Selective Structural Change by Spermidine in the Bulged-out Region of Double-stranded RNA and Its Effect on RNA Function*

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Polyamines play important roles in cell growth mainly through their interaction with RNA. We have previously reported that polyamines stimulate the synthesis of oligopeptide-binding protein OppA in Escherichia coli and the formation of Ile-tRNA in rat liver (Igarashi, K., and Kashiwagi, K. (2000) Biochem. Biophys. Res. Commun. 271, 559–564). These effects involve an interaction of polyamines with the bulged-out region of double-stranded RNA in the initiation region of OppA mRNA and in the acceptor stem of rat liver tRNA1le. In this study, the effects of polyamines on E. coli OppA synthesis and rat liver Ile-tRNA formation were compared using OppA mRNA and tRNA1le with or without the bulged-out region of double-stranded RNA. The results indicate that the bulged-out region is involved in polyamine stimulation of OppA synthesis and Ile-tRNA formation. A selective structural change by spermidine in the bulged-out region of double-stranded RNA was confirmed by circular dichroism.

Polyamines (putrescine, spermidine, and spermine) are essential for normal cell growth due to effects mainly at the level of translation (1–3). It is known that polyamines bind preferentially to double-stranded RNA rather than single-stranded RNA and double-stranded DNA (4). Indeed, polyamines were found mostly in polyamine-RNA complexes when measured in rat liver, bovine lymphocytes, and Escherichia coli (5, 6). We reported previously that polyamines have not only a sparing effect on the Mg2+ requirement of polyphenylalanine and globin synthesis but also a stimulatory effect that cannot be fulfilled by any amount of Mg2+ in the absence of polyamines (7, 8). It has also been reported that polyamines enhance, at the level of translation, the synthesis of several kinds of proteins that are important for cell growth in E. coli (3, 9–11). We propose that a group of genes whose expression is enhanced by polyamines at the level of translation be referred to as the “polyamine modulon” (3).

There are three different mechanisms underlying polyamine stimulation of the synthesis of various members of the polyamine modulon. First, polyamine stimulation of protein synthesis can occur when a Shine-Dalgarno sequence in the mRNA is obscure or is distant from the AUG initiation codon. In this case, polyamines cause structural changes in a region of the Shine-Dalgarno sequence and the AUG initiation codon of the mRNA, facilitating formation of the initiation complex, and examples include OppA, a periplasmic substrate-binding protein of the oligopeptide uptake system; FecI σ factor (σ18), for transcription of the iron transport operon; Fis, a global regulator of transcription of some growth-related genes, including those for rRNA and some tRNAs; RpoN σ factor (σ25), for transcription of genes for nitrogen metabolism; and H-NS, a transcription factor of many kinds of mRNAs, including ribosomal protein mRNAs and flagellar protein mRNAs. By a second mechanism, polyamines enhance the inefficient initiation codon UUG (or GUG)-dependent fMet-tRNA binding to Cya (or Cra) mRNA-ribosomes so that polyamines enhance the synthesis of Cya (adenylate cyclase) and Cra (a global regulator of transcription of glycolysis and glycogenesis). By a third mechanism, polyamines stimulate read-through of the amber codon UAG-dependent Gln-tRNAsupF on ribosome-associated RpoS mRNA or stimulate a +1 frameshift at the 26th UGA codon of RF2 mRNA, resulting in enhanced synthesis of RpoS σ factor (σ38) for transcription of stationary-phase genes, and RF2 (polypeptide-releasing factor 2). We also reported that rat liver Ile-tRNA formation is enhanced by polyamines (12). Among these effects, we extensively studied the mechanism of polyamine stimulation of OppA synthesis (9) and Ile-tRNA formation (12) because polyamine stimulation was most pronounced in these two systems. The results suggest that polyamines likely induce a structural change in the bulged-out region of double-stranded RNA and that this is different from changes caused by Mg2+ (9, 12). Recently, we have shown a selective conformational change in the bases in the bulged-out region of double-stranded RNA by spermidine using U6–34, a model RNA of U6 small nuclear RNA (13).
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In this work, we have studied correlations between the effects of polyamines on OppA synthesis and rat liver Ile-tRNA formation and their effects on the structure of the bulged-out regions of OppA mRNA and Ile-tRNA. We found that structural changes by spermidine in the bulged-out region of the initiation region of OppA mRNA and in the acceptor stem of rat liver tRNA^Ile are well correlated with polyamine stimulation of OppA synthesis and Ile-tRNA formation.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Culture Conditions—A polyamine-requiring mutant, E. coli MA261 oppA::Km, was prepared as described previously (14). These cells were grown at 37 °C in medium A (4 g of glucose, 7 g of K2HPO4, 3 g of KH2PO4, 500 mg of sodium citrate, 1 g of (NH4)2SO4, 100 mg of MgSO4·7H2O, 2 mg of thiamine, 10 mg of biotin, 3 mg of methionine, and 100 mg each of leucine, threonine, serine, glycine, and ornithine per liter of water) in either the presence or absence of putrescine dihydrochloride (100 μg/ml). Cell growth was monitored by measuring the absorbance at 540 nm.

