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ORIGINAL ARTICLE

Polyamine stimulation of eEF1A synthesis based on the unusual position of a complementary sequence to 18S rRNA in eEF1A mRNA

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Abstract It is thought that Shine–Dalgarno-like sequences, which exhibit complementarity to the nucleotide sequences at the 3′-end of 18S rRNA, are not present in eukaryotic mRNAs. However, complementary sequences consisting of more than 5 nucleotides to the 3′-end of 18S rRNA, i.e., a CR sequence, are present at −17 to −32 upstream from the initiation codon AUG in 18 mRNAs involved in protein synthesis except eEF1A mRNA. Thus, effects of the CR sequence in mRNAs and polyamines on protein synthesis were examined using control and polyamine-reduced FM3A and NIH3T3 cells. Polyamines did not stimulate protein synthesis encoded by 18 mRNAs possessing a normal CR sequence. When the CR sequence was deleted, protein synthetic activities decreased to less than 70 % of intact mRNAs. In eEF1A mRNA, the CR sequence was located at −33 to −39 upstream from the initiation codon AUG, and polyamines stimulated eEF1A synthesis about threefold. When the CR sequence was shifted to −22 to −28 upstream from the AUG, eEF1A synthesis increased in polyamine-reduced cells and the degree of polyamine stimulation decreased greatly. The results indicate that the CR sequence exists in many eukaryotic mRNAs, and the location of a CR sequence in mRNAs influences polyamine stimulation of protein synthesis.

Keywords Polyamines · Protein synthesis · mRNA · 5′-UTR · CR sequence

Abbreviations

APCHA	N^1-(3-aminopropyl)-cyclohexylamine
CD	Circular dichroism
CR sequence	Complementary sequence to 18S rRNA
DFMO	α-Difluoromethylornithine
GC_7	N^1-guanyl-1,7-diaminoheptane
PBS	Phosphate-buffered saline
5′-UTR	5′-Untranslated region of mRNA

Introduction

Polyamines (putrescine, spermidine and spermine) are present at millimolar concentrations in prokaryotic and eukaryotic cells and play regulatory roles in cell growth (Igarashi and Kashiwagi 2010; Pegg 2009). Since polyamines exist mainly as polyamine–RNA complexes (Miyamoto et al. 1993; Watanabe et al. 1991), polyamines enhance several kinds of protein synthesis, which are important for cell growth and viability (Igarashi and Kashiwagi 2006, 2010), stimulate general protein synthesis by inducing the in vivo assembly of 30S ribosomal subunits (Echandi and Algranati 1975; Igarashi et al. 1979b), and increase the fidelity of translation (Igarashi et al. 1979a; Jelenc and Kurland 1979). It was also shown recently that overexpression of spermidine/spermine N^1-acetyltransferase, a

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key catabolic enzyme of polyamines, causes a total arrest of translation and cell growth in mammalian cells because of the depletion of spermidine and spermine (Mandal et al. 2013).

We have extensively studied proteins in *Escherichia coli* whose synthesis is enhanced by polyamines using a polyamine-requiring mutant MA261 (Cunningham-Rundles and Maas 1975), and proposed that a set of genes whose expression is enhanced by polyamines at the level of translation can be classified as a "polyamine modulon" (Igarashi and Kashiwagi 2006). We have thus far identified 17 different genes as components of the polyamine modulon in *E. coli* (Igarashi and Kashiwagi 2010; Sakamoto et al. 2012). There are three different mechanisms underlying polyamine stimulation of the translation of various members of the polyamine modulon. First, polyamine stimulation of protein synthesis can occur when a Shine–Dalgarno (SD) sequence (Shine and Dalgarno 1974) in the mRNA is distant from the initiation codon AUG of the mRNA, facilitating formation of the initiation complex. This is the case for *oppA*, *fecI*, *fis*, *rpoN*, *hns*, *rpoE*, *stpA*, *rmf*, *rpoZ* and *cpxR*. Second, polyamines enhance the inefficient initiation codon UUG- or GUG-dependent fMet-tRNA binding to *cya*, *spoT* and *uvrY* mRNA- or *cra* and *frr* mRNA–ribosomes. Third, polyamines stimulate readthrough of the amber codon UAG on *rpoS* mRNA by Gln-tRNA^{supE}, or stimulate a +1 frameshift at the 26th UGA codon of *prfB* mRNA encoding RF2.

In polyamine-reduced eukaryotic cells, protein synthesis is more rapidly recovered by polyamines than DNA synthesis (Igarashi and Morris 1984), and protein synthesis is enhanced more rapidly than DNA synthesis in concanavalin A-activated lymphocytes in parallel with the increase of polyamine content (Kakinuma et al. 1988). It was also shown that eIF5A, containing hypusine derived from spermidine, is involved in cell proliferation (Jakus et al. 1993). We have shown that polyamines and eIF5A are independently involved in cell proliferation (Nishimura et al. 2005). Thus, it is thought that the effects of polyamines on eukaryotic cell growth are mediated through stimulation of the synthesis of specific proteins as well as activation of eIF5A. Using α-difluoromethylornithine (DFMO, an inhibitor of ornithine decarboxylase)-treated (polyamine-reduced) and untreated mouse mammary carcinoma FM3A cells, we identified three proteins (Cct2, Hnrpl and Pgam1) whose synthesis is enhanced by polyamines at the level of translation (Nishimura et al. 2009). In addition, it was found that polyamines enhance ribosome shunting, which involves discontinuous scanning by 40S ribosomal subunits (Chappell et al. 2006) in the synthesis of Cct2. In yeast, it was also shown that COX4 synthesis is stimulated by polyamines at the level of translation using an ornithine decarboxylase-deficient mutant through polyamine enhancement of ribosome shunting (Uemura et al. 2009).

In this study, we looked for other proteins whose synthesis is enhanced by polyamines at the level of translation using FM3A and NIH3T3 cells, and found that synthesis of eEF1A is enhanced by polyamines at the level of translation. Furthermore, the mechanism of polyamine stimulation of eEF1A synthesis was investigated. It was found that a complementary sequence consisting of more than 5 nucleotides to the 3′-end of 18S rRNA, i.e., a complementary sequence to 18S rRNA (CR sequence), exists at −17 to −32 upstream from the initiation codon AUG in many eukaryotic mRNAs, and the location of a CR sequence in eEF1A mRNA influences polyamine stimulation of eEF1A synthesis.

Materials and methods

Cell culture of mouse mammary carcinoma FM3A cells and preparation of cell lysate

FM3A cells (Japan Health Science Foundation) were cultured in Dulbecco's modified Eagle's medium (D-MEM) (Wako) supplemented with 2 % heat-inactivated fetal bovine serum (FBS) at 37 °C in an atmosphere of 5 % CO_2 in air. To make polyamine-reduced FM3A cells, 50 μM DFMO was added to the medium. In some cases, FM3A cells were treated with an inhibitor of deoxyhypusine synthase, GC_7 (N^1-guanyl-1,7-diaminoheptane) (Jakus et al. 1993), or with DFMO plus an inhibitor of spermine synthase, APCHA [N^1-(3-aminopropyl)-cyclohexylamine] (Nishimura et al. 2005). Synthesis of active eIF5A was inhibited in GC_7-treated cells without the significant change of polyamines in cells, and the level of hypusinated eIF5A was maintained at normal levels in DFMO plus APCHA-treated cells because the decrease in spermidine content was small (Nishimura et al. 2005). FM3A cells (2×10^6 cells) were treated with 200 μl of 5 % (w/v) trichloroacetic acid (TCA), and centrifuged at 12,000×g for 10 min. The precipitate was dissolved with 100 μl of a buffer containing 25 mM Tris–HCl, pH 6.8, 1 % 2-mercaptoethanol, 5 % glycerol and 1 % SDS, and pH of the homogenate was adjusted by 1 M Tris-HCl, pH 8.0. After standing at room temperature overnight, supernatant was obtained by centrifugation at 17,000×g for 15 min and used as cell lysate. Protein content was determined by the method of Bradford (1976).

