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Increase in Cap- and IRES-Dependent Protein Synthesis by Overproduction of Translation Initiation Factor eIF4G

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The role of eIF4G during the initiation of protein synthesis was studied using mouse mammary carcinoma FM3A cells and FM4G cells that overproduce an N-terminally truncated form of eIF4G, which lacks the binding site of poly(A)-binding protein. An increase in eIF4G was correlated with an increase in protein synthesis and RNA helicase activity. Translation of mRNAs having both short and long 5′-untranslated regions (5′-UTR) increased significantly in FM4G cells compared to that in FM3A cells. Both full-length and N-terminally truncated eIF4G transfectants of NIH3T3 cells formed colonies in soft agar and increased the saturation density of cell growth, indicating that both eIF4Gs function similarly. We also found that an internal ribosome entry site (IRES) exists in the 5′-UTR of ornithine decarboxylase mRNA and that IRES-dependent protein synthesis increased in FM4G cells. Our results indicate that an increase in eIF4G contributes to the formation of active eIF4F similarly to that caused by an increase in eIF4E, as well as to a stimulation of IRES-dependent protein synthesis.

Key Words: **translation initiation; eIF4G; eIF4F; cap; IRES; RNA helicase; ODC mRNA.**

Translational control plays an important role in the regulation of gene expression in eukaryotes (1, 2). This occurs mainly at the level of initiation involving Met-tRNA$_i$ binding to 40 S ribosomal subunit and the subsequent subunit scanning to the initiation codon AUG on mRNA initiated by m^{7}G cap recognition. The latter is especially important for the translation of mRNAs having a long 5′-untranslated region (5′-UTR). Until recently, subunit scanning was considered to be regulated by eIF4E, since this is the cap recognition factor and the least abundant protein among initiation factors (3). However, it was reported that eIF4E exists in an inactive form, in which the factor binds tightly to 4E-BP proteins (4–8). When 4E-BP proteins are phosphorylated in response to insulin and some other growth factors, 4E-BPs dissociate from eIF4E, allowing eIF4E to interact with eIF4G to form the eIF4F complex and to initiate translation (9). It is also known that eIF4E is phosphorylated in response to a wide variety of extracellular stimuli and that the phosphorylation correlates with increased translation and cell proliferation (1, 2). Furthermore, cells that overproduce eIF4E efficiently translate mRNAs having a long 5′-UTR (10), and the overexpression causes malignant transformation (11). As the mRNA for ornithine decarboxylase (ODC), one of the key enzymes for polyamine biosynthesis, has a long GC-rich 5′-UTR (12, 13), ODC mRNA also was efficiently translated in the cells overproducing eIF4E (14). Elevated expression of eIF4E has been also reported in several cancers and tumors (15, 16). In addition, we recently observed that the overproduction of eIF4G protein in NIH3T3 cells caused malignant transformation (17). The importance of eIF4G like eIF4E as a key protein in the formation of active eIF4F has been also pointed out (18–20).

We previously isolated mouse FM3A ODC-overproducing cells, termed EXOD-1 cells (21). Since ODC mRNA was translated efficiently in EXOD-1 cells, we examined the amount and properties of eIF4E and found an increase in the phosphorylation of eIF4E, its association with eIF4G, and an enhancement of RNA helicase activity (22). In this study, we tried to identify the role of eIF4G during the initiation of protein synthesis by using FM3A cells and a derivative cell line that overproduces eIF4G. Our results show that an increase in eIF4G is correlated with an increase in protein synthetic activity similar to that caused by the increase in eIF4E, suggesting that active eIF4F is formed by an increase in either eIF4G or eIF4E. During our study, we realized that the eIF4G used was N-terminally truncated eIF4G (23). Overproduction of

Abbreviations used: IRES, internal ribosome entry site; ODC, ornithine decarboxylase; PABP, poly(A)-binding protein.

