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Minor contribution of an internal ribosome entry site in the 5′-UTR of ornithine decarboxylase mRNA on its translation

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Abstract

The mechanism of synthesis of ornithine decarboxylase (ODC) at the level of translation was studied using cell culture and cell-free systems. Synthesis of firefly luciferase (Fluc) from the second open reading frame (ORF) in a bicistronic construct transfected into FM3A and HeLa cells was enhanced by the presence of the 5′-untranslated region (5′-UTR) of ODC mRNA between the two ORFs. However, cotransfection of the gene encoding 2A protease inhibited the synthesis of Fluc. Synthesis of Fluc from the second cistron in the bicistronic mRNA in a cell-free system was not affected significantly by the 5′-UTR of ODC mRNA. Synthesis of ODC from ODC mRNA in a cell-free system was inhibited by 2A protease and cap analogue (m^7GpppG). Rapamycin inhibited ODC synthesis by 40–50% at both the G_1/S boundary and the G_2/M phase. These results indicate that an IRES in the 5′-UTR of ODC mRNA does not function effectively.

Keywords: ODC synthesis; IRES; 5′-UTR of mRNA

Ornithine decarboxylase (ODC) is one of the rate-limiting enzymes in the biosynthesis of polyamines, which are essential for cell growth [1]. Thus, it is important to clarify how synthesis of ODC is regulated. We previously reported that the level of ODC is mainly regulated by three steps, namely the level of mRNA, the translational efficiency of mRNA, and the stability of the enzyme [2,3]. With regard to translational efficiency, the ODC mRNA contains a GC-rich 5′-UTR, rendering it poorly translated [4,5]. Overexpression of eIF4E in cells leads to a 30-fold increase in ODC levels [6]. Thus, it is thought that ODC is synthesized mainly by a cap-dependent mechanism. However, it has been reported that an increase in ODC activity is observed at both G_1/S boundary and G_2/M transition, and that ODC synthesis at G_2/M in HeLa cells is achieved by internal ribosome entry site (IRES)-dependent translation [7]. We also found that the 5′-UTR of mouse ODC mRNA enhances expression of the second open reading frame (ORF) in a bicistronic construct, suggesting that the 5′-UTR of ODC mRNA contains an IRES element [8].

In this study, we reexamined the question of whether an IRES in the 5′-UTR of mouse ODC mRNA functions effectively using FM3A and HeLa cell culture systems, and FM3A and rabbit reticulocyte cell-free protein synthesis systems. We found that an IRES in the 5′-UTR of ODC mRNA is different from typical IRES elements such as the IRES in encephalomyocarditis virus (EMCV) RNA [9].

Materials and methods

Cell culture and synchronization. Mouse mammary carcinoma FM3A cells were cultured as described previously [10]. HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 50 U/ml streptomycin, 100 U/ml penicillin G, 50 μg/ml gentamycin, and 5% fetal calf serum at 37 °C in an atmosphere of 5% CO_2. HeLa cells were synchronized at the

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G_1/S boundary by sequential 2 mM thymidine and 5 μg/ml aphidicolin treatment according to the method of Heintz et al. [11], and in metaphase by the addition of 0.03 μg/ml nocodazole after treatment of 2 mM thymidine according to the method of Zieve et al. [12]. Synchronization was monitored by flow cytometry analysis of cellular DNA stained with propidium iodide using EPICS ELITE flow cytometer (Beckman Coulter).

