-4E lies on the same signal transduction pathway as does p21 Ras.

The observation that eIF-4E alone is capable of transforming REFs when G418 selection is applied is not without precedent, inasmuch as an activated Ha-c-*ras* gene alone was also shown to transform primary cells under similar conditions (14, 35; this study). One explanation for this phenomenon is that when primary cells are transfected with a single oncogene, the small number of cells that become transformed are incapable of clonal expansion because of the growth-suppressing activity exerted by the surrounding normal cells (14). This could be due to inhibitory cytokines secreted by normal cells. However, when the normal cells are killed by G418, the growth-inhibitory influence of the normal cells is removed and the single oncogene-transformed cells (in our case, eIF-4E) are capable of vigorous proliferation. Two cooperating oncogenes are able to transform primary cells irrespective of the normal surrounding cell population, perhaps because the second immortalizing gene provides a function that overrides the growth-inhibitory activity of the normal cells (for reviews, see references 11, 36, and 37).

Transformation of primary rodent cells by the cooperating activated Ha-c-*ras* and v-*myc* oncogenes results in transformed cells possessing limited in vivo tumorigenic potential (15). When these cells are injected subcutaneously into nude mice, tumors develop that exhibit limited growth (15), unlike fully malignant cells, which form tumors that kill the host. In contrast to the phenotype produced by activated Ha-c-*ras* and v-*myc*, REFs transformed by eIF-4E alone or in cooperation with v-*myc* formed tumors exhibiting unlimited growth. It is therefore possible that eIF-4E activates a larger complement of genes than does p21 Ras, and these genes are required for progression of the cells to the tumorigenic phenotype.

The mechanism by which eIF-4E transforms cells is not clear. On the basis of the function and the biochemical characteristics of eIF-4E, the most plausible hypothesis is that overproduction of eIF-4E results in translational activation of one or several effector oncogenes, whose translation is tightly regulated. It is of interest in this respect that many proteins that play key roles in regulation of cell growth and differentiation, such as transcription factors, growth factors, growth factor receptors, and tyrosine kinases, are translated from mRNAs with long 5′ noncoding regions (e.g., references 20 and 24). Translation of mRNAs with long 5′ untranslated regions is inefficient, presumably because of their increased secondary structure (34; for examples, see references 3 and 18). Overexpression of eIF-4E, which is involved in the unwinding of secondary structure as part of the eIF-4F complex, should facilitate the unwinding and subsequent translation of these inefficient mRNAs. In support of this hypothesis, we have found that translation of mRNAs containing extensive secondary structure in their 5′ ends is enhanced in cells that overexpress eIF-4E (13b). Furthermore, translation of ornithine aminotransferase in a retinoblastoma cell line could be enhanced by overexpression of eIF-4E (6).

ACKNOWLEDGMENTS

We are grateful to M. Bastin for his generous and continuous supply of REFs. We thank Vinita Adkar for expert technical assistance. We thank R. A. Weinberg and G. Matlashewski for plasmids, and we thank P. Branton, M. Szyf, A. Veillette, and S. Roy for critical evaluation of the manuscript.

This work was supported by a grant from the National Cancer Institute of Canada N.S. A.L.-K. is a recipient of a predoctoral studentship from the Cancer Research Society (Montreal).

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