Western blot analysis

Western blot analysis was performed by the method of Nielsen et al. (1982), using horseradish peroxidase conjugated anti-rabbit IgG (GE Healthcare Bio-Sciences) as secondary antibody and ECL Western blotting reagents (GE Healthcare Bio-Sciences). Antibodies against eIF1, eIF4B,

eIF4E, eIF5, eEF1A and β-actin were purchased from Santa Cruz Biotechnology Inc., USA. Antibodies against eIF1A and eRF1 were from Abcam, UK. Antibodies against eIF2α, eIF3A, eIF3C, eIF4A, eIF4G and eIF6 were from Cell Signaling Technology, Japan. Antibodies against eIF3B and eEF2 were from EPITOMICS, USA. Antibody against eIF5B was from Sigma-Aldrich. Antibodies against eEF1B and GSPT1 (eRF3) were from Protein Tech, USA. Antibody against EGFP was from Clontech, USA. Antibody against eIF5A was prepared as described previously (Nishimura et al. 2005). Antibody against hypusinated eIF5A was kindly supplied by R. G. Mirmira, Indiana University School of Medicine (Nishiki et al. 2013). The level of protein on the blot was quantified with an LAS-3000 luminescent image analyzer (Fuji Film).

Northern blot analysis

Total RNA was isolated from 2.5 × 10^7 cells using TRIzol reagent (Invitrogen). Northern blot analysis was performed according to standard methods (Sambrook et al. 2001) using the ECL direct nucleic acid labeling and detection systems (GE Healthcare Bio-Sciences) with 10 μg of total RNA. Chemical luminescence was detected by an LAS-3000 luminescent image analyzer (Fuji Film). The cDNA used for template DNA was prepared using SuperScript™ II RNase H^- Reverse Transcriptase (Life Technologies) according to the accompanying manual. Genes for eEF1A and EGFP were amplified by PCR using primer sets, 5′-TAATCGGACACGTAGATTCCGGCA-3′ (eEF1A F), 5′-TTCACA-CCCAGGGTGTAAGCCAGA-3′ (eEF1A R), 5′-CGGGGTAC-CATGGTGAG-CAAGGGCG AGG-3′(EGFP F), 5′-CCGGAATTCTTACTTGTACAGCT CGTCCATG-3′(EGFP R), and the cDNA as templates. PCR products thus obtained were used as probes for Northern blot analysis.

Plasmids

To make peEF1A-EGFP fusion plasmids, PCR was performed using P1 and P2 as primers, and eEF1A cDNA as template. All primers used for construction of various eEF1A-EGFP fusion plasmids are listed in Table S1. The amplified *eEF1A* gene (a 120-nucleotide 5′-upstream region and a 189-nucleotide open reading frame) was digested with EcoRI and BamHI, and inserted into the same restriction site of plasmid pEGFP-N1 (Clontech). To make peEF1A(ΔHairpin 1)-EGFP encoding the fusion protein consisting of the NH_2 terminal 63 amino acids of eEF1A and full length of EGFP, but lacking 77–94 nucleotides in the 5′-untranslated region (5′-UTR) of eEF1A gene, overlap extension PCR was performed using the following four primers, P1 and P4, P2 and P3 for first PCR and peEF1A-EGFP as a template. The second PCR was performed using the first PCR products as templates and P1 and P2 as primers. PCR products thus obtained were digested with EcoRI and BamHI, and inserted into the same restriction site of plasmid pEGFP-N1. To make peEF1A(ΔHairpin 2)-EGFP lacking 1-60 nucleotides in the 5′-UTR of eEF1A mRNA, PCR was performed using P5 and P2 primers, and peEF1A-EGFP as a template. Other plasmids {peEF1A[−29(G→C)]-EGFP, peEF1A[−42(G→C)]-EGFP, peEF1A[−29, −42(G→C)]-EGFP, peEF1A(NC-18S rRNA)-EGFP, peEF1A(CR1)-EGFP, peEF1A(CR2)-EGFP, and peEF1A(CR3)-EGFP} were prepared by site-directed mutagenesis employing overlap extension using PCR (Ho et al. 1989). The plasmids peIF2α-EGFP, peIF4B-EGFP, peRF1-EGFP and their mutants were constructed similarly as described above. Primers used for construction of peIF2α-EGFP, peIF4B-EGFP, peRF1-EGFP and their mutants are listed in Table S1. The nucleotide sequence of the plasmids was confirmed by the 3130 Genetic Analyzer (Applied Biosystems).

Transient transfection of eEF1A-EGFP, eIF2α-EGFP, eIF4B-EGFP and eRF1-EGFP fusion plasmids into NIH3T3 cells and measurement of their mRNA and protein levels

NIH3T3 cells (3 × 10^5/10 ml) were cultured in D-MEM supplemented with 50 U/ml streptomycin, 100 U/ml penicillin G, and 10 % FBS at 37 °C in an atmosphere of 5 % CO_2 in air for 36 h. Then, cells were cultured in the presence and absence of 500 μM DFMO for 12 h. After changing the medium with a fresh one without FBS, cells were transfected with 4 μg of various fusion plasmids by Lipofectamine™ Reagents (Invitrogen) according to the manufacturer's instructions and cultured for 3 h. After changing the medium with a fresh one containing FBS, cells were cultured in the presence and absence of 500 μM DFMO for further 24 h. NIH3T3 cells attached to the culture dish were washed twice with 5 ml of phosphate-buffered saline (PBS), incubated with 0.4 ml of 0.25 % trypsin–0.02 % EDTA–4Na solution at 37 °C for 3 min, and 5 ml of D-MEM containing 10 % FBS was added to the culture dish. Dispersed cells were collected by centrifugation at 300×*g* for 5 min, washed twice with PBS, and used for Northern or Western blotting as described above.

