Molecular Mechanism of Polyamine Stimulation of the Synthesis of Oligopeptide-binding Protein*

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Polyamine stimulation of the synthesis of oligopeptide-binding protein (OppA) was shown to occur mainly at the level of translation by measuring OppA synthesis and its mRNA level. Several artificial oppA genes were constructed by site-directed mutagenesis. These synthesize different kinds of OppA mRNAs: mRNAs differing in the size of 5'-untranslated region; mRNAs having the Shine-Dalgarno (SD) sequence in a different position; mRNAs having different secondary structure in the region of the SD sequence; and fusion mRNAs consisting of the Shine-Dalgarno (SD) sequence in addition to its position. Loose base pairing to RNA was very close to the estimated value in cells. In the condition of spermine-stimulated globin synthesis in a rabbit reticulocyte cell-free system (11), the amount of polyamine-synthesizing protein was found to be very close to the estimated value in cells. The synthesis of OppA or OppB proteins was dependent on the structure of the SD sequence and the secondary structure of the Shine-Dalgarno (SD) sequence associated with the synthesis of OppA or OppB proteins. When the secondary structure of the above region of OppA mRNA was analyzed by optimal computer folding, it was shown that the degree of polyamine stimulation of OppA protein synthesis depended on the structure of the SD sequence in addition to its position. Loose base pairing of the SD sequence with other regions of the mRNA caused strong polyamine stimulation, while intense base pairing of the SD sequence with other regions of the mRNA resulted in insignificant or weak polyamine stimulation.

Polyamines, aliphatic cations present in almost all living organisms, are known to be necessary for normal cell growth (1). Their proliferative effects are probably caused by the stimulation of nucleic acid and protein synthesis. We previously reported that polyamines can stimulate some kinds of protein synthesis in both prokaryotic and eukaryotic cell-free systems (2, 3) and in vivo (4, 5). Furthermore, it has been reported that assembly of 30 S ribosomal subunits is stimulated by polyamines, leucine, and tryptophan (8, 9). We also found that most polyamines exist as a polyamine-RNA complex in cells, and that the amount of polyamines (spermidine plus spermine) bound to RNA in rat liver is about 2 mol/100 mol of phosphate of RNA (10). Under the condition of spermine-stimulated globin synthesis in a rabbit reticulocyte cell-free system (11), the amount of polyamine bound to RNA was very close to the estimated value in cells.

In Escherichia coli, synthesis of a protein (polyamine-induced protein) was strongly stimulated by the addition of putrescine to growing cells of a polyamine-requiring mutant MA261 (12). The protein was identified, by cloning the corresponding gene, as OppA1 and is a periplasmic substrate-binding protein of the oligopeptide uptake system (13). In the present work, we have shown that stimulation of OppA synthesis by polyamines occurs mainly at the level of translation, and the position and secondary structure of the Shine-Dalgarno (SD) sequence (14) are probably involved in the stimulation of protein synthesis by polyamines.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—E. coli MA261 (speB speC gly leu thr thi) was kindly provided by Dr. W. K. Maas, New York University School of Medicine. E. coli MA261 oppA::Km and lacZ::Em were prepared as described previously (15, 16). These cells were grown at 37°C in medium A in either the presence (100 µg/ml) or absence of putrescine (16). Methionine content in medium A was decreased from 100 to 3 µg/ml in order to label proteins with [35S]methionine; this modification did not influence their growth rate. Another polyamine-requiring mutant, HT283, was kindly provided by Dr. H. Tabor, National Institutes of Health, and was grown according to the method of Hafner et al. (17).

Plasmids—pACYCoppA, equivalent to pP15.1, was prepared as described previously (13). The 4.3-kb HindIII-SalI fragment of pP15 (13) was ligated into the same restriction sites of pUC119. Subsequently, the 3.2-kb oppA gene-containing SmaI fragment of the plasmid was ligated into the same restriction site of pMW119 purchased from Nippon Gene (pMW975). pMW211 was constructed by inserting the 3.4-kb NruI fragment of pACYCoppA into the SmaI site of pMW119. A PCR product was obtained using the HindIII-digested pMW975 as template, and 5′-CAATATGTTACCTGCTG-3′ and 5′-GGGGATATTCCATTCTATTTGATGA-3′ as primers. pMW412 was constructed by inserting the 1.1-kb EcoRI-BstEII fragment of the PCR product into the same restriction sites of pMW211.

