Correlation between RNA Synthesis and Basal Level Guanosine 5'-Diphosphate 3'-Diphosphate in Relaxed Mutants of *Escherichia coli*

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The ability of wild type bacteria to reduce their production of stable RNA in response to amino acid limitation has been linked to their capacity to synthesize the unique nucleotide guanosine 5'-diphosphate 3'-diphosphate (ppGpp) when subjected to amino acid starvation. Although relA mutants do not produce ppGpp, they do maintain basal levels of this nucleotide during balanced growth (5-7) and, like stringent bacteria, they are able to adjust their intracellular content of ppGpp in response to various physiological conditions (5, 6, 8-10). Studies conducted with relA and relA strains containing spoT mutations have shown that the basal level of ppGpp which a cell maintains directly influences both its metabolic activity and its growth behavior (2-4, 8, 11-13).

An inverse correlation between RNA synthesis and the ppGpp level of the cell has been well documented in stringent bacteria (14-16), but until recently, this has not been the case for relaxed mutants (17). The ability to recover substantially more ppGpp from relaxed mutants during balanced growth than had been previously reported in these strains has enabled us to demonstrate that both relA mutants and phenotypically relaxed relA* rplK (relC) strains display an immediate and sustained decline in their ppGpp content to amounts much less than their original basal levels (17). These diminished levels of ppGpp are maintained throughout the course of the starvation, increase rapidly upon addition of the required amino acid, and temporarily exceed their prestarvation basal level amounts before eventually returning to their preexisting basal levels (17). A rough inverse correlation between RNA accumulation and the ppGpp level during amino acid starvation and subsequent resupplementation has also been observed in these relaxed mutants (17). In the present report, we have investigated the above correlation further and found that a precise inverse coupling exists between cellular ppGpp levels and initial rates of RNA synthesis. We have also characterized the ribosomal RNA species that are overproduced during amino acid starvation and studied their fate after amino acid resupplementation. The significance of these findings is discussed in relation to the role of ppGpp as a modulator of RNA synthesis during all phases of cell growth.

EXPERIMENTAL PROCEDURES

Materials—Carrier-free orthophosphoric acid H33PO4, [14C]uracil, [14C]arginine, and Formula 949 scintillation fluid were obtained from New England Nuclear Co., Boston, MA. Polyethyleneimine cellulose thin layer chromatography sheets (Polygram Cel 500 - precoated plastic sheet, 20 × 20 cm) were purchased from Brinkmann Instruments, Inc., Westbury, NY. Radioautography and fluorography were carried out with Kodak x-ray film (X-Omat R). Nonradioactive nucleoside phosphates were obtained from P-L Biochemicals, Inc., Milwaukee, WI. All other reagents were of analytical grade and were obtained from local sources.

Bacterial Strains—The following *Escherichia coli* K12 strains were used in this study: KL99 (Hfr P042, thi-1, relA1, spoT1, lac-42) (18), NF161 (F-, metB1, argA2, relA*, spoT1) (19), NF859 (F-, metB1, argA2, relA*, spoT*) (19), and PL10 (F-, metB1, relA1, spoT*) (17). PL10 was derived from an interrupted Hfr mating between KL99 and a spontaneous streptomycin resistant mutant of NF859. It carries the relA marker from KL99 and is streptomycin-resistant.

Media, Growth Conditions, and Radioactive Labeling—All bacterial strains were grown at 37 °C in Hensley's Tris minimal medium (20) containing 0.33 mM phosphate, 0.3% glucose, thiamin (2 μg/ml), and 50 μg/ml of each required amino acid or nucleotide. Amino acid starvation was initiated by the addition of 500 μg/ml of valine and was reversed by adding 500 μg/ml of isoleucine.

RNA accumulation was measured by labeling cells with carrier-free H33PO4 (1.5 μCi/ml) which was added two generations prior to sampling. DNA content was also assayed and as indicated below, the...
RNA values were corrected for DNA content. The production of ppGpp was also monitored directly from these same cultures using the lysozyme-deoxycholate freeze-thaw procedure (21).

Extraction and quantitation of accumulated H$_2$PO$_4$-labeled RNA was as described previously (17). The DNA content of these H$_2$PO$_4$-labeled cultures was also measured as detailed elsewhere (22).

The H$_2$PO$_4$-labeled RNA and DNA samples were normalized and expressed as nanomoles of phosphate per A$_260$ of bacterial culture at 720 nm (nanomoles of PO$_4$/A$_{720}$). There are approximately 1 x 10$^9$ cells/A at 720 nm. The H$_2$PO$_4$-labeled, trichloroacetic acid-precipitated RNA samples in Fig. 1 were corrected for DNA content by subtracting alkali resistant, trichloroacetic acid-precipitable nanomoles of radioactivity from total trichloroacetic acid-precipitable nanomoles of phosphate per A$_{720}$.