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full-length eIF4G also formed colonies in soft agar and increased the saturation density of cell growth. Thus, we judged that N-terminally truncated eIF4G functions similarly to full length eIF4G except in the interaction with poly(A)-binding protein (PABP), and therefore it was used in this study. We also found that an internal ribosome entry site (IRES) exists in the 5′-UTR of ODC mRNA and that IRES-dependent protein synthesis increases in FM4G cells compared to FM3A cells.

MATERIALS AND METHODS

Cell culture. Mouse mammary carcinoma FM3A cells (Japan Health Science Foundation) were cultured in ES medium (Nissui Pharmaceutical Co., Ltd.) supplemented with 50 U/ml streptomycin, 100 U/ml penicillin G, 50 μg/ml gentamicin and 2% heat-inactivated fetal calf serum at 37°C in an atmosphere of 5% CO_2 (24). NIH3T3 cells were cultured in DMEM (Nissui Pharmaceutical Co., Ltd.), supplemented with 2 mM glutamine, 40 μg/ml gentamicin and 10% heat-inactivated fetal calf serum at 37°C in an atmosphere of 5% CO_2.

Plasmid construction. The plasmid containing human eIF4G cDNA SK4G (Ref. 25; kindly provided by Dr. R. E. Rhoads, Louisiana State University Medical Center) was digested with *Xba*I–*Xho*I and inserted into the same restriction sites of pMAM2-*BSD* (Funakoshi, Japan). The plasmid was termed pMAM-4G and transfected into FM3A cells by electroporation according to the method of Kimura *et al.* (26). Stable transfectant cells thus obtained were named FM4G cells. The eIF4G was induced by treatment of the cells with 1 μM dexamethasone (27, 28).

To make a long GC-rich 5′-UTR, *Hin*dIII sites were created on pODC188 (29) at the positions −3 and −192 (−3 and −192 nucleotides upstream from the initiation codon ATG) by PCR using 5′-ACTGAAGCTTGAATACACGG-3′ and 5′-TAGCAAGCTTCTCGATGTGC-3′ as primers. The 189 nt *Hin*dIII fragment was inserted into the *Hin*dIII site of pGL2-Control vector (Promega), containing the gene for luciferase (LUC). After determination of the nucleotide sequence, the plasmid, which has the reporter gene in the same orientation as 5′-UTR of ODC, was termed pGL2-279 since the size of 5′-UTR was 279 nt. The other one was termed pGL2-279rev since the orientation of the 5′-UTR was reversed. pGL2-Control vector was termed pGL2-89 since the size of 5′-UTR of LUC mRNA was 89 nt. pGL2-34 was constructed from pGL2-89 by removing 55 nt of 5′-UTR of LUC mRNA using PCR.

The various pGL2 constructs (pGL2-279ODCΔ1-37, pGL2-279ODCΔ53-88 and pGL2-279ODCΔ124-150), in which some portion of the 5′-UTR of ODC mRNA are deleted, were constructed from pGL2-279 using PCR. Bicistronic pRL-luc constructs (pRL-luc, pRL-ODC-luc, pRL-ODCrev-luc, pRL-ODCΔ1-37-luc, pRL-ODCΔ53-88-luc and pRL-ODCΔ124-150-luc) were made by replacing the *Bam*HI–*Xba*I fragment of pRL-SV40 (Promega) with the *Bam*HI–*Bln*I fragment (3.1 to 3.2 kb) of the pGL2 constructs described above.

pCMV-eIF4G, encoding N-terminally truncated eIF4G (25), was constructed as described previously (17). pcDNA3-HA-extended eIF4G, encoding full-length eIF4G (23), was kindly supplied by Dr. Sonenberg.

The nucleotide sequence of the plasmid DNA was confirmed using the Gene Rapid System (Amersham Pharmacia Biotech).

