JOURNAL OF BACTERIOLOGY, Mar. 1973, p. 1177-1183

Vol. 113, No. 3
Printed in U.S.A.

Role of the Translocation Factor G in the Regulation of Ribonucleic Acid Synthesis

ELAZAR RABBANI AND P. R. SRINIVASAN

Department of Biochemistry, Columbia University, College of Physicians and Surgeons, New York, New York, 10032

Received for publication 12 October 1972

In an *Escherichia coli rel*$^+$ *arg* strain (ES-2) which carries a temperature-sensitive "G factor," the synthesis of ribonucleic acid (RNA) continues at the nonpermissive temperature (42 C) even though protein synthesis is terminated. However, at 32 C, the strain exhibits a stringent control of RNA synthesis in the absence of arginine. The stringent control of RNA synthesis imposed by trimethoprim (an inhibitor of initiation of protein synthesis) at 32 C is released at the nonpermissive temperature. Even the diauxie lag in RNA synthesis, which is regulated independently of the allelic state of the *rel* gene, is overcome by inactivation of the G factor. The unusual guanosine nucleotide, guanosine 5′-diphosphate 2′ or 3′-diphosphate (ppGpp), is produced in small amounts during growth in strain ES-2. Withdrawal of arginine results in a greater accumulation of this compound at 32 C. At 42 C, the synthesis of ppGpp is abolished and is considerably lower than the level found in ES-2 under normal growth conditions. These results indicate that the translocation factor G plays an important role in the regulation of RNA synthesis and in the synthesis of ppGpp.

The synthesis of stable ribonucleic acid (RNA) species in *Escherichia coli* normally depends on the presence of all amino acids and their aminoacylated transferRNAs (tRNAs), and a genetic locus RC (or *rel* gene) has been implicated in this regulation. Upon starvation of a *rel*$^+$ strain for a required amino acid, both protein and RNA synthesis are simultaneously terminated (stringent control). A mutation to *rel*$^-$ uncouples these reactions, and during amino acid starvation protein synthesis ceases while synthesis of stable RNAs continues (relaxed control) (1, 7, 21).

During amino acid starvation of a stringent (*rel*$^+$) strain of *E. coli* K-12, Cashel and Gallant (4) observed the accumulation of two novel guanine nucleotides, MSI and MSII, in the nucleotide pool of the cells. MSI has been identified as guanosine 5′-diphosphate 2′ or 3′-diphosphate (ppGpp), whereas the exact nature of MSII, also a guanosine derivative, is unknown. Cashel and his associates have provided strong evidence that ppGpp is probably the actual effector of many aspects of amino acid control. This nucleotide inhibits *E. coli* RNA polymerase (3), antagonizes the ψ factor which mediates *in vitro* synthesis of ribosomal RNA (22) and also inhibits inosine 5′-monophosphate dehydrogenase and adenyl succinate synthetase, the first enzymes in the pathways leading to the synthesis of guanosine 5′-triphosphate (GTP) and adenosine 5′-triphosphate (ATP) (9). However, recent studies by Haseltine (10) have failed to corroborate the inhibition of ribosomal RNA synthesis by ppGpp, and Zubay et al. (23) have reported that it did not affect the in vitro synthesis of a tRNA. Moreover, Lazzarini et al. (10) have shown that MSI and MSII cannot be the immediate gene products of the *rel*$^+$ allele because they are accumulated in both *rel*$^+$ and *rel*$^-$ auxotrophs under other stress conditions, such as carbon deprivation or step-down transitions. Lund and Kjeldgaard (15) have carried out an exhaustive investigation of the effect of a variety of antibiotics (which inhibit protein synthesis) on the formation and degradation of ppGpp and arrived at the conclusion that the synthesis of this compound may be independent of RNA synthesis and that a coupling may exist between MSI formation and an idling reaction of one of the initiation steps of protein synthesis. That some step in the initiation of protein synthesis may be involved in the

cellular control of RNA synthesis has been proposed earlier by Shih et al. (19) and more recently by Patterson and Gillespie (18).