Plasmids—Site-directed mutagenesis for the construction of pMW-OppA(Stem I + U) was performed using pMW-OppA (pMW975 in Ref. 14) as template and 5′-GTCCCAATCCGGTGATTACACATTGCTGG-3′ (P1) and 5′-CCAGCATGTGTAATCACCAGGTGAGAC-3′ (P2) as primers with the QuikChange site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol, i.e. PCR product was transformed into E. coli DH5α, and pMW-OppA(Stem I + U) was isolated from the cells by the standard method (15). The nucleotide sequence of the plasmids was confirmed using the CEQ8000 DNA genetic analysis system (Beckman Coulter).

Measurement of [35S]Methionine-labeled OppA—E. coli MA261 oppA::Km/pMW-OppA and MA261 oppA::Km/pMW-OppA(Stem I + U) were grown in medium A. When A540 reached 0.15, the cells were divided into aliquots of 5 ml and grown in the presence or absence of putrescine (100 μg/ml) for 10 min. Then, [35S]methionine (1 MBq) was added to each 5-ml aliquot, and the cells were allowed to grow for 20 min. They were harvested after the addition of methionine at a final concentration of 20 mM; resuspended in 1 ml of buffer containing 10 mM sodium phosphate (pH 7.4), 100 mM NaCl, 1% Triton X-100, and 0.1% SDS; and disrupted with a French press at 20,000 p.s.i. The amount of radioactive OppA was determined by counting the harvested samples in a liquid scintillation counter (Beckman). The radioactivity of labeled OppA was quantified using a BAS2000II imaging analyzer (FujiFilm).

Dot Blot Analysis—E. coli MA261 was cultured at A540 = 0.03 in the presence and absence of putrescine and harvested at A540 = 0.15. Total RNA was prepared from these cells according to the method of Emory and Belasco (17). The oppA gene was amplified by PCR using pMW-OppA as a template and 5′-GAACACCATGCTTTGCAGAAACGCTATGAA-3′ (P3) and 5′-GGAGCCAGATCCTCTCTGCCCACCTGATATTG-3′ (P4) as primers for the probe. Dot blot analysis was performed using the ECL direct nucleic acid labeling and detection system (GE Healthcare). Chemiluminescence was detected using a LAS-1000 Plus luminescent image analyzer (FujiFilm).

Purification of Rat Liver Aminoacyl-tRNA Synthetase Complex and tRNA from Rat Liver and Torula Yeast—Rat liver aminoacyl-tRNA synthetase complex was prepared as described previously (18). The bands included at least eight aminoacyl-tRNA synthetases, specific for Gln (M, 155,000), Ile (M, 145,000), Leu (M, 140,000), Met (M, 100,000), Glu (M, 92,000), Lys (M, 70,000), Arg (M, 69,000), and Asp (M, 51,000). Unfractionated tRNA was prepared from the 100,000 × g supernatant fraction of rat liver and torula yeast according to the method of Zubay (19). The tRNA^Ile was purified by successive column chromatographies of DEAE-Sephallex A-50, hydroxylapatite, and Sepharose 4B reverse salt gradient as described previously (12).

Assay for Ile-tRNA^Ile Formation—The reaction mixture (0.05 ml) for Ile-tRNA^Ile formation contained the following: 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM dithiothreitol, 2 mM ATP, 1.85 kBq of [14C]isoleucine (12.1 GBq/mmol), 5 μg of tRNA^Ile, and 0.8 μg of aminoacyl-tRNA synthetase complex. Magnesium acetate and/or spermidine was added at the concentrations shown in Fig. 2. After the reaction mixture was incubated at 30 °C for 5 min, the [14C]Ile-tRNA formed was measured as described previously (18).

