The effect of the 5'-untranslated region of ornithine decarboxylase (ODC) mRNA and of spermidine on the translation of ODC mRNA was studied in a rabbit reticulocyte cell-free system. The ODC mRNAs, possessing different sizes of the 5'-untranslated region and named mODC241, mODC188, mODC85, and mODC24 according to the number of nucleotides in the 5'-untranslated region, were synthesized by in vitro transcription techniques through the use of plasmids containing various sizes of mouse ODC cDNA. As the size of the 5'-untranslated region of ODC mRNA increased, the efficiency of the translation decreased and the degree of stimulation of the translation by 0.2 mM spermidine increased. The inhibition of ODC mRNA translation by high concentrations of spermidine (0.6-1 mM) also increased with an increase in the size of the 5'-untranslated region. The spermidine effects were increased greatly when the size of the 5'-untranslated region of ODC mRNA was increased from 85 to 188 nucleotides. These results suggest that the nucleotides 70-220 upstream from the initiator AUG are involved in the decrease in translational efficiency. In addition, at least the nucleotides in the 70-170 region upstream from the initiator AUG are important in the strong stimulation of ODC synthesis by low spermidine concentrations and in the inhibition of ODC synthesis at high spermidine concentrations.

Polyamine biosynthesis is highly regulated in mammalian cells; polyamines themselves participate in this regulation (1, 2). It is known that the amount of ornithine decarboxylase (ODC) is decreased by polyamines increasing the degradation rate of ODC (3-7) and reducing the rate of ODC synthesis (6-11). The finding that the reduction in the rate of ODC synthesis does not involve a decrease in ODC mRNA content (9-11) suggests that polyamine regulation of ODC synthesis is at the translational level. In our studies of the polyamine effect on protein synthesis, we have shown that a low concentration of polyamines stimulates protein synthesis mainly at the level of initiation (12, 13). However, the degree of polyamine stimulation differs with individual proteins (12-16).

Spermidine can stimulate ODC synthesis at a concentration lower than that necessary for stimulating total protein synthesis. Higher concentrations of spermidine were inhibitory, and its inhibitory effect on ODC synthesis was greater than on protein synthesis in general (17-19). This indicates that at least part of the feedback regulation of ODC synthesis exerted by polyamines is due to their direct inhibition of ODC mRNA translation.

In this study, we have prepared various ODC mRNAs having different sizes of the 5'-untranslated region (5'-UTR) and examined the contribution of this region to ODC synthesis in the presence and absence of spermidine. The results suggest that the size of the 5'-UTR is inversely correlated to ODC synthetic activity and that at least the nucleotides in the 70-170 region upstream from the initiator AUG are important for polyamine stimulation and inhibition of ODC synthesis.

EXPERIMENTAL PROCEDURES

Construction of Plasmids and Preparation of Plasmid DNA—Plasmid pOD20.7 containing mouse ornithine decarboxylase cDNA (20) was kindly provided by Dr. P. Coffino (University of California, San Francisco). A fragment containing 89 base pair of the 5'-leader sequence, the complete coding sequence, and 240 base pair of the 3' nontranslated sequence, was obtained by digestion of pOD20.7 with endonucleases SalI and PvuII. The SalI and PvuII sites were converted into EcoRI and BamHI sites, respectively, by the addition of linkers, and then the fragment was inserted into the multiple cloning site of plasmid pSP65 (Promega Corp.) cut with EcoRI and BamHI (pODC85). The plasmid pODC20.7 was digested with PvuII and a 4.66-kb fragment was isolated. After a BamHI linker was ligated, the fragment was digested with Avai to yield a 1.79-kb fragment. The Avai site of the 1.79-kb fragment was converted into an EcoRI site by the addition of a linker, and the fragment was inserted into the EcoRI and BamHI sites of pSP65 (pODC188). The plasmid pODC188 was digested with TaqI and a 1.66-kb fragment was isolated. After the TaqI site was converted to an EcoRI site, the fragment was digested with EcoRI and BamHI and inserted into the EcoRI and BamHI sites of pSP65 (pODC24). The plasmid pODC20.7 was digested with PstI, a 0.54-kb fragment was inserted containing the complete coding sequence with the silent mutation, and then the fragment was inserted into the multiple cloning site of plasmid pSP65 (pSP64PstI). The 1.7-kb SalI-BamHI fragment of pODC188 was inserted into the SalI and BamHI sites of pSP64PstI (pODC241).