In this communication we report on our studies with strain of *E. coli* K-12 *rel*$^+$ *arg* which carries a temperature-sensitive mutation in the translocation factor G. The results obtained suggest that the G factor plays an important role in the regulation of RNA synthesis and in the synthesis of ppGpp.

MATERIALS AND METHODS

Bacterial strain. *E. coli* K-12 ES-2 *rel*$^+$ *argA thi* with temperature-sensitive "G factor" was constructed by P1 transduction of mutation producing the altered G factor from strain *E. coli* K-12 *rel*$^-$ *strA* (12) (obtained from David Schlessinger) into *E. coli* K-12 MA-5, *rel*$^+$ *argA*, *strA*$^+$ (from Werner Maas) and selecting for streptomycin-resistant colonies. The strain ES-2 shows normal growth at 32 C but fails to grow above 40 C due to the inactivation of the G factor.

Biochemicals. Chloramphenicol was obtained from Parke-Davis and Co., Detroit, Mich.; trimethoprim [2,4 diamino-5 (3',4',5',trimethoxybenzyl) pyrimidine] was provided by G. H. Hitchings, (Burroughs Wellcome & Co.); uracil-5-^3H (2 mCi/μmol), L-leucine-*U*-^{14}C (312 μCi/μmol) were purchased from Schwarz BioResearch (Orangeburg, N. Y.) and carrier-free ^{32}PO from New England Nuclear Corp.

Media and growth conditions. Bacterial cultures were grown with vigorous shaking at 32 C in the minimal medium described by Davis and Mingioli (6) supplemented with 0.4% glucose. Thiamine was present at 1 μg/ml, and when needed arginine was added to the medium at a final concentration of 50 μg/ml. The absorbance of the cultures was measured at 580 nm in a Bausch & Lomb, Inc. spectrophotometer. An absorbance of 0.2 corresponds to 2×10^8 bacteria per ml. For the experiments, overnight cultures were diluted to an absorbance of about 0.1 into fresh medium containing 0.4% glucose, thiamine, and arginine and were allowed to grow to an absorbancy of 0.4. The cells were harvested, washed twice with salts medium at 15 C, and resuspended in the glucose salts medium with the indicated additions to achieve an absorbancy of 0.2. Chloramphenicol and trimethoprim were used at a concentration of 100 μg/ml and 50 μg/ml, respectively. In the experiments with trimethoprim, the cells were also supplemented with adenine, guanine, cytosine (25 μg/ml), and thymine (20 μg/ml).

Measurements of RNA and protein syntheses. The syntheses of RNA and protein were followed with uracil-5-^3H (0.25 μCi per 10 μg per ml) and ^{14}C-leucine (0.05 μCi per 5 μg per ml), respectively, at either 32 or 42 C as outlined. At intervals, samples of 0.2 ml were removed, precipitated with 10% trichloroacetic acid, and filtered through glass fiber paper discs (Whatman GF/C). The filters were dried and counted in a toluene scintillation mixture in a Beckman scintillation counter.

Assay of ppGpp. The accumulation of ppGpp under various conditions was followed by the procedure of Cashel (16). Cultures grown in tris(hydroxymethyl)aminomethane (Tris)-glucose minimal medium containing 2×10^{-3} M KH_2PO_4 were washed twice with 0.9% NaCl and resuspended in Tris-glucose minimal medium containing 2×10^{-4} M KH_2PO_4. A concentration of 10 to 20 μCi of $^{32}PO_4$ per ml was then added to the cultures. At appropriate intervals, 0.2-ml samples were mixed with 0.2 ml of 2 M formic acid, and the nucleotide concentrations were determined by chromatography on polyethyleneimine-cellulose thin-layer sheets.