Cell fractionation. Cells (5×10^5 cells/ml, 1 L) were suspended in 2 ml Buffer A containing 20 mM Mops/KOH, pH 7.6, 2.5 mM magnesium acetate, 10 mM KCl, 0.5 mM dithiothreitol, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 50 mM NaF, 1 μM okadaic acid, an inhibitor of protein phosphatase (30), and 20 μM 6-amino-2-naphthyl-4-guanidinobenzoate dihydrochloride (FUT-175), a strong proteinase inhibitor (31). They were frozen, thawed, then homogenized with a Teflon homogenizer. The homogenate was centrifuged for 10 min at 17,000*g*. The supernatant (S17) was then centrifuged for 2 h at 200,000*g* to obtain the S200 fraction and ribosomes. The precipitated ribosomes were homogenized with 2 ml of Buffer B containing 20 mM Mops/KOH, pH 7.6, 10% glycerol, 450 mM KCl, 50 mM NaF, 2.5 mM magnesium acetate, 0.5 mM dithiothreitol, 0.1 mM EDTA, 1 μM okadaic acid and 20 μM FUT-175, and the homogenate was again centrifuged for 2 h at 200,000*g* to obtain the ribosome associated fraction (RAF). Protein was determined by the method of Lowry *et al.* (32).

Western blot analysis of eIF4G and eIF4E. Antibodies for eIF4E and eIF4G were prepared as described previously (22). Thirty micrograms of protein in RAF was separated by SDS/PAGE on a 7.5% acrylamide gel, and transferred to Immobilon transfer membranes (Millipore). eIF4G was detected with Western Exposure Chemiluminescent Detection System (CLONTECH). Phosphorylated and unphosphorylated eIF4Es in RAF were separated by two dimensional electrophoresis using 30 μg of protein according to the method of O'Farrell (33). Separated eIF4E proteins were transferred to Immobilon transfer membranes and detected with ProtBlot Western blot AP System (Promega) (34).

Northern blot analysis. Total RNA was isolated from 1×10^7 cells using the RNeasy Kit (Qiagen), and Northern blot analysis was performed as described previously (35), using 20 μg total RNA and ^{32}P-labeled eIF4G cDNA as a probe.

Assay for protein synthesis. The cells were cultured for 72 h as described, and then the 25 ml cell suspension (5×10^5/ml) was prepared. To the cell suspension, 366 kBq [^{3}H]leucine (1480 GBq/mmol) was added and incubated at 37°C. A 3 ml aliquot was taken out at the designated time, and hot trichloroacetic acid-insoluble radioactivity was measured by a liquid scintillation spectrometer.

Cap-independent RNA helicase assay. The unwinding activity of the RAF was measured according to the method of Rozen *et al.* (36) with some modifications (22). The reaction mixture (20 μl) contained 20 mM Tris–HCl, pH 7.6, 100 mM KCl, 1.5 mM magnesium acetate, 1.5 mM dithiothreitol, 0.5 mM ATP, 40 U of RNase inhibitor (TOYOBO, Japan), 3500 cpm ^{32}P-labeled uncapped double-stranded RNA and RAF at the specified amount. The structure of double-stranded RNA is as follows: 5′-GCGCGCGCAACAACAUUACAAUUUACAAUCC-3′ and 3′-CCUAACAUUUAACAUUACAACAACGCGCGCGCG-5′. After incubating the reaction mixture at 37°C for 20 min, the reaction was terminated by the addition of 2.5 μl of a buffer containing 30% glycerol, 3% SDS, 150 mM EDTA, 0.5% bromophenol blue and 0.5% xylene cyanol. The products of unwinding were analyzed directly by electrophoresis on a native 10.5% polyacrylamide gel, followed by autoradiography.

Stable transfection. Trypsin-treated NIH3T3 cells (2×10^6 cells/0.2 ml) and pCMV · SPORT containing eIF4G genes (10 μg) were mixed gently in a disposable cuvette (Bio-Rad; interelectrode distance, 0.4 cm) and a single electrical pulse was delivered from a 950-μF capacitor, charged at 0.5 kV/cm. The number of stable transfectants in soft agar was counted after 20 days as described previously (17).

