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Preferential Stimulation of the in Vivo Synthesis of a Protein by Polyamines in *Escherichia coli*: Purification and Properties of the Specific Protein†

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ABSTRACT: The possibility that polyamines can stimulate the synthesis of special kinds of proteins has been examined by using a polyamine-requiring mutant of *Escherichia coli*. It was found that the synthesis of some proteins, particularly one with a molecular weight (M_r) of 62K, was significantly stimulated following polyamine supplementation of polyamine-starved cells. The preferential stimulation of the synthesis of this polyamine-induced protein of M_r 62K (PI protein) was followed by the stimulation of overall protein synthesis by polyamines. PI protein was purified to homogeneity and some of its properties were examined. From studies on the effect of PI protein on MS2 RNA directed protein synthesis, it was shown that this protein stimulated the synthesis of RNA replicase by 2.2-fold in the presence of 1 mM spermidine.

Polyamines have been implicated in numerous growth processes (Cohen, 1971; Tabor & Tabor, 1976). Since polyamines are polycationic, they probably participate in many cellular processes through their binding to DNA, RNA, ribosomes, and phospholipid (Igarashi et al., 1982). It is well-known that polyamines exhibit not only a sparing effect on the Mg^{2+} requirement for polypeptide synthesis but also a stimulating effect, which cannot be equalled by any amount of Mg^{2+} (Igarashi et al., 1974; Atkins et al., 1975; Hunter et al., 1977). The stimulation of polypeptide synthesis by polyamines occurs mainly due to a stimulation of the initiation complex formation of polypeptide synthesis (Konecki et al., 1975; Salden & Bloemendal, 1976; Igarashi et al., 1980a). In addition, we have recently shown that, in QB RNA and MS2 RNA directed protein synthetic systems, spermidine caused a marked stimulation in the synthesis of RNA replicase without a significant effect on the coat protein (Watanabe et al., 1981). Therefore, we have examined the possibility of polyamines enhancing the synthesis of special kinds of proteins in vivo using a polyamine-requiring mutant of *Escherichia coli*.

In this paper, we present evidence for the preferential synthesis of a M_r 62K protein under the influence of polyamines, along with its purificatiion and properties.

Materials and Methods

Bacterial Strain and Culture Conditions. A polyamine-requiring mutant of *E. coli* used in this experiment was MA261 (*thr*, *leu*, *ser*, *thi*, *speB*, *speC*), which was kindly supplied by Dr. W. K. Maas. The cells were cultured in the presence or absence of 100 μg/mL putrescine (media A and B, respectively) by the method of Algranati et al. (1975). When growth was sufficient to give an absorbance of 0.45 at 540 nm, the cells were harvested by centrifugation at 15000*g* for 15 min. About 7 and 15 h of incubation were required when media A and B were used, respectively. The cells were washed once with a buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 60 mM NH_4Cl, and 6 mM 2-mercaptoethanol, centrifuged as above, and stored at −80 °C until used. The cells cultivated in media A and B were named, tentatively, unstarved and starved bacteria, respectively.

Materials. The fractions containing elongation factors and aminoacyl-tRNA synthetases (S-100 protein) and 0.3 M NH_4Cl washed ribosomes were prepared from *E. coli* Q13 as described previously (Watanabe et al., 1981). Initiation factor 2 was purified according to the method of Hershey et al. (1977). Bacteriophage MS2 was grown and purified according to the method described by Loeb & Zinder (1961). MS2 RNA was prepared from the phage by phenol extraction and alcohol precipitation according to the procedure of Gierer & Schramm (1956). The ribosomal S1 protein was purified as described previously (Igarashi et al., 1981) according to the

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method developed by Tal et al. (1972) and Carmichael (1975).

Assay of MS2 RNA Directed Polypeptide Synthesis. The polypeptide synthesis was performed as described previously (Watanabe et al., 1981) except that 0.3 M NH_4Cl washed ribosomes were dialyzed before use against a buffer containing 10 mM Tris-HCl (pH 7.5), 60 mM NH_4Cl, 0.1 mM magnesium acetate, and 6 mM 2-mercaptoethanol to remove the polyamine-induced protein of M_r 62K (PI protein[1]). To quantify the amount of [^{35}S]methionine incorporated into RNA replicase, A protein, or coat protein, sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and fluorography were performed by the methods of Laemmli (1970) and Bonner & Laskey (1974), respectively. The radioactivity in each protein band was measured as described previously (Watanabe et al., 1981).