Carbon and nitrogen shift-down experiments. The shift-down experiments in carbon source were performed by harvesting cells of strain ES-2 grown on glucose by centrifugation at 4 C and resuspending the cell pellet in arginine, thiamine, and salts medium with either glucose or succinate at a concentration of 0.4%. For the shift-down experiments in nitrogen, source strain ES-2 was grown on basal salt solution P (17) supplemented with $(NH_4)_2SO_4$ (0.1%) and glucose (0.4%). The cells were centrifuged, washed once with basal solution P, and the cell pellet was resuspended in arginine, thiamine, basal salt solution P, and glucose with either $(NH_4)_2SO_4$ (0.1%) or L-tryptophan (0.2%). The syntheses of RNA and protein were followed at 32 C with ^{3}H-uracil and ^{14}C-leucine, respectively. After 20 min, a portion of the culture from each flask was raised to 42 C, and syntheses of RNA and protein were studied.

RESULTS

RNA synthesis during inhibition of protein synthesis. The parent strain MA-5 grows well at 32 and 42 C, and withdrawal of arginine at these two temperatures results in cessation of RNA and protein synthesis. The behavior of strain ES-2 in the presence of arginine at 32 and 42 C is illustrated in Fig. 1. At 32 C, the incorporation of ^{3}H-uracil and ^{14}C-leucine into RNA and protein, respectively, is similar to that observed in MA-5. However, at 42 C, the incorporation of ^{14}C-leucine into trichloroacetic acid-precipitable material is arrested immediately, but the incorporation of ^{3}H-uracil into RNA continues unabated and finally levels off.

Withdrawal of arginine from the medium elicits a stringent response of RNA synthesis at 32 C (Fig. 2), and addition of chloramphenicol overcomes the stringent behavior and promotes RNA synthesis. At 42 C, deprivation of arginine from the medium does not alter the synthesis of RNA noted earlier in arginine-containing medium. Inclusion of chloramphenicol at 42 C in the amino acid starvation medium neither increases nor diminishes the level of RNA synthesis. Thus the strain ES-2 shows a stringent control of RNA synthesis at 32 C and

a relaxed control at 42 C, even though it carries the *rel*$^+$ gene and differs from the parent strain MA-5 only in possessing a temperature-sensitive lesion in the function of the G factor.

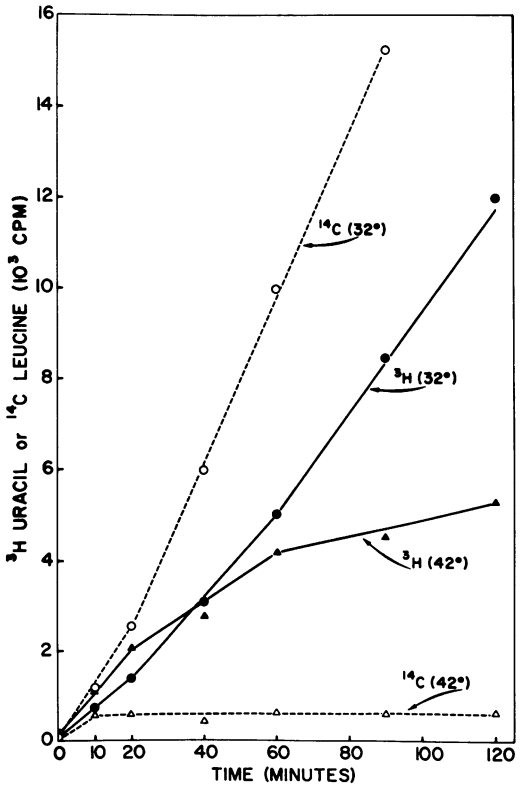

FIG. 1. *Kinetics of RNA and protein synthesis in strain ES-2 at 32 and 42 C.*

The drug trimethoprim, which blocks the initiation of protein synthesis by interfering with the formylation of methionyl tRNA^{met}, causes an arrest of net RNA synthesis in *rel*$^+$ but not in *rel*$^-$ strains (19). The effect of trimethoprim on the synthesis of RNA in strain ES-2 is shown in Fig. 3. While RNA synthesis (as measured by ^{3}H-uracil incorporation) is completely blocked at 32 C, it is only slightly diminished at 42 C from the level of RNA synthesis observed in the absence of trimethoprim. Thus the strain fails to manifest a stringent response at the nonpermissive temperature.