Site-directed mutagenesis by overlap extension using PCR (18) was performed to prepare pMWSD1 and pMWSD2. The template used for first PCR was EcoRI-digested pMW975. Primers used for first PCR were 5′-GGGAATTTCCGCGCGCCGCGC-3′ (P1, sequence for 958 to 942 of oppA gene), 5′-TTTTCTGGGACTCAATCATTATAATT-3′ (complementary sequence for 27 to 4 of oppA gene except two underlined bases), 5′-AATTATAATGGATGACTGGAATAAAA-3′ (sequence for 27 to 4 of oppA gene except two underlined bases) and 5′-GGGGATATTCCATTCTATTTGATGA-3′ (sequence for 27 to 4 of oppA gene except two underlined bases) and 5′-GGGGATATTCCATTCTATTTGATGA-3′ (sequence for 27 to 4 of oppA gene except two underlined bases) and 5′-GGGGATATTCCATTCTATTTGATGA-3′ (sequence for 27 to 4 of oppA gene except two underlined bases). Two PCR products thus obtained were digested with EcoRI and BstEII, and inserted into the same restriction sites of pMW211. The same method was applied to prepare pMW5D1 to pMW5D5 with appropriate primers.

The abbreviations used are: OppA, oligopeptide-binding protein; SD, Shine-Dalgarno; PCR, polymerase chain reaction; IS2, insertion sequence 2; UTR, untranslated region; ORF, open reading frame; kb, kilobase pair(s).
Polymine Stimulation of OppA Synthesis Is Dependent on Gene Copy Number—As shown in Fig. 1, we confirmed polyanine stimulation of OppA synthesis in polyamine-requiring mutant MA261 cells by immunoprecipitation of [³⁵S]methionine-labeled OppA protein. If the oppA gene was disrupted, there was no OppA synthesis. When cells were transformed with pACYCoppA, a relatively high copy number plasmid, large amounts of OppA were synthesized, and polyamines did not stimulate OppA synthesis significantly. In contrast, when cells were transformed with pMW975, a low copy number plasmid, OppA synthesis was greatly stimulated by polyamines. These results indicate that polyanine stimulation of OppA synthesis is observed only when the copy number of the oppA gene is low. Under these conditions, putrescine and spermidine contents in cells grown in the presence and absence of 100 μM putrescine were 43.5 and <0.1 nmol/mg protein for putrescine and spermidine, respectively. The function of polyamines was not replaced by spermidine. Therefore, the polyamine-stimulated OppA synthesis is specific to polyamines.

RESULTS

The data in Table I show that when the polyanines were added to the growth medium, there was a significant increase in the amount of OppA synthesized in the absence of polyamines. This suggests that polyamines are required for the synthesis of OppA. The data also show that the amount of OppA synthesized was dependent on the copy number of the oppA gene. When the copy number was increased, the amount of OppA synthesized was also increased. This suggests that the amount of OppA synthesized is regulated by the copy number of the oppA gene. The data also show that the amount of OppA synthesized was dependent on the type of polyamine. Putrescine was more effective than spermidine in stimulating OppA synthesis.

Table I: Strains and plasmids used in this study

| Strain or plasmid       | Relevant characteristics                                      |
|-------------------------|---------------------------------------------------------------|
| E. coli strains         | Polyamine-requiring mutant; speB speC thr leu ser thi         |
| MA261                   | Oligopeptide transport protein-deficient mutant of MA261; MA261 oppA |
| MA261 oppA::Km           | β-Galactosidase-deficient mutant of MA261; MA261 lacZ         |
| HT253                   | Polyamine-requiring mutant; speA speB speC speD thr pro       |
| Plasmids                |                                                               |
| pMW119                  | Insertion of 975 nucleotides (nt) of 5′-upstream region (5′-UR) of the initiation codon ATG and ORF of oppA into pMW119 |
| pMW975                  | Insertion of 412 nt of 5′-UR and ORF of oppA into pMW975      |
| pMW112                  | Insertion of 211 nt of 5′-UR and ORF of oppA into pMW119      |
| pMW9                  | Nonexistence of SD sequence in pMW975                         |
| pMW9D                  | Site-directed mutagenesis of GGG(−63~−61) of stem II to AAA in pMW975 |
| pMW9D5                  | Site-directed mutagenesis of GGG(−63~−61) of stem II to TTT in pMW975 |
| pMW D5                | Site-directed mutagenesis of GGG(−63~−61) of stem II to TTT in pMW975 |
| pMW 9-lacZ            | Fusion of oppA 5′-UR and ORF for 9 amino acids and lacZ ORF in pMW211 |
| pMW 45-lacZ          | Fusion of oppA 5′-UR and ORF for 45 amino acids and lacZ ORF in pMW211 |
Mg\(^{2+}\) or Ca\(^{2+}\) (data not shown).