Circular dichroism (CD) measurement of RNA

The 26 nucleotides in hairpin 1 (−23 to −48 of 5′-UTR) of eEF1A WT RNA (5′-CCGUCAGAACGCAGGUGUUG UGAA-AA-3′) and eEF1A-29(G→C) RNA (5′-CCGUC AGAACGCAGGUGUUCUGAAAA-3′) were obtained from Hokkaido System Science, Japan. Optimal computer

Table 1 Polyamine content in FM3A and NIH3T3 cells

Cell	FM3A								NIH3T3	
Addition										
DFMO	50 μM	50 μM	–	–	50 μM	50 μM	–	500 μM	–	
APCHA	–	150 μM	–	–	–	150 μM	–	–	–	
GC_7	–	–	–	2 μM[†]	–	–	–	–	–	
Spermidine	–	–	–	–	25 μM	25 μM	25 μM	–	–	
	Polyamine content (nmol/mg protein)								Polyamine content (nmol/mg protein)	
Putrescine	ND	ND	3.20 ± 0.60*	4.05 ± 0.50*	ND	ND	ND	ND	ND	1.65 ± 0.15*
Spermidine	0.33 ± 0.13	11.5 ± 0.75*	17.4 ± 1.40**	18.5 ± 1.50**	29.8 ± 2.30**	33.5 ± 1.53**	27.8 ± 2.12**	0.45 ± 0.05	11.3 ± 1.30*	
Spermine	7.55 ± 0.85	2.83 ± 0.50*	14.5 ± 0.50*	12.1 ± 0.95*	9.73 ± 0.80*	2.32 ± 0.45*	10.8 ± 0.93*	5.21 ± 0.51	9.70 ± 0.50*	

Values are mean ± SE of triplicate determinations

Student's *t* test was performed versus the value of DFMO-treated cells in each cell line

ND not detectable

* $p < 0.05$, ** $p < 0.01$

[†] Where indicated, 2 μM GC_7 was added to the medium every 24 h

folding of RNAs was performed by the method of Zuker (2003). Free energy (ΔG) for the formation of the secondary structure was calculated on the basis of the data of Turner et al. (1988). CD spectra were recorded over 200–300 nm on a Jasco J-820 spectropolarimeter (Jasco International Co.) using a 0.1 cm path-length cuvette at 37 °C (Nakano et al. 2004). Scan speed was 100 nm/min, and CD samples contained 10 mM Tris-HCl (pH 7.5), 50 mM KCl, and 50 μM RNA. Where indicated, Mg^{2+} and/or spermine were added to the CD samples. Typical spectra at 37 °C corresponded to the average of three scans.

Results

Stimulation of synthesis of eEF1A by polyamines at the level of translation

Proteins whose synthesis is enhanced at the level of translation were searched for among proteins involved in protein synthesis by comparing the level of proteins in mouse FM3A cells cultured with or without DFMO for 72 h. Under these conditions, putrescine and spermidine content became negligible and spermine content decreased to approximately 50 % of the control cells (Table 1). Cell number decreased to approximately 25 % of control cells on day 3.

As shown in Fig. 1, the level of translation factors (eIF1, eIF1A, eIF2α, eIF3A, eIF3B, eIF3C, eIF4A, eIF4B, eIF4E, eIF4G, eIF5, eIF5A, eIF5B, eIF6, eEF1B, eEF2, eRF1 and eRF3) was nearly equal in both control and DFMO-treated cells. However, the level of eEF1A in control cells was 3.1-fold higher than that in DFMO-treated cells (Fig. 2a), but the level of eEF1A mRNA measured by Northern blotting was nearly equal in control and DFMO-treated cells (Fig. 2b). In DFMO-treated cells, the level of hypusinated eIF5A decreased significantly (Fig. 2a), as we reported previously (Nishimura et al. 2005). To confirm that the decrease in eEF1A in DFMO-treated cells is caused by the decrease in polyamines, effect of DFMO plus APCHA, an inhibitor of spermine synthase, or GC_7, an inhibitor of active eIF5A formation, was examined. The level of eEF1A was more clearly decreased in cells treated with DFMO plus APCHA compared to control cells designated as none. Under these conditions, the level of hypusinated eIF5A was similar to that of control cells because of the existence of a significant amount of spermidine in DFMO plus APCHA-treated cells (Fig. 2; Table 1). The level of eEF1A in cells treated with GC_7 was nearly equal to that in control cells, although the level of hypusinated eIF5A decreased greatly (Fig. 2). Under these conditions, the level of polyamines was similar to control cells (Table 1). By the addition of 25 μM spermidine to DFMO- or DFMO

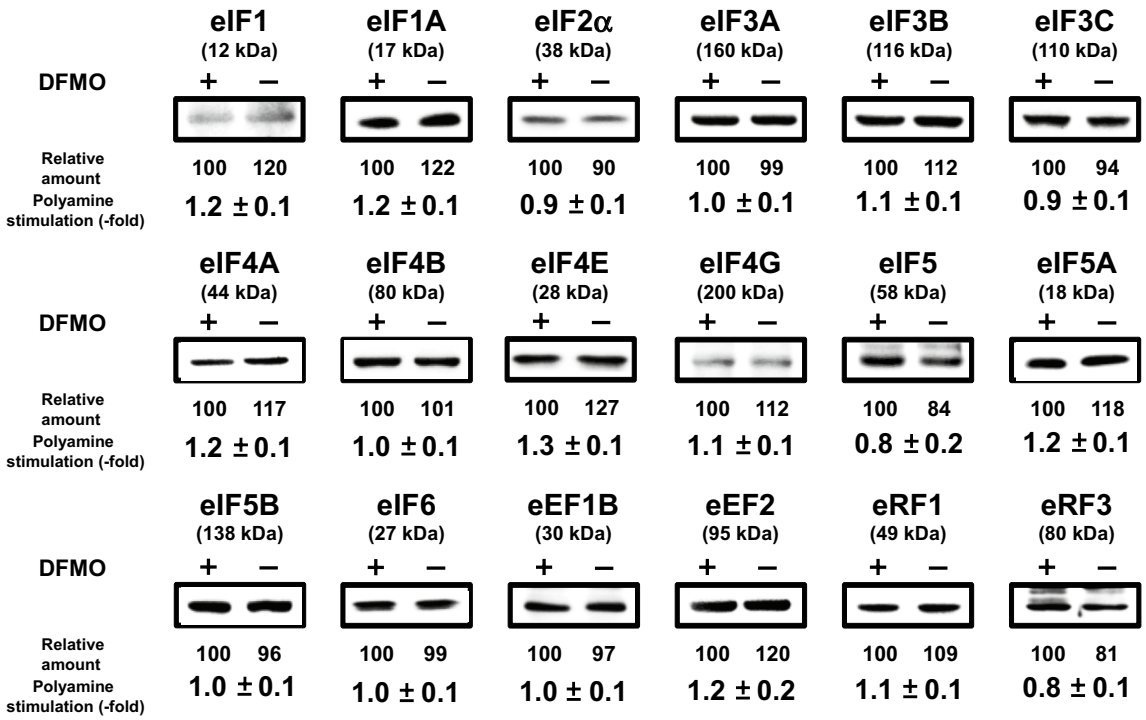

Fig. 1 Polyamine effect on the level of translation factors. The levels of 18 kinds of initiation, elongation and termination factors involved in translation were evaluated by Western blotting using 20 μg protein. Molecular mass of each protein is shown in parenthesis. Values are means ± SE of triplicate determinations

plus APCHA-treated cells, the level of eEF1A recovered to the normal level, but the levels of eIF5A and hypusinated eIF5A in these cells were similar to those of control cells (Fig. 2a). β-Actin was used as a control and the level of the protein was not altered in any of the conditions. The results indicate that synthesis of eEF1A is enhanced by polyamines at the level of translation, but not by active eIF5A, i.e., the gene encoding eEF1A is a member of polyamine modulon.

