JOURNAL OF BACTERIOLOGY, Aug. 1984, p. 647–651
0021-9193/84/080647-05$02.00/0

Vol. 159, No. 2

Positive Control of Expression of the *argECBH* Gene Cluster In Vitro by Guanosine 5′-Diphosphate 3′-Diphosphate

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Received 13 January 1984/Accepted 21 May 1984

By using a cell-free system derived from *Escherichia coli*, it was found that guanosine 5′-diphosphate 3′-diphosphate (ppGpp) was a positive effector for expression of both wings of the bidirectionally transcribed *argECBH* gene cluster. A 7- to 20-fold increase in the synthesis of both argininosuccinase (the *argH* enzyme) and *N*-acetylornithinase (the *argE* enzyme) resulted with added ppGpp (0.2 mM optimum). Synthesis of hybridizable *argECBH* mRNA was enhanced only 30 to 100% by added ppGpp. Of the various guanosine nucleotides tested, only pppGpp mimicked ppGpp. Added ppGpp had no important effect upon (i) measurable *argE* or *argH* enzyme activity, (ii) total protein synthesis in the cell-free system, or (iii) the rate of decay of hybridizable *argECBH* mRNA. With extracts of an *argR*$^+$ strain, added ppGpp had no effect on the repression of enzyme or mRNA synthesis by L-arginine. By using a two-stage system in which the bulk of *argECBH* mRNA was synthesized while protein synthesis was delayed, we showed that ppGpp acted at some point during transcription.

Arginine is synthesized in *Escherichia coli* by eight enzymes coded for by nine genes. Four of the genes, *argECBH*, are clustered and are transcribed divergently from an internal control region between *argE* and *argC* (3). The *argECBH* cluster is negatively controlled by arginine and a repressor protein coded for by the *argR* gene (3). Previous work has shown that repression by arginine of *argE* and *argH* enzyme synthesis is discoordinate from and much greater than repression of *argECBH* mRNA synthesis (9, 10, 12). In the accompanying paper, we demonstrate cell-free synthesis and repression by arginine of the *argE* and *argH* gene products and of *argECBH* mRNA (18). We also showed that uncoupling of transcription and translation in the in vitro system resulted in the synthesis of small *argECBH* mRNA and much reduced enzyme synthesis. We interpret this to indicate translation-sensitive transcription termination sites in the *argECBH* gene cluster (18).

The cell-free system has allowed us to explore the role of another regulatory element, guanosine 5′-diphosphate 3′-diphosphate (ppGpp), in the expression of the *argECBH* cluster. The nucleotide ppGpp, first described in 1969 by Cashel (1), is now well known as a regulatory molecule promoting the stringent response (5). This appears to be a cellular control mechanism, coordinating cell metabolism with protein synthesis. But ppGpp has also been shown to enhance cell-free enzyme synthesis from the *lac*, *his*, *ara*, and *trp* operons (11, 13, 17). Reports of the effect of ppGpp on arginine biosynthetic genes are conflicting, for it has been shown to inhibit *argE* (17) and stimulate *argA* expression in vitro (7). We report here that ppGpp added to an in vitro system increases the expression of the *argECBH* cluster in both the leftward and the rightward directions. We also present evidence that the ppGpp effect is distinct from regulation by the *argR* protein-arginine system and that ppGpp exerts an effect at some point during transcription of the *argECBH* region.

MATERIALS AND METHODS

Bacteria and bacteriophage. *E. coli* RM179, which is *trp* Δ*lac* Δ(*ppc-argECBH*) Rif^r *rpsL argR*, and RM178, which is *trp* Δ*lac* Δ(*ppc-argECBH*) Rif^r *strA argR*$^+$ (both from N. Kelker), are deleted for the *argECBH* region and were used to prepare *argR* and *argR*$^+$ S30 extracts, respectively. Both *E. coli* strains used were *relA*$^+$ (5). The *argR* mutation renders whole cells and extracts insensitive to arginine repression because of an altered *argR* repressor protein. *E. coli* C600Rgdel, which is *met* Δ(*ppc-argECBH*) *rpsL*, (from N. Glansdorff), was made doubly lysogenic for the bacteriophages φ80 d*ppc argECBH* and φ80 (from N. Kelker) or singly lysogenic for φ80 and was used for production of these bacteriophages. Strain N49, which is Hfr *met* Δ(*ppc-argECBH*) (λ *c*I857 *S7*) (λ *c*I857 *S7* d*ppc argECBH14*) (from N. Glansdorff) and is doubly lysogenic for bacteriophage λ and λ d*argECBH*, and strain P603, which is singly lysogenic for λ *c*I857 *S7* (our collection), were used for production of these bacteriophages. Strain QD5003, which is SuIII$^+$ and λ sensitive (from J. Zissler), was used for plaque assay of phage carrying the supressible *S7* mutation.