The synthesis and degradation of ppGpp. In view of the apparent importance of the

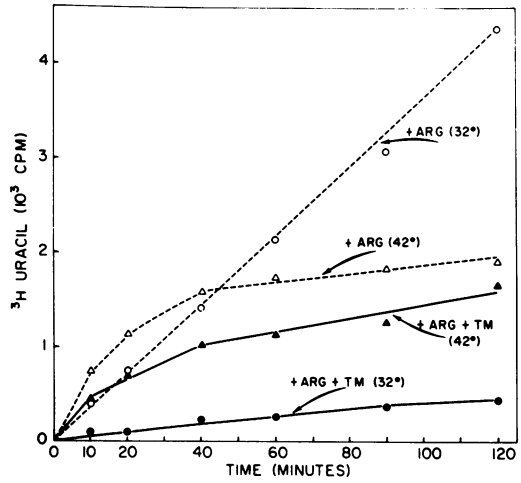

FIG. 3. *Effect of trimethoprim (TM) on RNA synthesis in strain ES-2 at 32 and 42 C.*

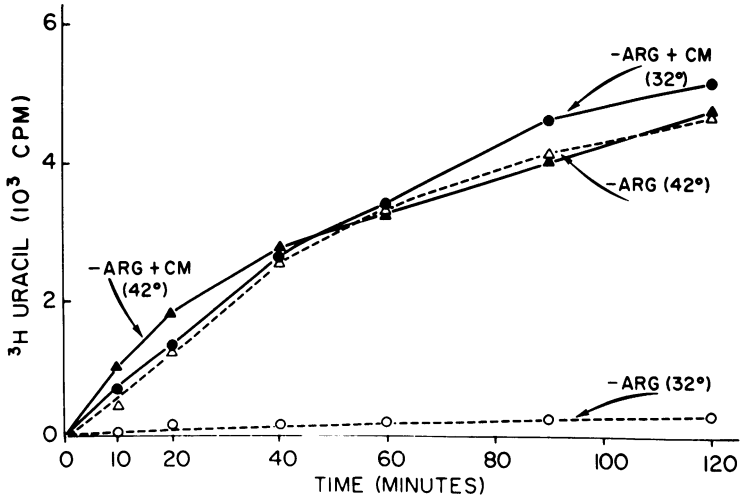

FIG. 2. *RNA synthesis at 32 and 42 C in strain ES-2 during arginine starvation and during arginine starvation in the presence of chloramphenicol (CM).*

guanine nucleotide MSI in the regulation of metabolic functions, its formation and degradation was studied. In the low phosphate medium, the strain ES-2 accumulates some ppGpp in the presence of arginine even though the synthesis of RNA and protein, monitored in a parallel set of experiments, continues. Removal of arginine results in elevated levels of ppGpp (Table 1). At 42 C, the accumulation of ppGpp is drastically reduced either in the presence or absence of arginine and is approximately 10 to 15% of the levels observed at 32 C in the absence of arginine. The unexpected accumulation of ppGpp in the presence of arginine at 32 C in ES-2 necessitated a study of the parent strain MA-5, and these results are presented in Table 2. The strain MA-5 was also found to accumulate ppGpp at 37 C in the presence of arginine, and again the level of this compound is considerably less than that observed in cells starved for arginine.

TABLE 1. *Accumulation of ppGpp by E. coli K 12 ES-2 at 32 and 42 C in the presence and absence of arginine*[a]

Time (min)	32 C		42 C	
	With arginine	Without arginine	With arginine	Without arginine
30	0.43[b]	1.1	0.16	0.12
45	0.91	1.25	0.21	0.14
60	0.91	1.7	0.17	0.13

[a] The washed cells were resuspended in Tris-glucose minimal medium containing 2×10^{-4} M $KH_2{}^{32}PO_4$ (10 μCi/ml), and the cell density was adjusted to yield an A_{580} of 0.3. The culture was then immediately divided into four equal portions, two of which were placed at 32 C, one with arginine and the other without arginine. The other two flasks were placed at 42 C, again one flask received arginine. At the indicated intervals, 0.2-ml samples were removed and mixed with 0.2 ml of 2 M HCOOH. The cell-free supernatant fluids (25 μliters) were spotted directly and chromatographed. The results are expressed as nanomoles per milliliter of culture.