Mechanism of polyamine stimulation of eEF1A synthesis

In mouse FM3A cells and yeast cells, polyamine stimulation of synthesis of Cct2, T-complex protein 1, β-subunit, and COX4, one of the subunits of cytochrome C oxidase (complex IV), was due to the stimulation of ribosome shunting of the stem-loop structures (hairpin structures) during the scanning of the 5′-untranslated region (5′-UTR) of Cct2 and COX4 mRNAs by the 40S ribosomal subunit-Met-tRNA$_i$ complex (Nishimura et al. 2009; Uemura et al. 2009). Thus, it was first examined whether hairpin 1 and hairpin 2 existing on the 5′-UTR of eEF1A mRNA (Fig. 3b) are involved in polyamine stimulation of eEF1A synthesis, using NIH3T3 cells transfected with eEF1A-EGFP (enhanced green fluorescent protein) fusion plasmids (Fig. 3a). As shown in Fig. 3c, polyamine stimulation of eEF1A-EGFP synthesis from wild-type mRNA was observed at a similar extent to eEF1A synthesis in FM3A cells (see Fig. 2a), whereas polyamine stimulation of eEF1A-EGFP synthesis disappeared by removing hairpin 1 but not hairpin 2. Since the level of eEF1A-EGFP mRNA did not change by removing hairpin 1 or hairpin 2 in the presence and absence of DFMO (Fig. 3d), the results strongly suggest that structural change of hairpin 1 by polyamines is involved in stimulation of eEF1A-EGFP synthesis by polyamines. For ribosome shunting, take-off and landing sites, which are complementary to the nucleotide sequences in the 3′-end of 18S rRNA, are required in the 5′-UTR of mRNA (Yueh and Schneider 1996). However, these nucleotide sequences are not found in the 5′-UTR of eEF1A mRNA.

The properties of nucleotide sequence of hairpin 1 on eEF1A mRNA were then studied. In the case of polyamine stimulation of OppA (oligopeptide-binding protein) synthesis in *E. coli*, a structural change by polyamines of the bulged-out region close to the initiation codon AUG of *oppA* mRNA was strongly involved (Yoshida et al. 1999). So, it was tested whether a structural change of the bulged-out region of hairpin 1 is involved in polyamine stimulation of eEF1A synthesis. As shown in Fig. 4a, −29G and −42G cannot make a hydrogen bond in hairpin 1. So, each G was converted to C, and the polyamine effect on

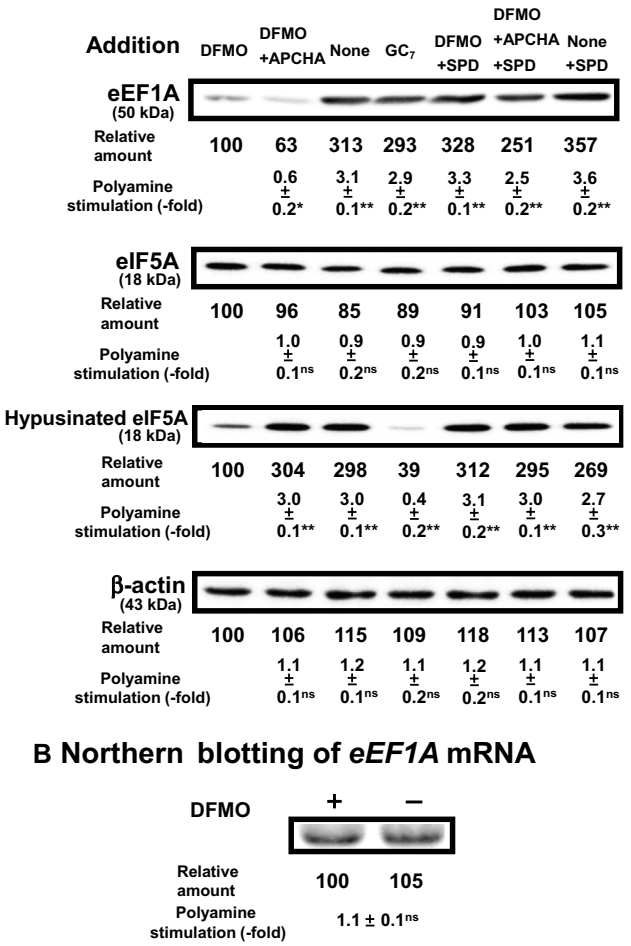

Fig. 2 Polyamine stimulation of the synthesis of eEF1A at the level of translation. The levels of eEF1A protein and its mRNA were measured by Western and Northern blotting using 20 μg protein and 10 μg total RNA, respectively. Where indicated, 50 μM DFMO, 150 μM APCHA, 2 μM GC_7 or 25 μM spermidine (SPD) was added to the culture medium. When SPD was added to the medium, 1 mM aminoguanidine was added to inhibit amine oxidase in FBS. As a control, the levels of eIF5A, hypusinated eIF5A and β-actin were examined. Values are means ± SE of triplicate determinations. Student's *t* test was performed for the value obtained in various cells treated as shown in the figure versus in DFMO-treated cells. *ns*, $p \geq 0.05$; *$p < 0.05$; **$p < 0.01$

eEF1A-EGFP synthesis was evaluated. In both −29(G→C) and −42(G→C) converted mRNAs, in which the bulged-out region was eliminated, polyamine stimulation of eEF1A-EGFP synthesis decreased greatly, but synthesis of eEF1A-EGFP in polyamine-reduced cells increased about 2.6-fold (Fig. 4b). When both −29G and −42G were converted to C in eEF1A-EGFP mRNA, in which the bulged-out structure was maintained, eEF1A-EGFP synthesis was stimulated by polyamines (Fig. 4b). The level of the converted eEF1A-EGFP mRNAs was nearly equal in the presence and absence of DFMO (Fig. 4c). These results indicate that structural change of the bulged-out region in hairpin 1 is involved in polyamine stimulation of eEF1A synthesis. The results also support an idea that a structural change by polyamines in the bulged-out region of double-stranded RNA causes stimulation of the synthesis of some proteins (Higashi et al. 2008).

The nucleotide sequence on the loop structure of hairpin 1 was complementary to the nucleotide sequence of 1,851–1,857 of 18S rRNA, which is located at the 3′-end of 18S rRNA (Fig. 4a). In *E. coli*, the nucleotide sequence at the 3′-end of 16S rRNA is complementary to the Shine–Dalgarno (SD) sequence of mRNAs, and this interaction is important for efficient translation (Chen et al. 1994). When the nucleotide sequence of the loop structure in hairpin 1 was modified from complementary to non-complementary sequence to the 18S rRNA, polyamine stimulation of eEF1A-EGFP synthesis disappeared and protein synthetic activity decreased (Fig. 4a, b), suggesting that the complementary sequence to the 3′-end of 18S rRNA (CR sequence) is involved in protein synthetic activity and the degree of polyamine stimulation.