Plasmid construction. Bicistronic pRL (*Renilla* luciferase)-luc (firefly luciferase) constructs (pRL-luc, pRL-ODC-luc and pRL-ODCrev-luc) were prepared as described previously [8]. pRL-ODC(-ORF)-luc, which is the direct fusion of 5′-UTR of ODC and Fluc, and pRL-ODC(+ORF)-luc, which is the fusion of 5′-UTR and nucleotides encoding first 17 amino acids of ODC with Fluc, were made by overlap extension using PCR [13]. The templates used were pODC188 [5] and pGL2-control (Promega). A list of primers used for PCR has not been included but is available from the authors upon request. The HindIII/BamHI fragments of the PCR products were inserted to the same site of pGL2-control. The BlnI/BamHI fragments of the pGL2 constructs were then inserted to the XbaI/BamHI site of pRL-SV40 (Promega). pRL-EMCV(+ORF)-luc, which is the fusion of 5′-UTR and nucleotides encoding first 17 amino acids of EMCV protein with Fluc, was similarly made by overlap extension using PCR [13]. The templates used were pBS-EMCV(+ORF)-CAT, a kind gift of Dr. A. Nomoto, University of Tokyo, and pGL2-control. A list of primers used for PCR is available from the authors upon request. The NotI/BamHI fragment of the PCR product was inserted to the same site of pRL-SV40. To make the SV40 promoter-less pRL-ODC(+ORF)-luc, the BglII–PstI fragments containing SV40 promoter were removed from the constructs. Then, BglII and PstI sites were ligated after these sites were blunted by T_4 DNA polymerase. The nucleotide sequence of the plasmid DNA was confirmed using a CEQ8000 DNA genetic analysis system (Beckman Coulter).

Plasmids pET8C/HRV2 2A [14], which expresses 2A protease in *Escherichia coli*, and pIND-2A [15], which produces 2A protease in animal cells, were kind gifts of Dr. T. Skern, University of Vienna, and Dr. C. Kahana, Weizman Institute of Science, respectively. To make the pTracer-CMV-2A, the EcoRI–ApaI fragments of pIND-2A encoding 2A protease were inserted into the same site of pTracer-CMV (Invitrogen).

Transient transfection and luciferase assay. Ten micrograms of pRL constructs was transfected into 1×10^7 FM3A cells by electroporation as described previously [8]. Twenty-four micrograms of pRL constructs were transfected into 1×10^7 HeLa cells by lipofectamine 2000 according to the manufacturer's instructions (Invitrogen). Luciferase activities of the cell lysate were determined at 30 h after transfection using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. Where indicated, 10 or 24 μg of pTracer-CMV-2A or pTracer-CMV vector was cotransfected to FM3A or HeLa cells.

In vitro protein synthesis in FM3A and rabbit reticulocyte cell-free systems. FM3A and rabbit reticulocyte nuclease-treated cell lysates were prepared as described previously [5]. *In vitro* protein synthesis in FM3A and rabbit reticulocyte cell-free systems was performed as described previously [16]. After incubation at 33 °C for 60 min, the amount of [^{35}S]methionine-labeled protein was determined by 13.5% SDS–PAGE followed by quantification with a Fujix BAS 2000II imaging analyzer. ODC188 and SAMDC346 mRNAs were synthesized as described previously using SP6 RNA polymerase in the presence and absence of m^7GpppG, cap analogue (GE Healthcare) [5,17]. EMCV(+ORF)-CAT, RL-ODC(+ORF)-luc, and RL-EMCV(+ORF)-luc mRNAs were synthesized from the respective plasmids using T7 High Yield Transcription Kit (Ampliscribe) according to the manufacturer's protocol. Where indicated, 0.5 mM m^7GpppG was added to the reaction mixture of protein synthesis, or cell lysate was pretreated with 60 ng of 2A protease purified by the method of Liebig et al. [14] for 60 min at 0 °C.

Assay for ODC and measurement of putrescine. ODC activity was measured using 2×10^7 HeLa cells and putrescine was measured by high-per-