Transient transfection and luciferase assay. The 5 μg of pGL2 constructs was transfected into 1×10^7 FM3A and FM4G cells by electroporation as described above, except that a single electrical pulse was delivered from a 950-μF capacitor, charged at 0.75 kV/cm. Bicistronic pRL-luc constructs were transfected by the same method, and LUC activity of the cell lysate was determined at 30 h after transfection using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol.

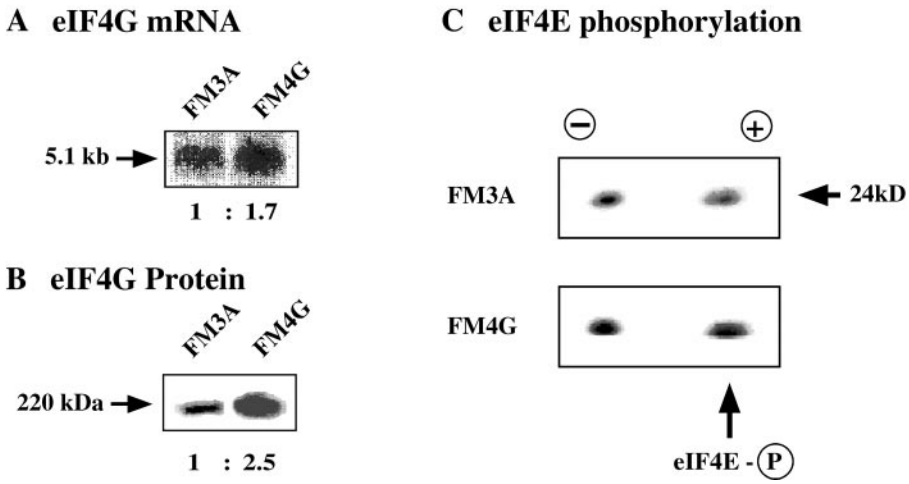

FIG. 1. Levels of eIF4G mRNA (A), eIF4G protein (B) and phosphorylated eIF4E (C) in FM3A and FM4G cells. FM3A and FM4G cells were cultured for 36 h in the presence of 1 μM dexamethasone. Level of eIF4G mRNA was determined by Northern blot analysis. Radioactivity of each band was quantified using a Fujix Bas 2000II imaging analyzer. Levels of eIF4G protein and phosphorylated eIF4E were determined by Western blot analysis using RAF. Intensity of each spot was measured using Bio Image Densitograph with Intelligent Quantifier 2.0.2.

RESULTS

Cellular Distribution of eIF4E and eIF4G in FM3A and Its eIF4G Overproducing FM3A Cells

FM4G cells, designed to overproduce eIF4G, were created by transfection of FM3A cells with pMAM-4G under the control of a dexamethasone-inducible promoter. Treatment of FM4G cells with 1 μM dexamethasone leads to expression of eIF4G (27, 28). The synthesis of eIF4G mRNA in FM4G cells was higher by 1.8-fold than that in FM3A cells (Fig. 1A). Under these conditions, the amount of eIF4G protein in FM4G cells was higher by 2.5-fold than in FM3A cells (Fig. 1B). Our results confirmed previous results indicating the exclusive distribution of eIF4G on ribosomes (37). eIF4E was mainly located in cytoplasm, and about 15% of eIF4E was associated with ribosomes (data not shown). The amount of phosphorylated and unphosphorylated eIF4E in the RAF from FM4G cells was nearly equal to that in the RAF from FM3A cells (Fig. 1C).

Increase in Protein Synthesis and Cap-Independent RNA Helicase Activity in eIF4G Overproducing Cells

Protein synthetic activity was measured by incorporation of [^{3}H]leucine into the hot trichloroacetic acid-insoluble fraction. As shown in Fig. 2A, protein synthetic activity of FM4G cells treated with dexamethasone was higher than that of FM4G cells untreated with dexamethasone. Protein synthetic activity of FM3A cells treated or untreated with dexamethasone was nearly equal to that of FM4G cells untreated with dexamethasone.