Growth of bacteria and preparation of extracts. Bacteria were grown for preparation of extracts, and cell extracts were prepared as described in the accompanying report (18).

Preparation of bacteriophages and bacteriophage DNA. Bacteriophages were prepared from lysogenic strains of *E. coli* grown in four 1-liter lots for λ, and in a 10-liter lot for φ80, in a New Brunswick fermenter as described previously (9, 10, 12). Strains N49 and P603, lysogenic for λ phages with a temperature-sensitive repressor (*c*I857), were induced at 37°C. Lysogenic strains containing phages φ80 d*argECBH* or φ80 were induced by UV irradiation (9). The isolation and purification of these bacteriophages and the extraction and purification of bacteriophage DNAs have been described in detail (9, 10).

Synthesis and extraction of [^{3}H]RNA and DNA-RNA hybridization. The procedures for synthesis and extraction of [^{3}H]RNA by using cell extracts from strains RM179 and RM178 with added λ d*argECBH* or φ80 d*argECBH* DNA are described in the accompanying report (18). DNA-RNA hybridization was carried out as we described elsewhere (9, 10).

Decay of total [^{3}H]RNA and *argECBH* mRNA was studied by incubating 1 ml of the complete in vitro synthesis mixture with [5-^{3}H]UTP. After 20 min of incubation at 37°C,

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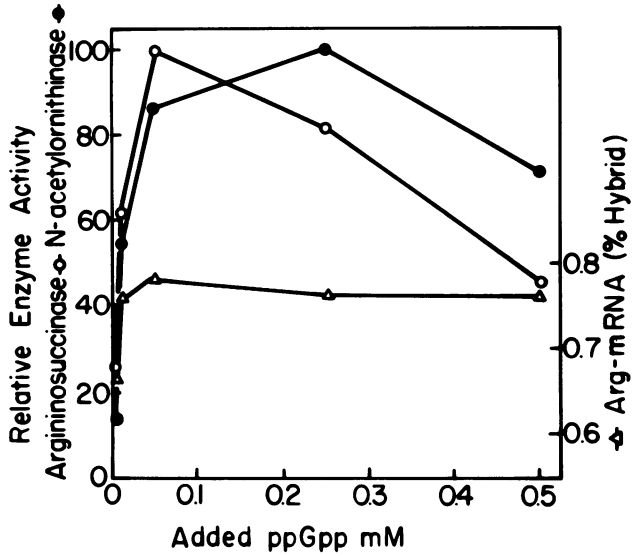

FIG. 1. Effect of ppGpp concentration on the in vitro synthesis of *argE* and *argH* enzymes and *argECBH* mRNA. ppGpp was added to the various concentrations shown to a 300-μl in vitro reaction mixture which contained [^{3}H]UTP and an *argR* extract (*E. coli* RM179). After 20 min of incubation for synthesis with a λ d*argECBH* template, a 100-μl sample was drawn from each reaction mixture, phenol extracted, and hybridized to φ80 d*argECBH* DNA. The percentage of the total input [^{3}H]RNA that was hybridized (% hybrid) is plotted as *arg* mRNA. After 80 min of incubation, 40-μl samples were assayed for argininosuccinase, and 50-μl samples were assayed for *N*-acetylornithinase. Relative enzyme activity is plotted where the maximum *N*-acetylornithinase activity was 2.8 U/ml and maximum argininosuccinase activity was 2.6 U/ml.

rifampin to 10 μg/ml was added. Samples (100 μl) were withdrawn at various times to determine hybridizable *argECBH* mRNA and were immediately iced and treated with DNase. Samples (10 μl) were withdrawn to determine total [^{3}H]RNA as described in the accompanying paper (18).