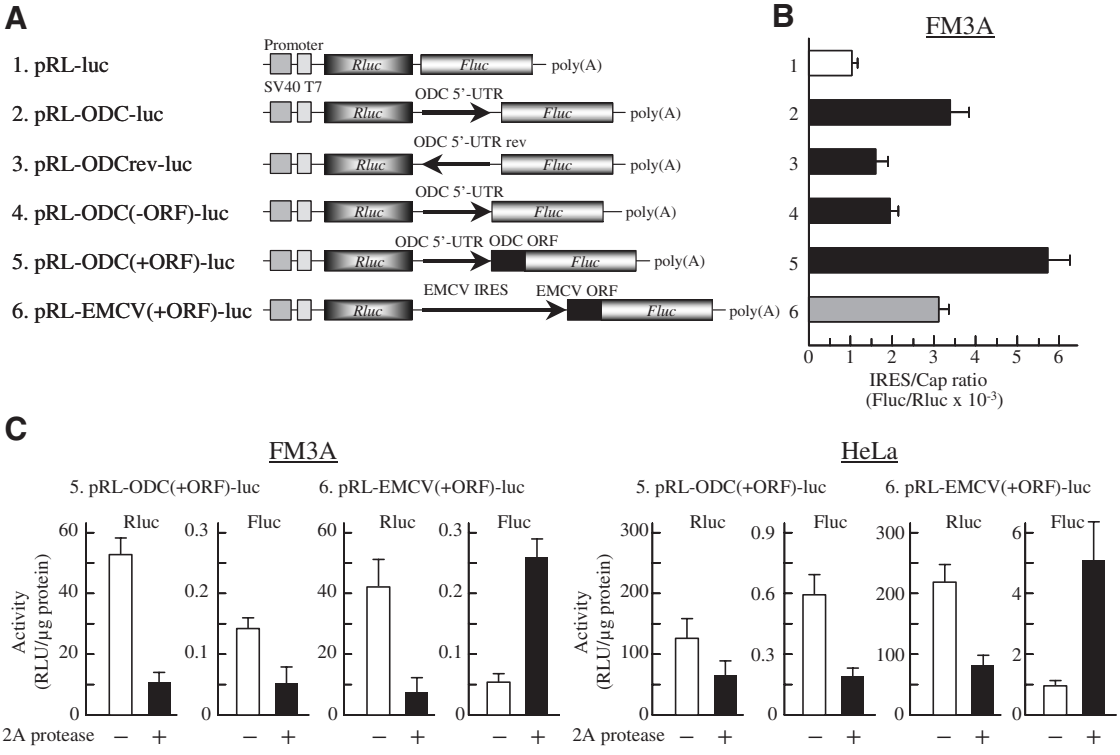

Fig. 1. Translational efficiency of Luc mRNAs synthesized from various bicistronic pRL-luc constructs in FM3A and HeLa cells. (A) Structure of bicistronic pRL-luc constructs. (B) Rluc and Fluc activities of FM3A cells measured were shown as the ratio of Fluc/Rluc. (C) Effect of 2A protease on Rluc and Fluc activities was examined by cotransfection of pTracer-CMV-2A or pTracer-CMV (control) with pRL-ODC(+ORF)-luc or pRL-EMCV(+ORF)-luc in FM3A cells (left figure) and HeLa cells (right figure). Values [RLU (relative light units)/μg protein] are means ± SD of triplicate determinations.

formance liquid chromatography using 6×10^6 HeLa cells as described previously [10,18]. Protein concentration was determined by the method of Lowry et al. [19].

Northern blot analysis of Rluc, Fluc and GAPDH (glyceraldehyde-phosphate dehydrogenase) mRNAs. HeLa cells transiently transfected with pRL-ODC(+ORF)-luc or promoter-less pRL-ODC(+ORF)-luc were cultured for 20 h. RNA was extracted from the cells with an TRIzol® Reagent (Invitrogen), and treated with RNase-free DNase (Promega). Northern blot analysis was performed as described previously [20] using the ECL direct nucleic acid labeling and detection system (GE Healthcare) with 20 μg RNA. Rluc, Fluc and GAPDH genes were amplified by PCR and the products were used for probes. Chemical luminescence was detected by a LAS-1000 plus luminescent image analyzer (Fuji Film).

Western blotting. Western blotting was performed by the method of Nielsen et al. [21] using ECL™ Western blotting reagents (GE Healthcare). Antibody against eIF4E was prepared as described previously [22]. Antibodies against eIF4GI [23] and 4E-BP1 [24] were kindly supplied by Dr. N. Sonenberg, McGill University, Canada. Antibodies against phosphorylated eIF4E and 4E-BP1 were purchased from Cell Signaling Technology. The protein corresponding to 30 μg of the HeLa cell lysate was used for the blotting.