**Measurement of the Rate of RNA Synthesis**—The initial rates of RNA synthesis were measured by pulsing individual 0.5-ml aliquots of unlabeled culture with 0.6 pCi each of [$^{14}$C]uracil (0.203 Ci/mmol of uracil) in the presence of unlabeled cytidine (1 x 10$^{-4}$ M, final concentration) for 4 min (23). The labeling was stopped by adding an equal volume of ice-cold 20% trichloroacetic acid in the presence of 100 µg of unlabeled carrier E. coli tRNA. Millipore filtration and determination of radioactivity were the same as stated previously. The initial rates of RNA synthesis were expressed as the counts per min of [$^{14}$C]uracil incorporated into cold acid-insoluble material per min of exposure to the radioactive pulse per A$_{720}$.

**Measurement of the Rate of Protein Synthesis**—The initial rates of protein synthesis were measured by pulsing individual 0.5-ml aliquots of unlabeled culture with 1.5 µCi each of [$^{14}$C]arginine (0.203 Ci/mmol of arginine) for 4 min (23). Labeling was stopped by the addition of ice-cold 20% trichloroacetic acid and all samples were subsequently boiled at 100 °C in a water bath for 20 min. Millipore filtration and quantitation were the same as for the RNA samples measured above. The initial rates of protein synthesis were expressed as the counts per min of [$^{14}$C]arginine incorporated into hot trichloroacetic acid-insoluble material per min of exposure to the radioactive pulse per A$_{720}$.

**Extraction of H$_2$PO$_4$-labeled RNA for Gel Electrophoresis—**H$_2$PO$_4$-labeled RNA samples for use in gel electrophoresis were each derived from the cell pellets of 5 ml of whole culture. All samples were extracted as detailed by Lindahl (24) except the sucrose gradient fractionation step was eliminated. After DNase treatment, the whole RNA extract were underlaid per slot and were run at 4 V/cm. With this procedure, small RNA molecules are lost, but 17.5 S and 16 S RNA are clearly separated (24).

**RNA Gel Electrophoresis—**H$_2$PO$_4$-labeled RNA whole cell extracts were analyzed by electrophoresis on 3% polyacrylamide, 0.3% agarose composite gels following the procedure of Peacock and Dingman (25). These gels were prepared as vertical slabs (20 cm x 20 cm x 3 mm) with 20 sample slots (24). Ten of each whole cell H$_2$PO$_4$-labeled RNA extract were underlaid per slot and were run at 9 V/cm at 4 °C. With this procedure, small RNA molecules are lost, but 17.5 S and 16 S RNA are clearly separated (24). The gels were subsequently fixed, stained (25), and dried onto Whatman No. 3 paper. Following radioautography, the radioactive areas of the dried gel were then cut out and counted in 5 ml of Bray's Solution (26). All samples were quantitated as nanomoles of phosphate per A$_{720}$.

**RESULTS**

**Correlation between ppGpp Synthesis and RNA Accumulation**—Using an extraction procedure which recovers approximately 4 times more ppGpp from the cell than the conventional formic acid method (21), we had previously shown that the basal levels of ppGpp in all relaxed strains of E. coli decreased in response to amino acid starvation (17). A rough inverse correlation between RNA accumulation and the ppGpp level during amino acid starvation and subsequent resuspension had also been observed in these relaxed mutants (17). In our previous studies, however, RNA accumulation was quantitated as radioactive counts per min using [$^{14}$C]uracil while ppGpp was assayed in separate cultures labeled with H$_2$PO$_4$ (17). To more accurately test the relationship between ppGpp content during and after amino acid starvation and its influence on RNA accumulation, both parameters were measured in the same H$_2$PO$_4$-labeled culture and both were quantitated as moles of phosphate per A$_{720}$. The DNA content of this culture was also assayed in order to accurately quantitate the amount of RNA in each sample. Fig. 1 (bottom panel) reveals that in the E. coli K12 strain KL99 (relA1, spoT1) RNA accumulation continues at the same rate after addition of isoleucine to a valine-treated culture (at 41 min) as it did before resuspension until the ppGpp content of the culture surpasses its starvation basal level (at 60 min). Net RNA accumulation ceases after this time and, following a lag of approximately 15 min, a net decrease in RNA content is observed. The degradation of RNA proceeds while the ppGpp content is high (Fig. 1, top panel; 75 to 90 min) and only after the RNA content has decreased significantly does the ppGpp level begin to decline (after 90 min). The ppGpp content eventually returns to its prestarvation basal level (at approximately 120 min), and concomitantly, RNA accumulation resumes. Despite the apparent unresponsiveness of the rate of accumulation of RNA to a large change in the ppGpp level following the first 20 min of resuspension (41 to 60 min), these results appear to indicate that overall RNA accumulation in KL99, during its recovery from amino acid starvation, is inversely related to its ppGpp content (see below).