[b] Measured in nanomoles.

TABLE 2. *Accumulation of ppGpp by E. coli K12 MA-5 at 37 C in the presence and absence of arginine*[a]

Time (min)	With arginine (nanomoles)	Without arginine (nanomoles)
30	0.28	0.49
45	0.25	0.70
60	0.31	0.76
75	0.29	0.77

[a] The experimental conditions were similar to that described in Table 1 except that the density of cells before distribution was adjusted to an A_{580} of 0.2.

Readdition of a required amino acid or chloramphenicol to an amino acid-starved *rel*$^+$ culture results in rapid disappearance of ppGpp from the nucleotide pool followed by an immediate resumption of net RNA synthesis (2). The synthesis of RNA and decay of ppGpp in strain ES-2 was investigated by raising the temperature of an arginine-starved culture from 32 to 42 C (Fig. 4). During arginine starvation the level of ppGpp gradually increases and reaches a maximum after 60 min of starvation. During this period, very little synthesis of RNA occurs. A portion of this culture was placed at 42 C, and the syntheses of RNA and ppGpp were monitored. After a lag of 4 min, the level of ppGpp decreases progressively, reaching its lowest level around 18 min, and during this period there is a slow resumption of RNA synthesis. At a time when the synthesis of RNA is exponential, the level of ppGpp is negligible.

Regulation of RNA synthesis during shift-down of carbon or nitrogen source. Unlike *rel*$^+$ strains, strains which carry the *rel*$^-$ gene fail to regulate RNA synthesis only during

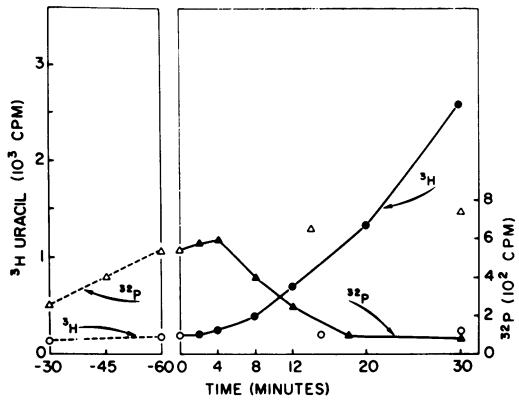

FIG. 4. *The synthesis RNA and metabolism of ppGpp during arginine starvation of strain ES-2 at 32 and 42 C. Exponentially grown cells were washed and resuspended in Tris-glucose minimal medium containing 2×10^{-4} M KH_2PO_4 and divided into two equal portions. To one ^{3}H-uracil was added to monitor RNA synthesis (O----O) and the other received $^{32}PO^4$ for studying the synthesis of ppGpp (Δ----Δ). After 60 min of arginine starvation at 32 C, a portion from each flask was raised to 42 C, and samples were again removed for analyzing the synthesis of RNA (●——●) and the level of ppGpp (▲——▲). The level of ppGpp is expressed as counts per minute in 25 μliters of the sample. The incorporation of ^{3}H-uracil is given as counts per minute per 0.2 ml of sample.*

amino acid starvation. The *rel*⁻ strains are capable of regulating RNA synthesis when subjected to a shift from a rich medium to a poor one (16).