Results

Re-estimation of an IRES-like element in the 5′-UTR of mouse ODC mRNA in a cell culture system

In animal cells, proteins are synthesized by a cap-dependent mechanism or, in rare cases, by an IRES-dependent mechanism [25,26]. IRESes are located in the 5′-UTR of mRNA, and IRES-dependent protein synthesis takes place without the aid of a cap structure. Accordingly, intact eIF4G is not necessary for IRES-dependent protein synthesis. We previously reported that an IRES-like element is present in the 5′-UTR of mouse ODC mRNA, based on results showing an increase in the expression of the second ORF in the *Renilla* luciferase–firefly luciferase (Rluc–Fluc) bicistronic construct [8]. We have now extended that work by measuring the expression of the second *Fluc* ORF in a variety of bicistronic Rluc–Fluc constructs. We compared four kinds of constructs containing the 5′-UTR of ODC with a construct having the IRES element of EMCV RNA (RL-EMCV(+ORF)-luc). Those constructs were: the *Fluc* ORF having its own 5′-UTR fused with the 5′-UTR of ODC mRNA (RL-ODC-luc) or with the reverse orientation of the 5′-UTR of ODC mRNA (RL-ODCrev-luc); the *Fluc* ORF directly fused with the 5′-UTR of ODC mRNA (RL-ODC(-ORF)-luc); and the *Fluc* ORF fused with the 5′-UTR and 51 nt of ORF of ODC mRNA (RL-ODC(+ORF)-luc) (Fig. 1A). As shown in Fig. 1B, an increase in Fluc/Rluc activity in FM3A cells was observed in the order RL-ODC(+ORF)-luc > RL-ODC-luc = RL-EMCV(+ORF)-luc > RL-ODC(−ORF)-luc > RL-ODC-rev-luc. An increase in Fluc/Rluc activity derived from RL-ODC(+ORF)-luc and RL-EMCV(+ORF)-luc constructs was confirmed with HeLa cells (Fig. 1C). Then, the effect of 2A protease was tested to clarify whether the 5′-UTR of ODC mRNA has a typical IRES element. The 2A protease is known to hydrolyze eIF4G specifically and inhibits cap-dependent protein synthesis, but not IRES-dependent protein synthesis [27]. As shown in Fig. 1C, Fluc activity was enhanced by cotransfection of a plasmid encoding 2A protease in RL-EMCV(+ORF)-luc, but it was decreased in RL-ODC(+ORF)-luc. The expression of Rluc was inhibited by 2A protease in both RL-EMCV(+ORF)-luc and RL-ODC(+ORF)-luc. The results suggest that enhancement of Fluc activity in RL-ODC(+ORF)-luc is not due to a typical IRES element.

We next determined whether a new promoter was created in between the *Rluc* and *Fluc* ORFs in the RL-ODC(+ORF)-luc construct. When HeLa cells were transfected with a pRL-ODC(+ORF)-luc construct that lacked the SV40 promoter (Fig. 2A), both Rluc and Fluc activities decreased greatly (Fig. 2B). The levels of mRNA generated from these constructs were evaluated using *Rluc* or *Fluc* ORF as probes. Although a broad band for Rluc-ODC(+ORF)-luc mRNA was observed in HeLa cells transfected with the pRL-ODC(+ORF)-luc construct, the band was not observed in cells transfected with the promoter-less construct (Fig. 2C). Expression of GAPDH mRNA from host gene was equal in both cells transfected