In Fig. 5, the position of the CR sequence of the 19 mRNAs encoding translation factors, in which polyamine effect on their synthesis was tested (see Figs. 1, 2), was shown. Except for eEF1A mRNA, CR sequences in various mRNAs were located between −17 and −32 from the initiation codon AUG. The position of the CR sequence of eEF1A mRNA was located between −33 and −39 from the initiation codon AUG. Accordingly, similar to the SD sequence in *E. coli*, polyamine stimulation of protein synthesis may occur if the distance between the CR sequence and the initiation codon AUG is relatively distant. The position of the CR sequence of eEF1A mRNA was then shifted from −33 to −39 to normal position −22 to −28 or closer position −15 to −21 or −8 to −14 from the initiation codon AUG. As shown in Fig. 6, when the position of CR sequence was shifted to the normal position (CR1 in Fig. 6), the degree of polyamine stimulation of eEF1A-EGFP synthesis decreased from 3.1-fold to 1.1-fold, but synthetic activity in the presence of DFMO increased greatly. When the position of the CR sequence was shifted to the closer position (−15 to −21 and −8 to −14) from the initiation codon AUG, the degree of polyamine stimulation became 1.2-fold and 3.5-fold, respectively, and the protein synthetic activity in the presence of DFMO became 2.6-fold and 0.8-fold, respectively, compared with the wild-type eEF1A-EGFP mRNA (Fig. 6b). When the CR sequence was deleted, protein synthetic activity decreased to 60 %. These results indicate that the normal position of the CR sequence is located at −17 to −32 on the 5′-UTR from the initiation codon AUG of mRNA, and that the degree of polyamine stimulation is enhanced in the case where the position of CR sequence varies, either closer or further from the AUG, relative to the normal position of the CR sequence.

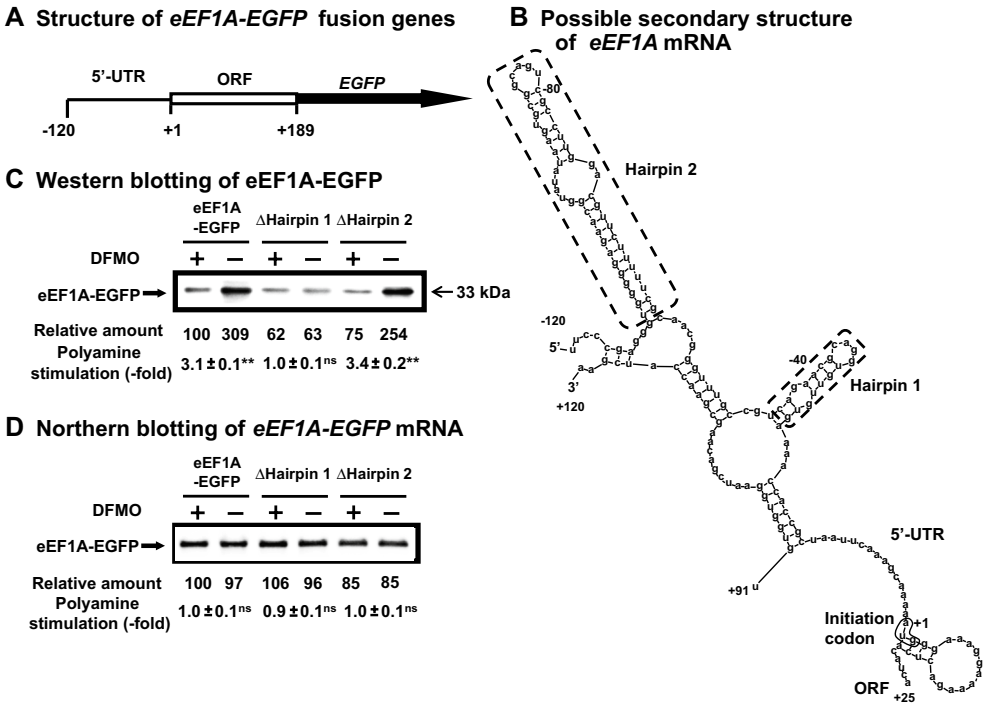

Fig. 3 Identification of the locus in the 5′-UTR of eEF1A mRNA necessary for polyamine stimulation of eEF1A synthesis. **a** and **b** Structure of eEF1A-EGFP fusion gene and the possible secondary structure of the 5′-UTR of eEF1A mRNA are shown. **c** and **d** The levels of protein and mRNA synthesized from control, ΔHairpin 1 or ΔHairpin 2 eEF1A-EGFP fusion genes were measured by Western and Northern blotting, respectively. Values are mean ± SE of triplicate determinations. *ns*, $p \geq 0.05$; $^{**}p < 0.01$

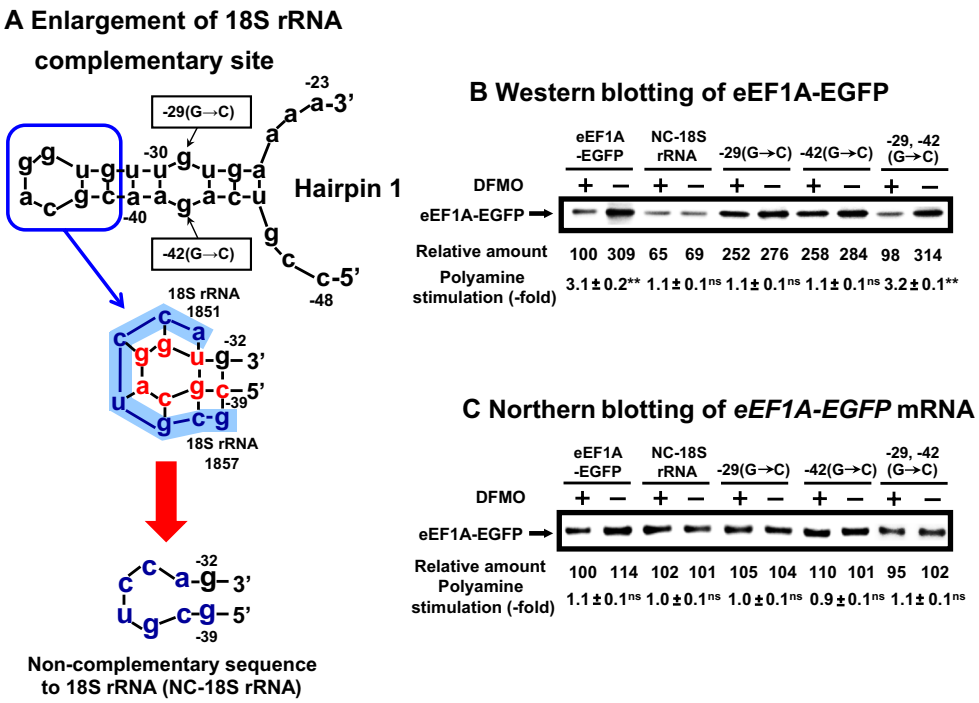

Fig. 4 Necessity of complementary sequence to the 3′-end of 18S rRNA in the 5′-UTR of eEF1A mRNA for polyamine stimulation of eEF1A synthesis. **a** The nucleotide sequence of hairpin 1 in eEF1A mRNA and its complementary sequence in 18S rRNA are shown. The nucleotide sequence of non-complementary sequence to 18S rRNA (NC-18S rRNA) in the modified hairpin 1 is also shown. **b** and **c** The levels of protein and mRNA synthesized from control and various mutant eEF1A-EGFP fusion genes are shown. Values are mean ± SE of triplicate determinations. *ns*, $p \geq 0.05$; $^{**}p < 0.01$

Next, the normal position of the CR sequence in eIF2α, eIF4B and eRF1 mRNAs was either deleted or shifted to a position distant from the initiation codon AUG. As shown in Fig. 7, protein synthetic activity was decreased to less than 70 % when the CR sequence was deleted, and the degree of polyamine stimulation became 1.8- to 2.5-fold when the CR