Cap-independent helicase activity was then measured using RAF prepared from FM3A and FM4G cells. As shown in Fig. 2B, RNA helicase activity was enhanced through the increase in eIF4G. These results clearly show that an increase in eIF4G enhances general protein synthesis as well as RNA helicase activity.

We next examined whether an increase in eIF4G equally enhances protein synthetic activity of mRNAs having short and long 5′-UTRs. FM3A and FM4G cells were transfected with plasmids containing the *luc* gene with the different sizes of 5′-UTR (34, 89, and 279 nt). A 190 nt portion of the 279 nt 5′-UTR was derived from

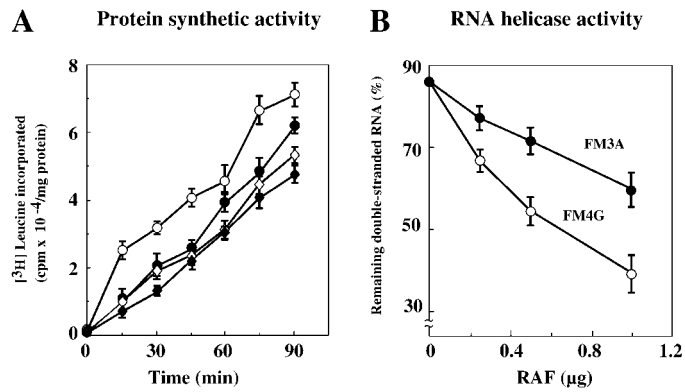

FIG. 2. Protein synthesis (A) and RNA helicase activity (B) in FM3A and FM4G cells. (A) Protein synthetic activity was measured as described under Materials and Methods. ○ and ●, FM4G cells treated and untreated with dexamethasone, respectively; ◇ and ◆, FM3A cells treated and untreated with dexamethasone, respectively. (B) RNA helicase activity. Percentage of remaining double-stranded RNA was quantified using a Fujix Bas 2000II imaging analyzer. ●, RAF from FM3A cells; ○, RAF from FM4G cells. Values are means ± SD of triplicate determinations.

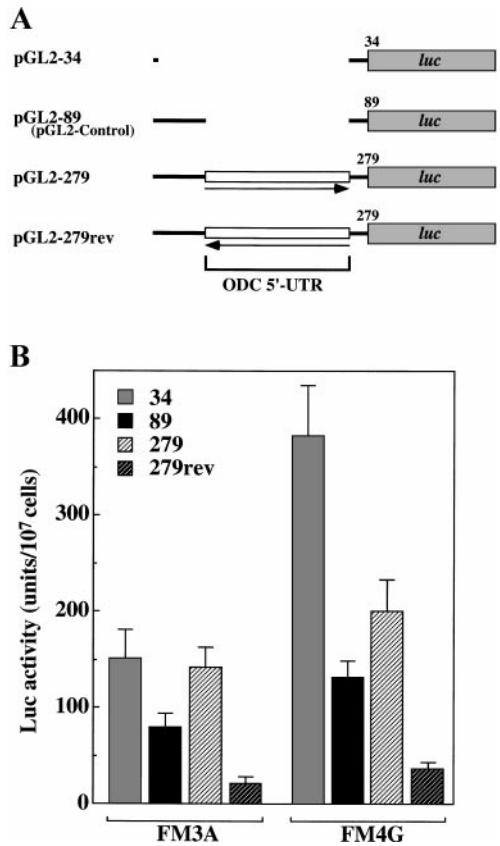

FIG. 3. Translational efficiency of LUC mRNAs having different sizes of 5′-UTR in FM3A and FM4G cells. (A) Structure of plasmids encoding *luc* gene. The black portion is 5′-UTR of LUC mRNA and the white portion is 5′-UTR of ODC mRNA, which was inserted into the 5′-UTR of LUC mRNA in either correct or reverse orientation. The figure on the gene indicates the number of nucleotides in the 5′-UTR. (B) The *luc* genes described in A were transfected into FM3A and FM4G cells, and LUC activity was detected as described under Materials and Methods. No LUC activity was observed with untreated FM3A and FM4G cells. Values are means ± SD of triplicate determinations.