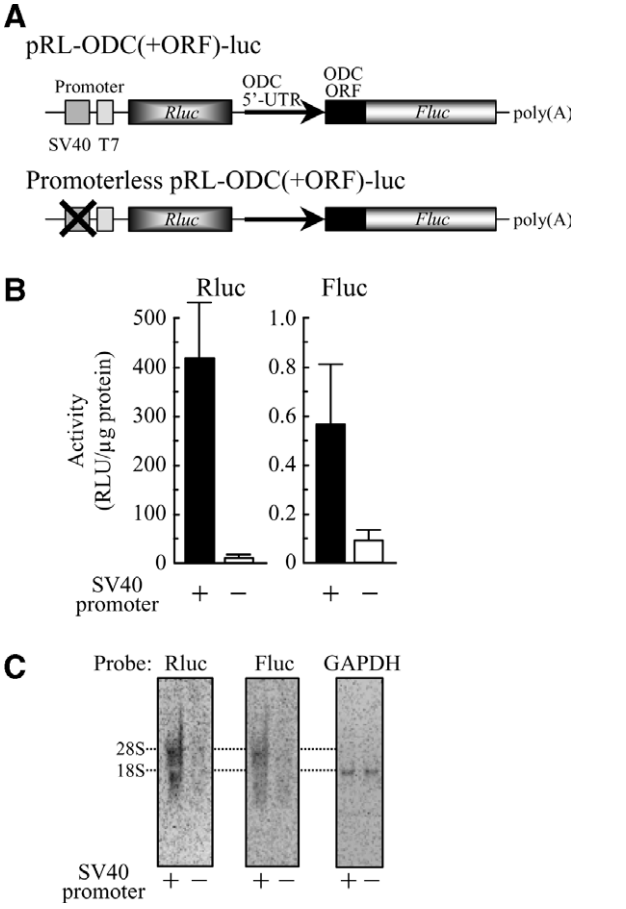

Fig. 2. Translational efficiency of Luc mRNAs synthesized from promoter-less pRL-luc constructs in HeLa cells. (A) Structure of pRL-ODC(+ORF)-luc and its promoter-less construct. (B) Comparison of Rluc and Fluc activities derived from pRL-ODC(+ORF)-luc and promoter-less pRL-ODC(+ORF)-luc. (C) Northern blot analysis of bicistronic Rluc–Fluc mRNA was performed using probes for Rluc and Fluc genes. As a control, Northern blotting for GAPDH mRNA was performed.

with pRL-ODC(+ORF)-luc and its promoter-less construct (Fig. 2C). The results suggest that synthesis of Fluc from the second cistron is dependent on the bicistronic mRNA, but the synthesis is dependent on intact eIF4G.

Cap-dependent ODC synthesis in FM3A and rabbit reticulocyte cell-free systems

In FM3A and HeLa cell culture systems, expression of the second ORF in a bicistronic construct was estimated by the activity, but not by the amount of protein synthesized (Fig. 1). Thus, the amount of protein synthesized from the second cistron in the bicistronic mRNA was determined in an FM3A cell-free system. As shown in Fig. 3A, Rluc was synthesized, but Fluc was not synthesized from the RL-ODC(+ORF)-luc mRNA. Synthesis of Rluc was inhibited by 2A protease pretreatment of the cell lysate. In contrast, both Rluc and Fluc were synthesized from the RL-EMCV(+ORF)-luc mRNA. Synthesis of Rluc was inhibited by 2A protease treatment, but that of Fluc from the second cistron was enhanced by 2A protease treatment, confirming that the 5′-UTR of EMCV mRNA, but not the 5′-UTR of ODC mRNA, has a typical IRES element. The C-terminal fragment of eIF4G produced by 2A protease is also shown in Fig. 3A.

The effect of 2A protease and cap analogue (m^7GpppG) on protein synthesis was examined using monocistronic mRNAs with cap structures in a rabbit reticulocyte cell-free system. As shown in Fig. 3B-1, the synthesis of *S*-adenosylmethionine decarboxylase was inhibited by 2A protease treatment and cap analogue. In the case of EMCV IRES-dependent protein synthesis (Fig. 3B-2), neither cap analogue nor 2A protease inhibited the synthesis. Treatment with 2A protease actually enhanced EMCV IRES-dependent protein synthesis. Synthesis of ODC was cap-dependent like synthesis of *S*-adenosylmethionine decarboxylase, because 2A protease and cap analogue markedly inhibited synthesis (Fig. 3B-3).

Cap-dependent ODC synthesis at both G_1/S boundary and G_2/M phases

Rapamycin causes a shutoff of cap-dependent protein synthesis, but does not inhibit EMCV RNA-directed