The effect of a shift down of carbon source on RNA synthesis in strain ES-2 at 32 C is depicted in Fig. 5. In succinate medium, the rate of RNA synthesis is slower compared to the rate observed in glucose medium, and after 40 min the cells adjust to a new rate of RNA synthesis. On the other hand, RNA synthesis in glucose medium is characteristic of cells in exponential growth. After 20 min, a portion of the succinate and glucose cultures was raised to 42 C. The synthesis of protein is immediately arrested at 42 C in this mutant, yet RNA synthesis is markedly enhanced in the succinate-grown cells. A similar response in RNA synthesis can also be observed with a shift down in nitrogen source (Fig. 6). With tryptophan as nitrogen donor, the rate of RNA synthesis is very poor compared to the rate obtained with NH_4^+. Elevation of the tryptophan culture to 42 C again results in a marked

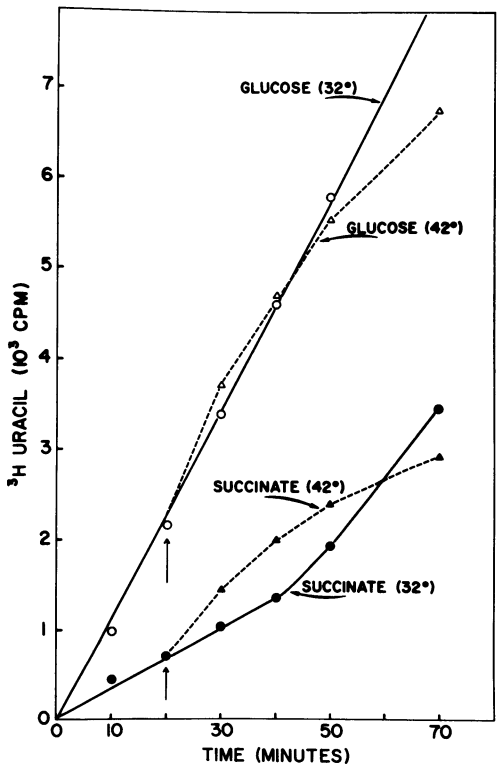

FIG. 5. *RNA synthesis in strain ES-2 during shift down in carbon source. At 20 min, a portion of the culture from each flask was raised to 42 C (indicated by arrow).*

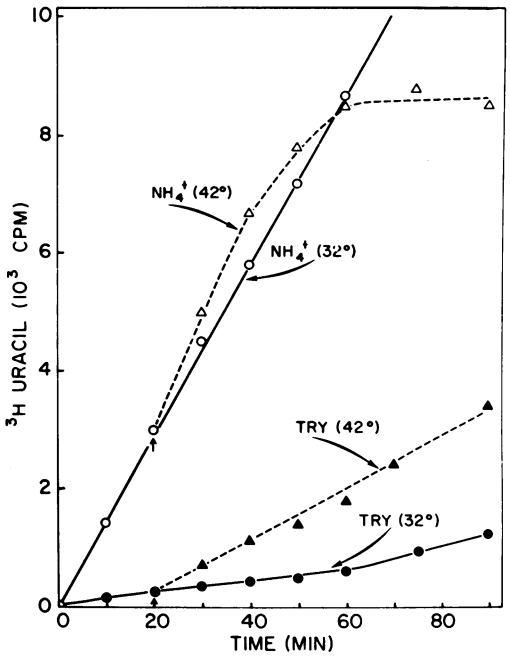

FIG. 6. *RNA synthesis in strain ES-2 during shift down in nitrogen source. At 20 min, a portion of the culture from each flask was raised to 42 C (indicated by arrow).*

increase in RNA synthesis. This behavior is very similar to the effect evoked by chloramphenicol in cultures of *rel*⁺ and *rel*⁻ strains shifted down in carbon or nitrogen source (17, 20).

DISCUSSION

The elongation factor G participates in the translocation of the peptidyl tRNA from the aminoacyl site to the peptidyl site and is additionally characterized by a potent guanosine 5'-triphosphatase activity manifested only in the presence of the ribosomes (14). The G factor must therefore recognize peptidyl tRNAs, possibly the uncharged tRNAs in the peptidyl site and also be sensitive to the level of GTP in the cell. In view of these interesting properties of the G factor we felt that the regulation of RNA synthesis may be mediated through this key component of the elongation process in protein synthesis.