the 5′-UTR of mouse ODC mRNA, and it was inserted into the 5′-UTR region of the *luc* gene in the correct or reverse orientation. As shown in Fig. 3, LUC synthesized from LUC mRNAs having different sizes of 5′-UTR was always greater in FM4G cells than FM3A cells. LUC activity in both cells was decreased with the increase in the size of 5′-UTR with the exception of mRNA having 279 nt of 5′-UTR in the correct orientation of the 5′-UTR of ODC mRNA. The reason for this exception has been clarified subsequently. These results indicate that an increase in eIF4G enhances protein synthetic activity regardless of the size of 5′-UTR of mRNA.

Increase in IRES-Dependent Protein Synthesis in eIF4G Overproducing Cells

Translation of mRNA having the 5′-UTR of ODC mRNA in the correct orientation was much higher than that of mRNA having the 5′-UTR of ODC mRNA in the reverse orientation (Fig. 3). This behavior is suggestive of the presence of an IRES in the 5′-UTR of ODC mRNA. To examine whether an IRES exists in the 5′-UTR of ODC mRNA, bicistronic constructs, in which various sizes of the 5′-UTR of ODC are inserted upstream of the second *luc* open reading frame, were made, and LUC activity was measured in FM3A cells by transfecting the bicistronic constructs. As shown in Fig. 4, significant LUC activity was observed in the transfectant having the 5′-UTR of ODC mRNA in the correct orientation, but not in the reverse orientation. An IRES was located in the first stem and loop structure upstream of the initiation codon AUG of ODC mRNA, since LUC activity greatly decreased in the transfectant with pRL-ODCΔ124-154-luc (III). Comparable results were obtained with FM4G cells, although the activity was always greater compared to that of FM3A cells (Fig. 4). The results indicate that an IRES exists in the 5′-UTR of ODC mRNA. Similar results, indicating that an IRES of ODC mRNA functions at G2/M of the cell cycle, have been recently reported by Pyronnet *et al.* (38).

Comparison of the Function of Full Length and N-terminally Truncated eIF4G

Our data were obtained using N-terminally truncated eIF4G (23). To compare the function of full length and N-terminally truncated eIF4G, malignant transformation by overproduction of both full length and N-terminally truncated eIF4G was examined using NIH3T3 cells. As shown in Table 1, both full length and N-terminally truncated eIF4G overproducing cells exhibited a malignantly transformed phenotype, and both cells formed colonies in soft agar. Furthermore, both cells continued to grow after NIH3T3 cells became confluent on day 5, and the saturation density increased up to 1.2- to 1.4-fold. The increase in eIF4G mRNA in both cells was 2- to 3-fold (data not shown). Since the increase in protein synthetic activity is probably correlated with the malignant transformed phenotype, the results suggest that both full length and N-terminally truncated eIF4Gs function similarly.

DISCUSSION

We studied the role of eIF4G on the initiation of protein synthesis using eIF4G-overproducing cells. Our results clearly show that eIF4G stimulates cap-independent RNA helicase activity and translation of mRNAs having different sizes of 5′-UTR. The results suggest that RNA helicase activity of eIF4A is stimulated by forming the eIF4F (eIF4A, eIF4E and eIF4G) complex.