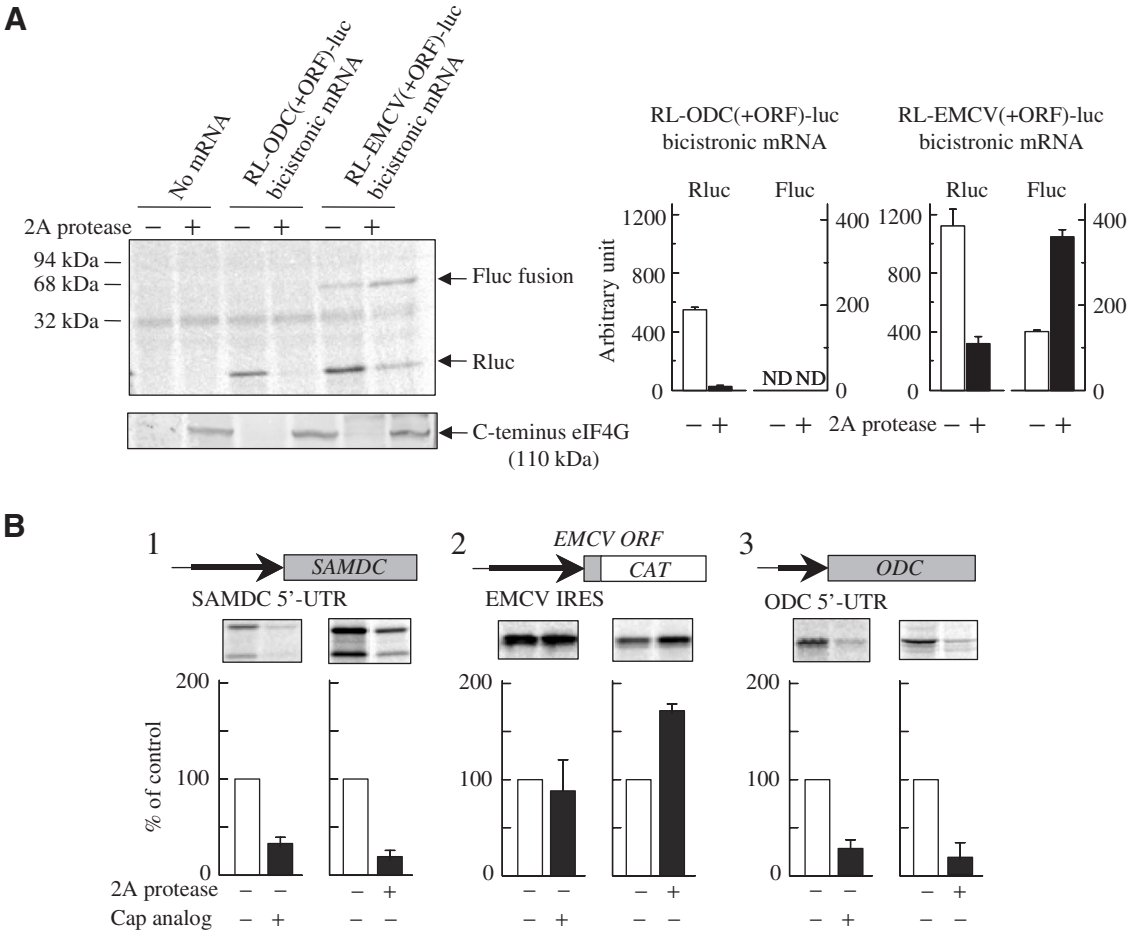

Fig. 3. Synthesis of Rluc and Fluc from bicistronic mRNAs in a FM3A cell-free system (A) and effect of 2A protease and cap analogue on protein synthesis in a rabbit reticulocyte cell-free system (B). (A) Fluorography of [^{35}S]methionine-labeled Rluc and Fluc and Western blot of the fragmented C-terminus eIF4G (110 kDa) were shown. [^{35}S]Methionine-labeled Rluc and Fluc were quantified with a Fujix BAS 2000II imaging analyzer. ND, not detectable. (B) [^{35}S]Methionine-labeled protein was shown as fluorography and quantified with a Fujix BAS 2000II imaging analyzer.