Purification of the Stem I Region of OppA mRNA and the Acceptor Stem Region of tRNA^Ile—OppA Stem I (5′-CCGG-GAUUUGCAUCCAAA-3′), OppA Stem I + U (5′-CCGG-UGAUUUGCAUCCAAA-3′, in which the underlined uracil was inserted in OppA Stem I), the acceptor stem of rat liver tRNA^Ile (5′-GGCCGGUUGCGGGCAGGCCACCA-3′), and the acceptor stem of torula yeast tRNA^Ile (5′-GGUCCCUUUGAGGGCACCACCA-3′) were obtained from Hokkaido System Science. Purification of RNAs was performed by PAGE using glass plates (30 × 40 cm; Nihon Eido Co.) under denaturing conditions. RNA samples in H2O were annealed by heating at 90 °C for 5 min, followed by snap-cooling on ice. The formation of the stem-loop structure was confirmed by native PAGE.

CD Measurement—CD spectra were recorded over 200–320 nm on a Jasco J-820 spectropolarimeter using a 0.1-cm path length cuvette at 25 °C (20). Scan speed was 100 nm/min, and CD samples contained 10 mM Tris-HCl (pH 7.5), 50 mM KCl, and 50 μM Stem I region of OppA mRNA and acceptor stem region of tRNA^Ile. Where indicated, magnesium acetate and/or spermidine was added to the CD samples. Typical spectra corresponded to the average of three scans.

RESULTS

Correlation between the Spermidine Effect and the Bulged-out Region of Double-stranded RNA in OppA mRNA and Rat Liver tRNA^Ile—We previously suggested that a selective structural change in the bulged-out region of double-stranded RNA produced by spermidine, but not by Mg2+, is important for the physiological functions of polyanines (13). To clarify the importance of the structural change produced by spermidine, the effects of spermidine were measured using E. coli OppA mRNA-dependent protein synthesis (14) and rat liver Ile-tRNA formation (12). The effects of spermidine on OppA synthesis were studied using a polyamine-requiring mutant, MA261.
oppA::Km, cultured in the presence and absence of putrescine. Strain MA261 is unable to synthesize putrescine, and cell growth is slow in the absence of exogenous putrescine (14). When putrescine is added to the culture medium, it is taken up into cells and serves as a substrate for synthesis of spermidine, leading to recovery of cell growth. The effective concentration of spermidine that influences RNA function is about one-twentieth that of putrescine (8). Thus, it is thought that the effects of polyamines (putrescine plus spermidine) are due mainly to spermidine. OppA synthesis was measured by immunoprecipitation using an anti-OppA antibody; the level of OppA mRNA was measured by dot blotting. As shown in Fig. 1B, polyamines enhanced OppA synthesis from wild-type OppA mRNA by 5.1-fold. This occurred mainly at the level of translation because the level of OppA mRNA was unaffected by the addition of putrescine to the medium (Fig. 1C). It has been suggested that spermidine produces a structural change in the bulged-out region of Stem I in OppA mRNA (Fig. 1A) and that this underlies spermidine stimulation of OppA synthesis (9). To study this further, the Stem I structure of OppA mRNA was modified to make intact double-stranded RNA (Stem I+U) (Fig. 1A). When OppA Stem I+U mRNA was used instead of wild-type mRNA, OppA synthesis in the absence of polyamines was enhanced, and the degree of polyamine stimulation was reduced from 5.1- to 1.5-fold (Fig. 1B). The results strongly suggest that a structural change in the bulged-out region of double-stranded RNA by spermidine is important for spermidine stimulation of OppA synthesis.

Similar results were obtained with Ile-tRNA formation in a cell-free system. We previously reported that polyamine stimulation of rat liver Ile-tRNA formation correlates with a structural change in the acceptor stem by polyamines (12). It is known that the acceptor stem of tRNAIle from rat liver has a bulged-out region, whereas that of tRNAIle from torula yeast does not (Fig. 2A). Thus, the effects of spermidine on Ile-tRNA formation by Ile-tRNA synthetase were compared using these two tRNAIle in the presence of spermidine and/or Mg2+ (Fig. 2B). As shown in Fig. 2B, Mg2+ was essential for Ile-tRNA formation from rat liver and torula yeast tRNAIle. Spermidine by itself did not stimulate Ile-tRNA formation, presumably because an isoleucyl-AMP-enzyme complex cannot be formed in the absence of Mg2+ (21). In the presence of 3 mM Mg2+, spermidine enhanced Ile-tRNA formation from rat liver tRNAIle, but had little effect on Ile-tRNA formation from torula yeast tRNAIle (Fig. 2, B and C). The results support the idea that a structural change in the bulged-out region of double-stranded RNA by spermidine is involved in the physiological function of polyamines. Similar results were obtained when assays were carried out in the presence of 100 mM K+ instead of 50 mM K+ (data not shown).