Cell-free synthesis and assay of enzymes. Extracts of strains RM179 and RM178 (*argR* and *argR*$^+$ extracts, respectively) were used for simultaneous cell-free synthesis of *N*-acetylornithinase (the *argE* enzyme) and argininosuccinase (the *argH* enzyme) with λ d*argECBH* or φ80 d*argECBH* DNA as the template as detailed in the accompanying report (18). After the incubation at 37°C for 80 min or for the time indicated for each experiment, synthesis was stopped by adding chloramphenicol and icing.

The active produced *N*-acetylornithine deacetylase (EC 3.5.1.16) was assayed by a colormetric procedure (18), and argininosuccinate lyase (EC 4.3.2.1) activity was assayed by a radiochemical method reported previously (19).

Materials. [5-^{3}H]UTP (26 mCi/mmol) was obtained from New England Nuclear Corp. or Amersham Corp. Nucleoside triphosphates, L-amino acids, trisodium phosphoenolpyruvate, and dithiothreitol were from Sigma Chemical Co. ppGpp, hexalithium salt, was from ICN Pharmaceuticals, Inc., and from P-L Biochemicals, Inc.

RESULTS

Effect of ppGpp on the levels of *argECBH* mRNA and of *argE* and *argH* enzymes synthesized in vitro. When ppGpp was added to the in vitro protein synthesis system, a 7- to 20-fold increase in detectable amounts of argininosuccinase and *N*-acetylornithinase was seen (Fig. 1). The optimal ppGpp concentration (0.05 to 0.2 mM) varied with the *relA*$^+$ S-30 extract used, probably owing to endogenous levels of ppGpp in the extract. The synthesis of *argECBH* mRNA, however, was enhanced only 30 to 100% by added ppGpp. Also, we found that added ppGpp enhanced *argECBH* mRNA synthesis only 15 to 50% over controls when purified *E. coli* RNA polymerase was used in place of an S-30 extract (G. Keller and P. Rogers, unpublished data). To determine whether these effects were due specifically to ppGpp, we added a variety of nucleotides to the in vitro system. The increase in measurable amounts of the *argE* and *argH* enzymes occurred upon the addition of ppGpp purchased from two different commercial sources (Table 1). The only other nucleotide tested which increased *N*-acetylornithinase and argininosuccinase was pppGpp, a metabolic precursor of ppGpp (5).

Determination of the ppGpp site of action. The rather small ppGpp enhancement of *argECBH* mRNA synthesis compared with enhancement of *argE* and *argH* enzyme synthesis suggested that the effect of ppGpp might not be simply at the level of transcription initiation. In addition, this difference was analogous to the data shown in the accompanying paper (18) for the repression by arginine of the synthesis of *argECBH* mRNA and the *argE* and *argH* enzymes.

We wished to pinpoint the stage, from initiation of mRNA synthesis to appearance of active enzyme, at which ppGpp exerted its effect on the *argECBH* system. The first target we looked at was a possible effect of ppGpp on *argECBH* mRNA stability. The apparent stimulation of *N*-acetylornithinase and argininosuccinase by ppGpp could be explained if the addition of ppGpp slowed the decay rate of *arg* mRNAs and thus allowed more translation per *argECBH* mRNA molecule. In the presence of ppGpp, the decay of hybridizable *argECBH* mRNA had a half-life of about 8.2 min (Fig. 2A). When the same experiment was repeated in the absence of ppGpp, *argECBH* mRNA had a half-life of approximately 9.0 min (Fig. 2B). These experiments demonstrate that the addition of ppGpp does not increase the stability of *arg*-specific message, nor does it have a general effect on total [^{3}H]RNA decay.