One-Dimensional Electrophoretic Analysis of Proteins from Starved and Unstarved Bacteria. E. coli MA261 was grown in the absence of putrescine until absorbance at 540 nm reached 0.10. Then, the cell suspension was divided into 5-mL aliquots and the culture was continued with or without 100 μg/mL putrescine. At the designated time, 15 μCi of [^{14}C]lysine (292 mCi/mmol) was added to the cell suspension and the culture was continued for 5 min. The labeling of protein was terminated by the addition of an equal volume of 10% trichloroacetic acid. Electrophoresis and fluorography were performed as described above.

Two-Dimensional Electrophoresis of Proteins from Starved and Unstarved Bacteria. E. coli MA261 was grown as described above except that the methionine concentration was 3 μg/mL instead of 100 μg/mL in the presence or absence of 100 μg/mL putrescine until absorbance at 540 nm reached 0.20. [^{35}S]Methionine (20 μCi, 1015 Ci/mmol) was then added to the cell suspension and the culture was continued for 5 min more. Preparation of samples, first-dimension isoelectric focusing, and second-dimension electrophoresis were carried out according to the method of O'Farrell (1975).

Separation of Protein-Synthesizing Ribosomes by Sucrose Density Gradient Centrifugation. MS2 RNA directed polypeptide synthesis was performed as described previously (Watanabe et al., 1981) except that the reaction mixture (2 mL) contained 20 μCi of [^{3}H]leucine (132 Ci/mmol) and 200 μM methionine instead of 12 μCi of [^{35}S]methionine and 200 μM leucine. The concentrations of Mg^{2+} and spermidine were 6 and 1 mM, respectively. After incubation at 37 °C for 10 min, sparsomycin was added at a final concentration of 25 μM to inhibit polypeptide synthesis. The reaction mixture was then layered on 32 mL of 10–30% sucrose gradient in 10 mM Tris-HCl (pH 7.5), 3 mM magnesium acetate, 100 mM NH_4Cl, and 6 mM 2-mercaptoethanol. The tube was centrifuged in a Hitachi RPS-27 rotor for 6 h at 27000 rpm, and 30-drop fractions were collected from the bottom of the tube. The hot trichloroacetic acid insoluble radioactivity was measured by using a 80-μL aliquot of each fraction. Absorbance at 260 nm of each fraction was measured after a 80-fold dilution with water. Under these conditions, ribosomes that do not have nascent peptidyl-tRNA were dissociated into 30S and 50S ribosomal subunits.

Results

Stimulation of the Synthesis of M_r 62K Protein (PI Protein) by Polyamines in a Polyamine-Requiring Mutant. The polyamine-deficient strain MA261 grew at decreased rate in the absence of putrescine (Figure 1). About a 2-fold increase in growth rate was observed after a 30-min lag period upon the addition of 100 μg/mL putrescine. In order to examine whether polyamines can stimulate the synthesis of some particular proteins, the radioactive proteins synthesized by starved and unstarved bacteria were analyzed by SDS–polyacrylamide gel electrophoresis. The labeling of proteins was performed by incubation with [^{14}C]lysine for 5 min at the designated time. As shown in Figure 2, the synthesis of some proteins (M_r 72K, 62K, and 57K), especially that of M_r 62K protein (PI protein), was stimulated by putrescine. When [^{14}C]lysine was added together with putrescine at $0.10A_{540}$, the synthesis of PI protein as well as that of total proteins was not stimulated significantly (Figure 2, 1S and 1U). The stimulation of the synthesis of PI protein occurred as early as 10 min after the addition of putrescine to the medium (Figure 2, 2S and 2U). This period is very similar to that (7 min) required for the appearance of the effect of polyamines on DNA synthesis in another polyamine-requiring mutant of *E. coli* (Geiger & Morris, 1980). The radioactivity incorporated

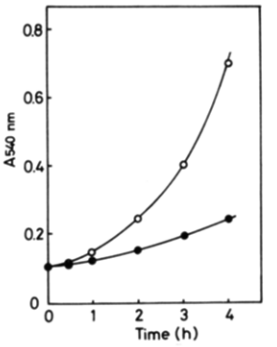

FIGURE 1: Growth of a polyamine-requiring mutant (MA261) in the presence and absence of putrescine. Cells were cultured as described under Materials and Methods. (○) 100 μg/mL putrescine; (●) no putrescine.