CD Analysis of the Stem I Region of OppA mRNA and the Acceptor Stem of tRNAIle—Structural changes in OppA Stem I and OppA Stem I+U induced by spermidine were studied by CD in the presence of 10 mM Tris-HCl (pH 7.5) and 50 mM KCl. With the OppA Stem I mRNA, a substantial increase in the relative intensity of the negative band at 208 nm, reflecting stabilization (or increase) of A-form double-stranded RNA (20),

**FIGURE 1.** Effect of polyamines on OppA synthesis in E. coli MA261. A, optimal computer folding of the initiation region (−65 to +65) of OppA mRNAs was performed by the method of Zuker and Stiegler (36). A uridine nucleotide was added to the Stem I structure to make intact double-stranded RNA (Stem I+U). AUG is the initiation codon. WT, the original wild-type initiation region of OppA mRNA; SD, Shine-Dalgarno sequence. B, OppA synthesis was measured as described under “Experimental Procedures.” The relative amount of OppA synthesized was quantified using a BAS2000II imaging analyzer. Values are the means ± S.E. for three determinations. C, dot blot analysis of OppA mRNA was performed as described under “Experimental Procedures.” The ratio (+/−) of the level of OppA mRNA indicates the ratio of chemiluminescence of the dots of RNA obtained from cells cultured with or without putrescine (PUT). Experiments were repeated three times, and the results were reproducible.
was observed in the presence of spermidine with or without Mg$^{2+}$, whereas Mg$^{2+}$ by itself had little effect (Fig. 3, B and C). In contrast, with the OppA Stem I+U mRNA, shifts in the negative band at 208 nm were small in the presence of spermidine (Fig. 3, F and G). The $K_d$ values for spermidine of OppA Stem I and OppA Stem I+U were 0.45 and 0.25 mM, respectively, confirming that spermidine binds to OppA Stem I+U more effectively than to OppA Stem I (Fig. 3, D and H). These results support the idea that the structure of the bulged-out region of OppA Stem I is selectively changed by spermidine.

Similar results were seen in CD studies of the acceptor stem of tRNA$^{\text{Ile}}$. A substantial increase in the relative intensity of the negative band at 208 nm in the acceptor stem of rat liver tRNA$^{\text{Ile}}$ was observed in the presence of spermidine with or without Mg$^{2+}$, whereas Mg$^{2+}$ by itself had a much smaller effect (Fig. 4, B and C). In contrast, spermidine produced only a small shift in the negative band at 208 nm in the acceptor stem of torula yeast tRNA$^{\text{Ile}}$ (Fig. 4, F and G). The $K_d$ values for spermidine of the acceptor stem of rat liver and torula yeast tRNA$^{\text{Ile}}$ were 0.40 and 0.26 mM, respectively, indicating that spermidine binds to the acceptor stem of torula yeast tRNA$^{\text{Ile}}$ more effectively than to that of rat liver tRNA$^{\text{Ile}}$. When the concentration of KCl was increased from 50 to 100 mM, essentially the same results were obtained in CD studies of both OppA and tRNA$^{\text{Ile}}$ (data not shown).

**DISCUSSION**

In this study, we carried out experiments to clarify how spermidine influences the structure of the bulged-out region of double-stranded RNA. In the bulged-out regions of both OppA Stem I and the acceptor stem of rat liver tRNA$^{\text{Ile}}$, spermidine produced structural changes that correlated with its biological effects. A selective structural change in the bulged-
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With regard to the proliferative effects of polyamines in eukaryotic cells, it is assumed that both the stimulation of protein synthesis by polyamines (23) and the function of active elf5A (eukaryotic translation initiation factor 5A) (24) are involved in cell proliferation; elf5A contains hypusine (Nε-(4-amino-2-hydroxybutyl)lysine) derived from spermidine. It has been recently shown that a decrease in either active elf5A or polyamines inhibits cell growth, indicating that elf5A and polyamines are independently involved in cell growth (25, 26). Mammalian cells contain spermine together with spermidine, and the effective concentration of spermine that influences RNA function is about one-fifth that of spermidine (8). We found that spermine, at lower concentrations than spermidine, also stabilized the bulged-out region of double-stranded RNA.