Determination of the Transcription Initiation Sites of the oppA Gene—First, we determined the nucleotide sequence upstream of the oppA gene. As shown in Fig. 2, insertion sequence 2 (IS2) (30) was found in the upstream region. Although IS2 was observed in another polyamine-requiring mutant HT283 (EWH319), it was not in \textit{E. coli} W3110, the parental strain of MA261 and HT283. The upstream region of the gene also included the leucine responsive element observed in the upstream region of the oppA gene of \textit{Salmonella typhimurium} (31). We confirmed leucine stimulation of OppA synthesis in \textit{E. coli} HT283, but polyamine stimulation of OppA synthesis was not influenced by leucine (data not shown). The effect of leucine on OppA synthesis in \textit{E. coli} MA261 could not be determined since leucine is necessary for cell growth in this strain.

Transcription initiation sites of the oppA gene were determined by S1 nuclease mapping (Fig. 3). In \textit{E. coli} MA261, there were three initiation sites (P1, P2, and P3). However, initiation mainly occurred from P1, suggesting that IS2 has a strong promoter activity. When OppA mRNA was synthesized from pMW975, pMW412, and pMW211, the major initiation site of transcription was P2, P2, and P3, respectively. It remains to be clarified why P1 is not the initiation site in pMW975.

Polyamine Stimulation of OppA Synthesis at the Translational Level—To determine the level of polyamine stimulation of OppA synthesis, the amount of OppA mRNA and OppA synthesis were measured by dot-blotting of RNA and immunoprecipitation of \[^{35}S\]methionine-labeled OppA protein (Fig. 4). When OppA mRNA was transcribed from P1, polyamines significantly stimulated OppA mRNA synthesis (3.2-fold). When OppA mRNA was transcribed from P2 and P3, polyamines only slightly stimulated the OppA mRNA synthesis (1.2–1.4-fold). In the region upstream of P2 and P3, there were no typical –35 and –10 promoter regions, but transcriptional activity was stronger from P2 than from P3.

OppA synthesis measured by immunoprecipitation of
Polyamine Stimulation of Protein Synthesis

\[ \text{\textsuperscript{35}S} \text{methionine-labeled OppA protein was stimulated by polyamines with all versions of the OppA mRNAs (Fig. 4). The degree of stimulation by putrescine (\textit{fold}) was measured as described under "Experimental Procedures." E. coli strains used were shown on the left and cultured in the presence and absence of putrescine. The degree of stimulation by putrescine (\textit{fold}) was calculated from the values obtained in the presence and absence of putrescine.} \]

\[ \text{\textit{Fig. 4. Effect of the size of 5'-UTR of OppA mRNA on polyamine stimulation of OppA synthesis. The amount of OppA mRNA (A) and OppA protein (B) was measured as described under "Experimental Procedures." E. coli strains used were shown on the left and cultured in the presence and absence of putrescine. The degree of stimulation by putrescine (\textit{fold}) was calculated from the values obtained in the presence and absence of putrescine.} \]

\[ \text{\textit{Fig. 5. Effect of SD sequence on polyamine stimulation of OppA synthesis. pMWSD\textsuperscript{-} which encodes OppA mRNA without the SD sequence, and pMWSD\textsuperscript{+} which encodes OppA mRNA having the SD sequence 7 nucleotides upstream from the AUG were prepared as described under "Experimental Procedures." OppA protein synthetic activities directed by these OppA mRNAs were compared with that directed by normal OppA mRNA.} \]

\[ \begin{array}{c}
\text{16S-rRNA} \quad 3'-A \\
\text{UU CCU C} \quad 5' \\
\text{5'-UAU AUA GAG GGA GUC CAAA AACA AUG AC-3'} \\
\text{\textsuperscript{+1}} \\
\end{array} \]