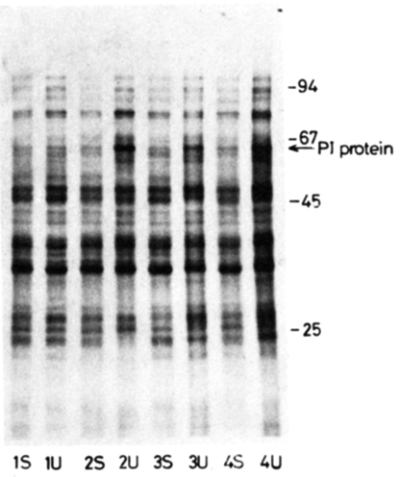

FIGURE 2: Stimulation of the synthesis of PI protein by putrescine. Bacterial culture, electrophoresis, and fluorography were performed as described under Materials and Methods. Equal amounts of radioactivity (50000 cpm) were applied to all lanes. 1S, [^{14}C]lysine was added at A_{540} = 0.10; 1U, putrescine and [^{14}C]lysine were added at A_{540} = 0.10; 2S, [^{14}C]lysine was added at 10 min after A_{540} reached 0.10; 2U, putrescine was added at A_{540} = 0.10 and [^{14}C]lysine was added at 10 min after A_{540} reached 0.10; 3S, [^{14}C]lysine was added when A_{540} reached 0.15; 3U, putrescine was added at A_{540} = 0.10 and [^{14}C]lysine was added when A_{540} reached 0.15; 4S, [^{14}C]lysine was added when A_{540} reached 0.20; 4U, putrescine was added at A_{540} = 0.10 and [^{14}C]lysine was added when A_{540} reached 0.20. Numbers on the right represent $M_r \times 10^{-3}$. PI protein is polyamine-induced protein.

[1] Abbreviations: PI protein, polyamine-induced protein of M_r 62K; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

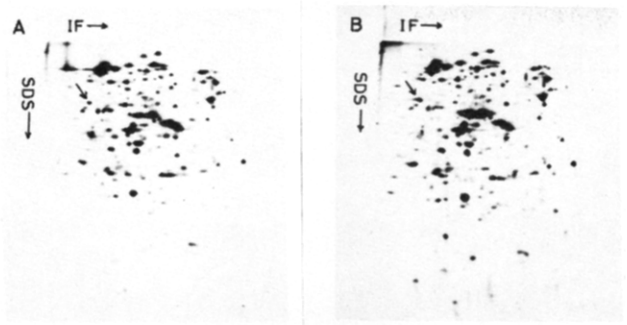

FIGURE 3: Identification of polyamine stimulation of the synthesis of PI protein by two-dimensional electrophoresis. Electrophoresis and fluorography were performed as described under Materials and Methods. (A) Protein from starved bacteria; (B) protein from unstarved bacteria. The same amounts of radioactivity (300000 cpm) were used in (A) and (B). Arrows indicate PI protein.

into total proteins was stimulated 1.2-fold by putrescine at this point. When absorbance at 540 nm reached 0.15, the stimulation by putrescine of total protein synthesis became 1.8-fold and the preferential stimulation of the synthesis of PI protein was still observed (Figure 2, 3S and 3U). These results show that the preferential stimulation of PI protein by putrescine was followed by the stimulation of overall protein synthesis by the polyamine. Similar results were obtained by the addition of spermidine (50 μg/mL) instead of putrescine (100 μg/mL). The [^{35}S]methionine-labeled proteins from starved and unstarved bacteria were also analyzed by two-dimensional electrophoretic analysis (Figure 3). When spots which migrated near 62K were examined, only one spot (indicated by the arrow in Figure 3) showed increased synthesis in the presence of polyamines. Therefore, this protein was identified as PI protein. PI protein was basic in nature and not strongly labeled with [^{35}S]methionine compared with that labeled with [^{14}C]lysine. Although one more spot just under PI protein showed increased synthesis, the molecular weight of the protein was 57K. It was also observed that some spots which migrate near 62K showed decreased synthesis in the presence of polyamines.

Purification of PI Protein. In order to purify the PI protein, intracellular localization and chromatographic behavior of PI protein were studied. As shown in Figure 4, this protein was found in the 100000*g* supernatant as well as in the crude initiation factor fraction and was not adsorbed by DEAE-Sephadex A-50 in the presence of 50 mM KCl. Since PI protein was a main protein in the 50 mM KCl eluate of the 100000*g* supernatant from DEAE-Sephadex A-50 column chromatography, the 100000*g* supernatant from unstarved *E. coli* MA261 was routinely employed as the source for purification of PI protein.