Polyamines have not only a sparing effect on the Mg$^{2+}$ requirement of polyphenylalanine and globin synthesis but also a stimulatory effect that cannot be fulfilled by any amount of Mg$^{2+}$ in the absence of polyamines (7, 8). One effect of polyamines is to bind to intact double-stranded RNA (approximate $K_d = 0.25$ mM), an interaction similar to that of Mg$^{2+}$ with RNA. Because levels of polyamines, but not of Mg$^{2+}$, change during the cell cycle (22), an increase in polyamine content during cell proliferation is presumably important to stabilize the structure of double-stranded RNA. A second effect of polyamines involves binding to the bulged-out region of double-stranded RNA (approximate $K_d = 0.4$ mM), and this represents an apparently unique effect of polyamines because Mg$^{2+}$ and K$^+$ cannot stabilize the bulged-out region of double-stranded RNA. These two effects likely represent the key roles for polyamines in prokaryotic cells (2, 3). Polyamines stimulate the growth of prokaryotic cells by enhancing the synthesis of specific kinds of protein that are important for cell growth. To date, we have found that the synthesis of nine kinds of proteins encoded by the polyamine modulon is enhanced by polyamines at the level of translation. It seems likely that a structural change in the bulged-out region of double-stranded RNA underlies polyamine stimulation of the synthesis of these proteins. Polyamine content is high when cell growth is rapid in both prokaryotic and eukaryotic cells (1, 2, 22). Accordingly, polyamine regulation of protein synthesis is a rational mechanism because the proteins involved in cell proliferation are synthesized when they are required in response to elevated levels of polyamines. RNA.$^2$ Thus, together with spermidine, spermine probably plays a predominant role in stabilizing RNA in mammalian cells.

It has been recognized that spermine and spermidine associate with and stabilize DNA oligonucleotides (27–29). However, many published reports are from studies carried out using relatively high concentrations of polyamines. The binding of spermine and spermidine to E. coli 16 S ribosomal RNA in the presence of 2 mM Mg$^{2+}$ and 100 mM K$^+$ was ~5–6-fold higher than that of calf thymus DNA (5). It is thought that the weaker binding of polyamines to DNA than to RNA may be due to a difference in the B- and A-forms of double-stranded nucleotides, respectively. The average distance between two adjacent phosphates of B-form DNA (Protein Data Bank code 1D29) is 6.65 Å (6.16–7.44 Å) for the major groove and 7.35 Å (6.52–8.35 Å) for the minor groove, and that of A-form RNA (Protein Data Bank code 157D) is 6.35 Å (4.97–7.61 Å) for the major groove and 5.20 Å (4.20–5.98 Å) for the minor groove. On the hand, the N-N distances of dianinobutane and dianinopropionamide moieties in all-trans-spermidine are ~6.2 and 5.0 Å, respectively (30). Actually, immunoelectron microscopy study of polyamines using monoclonal antibody against spermidine has indicated that polyamines are located predominantly on polysomes and ribosomes (31). Furthermore, the presence of spermidine in the crystal of a DNA/RNA duplex with unpaired ribonucleotides, with spermidine lying along the RNA strands, close to the phosphate groups, has been reported (32). This also supports the idea that polyamines bind preferentially to RNA.

Norspermidine (NH$_2$(CH$_2$)$_3$NH(CH$_2$)$_3$NH$_2$) and homospermidine (NH$_2$(CH$_2$)$_4$NH(CH$_2$)$_4$NH$_2$) are present in algae and...

$^2$K. Higashi, Y. Terui, A. Suganami, Y. Tamura, K. Nishimura, K. Kashiwagi, and K. Igarashi, unpublished data.
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thermophilic eubacteria, respectively, together with spermidine (NH₂(CH₂)₃NH(CH₂)₄NH₂) (33–35). Polyamine stimulation of OppA synthesis was observed at 30–42 °C, suggesting that the possible secondary structure of the initiation region of OppA mRNA is maintained at 30–42 °C. At 30–42 °C, norspermidine is probably able to bind to the minor groove of A-form RNA with similar efficiency compared with spermidine. Homospermidine may be easily bound to the flexible RNA at high temperature because the N-N distance (6.2 Å) is longer compared with that of norspermidine (5.0 Å). The data obtained thus far strongly suggest that polyamines function mainly through interaction with RNA and that selective structural change in the bulged-out region of double-stranded RNA by spermidine is important for the physiological functions of polyamines.

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