Plasmid pTK11 containing human thymidine kinase cDNA (21) was kindly provided by Dr. P. L. Deininger (Louisiana State University Medical Center). After ligation of a HindIII linker to the SalI site, the 1.2 kb SalI-BamHI fragment of pTK11 containing the complete coding sequence was inserted into the HindIII and BamHI sites of pSP65 (pSP64TK). This plasmid was digested with SmaI, and a 1.25-kb fragment was isolated. HindIII linkers were ligated, the fragment was digested with HindIII, and the 0.81-kb fragment was inserted into the HindIII site of pODC188 (pSP65ODC-TK). The plasmid pSP64TK was digested with SmaI and BseEI, and a 0.51-kb fragment of the 3'-untranslated region was isolated. The fragment was inserted into PvuII- and BseEI-digested 4.37-kb fragment.
calculated on the basis of the data of Salser (32). Free energy (ΔG) for the formation of the secondary structure was calculated on the basis of the data of Salser (32).

Preparation of ODC mRNA Containing Different Sizes Sequences of the 5'-Untranslated Region and of ODC-TK Fusion mRNA. The ODC and ODC-TK fusion mRNAs were prepared in vitro as described by Melton et al. (23) with a slight modification. The pODC188, pODC85, and pODC24 plasmids were digested with PvuII, and the pODC241 and pODC188-TK were digested with SmaI and BamHI, respectively. These linearized plasmid DNAs were used as templates for in vitro transcription. After the reaction mixture (0.18 ml containing 20 μM Tris-HCl, pH 7.5, 2 mM spermidine, 6 mM MgCl₂, 0.1 mg/ml bovine serum albumin, 0.5 mM each of ATP, CTP, and UTP, 0.05 mM GTP, 0.5 mM mGppG (Pharmacia L-Biochemicals), 10 mM diithiothreitol, 50 units of human placental ribonuclease inhibitor (Takara shuzo), 5 μg of template DNA, and 5 units of SP6 RNA polymerase (Takara shuzo)). After a 40-min incubation at 40 °C, the GTP concentration was increased to 0.5 mM, and the incubation was continued for 90 min at 40 °C. The reaction mixture was then incubated with 2 units of DNase I (Bethesda Research Laboratories) and 5 μg of rat liver tRNA (24) for 15 min at 37 °C. The sample was mixed with 0.06 μl of a buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 0.05% sodium dodecyl sulfate, and extracted with phenol/chloroform/3-methylbutan-1-ol (25:24:1 by volume). After the sample was extracted again with chloroform/3-methylbutan-1-ol (24:1, v/v), the mRNA was precipitated from the sample by the addition of 0.1 ml of 4 M ammonium acetate, pH 5.4, and 0.5 ml of cold ethanol. The amount of mRNA synthesized was estimated by measuring the incorporation of [35S]UTP into the mRNA.