All purification steps were carried out at 4 °C. Purification was started from 2340 mg of protein of the 100000*g* supernatant. One-third of the protein (780 mg) was dialyzed against buffer I (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 6 mM 2-mercaptoethanol, 50 mM KCl, and 10% glycerol), applied to a column of DEAE-Sephadex A-50 (4 × 20 cm) previously equilibrated with buffer I, and eluted with the same buffer. The fractions that contained PI protein (as determined by SDS–polyacrylamide gel electrophoresis) were concentrated by ultrafiltration and dialyzed against buffer I. Proteins from three batches of the above purification step were combined (160 mg of protein) and one-fifth of the protein (32 mg) was applied to a column of Sephadex G-100 (2 × 100 cm), equilibrated with buffer I. The protein was eluted with the same buffer (3-mL fractions; flow rate, 0.3 mL/min). The

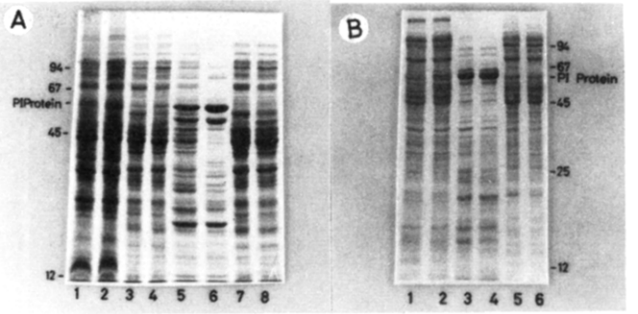

FIGURE 4: Intracellular localization and chromatographic behavior of PI protein. Electrophoresis was carried out as described under Materials and Methods. Proteins were stained with Coomassie brilliant blue R-250. Crude initiation factors were prepared as described previously (Igarashi et al., 1980b). (A) 1, total protein from starved bacteria (S); 2, total protein from unstarved bacteria (U); 3, 10000*g* supernatant (S-100) from S; 4, S-100 from U; 5, 50 mM KCl eluate of DEAE-Sephadex A-50 column chromatography (DEAE-chromato) of S-100 from S; 6, 50 mM KCl eluate of DEAE-chromato of S-100 from U; 7, 300 mM KCl eluate of DEAE-chromato of S-100 from S; 8, 300 mM KCl eluate of DEAE-chromato of S-100 from U. (B) 1, crude initiation factors (IF) from S; 2, IF from U; 3, 50 mM KCl eluate of DEAE-chromato of IF from S; 4, 50 mM KCl eluate of DEAE-chromato of IF from U; 5, 300 mM KCl eluate of DEAE-chromato of IF from S; 6, 300 mM KCl eluate of DEAE-chromato of IF from U.

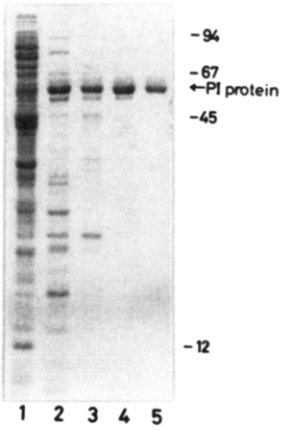

FIGURE 5: Sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis of proteins in each purification step. Electrophoresis was carried out as described under Materials and Methods. 1, 10000*g* supernatant; 2, DEAE-Sephadex A-50 (I) fraction; 3, Sephadex G-100 fraction; 4, DEAE-Sephadex A-50 (II) fraction; 5, Affi-Gel Blue fraction.

fractions that contained PI protein were concentrated by ultrafiltration and dialyzed against buffer II (20 mM Tris-HCl, pH 8.0, 1 mM EDTA, 6 mM 2-mercaptoethanol, 20 mM KCl, and 10% glycerol). Proteins from five batches of the preceding step were combined (72 mg of protein) and applied to a column of DEAE-Sephadex A-50 (2.5 × 12 cm) previously equilibrated with buffer II. The protein was eluted with a linear gradient of 20–150 mM KCl in buffer II. A total of 400 mL of the buffer was used for the gradient elution. The fractions containing PI protein were concentrated by ultrafiltration and dialyzed against buffer I. Twelve milligrams of proteins from the above step was applied to a column of Affi-Gel Blue (2 × 15 cm, Bio-Rad) previously equilibrated with buffer I. The protein was eluted with a linear gradient of 50–200 mM KCl in buffer I. A total of 200 mL of the buffer was used for the gradient elution. The PI protein was eluted at 80 mM KCl and concentrated by ultrafiltration. The amounts of PI protein obtained were 4 mg.