It has been reported that overproduction of eIF4E (11) or eIF4G (17) caused malignant transformation,

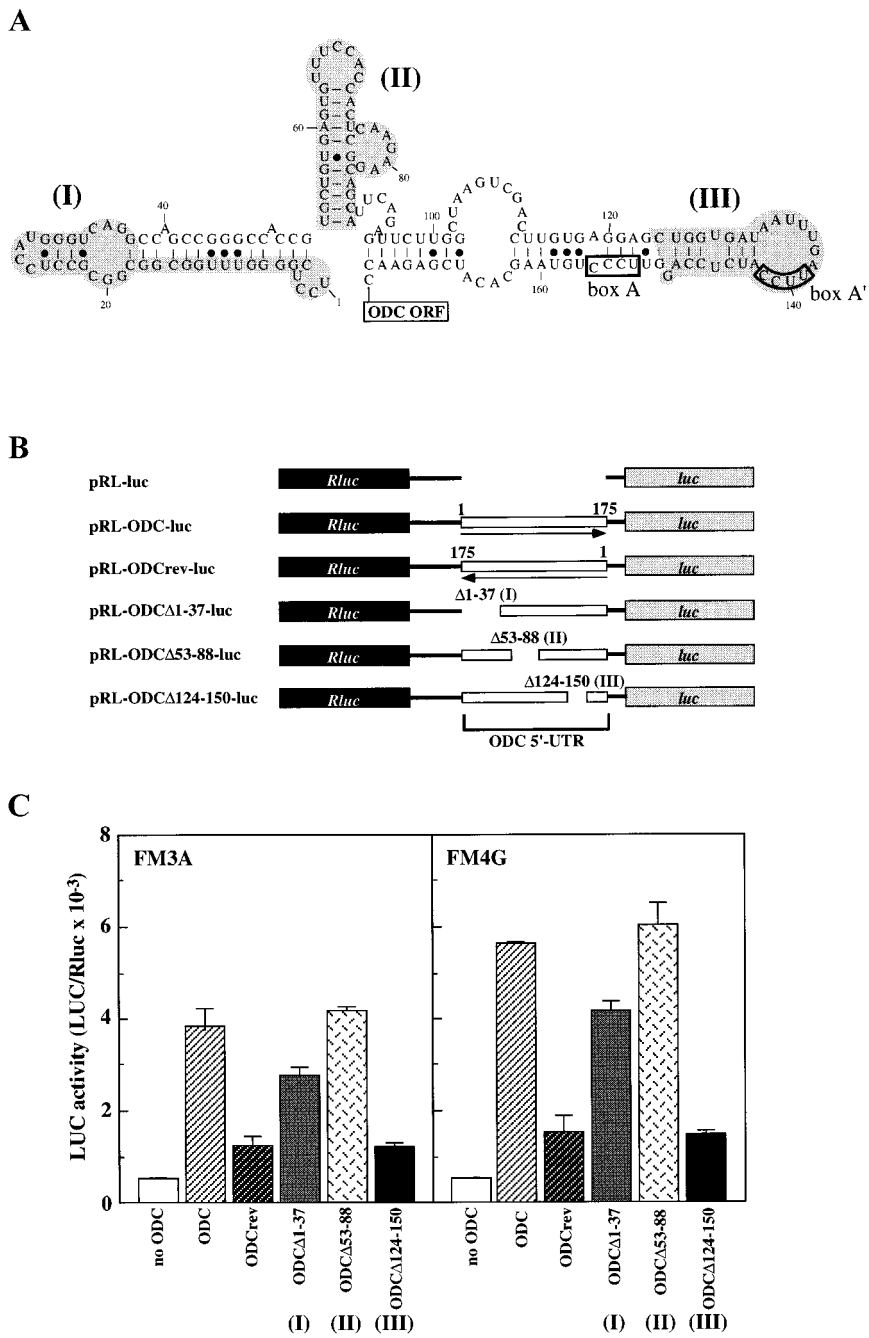

FIG. 4. Translational efficiency of LUC mRNAs synthesized from various bicistronic pRL-luc constructs in FM3A and FM4G cells. (A) Possible secondary structure of 5′-UTR of ODC mRNA. Optimal computer folding of ODC mRNA was performed by the method of Zucker and Stiegler (48). Two stretches of pyrimidines in boxes A and A′ were shown to be important for the IRES by Pyronnet *et al.* (38). (B) Structure of bicistronic pRL-luc constructs. Structure of the deletion constructs (I, II, and III) are shown by shadows in A. (C) LUC activity of FM3A and FM4G cells was shown as the ratio of LUC/RLuc. Values are means ± SD of triplicate determinations.