Synthesis of ODC and ODC-TK Fusion Protein in a Rabbit Reticulocyte Cell-free System—Rabbit reticulocyte lysate and nuclease-treated lysate were prepared as described by Pelham and Jackson (25). The nuclease-treated lysate was passed through a Sephadex G-25 column twice in order to remove polyamines, as described previously (26). The in vitro translation reaction mixture (0.02 ml) contained the following: 20 mM Hepes-KOH buffer, pH 7.8, 0.015 mM hemin, 100 mM potassium acetate, 2 mM diithiothreitol, 0.2 mM glucose 6-phosphate, 1.5 mM ATP, 0.5 mM GTP, 8 mM creatine phosphate, 3 μg of creatine kinase, 0.2 μg of mRNA synthesized as described above, 10 μCi of [35S]methionine (98 Ci/mmol), 0.03 mM each of the 19 other common amino acids, 9 μl of lysate filtered twice through Sephadex G-25 (0.68 mg of protein), and magnesium acetate and spermidine at the specified concentrations. After incubation for 34 °C for 30 min, the reaction was stopped by the addition of 0.03 ml of ice-cold 5 mM methionine. A 5-μl aliquot of each reaction mixture was plated on a paper disc (25-mm diameter) and the radioactivity insoluble in hot trichloroacetic acid was determined with a liquid scintillation spectrometer. To a 45-μl aliquot of the above reaction mixture, 45 μl of 2× gel sample buffer (125 mM Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, 10% 2-mercaptoethanol, 0.002% bromphenol blue) was added, and the mixture was boiled for 2 min. A 70-μl aliquot was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to the method of Laemmli (27) using 11% acrylamide slab gels. Fluorography was performed by the method of Laskey and Mills (28). To quantify the amount of radiolabeled incorporated into ODC or the ODC-TK fused protein, the corresponding bands were excised from the gel and dissolved in 0.5 ml of 30% H₂O₂ in a sealed glass scintillation vial at 65 °C for 2 h. Each sample was cooled, mixed with 10 ml of Triton-toluene scintillator (2.67 g of 2.5-diphenyloxazole, 67 mg of 2,2'-phenylenediamine-5-phenyl oxazole, 330 ml of Triton X-100, and 670 ml of toluene), and counted with a liquid scintillation spectrometer. Fluorography of [35S]methionine-labeled polypeptides precipitated with ODC-specific immunoglobulin (29) with the aid of Staphylococcus aureus Cowan I cells was performed as described previously (16).

The polyamine concentration in the translation reaction mixture was measured as described previously (30). The endogenous concentrations of putrescine, spermidine, and spermine in the reaction mixture were 0.34, 12.1, and 1.34 μM, respectively.

Free Energy Calculation for Formation of Secondary Structure—Optimal computer folding of the first 300 nucleotides of ODC mRNAs was performed according to the method of Zuker and Stiegler (31). Free energy (ΔG) for the formation of the secondary structure was calculated on the basis of the data of Salser (32).

RESULTS

Translational Activity of Various ODC mRNAs Having Different Sizes of 5'-UTR without Added Spermidine—Various ODC mRNAs with different sizes of 5'-UTR were synthesized using SP6 RNA polymerase and various DNA templates (Fig. 1). The 13-17 5'-terminal nucleotides were derived from the vector plasmid and linker used in plasmid construction (Fig. 2). The nucleotide sequences from the 5'-cap to the initiator AUG of the various ODC mRNAs used in these experiments are shown in Fig. 2. We named these mRNAs mODC241, mODC188, mODC85, and mODC24, respectively, according to the number of nucleotides in the 5'-UTR. Translation of ODC mRNA was examined using these mRNAs and a rabbit reticulocyte cell-free translation system (Figs. 3-6). ODC (M, 51,000) synthesis in the absence of spermidine was highest when mODC24 and mODC85 were used as templates, and the optimal Mg²⁺ concentration was 2.5-3 mM (Figs. 3 and 4, and Table I). When mODC188 was used as a template, the amount of ODC synthesized was approximately 5% of that with mODC24 (Table I). With mODC241, the amount of ODC synthesized was less than 0.5% of that with mODC24 (Table I). This could not be seen clearly by fluorography (Fig. 6). However, significant amounts of nascent polypeptide of ODC (M, 32,000) were synthesized (Fig. 6). Since the exposure time for this fluorography was 150 h rather than 20 or 50 h, a nonspecific protein band (M, 49,000) appeared in the figure. The same band was also observed in Fig. 5. When [35S]methionine-labeled polypeptides were precipitated with ODC-specific immunoglobulin, the nonspecific protein band (M, 49,000) disappeared and a faint band of ODC (M, 51,000) was observed (Fig. 7). The overall results suggest that the size of the 5'-UTR is very important for the translational efficiency of ODC synthesis. The shorter sequences were much more effective than the longer ones.