(IRES-dependent) protein synthesis [28]. It has been reported that ODC synthesis at the G_2/M phase was IRES-dependent although its synthesis at the G_1 phase was cap-dependent. This was based on the observation that rapamycin did not inhibit ODC activity and that phosphorylation of 4E-BPs changed greatly at the G_2/M phase [7]. We re-estimated the effect of rapamycin on ODC activity at both G_1/S boundary and G_2/M phase. HeLa cells were synchronized at the G_1/S boundary by treatment with thymidine followed by aphidicolin (Fig. 4A), or at M phase by the treatment with thymidine followed by nocodazole (Fig. 4D). At 8 h after synchronization, cells reached the G_2/M phase (Fig. 4A), or the G_1/S boundary (Fig. 4D). An increase in ODC activity and putrescine content was observed at both the G_1/S boundary (Fig. 4E and F) and the G_2/M phase (Fig. 4B and C). However, ODC activity in the G_2/M phase rapidly declined. When rapamycin was added to the medium, progress of the cell cycle was not significantly affected (Fig. 4G). Rapamycin inhibited ODC activity at both the G_1/S boundary and the G_2/M phase by approximately 40% (Fig. 4H). Inhibition by rapamycin of phosphorylation of 4E-BP1 at both the G_1/S boundary and the G_2/M phase was also observed (Fig. 4I). These results support the idea that most of ODC is synthesized in a cap-dependent mechanism at both the G_1/S boundary and the G_2/M phase. It has been also reported that the ODC IRES element is regulated by phosphorylation of eIF4E [29]. However, the degree of phosphorylation of eIF4E was nearly equal at G_1/S boundary and G_2/M phase (Fig. 4I).

Discussion

The level of ODC, which is a rate-limiting enzyme for polyamine biosynthesis, is regulated by transcription, translation, and degradation [2,3]. With regard to translation of ODC mRNA, it has been reported that ODC synthesis at the G_2/M phase is IRES-dependent, although