The strain ES-2 gives a stringent response of RNA synthesis on withdrawal of the required amino acid at 32 C, and inactivation of the G factor produces a relaxed phenotype either in the presence or absence of the required amino acid even though negligible synthesis of protein occurs. Trimethoprim, a known inhibitor of

initiation, fails to arrest the synthesis of RNA in this mutant at the nonpermissive temperature. These facts taken together suggest that either the G factor alone or in conjunction with the elongation machinery may be an important element in the sequence of events required for the regulation of RNA synthesis during inhibition of protein synthesis by amino acid starvation or by inhibition of initiation. The possibility that the effects on RNA synthesis are not due to the temperature-sensitive G factor mutation but due to a second mutation closely linked to either *str* or to temperature-sensitive G allele was ruled out by isolating temperature-stable revertants of ES-2 by plating the cells at 42 C. These revertants grew well at 32 and 42 C and showed a stringent response in RNA synthesis at both temperatures on starvation for arginine.

Controversy surrounds the role of the guanosine nucleotides in the regulation of RNA synthesis. The strain ES-2 accumulates a high level of ppGpp during amino acid starvation, and elevation of the amino acid-starved culture to 42 C causes a slow disappearance of ppGpp followed by a gradual increase in RNA synthesis. The correlation between the kinetics of RNA synthesis and disappearance of ppGpp indicates some kind of coupling or synchrony. The final level of ppGpp at 42 C is very low and is even lower than the level observed in this strain during growth at 32 C. The markedly decreased level implicates the G factor as one of the components required for its synthesis. The accumulation of ppGpp in strains MA-5 and ES-2 at 32 C during the growth phase is unexpected. This phenomenon has also been observed in another strain (5). Such accumulation of ppGpp may be governed by the growth rate of the strain and requires further study.

Since *rel*$^-$ strains are able to regulate RNA synthesis during shift down of carbon or nitrogen source but not during amino acid starvation, it has been suggested that the mechanisms underlying these two phenomena may be unrelated. This is unlikely because the inactivation of G factor in succinate- or tryptophan- grown cells promotes RNA synthesis in the absence of protein synthesis. Thus the regulation of RNA synthesis during changes in environment is probably again mediated through the G factor and other components of the elongation process. The function of the *rel*$^+$ gene in regulation of RNA synthesis is still unclear. Its gene product could be a ribosomal protein which senses the inhibition of protein synthesis caused by deprivation of amino acid (or by inhibition of initiation) and may express its effect through the G factor and the elongation machinery.

Recently, Haseltine et al. (11) have shown that the in vitro synthesis of ppGpp and MSII require the participation of the ribosomes and the G factor along with an acidic protein, stringent factor, which could be extracted only from the ribosomes of stringent and not from the relaxed strains. Although the precise role of ppGpp in the regulation of RNA synthesis is presently unclear, the involvement of the translocation factor G in the synthesis of ppGpp is in complete agreement with our observations reported here.

ACKNOWLEDGMENTS

We are extremely grateful to Werner Maas and David Schlessinger for providing the mutants.

This investigation was supported by grants from the National Science Foundation (GB-23802) and from the National Cancer Institute (Public Health Service no. CA-12235).

Elazar Rabbani is supported by Public Health Service Training Grant no. GM255 from the National Institute of General Medical Sciences.