Effect of Spermidine on Translation of Various ODC mRNAs—With all the mRNAs tested, the addition of spermidine lowered the optimal Mg²⁺ concentration for the translation of ODC mRNA. The optimal Mg²⁺ concentration in the presence of spermidine was 1.5-2 mM. When mODC24 was used as a template, 0.2 mM spermidine increased the ODC mRNA translation 9.1-fold compared with translations without added spermidine at 1.5 mM Mg²⁺ (Table I). The degree of spermidine stimulation was nearly equal to that observed for thymidine kinase and globin synthesis, and greater than that for protamine and bovine lymphocyte total protein synthesis in this translation system (data not shown). The inhibitory effect at high concentrations of spermidine (0.6-1 mM) was weak (Figs. 3 and 8). The amount of ODC synthesized in the presence of 0.8 mM spermidine was approximately 50% of

![Fig. 1. Structure of ODC mRNA possessing different sizes of 5'-UTR.](https://example.com/fig1.png)
Fig. 2. Nucleotide sequence of 5'-UTR of various ODC mRNAs and the calculated free energy for secondary structure formation within the first 300 nucleotides and of the cap to AUG sequence. The nucleotide sequence of the 5'-UTR was essentially taken from that of ODC cDNA (20) and slightly modified according to the sequence of ODC genomic DNA (33, 34, 45). Nucleotide sequences underlined are of vector and linker origin. A possible open reading frame is also marked (**).
TABLE I
Comparison of the degree of spermidine stimulation of ODC synthesis directed by ODC mRNA templates with different sizes of 5'-UTR

The amounts of ODC synthesized at the optimal Mg\(^{2+}\) concentrations (1.5 mM Mg\(^{2+}\) with spermidine and 2.5 mM Mg\(^{2+}\) only) and at 1.5 mM Mg\(^{2+}\) only were measured as described under "Experimental Procedures." The degree of spermidine stimulation is the ratio of the amount of ODC synthesized in the presence of 1.5 mM Mg\(^{2+}\) and 0.2 mM spermidine to that in the presence of 1.5 mM Mg\(^{2+}\) only. The [\(^{15}S\)]Met incorporated was calculated from the data of Figs. 3-7, as well as from two other experiments (data not shown). The values are expressed as mean ± S.D.

| mRNA      | Mg\(^{2+}\) | Spermidine | [\(^{15}S\)]Met incorporated | Degree of spermidine stimulation |
|-----------|------------|------------|-----------------------------|---------------------------------|
| mODC241  | 1.5        | 0.47 ± 0.02| 2.5 1.26 ± 1.08             | 19.9                           |
|           | 1.5        | 0.2        | 9.36 ± 8.05                |                                 |
| mODC188  | 1.5        | 7.43 ± 0.21| 2.5 22.4 ± 6.25            | 19.0                           |
|           | 1.5        | 0.2        | 141 ± 18                   |                                 |
| mODC188-TK| 1.5        | 7.31 ± 2.92| 2.5 19.2 ± 5.41            | 17.1                           |
|           | 1.5        | 0.2        | 125 ± 19                   |                                 |
| mODC85   | 1.5        | 128 ± 18   | 2.5 301 ± 38               | 10.3                           |
|           | 1.5        | 0.2        | 1315 ± 118                 |                                 |
| mODC24   | 1.5        | 165 ± 21   | 2.5 423 ± 35               | 9.1                            |
|           | 1.5        | 0.2        | 1495 ± 152                 |                                 |

FIG. 7. Fluorography of ODC241 mRNA-directed protein synthesis after the precipitation with ODC-specific immunoglobulin. The conditions of protein synthesis were exactly the same as those of Fig. 6. The polypeptides synthesized were then precipitated with ODC-specific immunoglobulin before gel electrophoresis as described under "Experimental Procedures." Exposure time, 230 h.