The effect of ppGpp on total in vitro protein synthesis was measured by the incorporation of [^{3}H]leucine. Added ppGpp had no effect on protein synthesis by the *argR* extract

TABLE 1. Effect of various nucleotides on in vitro synthesis of *argE* and *argH* enzymes[a]

Added nucleotide (0.25 mM)	Relative enzyme activity[b]	
	N-Acetylornithinase	Argininosuccinase
None	1	1
ppGpp (ICN)[c]	7	7
ppGpp (P-L)	11	7
pppGpp	4	7
pGpp	2	2
Gpppp	1	<1
ppApp	<1	2
Cyclic GMP	<1	<1
Cyclic AMP	2	<1
GDP	3	<1
GTP	1	<1
Guanosine	<1	1

[a] The synthesis reactions were incubated at 37°C for 80 min with φ80 d*argECBH* DNA as the template and an S-30 extract from strain RM179 (*argR*).

[b] Control values (no added nucleotide) were 0.12 U/ml for *N*-acetylornithinase (*argE*), 0.5 U/ml for argininosuccinase (*argH*).

[c] ppGpp was obtained from ICN Pharmaceuticals, Inc., and P-L Biochemicals, Inc., as indicated.

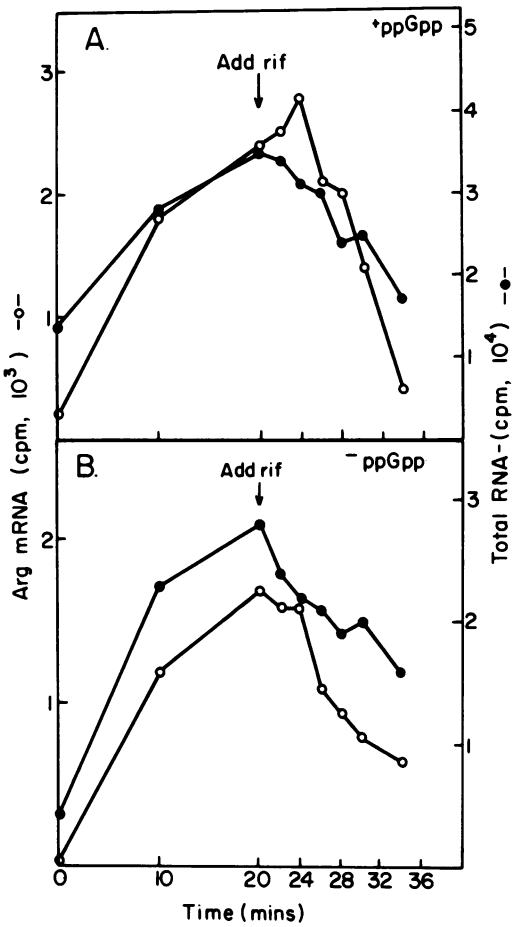

FIG. 2. Stability of in vitro-synthesized *argECBH* mRNA and total RNA. A 1.0-ml reaction mixture containing λd*argECBH* DNA as the template and an *argR* extract of strain RM179 was incubated in the presence of [^{3}H]UTP for 20 min, at which time rifampin to 10 μg/ml was added (rif). The experiment was performed in the presence (A) or absence (B) of ppGpp (final concentration, 0.25 mM). At the times shown, 5-μl samples were withdrawn and processed to determine total [^{3}H]RNA. At the same times, 100-μl samples were withdrawn, processed, and hybridized to φ80 d*argECBH* DNA. Total counts per minute hybridized were corrected as described in the text and plotted as *arg* mRNA.

whether excess arginine was present or not (data not shown).

ppGpp has been shown to affect directly the activities of a variety of enzymes. The effects of ppGpp on *N*-acetylornithinase and argininosuccinase activities were measured by first synthesizing these enzymes in vitro in the absence of added ppGpp, then assaying *argE* and *argH* enzyme activities in the presence and absence of added 0.25 M ppGpp. We found that the addition of ppGpp during the assays of these enzymes did not appreciably alter their activities (data not shown).

ppGpp involvement in the arginine-*argR* system. ppGpp could have affected the repression results by modification of the RNA polymerase or by interaction at a DNA site in the control region of the *argECBH* cluster. The effect of added ppGpp on *argECBH* message and enzyme synthesis with increasing amounts of arginine was measured (Fig. 3). If one compares the repression by increasing amounts of arginine of *N*-acetylorinthinase (Fig. 3A) and argininosuccinase (Fig. 3B), it is evident that the addition of ppGpp greatly increased the amount of measurable enzyme but did not effect repression by arginine. The addition of ppGpp did not affect the repression of *argECBH* mRNA by arginine either (Fig. 3C).