The most purified preparation was found to be homogeneous, as illustrated in Figure 5 showing the polyacrylamide

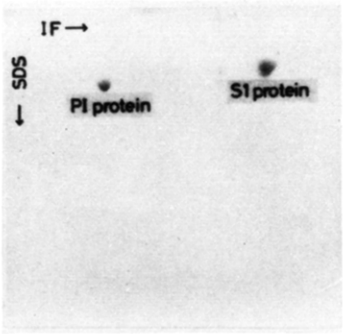

FIGURE 6: Two-dimensional gel electrophoresis of PI protein. Two-dimensional gel electrophoresis of purified PI protein and ribosomal S1 protein was performed as described under Materials and Methods.

Table I: Effect of PI Protein on MS2 RNA Directed Protein Synthesis with or without Spermidine[a]

PI protein (μg)	spermidine (mM)	[35S]methionine incorporated (cpm) coat protein	A protein	RNA replicase
0	0	80374	4496	669
	1	81731	4600	3164
1	0	80203	4269	751
	1	81348	4559	4886
2	0	82745	4185	891
	1	82799	4409	7010

[a] MS2 RNA directed protein synthesis was performed as described under Materials and Methods.

gel electrophoresis of samples from each purification step. The homogeneity of purified PI protein was confirmed by two-dimensional gel electrophoresis (Figure 6). When the purified protein was subjected to two-dimensional gel electrophoresis with pulse-labeled *E. coli* MA261 protein, it migrated as a single spot with the polyamine-induced protein observed in Figure 3.

Effect of PI Protein on Protein Synthesis. The differential effect of polyamines on the ratio of synthesis of various proteins directed by polycistronic mRNA (Watanabe et al., 1981) was recently demonstrated in our laboratory. Hence, using this model system, we have examined the spermidine stimulation of the synthesis of three kinds of proteins in the presence of various amounts of PI protein using MS2 RNA as a mRNA. The supernanant protein (S-100 protein) was essentially free of PI protein since this protein was removed by DEAE-cellulose column chromatography. To deplete PI protein from 0.3 M NH_4Cl washed ribosomes, the ribosomes were dialyzed against a low Mg^{2+} buffer as described under Materials and Methods. However, a small amount of PI protein still remained in the ribosomes. As shown in Table I, the stimulation of RNA replicase synthesis by 1 mM spermidine increased from 4.7- to 7.9-fold by the addition of 2 μg of PI protein. Thus, PI protein could stimulate RNA replicase synthesis by 2.2-fold in the presence of 1 mM spermidine. The synthesis of coat protein and A protein was not significantly influenced by PI protein.

Since PI protein was found in both the 100000*g* supernatant and the crude initiation factor fraction (1 M NH_4Cl ribosomal wash), the distribution of PI protein on ribosomes was examined by separating protein-synthesizing ribosomes and resting ribosomes. As shown in Figure 7, no IF-2α and IF-2β (M_r 118K and 90K, respectively) and PI protein were observed in protein-synthesizing ribosomes, but large amounts of IF-2 and PI protein were observed in 30S ribosomal subunits. It is