and translation of mRNA having different sizes of 5′-UTR was enhanced in both cells (10, 14 and this paper). We confirmed that protein synthesis in eIF4E overproducing NIH3T3 cells (11) was enhanced regardless of the size of 5′-UTR of mRNA. In both cells, protein synthesis is probably enhanced through eIF4F formation by competing with 4E-BPs because 4E-BPs and eIF4G recognize a common motif on eIF4E (9). Malignant transformation may be caused by increasing translation of oncogenes whose synthesis is not so efficient under normal conditions.

As for eIF4G, two kinds of eIF4G (I and II) exist, but the amount of eIF4GII is low (39). Thus, we judged that eIF4GI is more relevant than eIF4GII, and performed

TABLE 1

Efficiency of Growth in Soft Agar of eIF4G Transfectants of NIH3T3 Cells

Transfectant	Growth efficiency ($\times 10^{-2}$%)
None	1.89 ± 1.86
Vector	2.08 ± 4.20
eIF4G (N-terminally truncated)	15.5 ± 4.95
eIF4G (full length)	21.7 ± 6.72

Note. For growth in soft agar, 5×10^4 cells were resuspended in 2 ml of 0.35% (w/v) agar solution, containing DMEM plus 20% FCS, and overlaid onto a 0.5% (w/v) agar solution in a 35-mm plate. On day 2 after incubation, 2 ml of DMEM supplemented with 20% FCS were added. Colonies were counted 20 days after plating. Cloning efficiency in agar was calculated as the number of colonies × 100, divided by the number of cells plated. Values are means ± SD of triplicate determinations.

the experiments using N-terminally truncated eIF4GI, which lacks 156 amino acid residues in the N-terminus (23). PABP binds in this region of eIF4G and enhances protein synthesis by about 2-fold (23). Since both full length and N-terminally truncated eIF4G caused malignant transformation to a comparable degree, essential functions of eIF4G seem to be performed adequately by N-terminally truncated eIF4G.

Translation of mRNA having the 5′-UTR of ODC mRNA in the correct orientation was much higher than that of mRNA having the 5′-UTR of ODC mRNA in the reverse orientation (Fig. 3). We found an IRES in the 5′-UTR of ODC mRNA. Similar results have been obtained recently by Pyronnet *et al.* (38). Thus, ODC mRNA is probably translated by both cap-dependent and independent (IRES-dependent) mechanisms. It has been reported that an IRES exists on the 5′-UTR of several mRNAs like human immunoglobulin heavy chain binding protein (BiP) (40), Antennapedia (Antp) of *Drosophila* (41), fibroblast growth factor-2 (FGF-2) (42), vascular endothelial growth factor (VEGF) (43), X-linked inhibitor of apoptosis (XIAP) (44), and c-myc (45, 46). Thus, IRES-dependent translation probably plays important roles in some aspects of protein synthesis. Pyronnet *et al.* (38) proposed that IRES-dependent translation is likely to be a general mechanism to synthesize short-lived proteins even at mitosis, when cap-dependent translation is interdicted.

A common RNA structural motif in the IRES of cellular mRNAs has been analyzed (47). Our results indicate that an IRES exists in the first stem and loop structure upstream the initiation codon AUG of ODC mRNA (Fig. 4). In this structural motif, two stretches of pyrimidines (Box A and A′ in Fig. 4), which are shown to be important for IRES activity (38), are included. The IRES in ODC mRNA is very similar to that in Antp mRNA (47). Experiments are in progress to clarify how the IRES is recognized by 40 S ribosomal subunits.

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