Effect of ppGpp early by transcription. Our results so far have shown that the in vitro syntheses of *argECBH* mRNA and enzyme products are stimulated to different degrees by the addition of ppGpp. We have ruled out several sites as a target for ppGpp, which suggests that ppGpp may be acting at some time after transcription initiation or during translation.

In the accompanying paper, we show that omitting added L-amino acids and tRNA in the in vitro system effectively reduces protein synthesis by 90%, as measured by the *argE* and *argH* enzyme levels. However, in the presence of amino acids and tRNA, the bulk of *argECBH* mRNA suitable for translation is made in the first 15 min of incubation. By delaying the start of translation and allowing *argECBH* mRNA to accumulate, we could determine whether ppGpp had an effect during protein synthesis (Table 2). Table 2, line 1, shows the levels of *argE* and *argH* activity observed in the complete system with ppGpp added at time zero. After 15 min of incubation for in vitro synthesis, rifampin was added to 10 μg/ml to inhibit further RNA synthesis (Table 2, line 2); the amount of enzyme synthesized at 80 min was about the same as the control (Table 2, line 1). The data show that RNA synthesized before rifampin was added can be translated into arginine enzymes. It also shows that most of the *arg* mRNA was produced from 0 to 15 min, as was found in the accompanying paper (18).

N-Acetylornithinase and argininosuccinase were assayed after only 15 min of in vitro synthesis incubation, and it was clear that only about 20 to 30% of the translation of these enzymes had occurred by that time (Table 2, line 1). Thus, if ppGpp were added at 15 min rather than at the start of incubation, any effects of ppGpp on the translation process would be detected. In the experiment shown in Table 2, line 3, ppGpp addition was delayed for 15 min, at which time it was added together with rifampin. The amounts of *argE* and *argH* enzymes synthesized at 80 min (Table 2, line 3) were only slightly greater than the values we observed after 15 min with ppGpp added at time zero (Table 2, line 1). We interpret this to indicate that ppGpp has no direct effect on translation on the *argECBH* mRNA.

A further test of how ppGpp acts was made with added amino acids and tRNA omitted for the first 15 min. Amino acids and tRNA were added just after rifampin at 15 min

TABLE 2. Temporal study of ppGpp action[a]

Additions[b]		Enzyme activity (U/ml)			
		N-Acetylornithinase		Argininosuccinase	
0 min	15 min	15 min	80 min	15 min	80 min
ppGpp, aa, tRNA		1.1	3.3	1.1	4.7
ppGpp, aa, tRNA	Rif		3.0		5.5
aa, tRNA	Rif, ppGpp		1.5		1.3
ppGpp	Rif, aa, tRNA	0.2	1.6	0.26	1.8
	Rif, ppGpp, aa, tRNA		0.4		0.5

[a] The *in vitro* synthesis mixture was incubated with an *argR* extract (strain RM179) with λ d*argECBH* DNA as template.

[b] Abbreviations: aa, 19 amino acids (no arginine) added to 0.22 M; tRNA, *E. coli* K-12 tRNA added to 100 μg/ml; Rif, rifampin added to 10 μg/ml. Where indicated, ppGpp was added to 0.25 mM.