FIG. 8. Inhibitory effect by high spermidine concentration on ODC synthesis directed by various ODC mRNAs. The amount of ODC synthesized at the optimal Mg\(^{2+}\) concentration (1.5 mM Mg\(^{2+}\) with spermidine) was measured as described under "Experimental Procedures." Synthesis in the presence of 0.2 mM spermidine (SPD) was regarded as 100%.

FIG. 9. Effect of spermidine on ODC185-TK fusion mRNA-directed protein synthesis. Exposure time, 50 h. Lower bar on the left indicates the position of a nonspecific band (M, 49,000).

The amounts of ODC was synthesized (Figs. 6 and 7). The degree of spermidine stimulation at 0.2 mM spermidine was always more than 17-fold (Table I). The inhibition of ODC synthesis by high concentrations of spermidine was also observed, but the exact degree of inhibition could not be calculated. The synthesis of the nascent polypeptide of ODC (M, 32,000) was stimulated about 17-fold by 0.2 mM spermidine at 1.5 mM Mg\(^{2+}\) (data not shown), and the amount synthesized at 0.8 mM was approximately 7% of that at 0.2 mM (Fig. 6). The synthesis of the 39,000 protein was very low. These results suggest that the nucleotides 70-170 upstream from the initiator AUG are important both in the strong spermidine stimulation of ODC synthesis at low spermidine concentration and the inhibition of ODC synthesis by high spermidine concentration.

Next, we tried to determine whether or not a specific interaction between the 5'-side of ODC mRNA and the 3'-side of mRNA is necessary for the decrease in translational efficiency and the spermidine effect. For this purpose, an ODC-TK fusion mRNA was synthesized from template DNA, in which the 0.92 kb of the 5'-side of pODC188 was fused to the 1.24 kb of the 3'-side of pTK11 through a HindIII linker (Fig. 1). When protein synthesis was performed with this mRNA template, results similar to those obtained with mODC188 were obtained (Fig. 9). The translational efficiency was almost the same as with mODC188 (Table I). Spermidine stimulation at 0.2 mM was 17.1-fold, and the inhibitory effect by 0.8-1 mM spermidine was clearly observed (Fig. 8). These results show that the nucleotide sequence in the 5'-UTR itself is involved in the decrease of translational efficiency and the spermidine effect.

DISCUSSION

Mouse ODC mRNA has an unusually long 5'-UTR, which consists of 313 nucleotides (33, 34). The predicted secondary structures of the 5'-UTR may explain why ODC mRNA is difficult to translate in cell-free systems (2). To clarify the role of the 5'-UTR in the translational efficiency of ODC synthesis and the spermidine effect on this synthesis, we synthesized various ODC mRNAs with different sizes of 5'-UTR and constructed an ODC-TK fusion mRNA. From a comparison of the results with mODC188 and mODC188-TK fusion mRNAs (Table I and Fig. 8), we could show that the nucleotide sequence in the 5'-UTR itself is involved in the decrease of translational efficiency and the spermidine regulatory effect. Our data support the previously proposed role
of the 5′-UTR in the translational control of ODC synthesis (2).

A comparison of ODC synthetic activity with mODC241, mODC188, mODC85, or mODC24 shows that the decrease in the translational efficiency of ODC synthesis is proportional to the size of the 5′-UTR. The free energy of the potential secondary structures in the first 300 nucleotides are almost the same for the various mRNAs except that ΔG for mODC241 is only slightly lower (Fig. 2). However, when the free energy of the potential secondary structures in the region between the cap and the initiator AUG was calculated, ΔG correlated with ODC synthetic activity (Fig. 2 and Table I). We found that ODC synthetic activity decreases drastically if ΔG was lower than −50 kcal/mol. This is in good accordance with the results obtained with preproinsulin synthesis (35). These results suggest that the potential secondary structures may be a considerable obstacle for the scanning of 40S ribosomal subunits from the cap to the initiator AUG, but not much of an obstacle for the running of 80S ribosomes moving from the initiator AUG to the termination codon during protein synthesis. The same idea has been proposed recently from experimental results regarding the translational efficiency of aldehyde dehydrogenase mRNA (36).