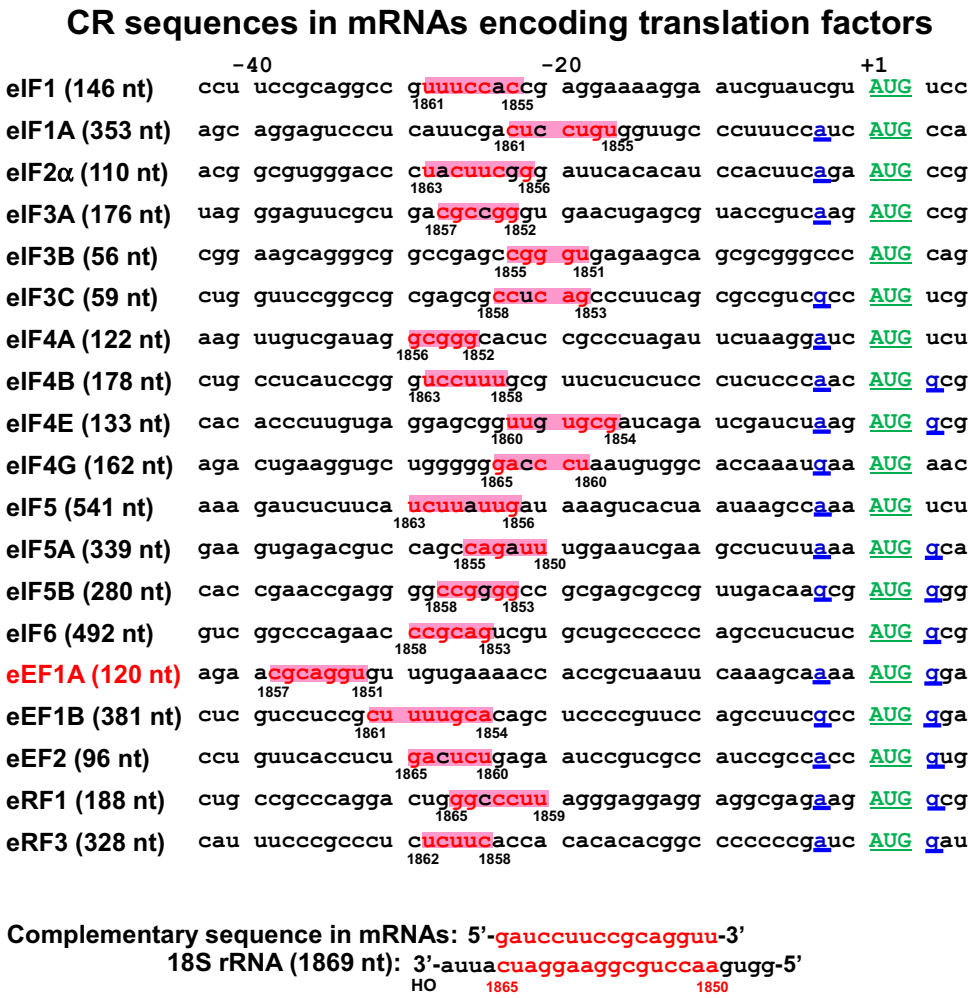

Fig. 5 Identification of complementary sequence to the 3′-end of 18S rRNA, i.e., CR sequence, in various mRNAs. The nucleotide sequences in the 5′-UTR of mRNAs were quoted from http://www.ncbi.nil.nih.gov/nucleotide/. The CR sequence was defined as more than five contiguous nucleotide sequences complementary to the sequences for 1,850–1,865 in 18S rRNA. Base pairing (G::U) was considered as complementary bases, and more than six contiguous nucleotide sequences including one non-complementary base (shown in *black*) were also defined as CR sequence. The complementary sequences to 18S rRNA in the CR sequence of various mRNAs are shown in red together with the numbers of the complementary nucleotide sequence of the 3′-end of 18S rRNA (Holmberg et al. 1994). Consensus Kozak sequences (Kozak 1991) are shown in blue. Number of nucleotides of the 5′-UTR of specified mRNA is shown in the *parentheses*

sequence was shifted to a position distant from the AUG. These results also support the idea that the CR sequence exists in eukaryotic mRNAs, which regulates protein synthetic activity and influences polyamine stimulation of protein synthesis.

Selective structural change of the bulged-out region in hairpin 1 existing on the 5′-UTR of eEF1A mRNA by spermine

Structural changes of the bulged-out region induced by spermine were studied using synthetic RNAs containing the wild type or the mutated [−29(G→C)] hairpin 1 on the 5′-UTR of eEF1A mRNA (Fig. 8a, d) in the presence of 10 mM Tris-HCl (pH 7.5) and 50 mM KCl at 37 °C. A substantial increase in the relative intensity of the negative band at 208 nm in CD reflects stabilization (or an increase) of the A-form double-stranded RNA (Nakano et al. 2004). There was a marked increase in the relative intensity of the negative band at 208 nm induced by 0.8 mM spermine in eEF1A WT RNA, and this was greater than the increase in eEF1A −29(G→C) RNA, which does not have the bulged-out region in RNA (Fig. 8b, c, e, f). In contrast, 1.6 mM Mg^{2+} had a smaller effect than 0.8 mM spermine on eEF1A WT RNA and eEF1A −29(G→C) RNA. A marked increase in the relative intensity of the negative band at 208 nm in eEF1A WT RNA by 0.8 mM spermine was also observed in the presence of 1 mM Mg^{2+}. The apparent dissociation constants (K_d) for spermine and Mg^{2+} are

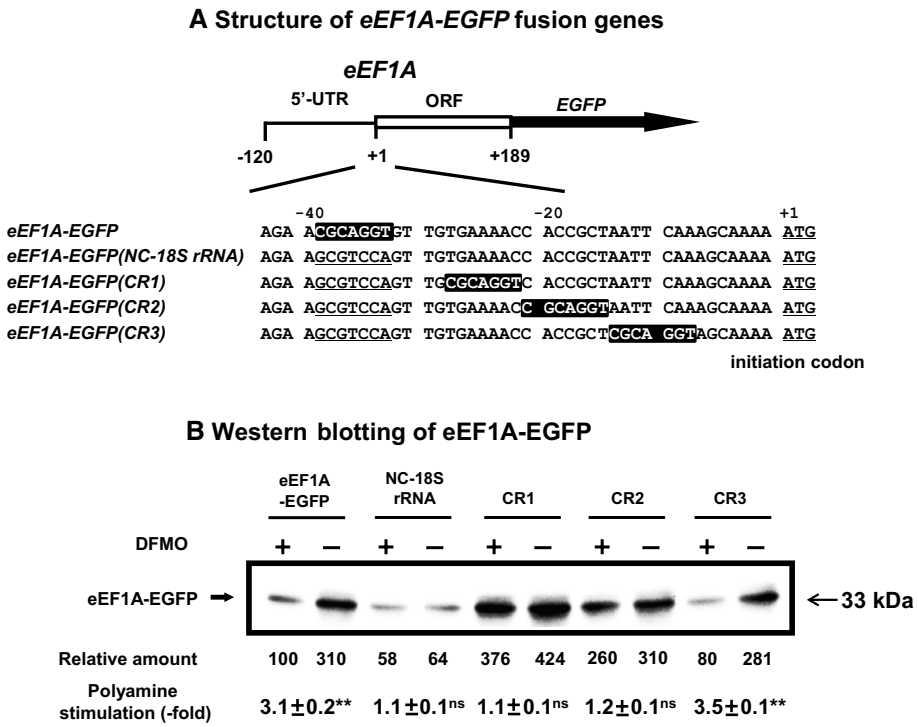

Fig. 6 Relationship between polyamine stimulation and the position of CR sequence in eEF1A mRNA. **a** Nucleotide sequences of various mutants at the region of CR sequence of eEF1A-EGFP gene are shown. The eEF1A mRNAs in which the position of CR sequence shifted were termed as CR1, CR2 and CR3, respectively. NC-18S rRNA, non-complementary sequence to 18S rRNA. **b** The levels of eEF1A-EGFP synthesized from various eEF1A-EGFP genes are shown. Values are mean ± SE of triplicate determinations. *ns*, $p \geq 0.05$; $^{**}p < 0.01$