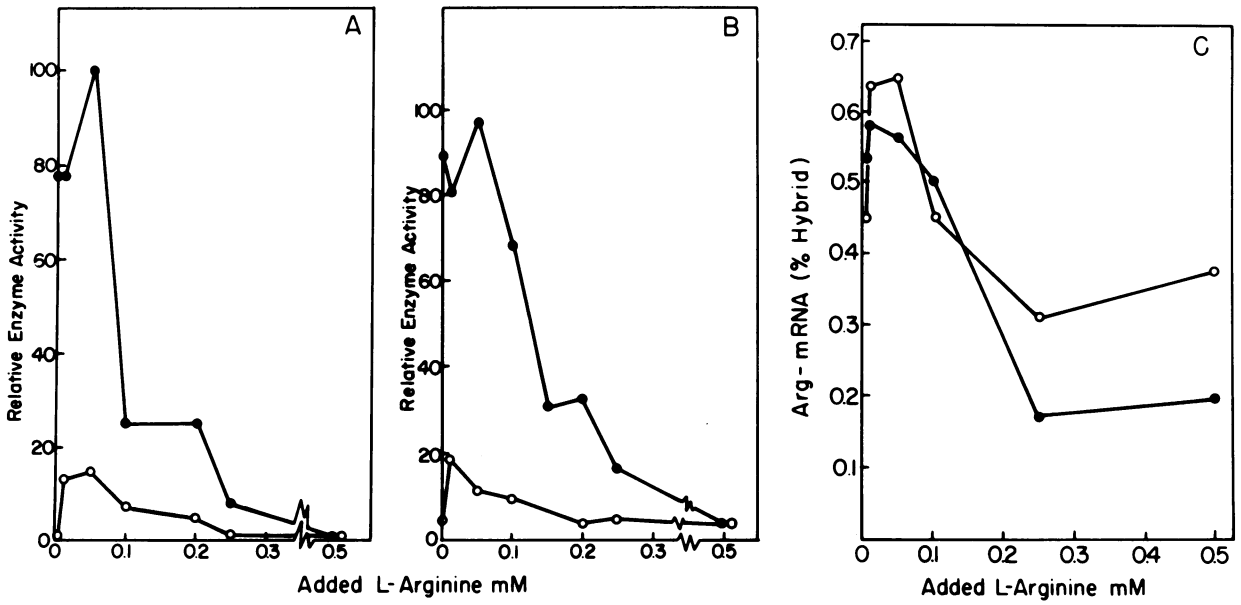

FIG. 3. Effect of ppGpp on the repression of the *argECBH* cluster by arginine. (A and B) A series of reaction mixtures was incubated with increasing amounts of L-arginine as shown, *argR*$^+$ extract (strain RM178), and φ80 d*argECBH* DNA either in the presence (●) or absence (○) of 0.25 mM ppGpp. After 80 min of incubation for synthesis, *N*-acetylornithinase (A) and argininosuccinase (B) were measured as described in the text. Relative enzyme activity is plotted where maximum *N*-acetylornithinase activity was 0.78 U/ml and maximum argininosuccinase activity was 2.1 U/ml. (C) In a separate series of reaction mixtures with the same additions as above but with added [^{3}H]UTP, 100-μl samples were extracted after 20 min of incubation for [^{3}H]RNA, which was hybridized to λ d*argECBH* DNA (see the text). The percentage of input radioactivity hybridized (% hybrid) is plotted as *arg* mRNA.

(Table 2, line 4). Enzyme levels observed were approximately one-half those of the control, showing the reduced translation of message reported elsewhere (18). The same experiment was performed, but with the addition of ppGpp delayed until 15 min (Table 2, line 5). Again, the amounts of *argE* and *argH* enzymes formed at 80 min were only slightly greater than those observed at 15 min when ppGpp was present at time zero (Table 2, line 4). We can see that under conditions of greatly reduced translation, ppGpp (during the first 15 min of incubation) enhances the final yield at 80 min of *N*-acetylornithinase and argininosuccinase synthesis about fourfold.

DISCUSSION

In this work, we show that ppGpp specifically stimulates by 7- to 20-fold the synthesis of the *argE* enzyme, *N*-acetylornithinase, and the *argH* enzyme, argininosuccinase, in a coupled *E. coli* cell-free system. This finding is in agreement with data reported by others for in vitro systems demonstrating a specific stimulation by ppGpp of the *his*, *lac*, *ara*, and *trp* operons (11, 13, 17). Approximately the same concentration of ppGpp (about 0.2 mM) yielded maximal stimulation for all the systems tested. The level of increase of the measured enzymes varied from about 2- to 3-fold for *trp* and *ara* (17) to 7- to 16-fold for *his*, *lac* (11), and the data reported here for *argE* and *argH*. In conflict with our data, Yang et al. described the inhibition of *argE* by ppGpp (17). Kelker and Eckhardt, however, found that ppGpp stimulated *argA* synthesis in an in vitro system (7). We also show here that ppGpp control does not perturb in any obvious way the arginine-*argR* repression system. The addition of ppGpp affected the synthesis of the *argE* and *argH* enzymes without affecting the sensitivity of this synthesis to repression by L-arginine (Fig. 3).

ppGpp was originally described by Cashel and Gallant in 1969 and is the pleiotrophic effector for the stringent response (2). In response to the levels of uncharged tRNA upon amino acid starvation, ppGpp acts as a global signal to reduce the synthesis of such diverse cellular components as ribosomal RNA and ribosomal protein, lipids, peptidoglycan, and nucleotides (5, 6).