Our results are similar to previous reports that the 5′-UTR region of the thymidine kinase mRNA of the herpes simplex virus (37) and of the porcine pro-opiomelanocortin mRNA (38) reduces translational efficiency but are contrary to the recent observation that deletion of the 5′-UTR region of phosphoglycerate kinase mRNA in yeast decreases translational efficiency (39).

When we used mODC241 as a template, ODC synthetic activity was extremely low, as reported in the experiments with intact ODC mRNA (2, 40). A factor which is necessary for the translation of ODC mRNA may be lacking from the rabbit reticulocyte lysate.

We have defined the M, 32,000 and 39,000 polypeptides observed in fluorography as the nascent polypeptides of ODC for the following reasons. 1) The polypeptides reacted with the polyclonal antibody for ODC. 2) If the polypeptides had been synthesized from the internal AUG instead of the initiator AUG, the translational efficiency would have been extremely low since, as mentioned above, the potential secondary structures from the cap to the internal AUG are thought to form an obstacle for the scanning of 40S ribosomal subunits. The ratio of M, 39,000 to M, 32,000 polypeptides synthesized increased with the decrease in the size of 5′-UTR. This also indicates that the decrease in the translational efficiency of ODC synthesis is proportional to the size of 5′-UTR and is consistent with the finding that the ratio of nascent polypeptide to intact polypeptide synthesized becomes smaller when protein synthetic activity is high.

Although the inhibition of ODC synthesis by polyamines at the translational level has been well documented (17–19), strong stimulation of ODC synthesis by a low concentration of spermidine (0.2 mM) has not been a consistent finding. We have examined the spermidine stimulatory effects on the synthesis of protamine, globin, thymidine kinase, ODC, and the total proteins of bovine lymphocytes in a rabbit reticulocyte cell-free system. Among these protein syntheses, the degree of spermidine stimulation of ODC synthesis was greatest. From our overall results, it can be concluded that spermidine regulates ODC synthesis positively at a low concentration (0.2 mM) and negatively at a high concentration (0.6–1 mM). Other investigators recently reported the stimulation of ODC synthesis by a low concentration of spermidine (19, 41).

When the size of the 5′-UTR of ODC mRNA increased from 65 to 198 nucleotides, both spermidine stimulation and inhibition were increased greatly (Figs. 4 and 5). A small open reading frame can be seen in this region (Met-Gly-Gln-Ala-Ser-Arg-Ala-Thr-Val-Leu) (Fig. 2). Such a potential leader peptide has been suggested as decreasing the translational efficiency of mRNA in another system (42). There is also a GC-rich region located in the 128–174 nucleotide sequence upstream from the initiator AUG. We do not yet know the precise mechanism(s) of the polyamine effect, but either the open reading frame or the GC-rich region is thought to be involved. A GC-rich region also exists in the 5′-terminus of the 5′-UTR of intact ODC mRNA. If we can construct a more efficient translation initiation site for ODC synthesis, we may be able to clarify whether or not the GC-rich region is really related to the polyamine effect. In this regard, we have reported previously that spermine binds more readily to GC-rich DNA than to AT-rich DNA (43).

Using a cell culture system, Coffino and his co-workers (44) have reported that there is little or no translational regulation of ODC by polyamines. However, in a cell-free system, we have shown that high concentrations of spermidine (0.8–1 mM) directly inhibit protein synthesis. Our ODC-TK fusion protein (M, 51,000) encoded by mODC188 TK does not contain PEST sequences, which are involved in the rapid degradation of ODC (5). The effect of spermidine on mODC188- and mODC188-TK-directed protein synthesis was almost the same, suggesting that spermidine is involved in the translation of ODC mRNA rather than the degradation of the ODC protein in this cell-free system. More detailed studies will be required to clearly determine how much of the polyamine effect on ODC synthesis is involved in the regulation of the ODC amount in cells, in addition to the polyamine effect on ODC degradation.

Acknowledgments—We thank Drs. P. Coffino and P. L. Derijnger for their kind gifts of mouse ODC cDNA and human TK cDNA, respectively, and Barbara K. Joyce for preparing the manuscript.

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