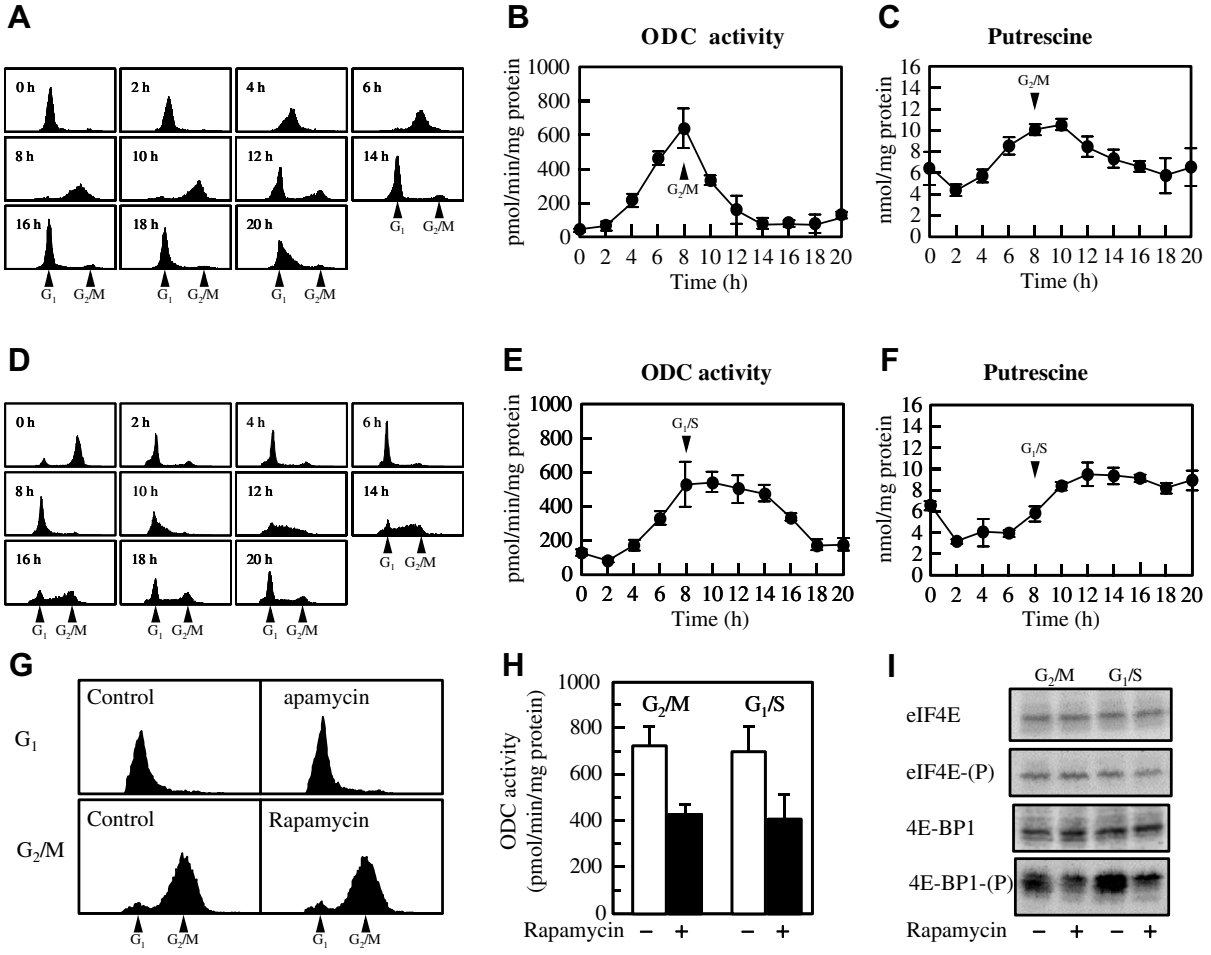

Fig. 4. Increase in ODC and putrescine at G_1/S boundary and G_2/M phase, and effects of rapamycin on ODC activity and levels of eIF4E, 4E-BP1, and their phosphrylated forms. HeLa cells were synchronized at the G_1/S boundary (A) or G_2/M phase (D) as described in Materials and methods. After synchronization, cells were cultured in a fresh medium at the designated time and collected with trypsin treatment. Then, cytometric analysis (A and D), ODC assay (B and E) and measurement of putrescine (C and F) were performed. (G) After the synchronized cells at the G_2/M phase and G_1/S boundary were cultured in the presence and absence of 100 ng/ml rapamycin for 8 h, cytometric analysis (G) and measurement of ODC activity (H) were performed. (I) Level of eIF4E, 4E-BP1, and their phosphorylated forms at G_1/S boundary and G_2/M phase and effect of rapamycin on their levels. Western blotting was performed using 30 μg protein of the HeLa cell lysate.

synthesis at the G_1 phase is cap-dependent [7]. In this study, we re-examined the issue of whether ODC synthesis is IRES-dependent. Some of the results and conclusions of this study differ from those reported elsewhere [7,8]. Our results indicate the absence of a typical IRES element in the 5′-UTR of mouse ODC mRNA for the following reasons: First, although synthesis from the second cistron of a bicistronic mRNA was enhanced by the 5′-UTR of ODC mRNA, the enhancement was abolished by treatment with 2A protease in a cell culture system. Second, synthesis from the second cistron of the bicistronic mRNA was not enhanced by the 5′-UTR of ODC mRNA in a cell-free system, and synthesis from monocistronic mRNA having the 5′-UTR of ODC mRNA was inhibited by treatment with 2A protease and cap analogue. Third, rapamycin inhibited ODC synthesis to the same degree at both the G_1/S boundary and G_2/M phase.

With regard to the different results obtained in this report and a previous study [7], it is noted that the 5′-UTR of ODC mRNA used in the bicistronic construct of the present work and our previous work [8] is different from the construct used by Pyronett's group [7] – they used the 5′-UTR of rat ODC mRNA whereas our construct is derived from mouse ODC. Since the nucleotide sequence of the 5′-UTR of ODC mRNA in rat and mouse is slightly different at the area of boxA, which is important as an IRES element, the 5′-UTR of rat ODC mRNA may have a typical IRES element.

It has been thus far reported that IRESes are present in more than 30 viral mRNAs and 50 cellular mRNAs including ODC mRNA [26]. There is also a report that the synthesis of several proteins at the G_2/M phase is IRES-dependent as evaluated by polysomal distribution of selective mRNAs using HeLa cells [30]. However, judging from the distribution of ODC mRNA on polysomes, it is suggested that ODC synthesis at G_2/M phase is not IRES-dependent [30]. Thus, careful analysis such as the effect of the treatment with 2A protease in a cell culture system, the mono- and bicistronic mRNA-dependent protein synthesis in a cell-free system, and/or comparison of polysomal distribution of selective mRNAs in addition to evaluation using a bicistronic construct is necessary to conclude that an IRES element exists in a specific mRNA.

Acknowledgments

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