It has been suggested that ppGpp acts as a supercontrol molecule coordinating the activities of the cell during amino acid starvation (11). Besides inhibiting the production of the protein synthesizing system, which is in excess during a shift-down, ppGpp may also activate the expression of certain metabolic genes. For example, raising the levels of amino acid biosynthetic operons and increasing breakdown makes more amino acids available for the cell to use. Thus, ppGpp may act to inhibit the expression of certain genes while stimulating the expression of others.

Strong indications that ppGpp may act as a positive effector for expression of the *argECBH* cluster in whole cells during amino acids starvation already exists. In work previously reported, Krzyzek and Rogers (9) measured the synthesis of *argE* and *argH* enzymes and of *argECBH* mRNA in whole cells. Upon the removal of arginine, they observed an immediate increase in arginine enzymes and message which then dropped to a steady-state level. This rapid increase in *argECBH* expression closely correlates with the burst of ppGpp synthesis observed during amino acid starvation (1). With the addition of arginine after starvation, the opposite effect is seen. A transient drop in *argECBH* mRNA and enzyme synthesis occurs before a steady-state level is reached. The level of ppGpp also drops and then readjusts upward to a steady-state level during an amino acid shift-up (4).

The next question which arises is the target site for ppGpp action. In this work, we have ruled out an effect of ppGpp on mRNA decay or on *N*-acetylornithinase and argininosuccin-

ase activity. It has been suggested that ppGpp binds with RNA polymerase and has a direct effect upon transcription initiation (14). This explanation would conflict with our mRNA results since the enhancement of *argECBH* mRNA by ppGpp (50%) is much less than ppGpp enhancement of *argE* and *argH* enzyme synthesis.

Kingston and Chamberlin have shown that ppGpp interacts with RNA polymerase to affect the rate of RNA chain elongation (8). They propose that ppGpp alters the pattern of DNA sequence-specific pausing of RNA polymerase, thereby causing a reduction in the overall rate of rRNA synthesis in whole cells. There is not enough evidence to suggest that ppGpp-influenced pausing of RNA polymerase is the mechanism for the observed enhanced expression of amino acid biosynthetic operons. Indeed, Winkler and Yanofsky (16) showed that ppGpp did not affect the pausing of RNA polymerase at a site about 50 nucleotides upstream from the attenuator during in vitro transcription of the tryptophan operon leader region.

ppGpp can also act during translation by reducing misincorporation under general starvation conditions (15). ppGpp slows down translation by inhibiting Ef-Tu and allowing buildup of the proper amino acyl tRNA and its incorporation into the protein chain. In our experiments, translation of the *argECBH* cluster was delayed by the omission of amino acids and tRNA (Table 2). If ppGpp was added at the start of transcription, even when translation was restricted for 15 min, a fourfold enhancement of synthesis of the *argE* and *argH* enzymes was seen. But if the addition of ppGpp was delayed to the point when translation was restarted and transcription was inhibited, no enhancement of argininosuccinase and *N*-acetylornithinase synthesis was observed. Thus, we can conclude that ppGpp enhances *argECBH* expression at some early point of the coupled transcription-translation process. Further work is required to identify the precise molecular mechanism by which ppGpp promotes expression of this gene cluster as well as of other biosynthetic operons.

ACKNOWLEDGMENTS

We thank Norman Kelker, Raymond Cunin, and Nicolas Glansdorff for bacterial strains.

This work was supported by Public Health Service grant GM-25320 from the National Institute of General Medical Sciences and by a grant from the Minnesota Medical Foundation. M.J.Z. was supported by a fellowship from the Bush Foundation.

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