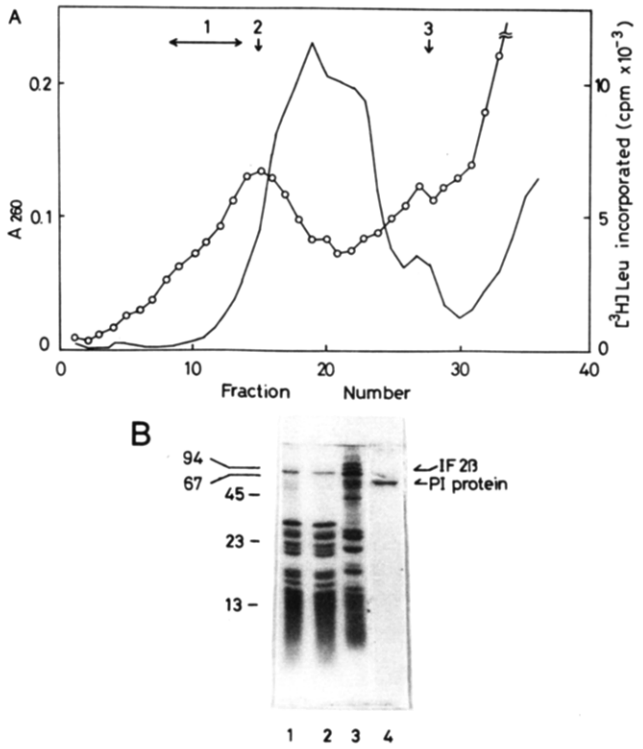

FIGURE 7: Nonexistence of PI protein in protein-synthesizing ribosomes. (A) Separation of protein-synthesizing ribosomes by sucrose density gradient centrifugation. Protein-synthesizing ribosomes were separated as described under Materials and Methods. (—) A_{260}; (O) amounts of [3H]leucine incorporated into hot trichloroacetic acid insoluble materials. (B) Sodium dodecyl sulfate–polyacrylamide slab gel electrophoresis of ribosomes: 1, protein-synthesizing ribosomes [fractions 8–14 of (A)]; 2, protein-synthesizing ribosomes [fraction 15 of (A)]; 3, 30S ribosomal subunits [fraction 28 of (A)]. Numbers on the right represent $M_r \times 10^{-3}$.

well-known that all initiation fractors are released from ribosomes after the completion of initation (Grunberg-Manago & Gros, 1977). Therefore, the above results suggest that PI protein may be released from ribosomes at the early stage of protein synthesis.

Discussion

The data presented show that the synthesis of M_r 62K protein (PI protein) was clearly stimulated by putrescine in a polyamine-requiring mutant of *E. coli* and the effect of putrescine on the synthesis of PI protein appeared significant even when total protein synthesis was not yet stimulated by putrescine. Therefore, we have presumed that this protein may play some roles for the stimulation of protein synthesis by polyamines. Although PI protein was not removed from the ribosomes completely, the addition of this protein to the MS2 RNA directed protein synthetic system caused the stimulation of RNA replicase synthesis by 2.2-fold in the presence of 1 mM spermidine. This suggests the potential of an amplification system in which polyamines first stimulate the synthesis of a particular 62K protein that then acts synergistically with polyamaines to stimulate the synthesis of other proteins. The stimulation of the synthesis of PI protein by polyamines was also observed in another polyamine-requiring mutant of *E. coli* (EWH319), which was constructed by Hafner et al. (1979).

In the presence of 2 μg of PI protein and 1 mM spermidine, the amount of RNA replicase synthesized in the MS2 RNA directed protein synthetic system was about 8.5% of coat protein synthesized. On the other hand, the amount of RNA replicase synthesized during the first 30 min after MS2 phage infection was about 15–30% of coat protein synthesized (un-

published data; Viñuela et al., 1967). Therefore, another unknown factor or factors may be necessary for the maximum synthesis of the replicase in addition to PI protein and spermidine. Otherwise, coat protein synthesized may inhibit the synthesis of RNA replicase more strongly in a in vitro system than in vivo. It also remains to be resolved whether or not this protein is absolutely necessary for the polyamine stimulation of protein synthesis since active ribosomes lacking PI protein have not been obtained thus far.

Recently, we have observed that the synthesis of T7 RNA polymerase and $\beta\beta'$ subunits of RNA polymerase was greatly stimulated by spermidine in the T7 DNA and $\lambda rif^{d}18$ DNA directed protein synthetic systems (Watanabe et al., 1983). The synthesis of T7 RNA polymerase was also stimulated by the addition of PI protein when dialyzed ribosomes were used (unpublished data).

Evidence has been adduced in a recent report for the possible existence of a factor or factors that enhanced polyamine stimulation of polyphenylalanine synthesis in an *E. coli* cell-free system (Rosano et al., 1983). Although this putative factor has not been purified yet, presumably it is similar to or identical with the PI protein described here.

It is of interest to know the molecular mechanism of how PI protein can stimulate the synthesis of RNA replicase in the presence of spermidine in a MS2 RNA directed protein synthesis. Experiments are now in progress to elucidate the above point.

Acknowledgments

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Registry No. Spermidine, 124-20-9; RNA replicase, 9026-28-2.

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