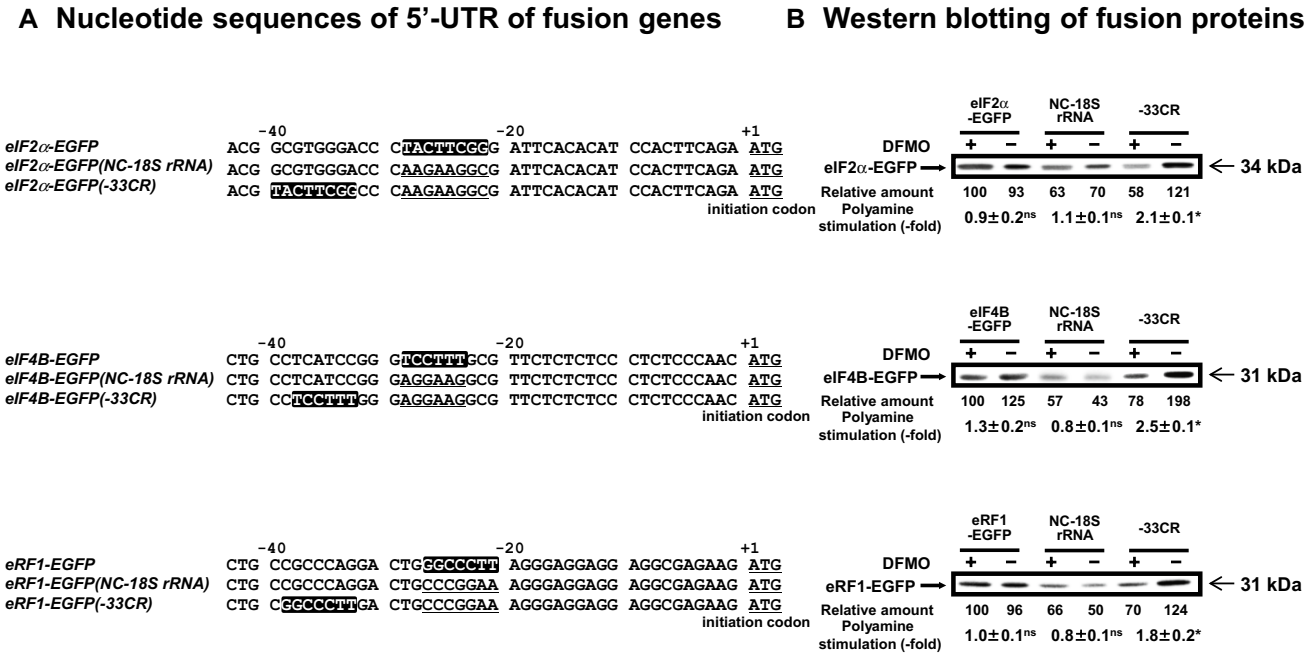

Fig. 7 Polyamine stimulation of eIF2α, eIF4B and eRF1 synthesis through the shift of a CR sequence to the unusual position in these mRNAs. **a** Nucleotide sequences of control and mutant fusion genes are shown. The CR sequence is shown with *white letters* in a *black box*. **b** The levels of proteins synthesized from various fusion genes (*eIF2α-EGFP*, *eIF4B-EGFP* and *eRF1-EGFP*) are shown. Values are mean ± SE of triplicate determinations. *ns*, $p \geq 0.05$; $^{*}p < 0.05$

shown in Fig. 8c, f. The K_d values for spermine to eEF1A WT RNA and eEF1A −29(G→C) RNA were decreased in the presence of 1 mM Mg^{2+}. Spermidine also had a similar effect as spermine, but the structural change of the bulged-out region in the double-stranded RNA by spermidine was less than that by spermine (data not shown).