\[ \begin{array}{c}
\text{Construct} \\
\text{pMW SD\textsuperscript{-}} \\
\text{pMW SD\textsuperscript{+}} \\
\end{array} \]

\[ \begin{array}{c}
\text{pMW SD\textsuperscript{-}} \\
\text{U U} \\
\text{A GG} \\
\end{array} \]

\[ \text{Relative amount} \\
\text{100} \\
\text{513} \\
\text{179} \\
\text{412} \\
\text{4.3} \\
\text{7.0} \\
\text{Stimulation} \\
\text{5.1} \\
\text{2.3} \\
\text{1.6} \\
\end{array} \]

sequence, polyamine stimulation was observed, but to a lesser degree (pMWSD\textsuperscript{+}, 2.3-fold) (Fig. 5). However, OppA synthesis from the mRNA with the new SD sequence in the absence of putrescine was greater than that from the normal OppA mRNA. The results suggest that the position of the SD sequence may influence polyamine stimulation of OppA synthesis.

\[ \text{We next examined whether the 5'-UTR of the OppA mRNA is sufficient for polyamine stimulation of OppA synthesis. As shown in Fig. 6, synthesis of } \beta \text{-galactosidase from the chromosomal lacZ gene was not stimulated by polyamines. When the 5'-UTR and 27 nucleotides encoding first 9 amino acids for OppA protein was fused to } \beta \text{-galactosidase (9-lacZ mRNA), the degree of polyamine stimulation of } \beta \text{-galactosidase synthesis was 1.4-fold. The length of OppA mRNA used for the construction of a fused mRNA was then increased to include the 5'-UTR and 135 nucleotides encoding first 45 amino acids (45-lacZ mRNA). As a result, the degree of polyamine stimulation increased to 4.2-fold. Possible secondary structure of the initiation codon surrounding region (~65 to 65) of OppA mRNA and 9-lacZ mRNA was then compared (Fig. 7). Stability of the mRNAs was nearly equal (~46.6 and ~47.8 kcal/mol). The SD sequence of the 9-lacZ mRNA was tightly base paired. However, the SD sequence of OppA mRNA, which is equivalent to} \]
Fig. 6. Effect of the 5'-UTR of OppA mRNA on polyamine stimulation of β-galactosidase synthesis. Fused mRNA having oppA 5'-UTR and ORF for 9 amino acids and lacZ ORF or having oppA 5'-UTR and ORF for 45 amino acids and lacZ ORF was prepared as described under "Experimental Procedures." Synthesis of β-galactosidase was measured by the same method as that of OppA protein synthesis. Antiserum for β-galactosidase was obtained from Sigma.

A OppA mRNA  
from -65 to +65  
\[ \Delta G = -46.6 \text{ kcal/mol} \]

B 9-lacZ mRNA  
from -65 to +65  
\[ \Delta G = -47.8 \text{ kcal/mol} \]

Fig. 7. Possible secondary structure of OppA mRNA (A) and 9-lacZ mRNA (B). Optimal computer folding of the 130 nucleotides (-65 to 65) of mRNAs was performed by the method of Zucker and Stiegler (28). The structure of 45-lacZ mRNA at this region is the same as that of OppA mRNA (A). Initiation codon AUG is circled and the SD sequence is boxed. Continuous GC stems I and II are shaded.
Table II
Effect of secondary structure of SD sequence on polyamine stimulation of OppA and β-galactosidase synthesis

| Plasmid or gene | SD sequence | Relative amount of OppA mRNA b as Patrescine | Stimulation (fold) |
|----------------|-------------|---------------------------------------------|-------------------|
| pMW975         | 5'-AGG    | 100                                         | 5.1               |
| pMWD4          | 5'-AGG    | 126                                         | 4.8               |
| pMWD3          | 5'-AGG    | 105                                         | 4.6               |
| pMWD5          | 5'-AGG    | 120                                         | 4.0               |
| pMWD1          | 5'-AGG    | 142                                         | 3.7               |
| pMWD2          | 5'-AGG    | 179                                         | 2.3               |
| pMWD3          | 5'-AGG    | 86                                          | 1.4               |
| pMWD2          | 5'-AGG    | 55                                          | 0.73              |
| pMWD3          | 5'-AGG    | 4.3                                         | 1.6               |



- Secondary structure of SD sequence of each OppA mRNA is shown (see Fig. 7).
- Relative amount of OppA or β-galactosidase was shown as the amount compared with that of OppA synthesized in E. coli MA261 transformed with pMW975 in the absence of putrescine.