LITERATURE CITED

1. Borek, E., J. Rockenbach, and A. Ryan, 1956. Studies of a mutant of *Escherichia coli* with unbalanced ribonucleic acid synthesis. J. Bacteriol. **71**:318-323.
2. Cashel, M. 1969. The control of ribonucleic acid synthesis in *Escherichia coli*. IV. Relevance of unusual phosphorylated compounds from amino acid-starved stringent strains. J. Biol. Chem. **244**:3133-3141.
3. Cashel, M. 1970. Inhibition of RNA polymerase by ppGpp, a nucleotide accumulated during the stringent response to amino acid starvation in *E. coli*. Cold Spring Harbor Symp. Quant. Biol. **35**:407-413.
4. Cashel, M., and J. Gallant. 1969. Two compounds implicated in the function of the RC gene of *Escherichia coli*. Nature (London) **221**:838-841.
5. Craig, E., D. Schlessinger, and C. Gurgo. 1972. Synthesis, no synthesis, or synthesis and degradation of ribosomal ribonucleic acid in various *Escherichia coli* strains starved for an amino acid. J. Bacteriol. **111**:66-72.
6. Davis, B. D., and E. S. Mingioli. 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B_{12}. J. Bacteriol. **60**:17-28.
7. Edlin, G., and P. Broada. 1968. Physiology and genetics of the "ribonucleic acid control locus in *Escherichia coli*. Bacteriol. Rev. **32**:206-226.
8. Fraenkel, D. G., and F. C. Neidhardt. 1961. Use of chloramphenicol to study control of RNA synthesis in bacteria. Biochim. Biophys. Acta **53**:96-110.
9. Gallant, J., J. Irr, and M. Cashel. 1971. The mechanism of amino acid control of guanylate and adenylate biosynthesis. J. Biol. Chem. **246**:5812-5816.
10. Haseltine, W. A. 1972. *In vitro* transcription of *Escherichia coli* ribosomal RNA genes. Nature (London) **235**:329-333.
11. Haseltine, W. A., R. Block, W. Gilbert, and K. Weber. 1972. MSI and MSII made on ribosome in idling step of protein synthesis. Nature (London) **238**:381-384.

12. Kuwans, M., D. Schlessinger, G. Rinaldi, L. Felicitti, and G. P. Tocchini-Valentini, 1971. G. factor mutants of *Escherichia coli*: map location and properties. Biochem. Biophys. Res. Commun. **42**:441-444.
13. Lazzarini, R. A., M. Cashel, and J. Gallant. 1971. On the regulation of guanosine tetraphosphate levels in stringent and relaxed strains of *Escherichia coli*. J. Biol. Chem. **246**:4381-4385.
14. Lucas-Lenard, J., and F. Lipmann. 1971. Protein biosynthesis. Annu. Rev. Biochem. **40**:409-448.
15. Lund, E., and N. O. Kjeldgaard. 1972. Mechanism of guanosine tetraphosphate in *Escherichia coli*. Eur. J. Biochem. **28**:316-326.
16. Neidhardt, F. C. 1963. Properties of a bacterial mutant lacking amino acid control of RNA synthesis. Biochim. Biophys. Acta **68**:365-379.
17. Neidhardt, F. C., and D. G. Fraenkel. 1961. Metabolic regulation of RNA synthesis in bacteria. Cold Spring Harbor Symp. Quant. Biol. **26**:63-74.
18. Patterson, D., and D. Gillespie. 1971. Stringent response of RNA synthesis of *E. coli* produced by a temperature shift up. Biochim. Biophys. Res. Commun. **45**:476-482.
19. Shih, An-Ya, J. Eisenstadt, and P. Lengyel. 1966. On the relation between ribonucleic acid synthesis and peptide chain initiation in *E. coli*. Proc. Nat. Acad. Sci. U.S.A. **56**:1599-1605.
20. Sokawa, Y., J. Sokawa, and Y. Kaziro. 1971. Function of the *rel* gene in *Escherichia coli*. Nature N. Biol. **234**:7-10.
21. Stent, G. S., and S. Brenner. 1961. A genetic locus for the regulation of ribonucleic acid synthesis. Proc. Nat. Acad. Sci. U.S.A. **47**:2005-2014.
22. Travers, A., R. Kamen, and M. Cashel. 1970. The *in vitro* synthesis of ribosomal RNA. Cold Spring Harbor Symp. Quant. Biol. **35**:415-418.
23. Zubay, G., L. Cheong, and H. Gefter. 1971. DNA directed cell-free synthesis of biologically active transfer RNA: Su_{III}^{+} tyrosyl-tRNA. Proc. Nat. Acad. Sci. U.S.A. **68**:2195-2197.