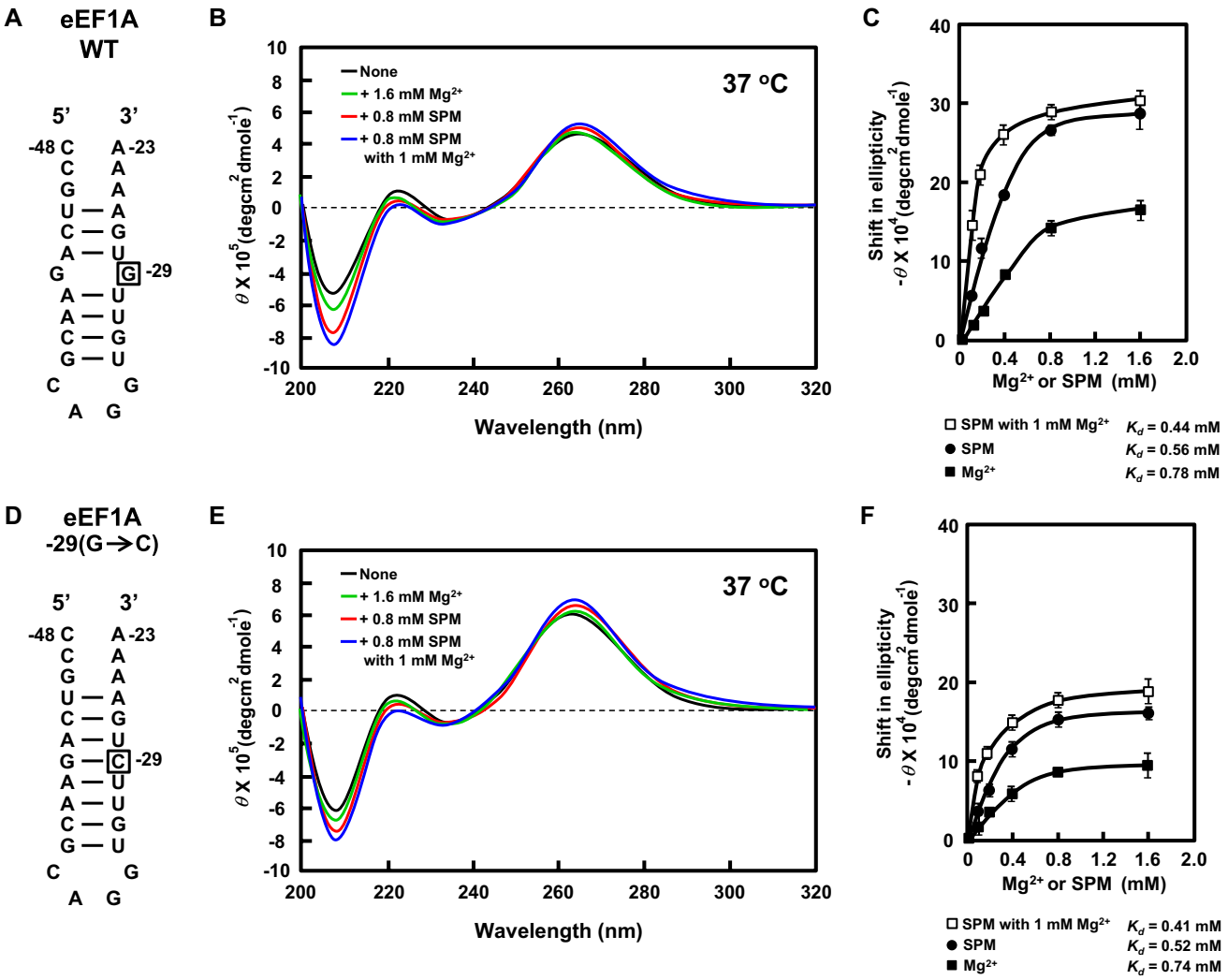

Fig. 8 CD spectra of eEF1A WT RNA and eEF1A −29(G→C) RNA. **a** and **d** Structures of eEF1A WT RNA and eEF1A −29(G→C) RNA are shown. The ΔG at 37 °C of eEF1A WT RNA and eEF1A −29(G→C) RNA was −5.8 and −9.9 kcal/mol, respectively. **b** and **e** CD spectra were recorded as described in "Materials and methods". **c** and **f** Concentration-dependent shifts induced by Mg^{2+} (*filled sqaure*), spermine (*filled circle*) and spermine with 1 mM Mg^{2+} (unfilled square) at 37 °C in magnitude at 208 nm are shown. Values are mean ± SE of triplicate determinations. The K_d values of spermine with 1 mM Mg^{2+}, spermine and Mg^{2+} for eEF1A WT RNA and eEF1A −29(G→C) RNA at 37 °C were determined according to the double reciprocal equation plot

Discussion

In this study, we have shown that the CR sequence, which exhibits complementarity to the nucleotide sequence at the 3′-end of 18S rRNA, exists in many mRNAs in eukaryotes and enhances protein synthesis. When the CR sequence was deleted, protein synthetic activity decreased to less than 70 % of normal mRNAs. It has been reported that base pairing between the 5′-UTR of mRNAs and 18S rRNA enhances protein synthesis in eukaryotes (Pisarev et al. 2008). However, the position of base pairing on the 18S rRNA is not with the 3′-end of 18S rRNA like base pairing between SD sequence of mRNA and the 3′-end of 16S rRNA in prokaryotes (Dresios et al. 2006; Meng et al. 2010). There is also a report that selection of the correct AUG initiation codon occurs through base pairing between the 5′-UTR of mRNAs and the 3′-end of 18S rRNA in internal ribosomal entry site (IRES) dependent but cap independent protein synthesis, in which more than 15 nucleotides are involved in base paring (Scheper et al. 1994). Interestingly, the complementary sequence in 18S rRNA to CR sequence is intensely overlapped with that of the IRES. Experiments are in progress to clarify how interaction between CR sequence in mRNAs and its complementary sequence in the 3′-end of 18S rRNA is involved in translation of mRNAs.

It is well known that polyamines are essential for normal cell growth (Igarashi and Kashiwagi 2010; Pegg 2009), and

polyamines mainly function at the level of translation (Igarashi and Kashiwagi 2006, 2010). In eukaryotes, we clarified that polyamines enhanced synthesis of Cct2 in FM3A cells and synthesis of COX4 in yeast through stimulation of the ribosome shunting of the hairpin structures during the scanning of the 5′-UTR of these mRNAs by the 40S ribosomal subunit-Met-tRNA$_i$ complex (Nishimura et al. 2009; Uemura et al. 2009), because polyamines are able to act at the regulated spaced intervals.

In this study, it was found that the CR sequence is located normally at −17 to −32 from the initiation codon AUG. If the CR sequence is located at the normal position, protein is synthesized efficiently in the absence of polyamines: a situation similar to the location of SD sequence in *E. coli*. If the CR sequence is located at a distant position from the initiation codon AUG (−33 to −39) such as eEF1A mRNA, polyamines are necessary for the efficient protein synthesis. When the position of the CR sequence of eIF2α, eIF4B and eRF1 mRNAs was shifted to a distant position from the initiation codon AUG, synthesis of these proteins was also stimulated by polyamines (see Fig. 7). Thus, the gene encoding eEF1A is a second type of polyamine modulon, in which protein synthesis is enhanced through the structural change by polyamines of the CR sequence, which is located at a distant position from the AUG.

Our results strongly suggest that the CR sequence is important for formation of the initiation complex correctly together with the Kozak sequence (Kozak 1991), but the role of the CR sequence was weaker than that of prokaryotic SD sequence, which is essential for protein synthesis (Igarashi et al. 1997). Although a typical SD sequence in prokaryotes consists of purine bases (GGAG) located at −8 to −12 from the initiation codon AUG, CR sequences are variable in each mRNA, consisting of a mixture of 5 to 8 purine and pyrimidine bases, and are slightly far away from the initiation codon AUG (−17 to −32).

In case of eEF1A mRNA, the structural change by polyamines in the bulged-out region of hairpin 1 was important for polyamine stimulation of eEF1A synthesis. However, in case of the mutated eIF2α, eIF4B and eRF1 mRNAs (see Fig. 7), the bulged-out structure was not present at the CR sequence region. Thus, a distant position of the CR sequence from the initiation codon AUG and its flexible secondary structure stabilized by polyamines may be necessary for polyamine stimulation of protein synthesis, and it is presumed that the existence of the bulged-out structure in the double-stranded RNA increases the degree of polyamine stimulation through the stabilization of the bulged-out structure by polyamines (Higashi et al. 2008).

The *eEF1A* gene was identified as polyamine modulon using DFMO-treated FM3A and NIH3T3 cells. In this case, putrescine and spermidine content became negligible, but spermine was present at 40–60 % of control cells. Putrescine, spermidine and spermine stimulated globin synthesis equally at the concentrations of 7.5, 0.4, and 0.08 mM, respectively, in a rabbit reticulocyte cell-free system (Ogasawara et al. 1989). Thus, if spermine content decreased more effectively, more distinct polyamine effect on protein synthesis would be obtained. Since spermine content decreased in DFMO plus APCHA-treated cells, the degree of polyamine stimulation of eEF1A synthesis increased from threefold to fivefold (see Fig. 2).

To establish the presence of the CR sequences in eukaryotic mRNAs, we searched for CR sequences in other randomly selected 45 mRNAs. In all mRNAs evaluated, a CR sequence was present (Fig. S1). Among these mRNAs, the CR sequence of PGK2, JAK2 and p16 mRNAs was distant from the initiation codon AUG. We confirmed that synthesis of JAK2 and p16 protein was stimulated by polyamines at the level of translation (data not shown). The results confirmed that the location of a CR sequence in mRNAs influences polyamine stimulation of protein synthesis.

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Conflict of interest The authors declare that they have no conflict of interest.

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