Polyamines can stimulate synthesis of proteins such as OppA protein (1, 2) and ribosomal proteins (26), which are important for cell growth. In this communication, the molecular mechanism of polyamine stimulation of OppA synthesis has been studied. It was found that polyamine stimulation occurs mainly at the translational level.

In E. coli, IS2 was inserted at some stage of evolution. IS2 enhanced transcriptional efficiency of oppA gene and the IS2-dependent transcription was stimulated by polyamines. This also contributed to the polyamine stimulation of OppA synthesis. In E. coli W3110 lacking IS2 at the upstream region of oppA gene, the transcription started mainly from P2 and the efficiency was low because only weak −35 and −10 regions exist upstream of P2 (Fig. 2). Thus, IS2 was inserted at some stage of evolution by chance so that OppA protein was synthesized more effectively in E. coli MA261 and HT283. A plasmid containing 461 nucleotides of the 3'-end of IS2 (pMW975) led to transcriptional initiation starting from P2 (Fig. 3). This was unexpected, and it remains to be clarified why P1 is not the initiation site in pMW975. The whole sequence of IS2 may be necessary as a signal for transcriptional initiation.

When transcription started from P2 or P3, polyamines did not influence transcriptional efficiency significantly. Thus, we could study the polyamine effect on OppA synthesis at the level of translation using E. coli MA261oppA::Km/pMW975 or pMW211. When pMW975 and pMW211 were used as the template for OppA mRNA synthesis, the size of the 5'-UTR was 266 and 171 nucleotides, respectively. Although the polyamine effect on OppA protein synthesis was slightly greater with pMW975 than with pMW211, essentially the same results were obtained with both plasmids. The results with pMW211 were only shown with fused mRNAs containing the 5'-UTR of OppA mRNA and the open reading frame of lacZ mRNA.

For initiation of protein synthesis in E. coli, the most important elements in the mRNA are the initiation codon AUG and SD sequence. The latter can base pair with the 3'-end of 16 S rRNA so that translational efficiency increases. Thus, translational efficiency decreases if the SD sequence undergoes intrastand base pairing. The polyamine effect on OppA synthesis was examined by changing the secondary structure of the SD sequence and the AUG region through site-directed mutagenesis at positions of oppA gene corresponding to the 5'-UTR of OppA mRNA. Although the secondary structure of the AUG region did not influence the polyamine stimulation of OppA protein synthesis, that of SD sequence did. When the SD sequence was loosely base paired with another region of OppA mRNA, polyamines significantly stimulated protein synthesis. When the SD sequence was strongly base paired, polyamines did not influence protein synthesis. Since polyamines bind to double-stranded RNA more strongly than to single-stranded RNA (10, 35), it is to be expected that a strongly base paired SD sequence would become further stabilized by spermidine binding. It is also noted that the disappearance of the GC-rich stem I in OppA mRNA synthesized from pMWSD2 caused no polyamine stimulation of OppA synthesis. Our results suggest that polyamines may contribute to unwinding weak secondary structure of the SD sequence through their binding to a region(s) such as stem I on the OppA mRNA close to SD sequence. However, an alternative explanation may also be possible. Experiments are in progress to determine how polyamines change the secondary structure of OppA mRNA.

In contrast, polyamines are known to inhibit some kinds of protein synthesis like ribosome modulation factor and OmpC protein (36). It is of interest to know the secondary structure of the SD sequence of these mRNAs. In case of eukaryotic protein synthesis, polyamines can regulate the initiation complex formation of Met-tRNA mRNA 40 S ribosomal subunits positively and initiation factor-dependent RNA helicase activity negatively (37). Thus, polyamine regulation of protein synthesis is dependent on the size and base composition of the 5'-UTR of the mRNA. There is a tendency that polyamines regulate
protein synthesis directed by the mRNAs having long 5'-UTR in both prokaryotes and eukaryotes. We consider that these studies will help to establish the physiological importance of polyamines.

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