**Influence of ppGpp on the Initial Rates of RNA Synthesis in KL99**—Since the rate of RNA accumulation represents the difference between the rate of RNA synthesis and the rate of its degradation (10, 27), the initial rates of RNA synthesis in KL99 were examined during balanced growth as well as in

![Fig. 1. Correlation of ppGpp synthesis with RNA accumulation in KL99. One culture of KL99 (relA1 spoT1) was grown in Hershey's Tria minimal medium with 0.33 mM phosphate and was labeled with H$_2$PO$_4$ (1.5 µCi/ml) which was added two generations before the first sample was taken. At -10 min, the culture was split and 1 part was maintained as an unstarved control. Five hundred µg/ml of valine was added to the other part at time zero and 500 µg/ml of isoleucine was added at 41 min. Samples for nucleotide content and RNA production were taken from the same culture flask and were processed according to procedures outlined under “Experimental Procedures.” The RNA measurements were corrected for DNA content by subtracting alkaline-resistant trichloroacetic acid-precipitable radioactive counts from duplicate culture samples as described under “Experimental Procedures.” Unstarved (□); starved and resupplemented (●).](image-url)
response to amino acid starvation and subsequent resupplementation in order to clarify the apparent discrepancy mentioned in the previous section. The results presented in Fig. 2 indicate that a 90% decline in basal level ppGpp content occurs during the first 20 min of starvation and that this corresponds to approximately a 60% increase in the initial rate of RNA synthesis in this strain. The ppGpp level remains severely depressed throughout the remainder of the starvation period with almost no additional change between 20 and 40 min. This is coordinately reflected in only a very slight additional increase in the level of the initial rates of RNA synthesis. It should be noted that the drop in ppGpp level precedes the increase in the rate of RNA synthesis. For example, 2 min after the addition of valine, the ppGpp level had dropped 50% whereas only a small increase in the rate of RNA synthesis was detected.

Immediately after resupplementation of the amino acid-starved KL99 culture with isoleucine, the ppGpp content begins to increase and within the next 20 min it accumulates to an amount which is equivalent to its original prestarved basal level. During this same 20-min time span (41 to 60 min), the rate of RNA synthesis progressively declines until it too reaches its prestarvation rate. The succeeding 30 min show the characteristic overaccumulation of ppGpp to an amount which is about twice the basal level present during balanced growth and this corresponds to a 40% decrease from the basal level rate of RNA synthesis. As the ppGpp content gradually returns to its prestarvation basal level, the initial rates of RNA synthesis again increase until they too attain a level which is characteristic of balanced growth for this strain. This precise inverse coupling between ppGpp content and the initial rates of RNA synthesis suggests that ppGpp may control the accumulation of RNA in relaxed mutants in response to amino acid starvation and subsequent recovery through its action on the rate of RNA synthesis. In the first 20 min following resupplementation (41 to 60 min), there is a difference in the responsiveness to changing levels in ppGpp between the initial rates of RNA synthesis and the rates of RNA accumulation. This difference could be caused by a change in the rate of RNA degradation (which was not measured) during this period of time. The correlation between ppGpp levels and the initial rates of RNA synthesis demonstrated in relA mutants, however, provides new evidence to substantiate the role of ppGpp as a control element of transcription and suggests that ppGpp uniformly serves as a modulator of RNA synthesis during balanced growth as well as under nutritionally unfavorable conditions.

Characterization of RNA Produced during Amino Acid Starvation in relA Mutants—The nature of the RNA produced in KL99 during amino acid starvation and its fate as a result of resupplementation of starved cultures was examined electrophoretically on 3% polyacrylamide, 0.3% agarose composite slab gels (24, 25). When H235PO4-labeled whole cell extracts were used as the RNA source large increases in precursor ribosomal RNA components (23 S and larger1 and 17.5 S) were noted immediately after starvation commenced (Fig. 3). To show that the increase in the precursor rRNA in the cell extracts of KL99 is not due to an artifact of the extraction procedure, whole cell RNA extracts of PL10 (relA spoT+), NF859 (relA spoT+), and NF161 (relA spoT7) were also subjected to polyacrylamide-agarose gel electrophoresis under the same experimental conditions. The results presented in Fig. 4 (top panel) indicate that the behavior of PL10 is similar to the response detailed above for KL99 except that the magnitude of the changes in its rRNA levels is not as great (as predicted from the fluctuations in the total RNA content of this strain during amino acid starvation (17)). More importantly, however, the results with NF859 and NF161 (Fig. 4, middle and bottom panel, respectively) are consistent with the response of stringent bacteria to amino acid starvation reported in the literature (4, 11, 15, 16). (i.e. no net accumulation of rRNA during the period of starvation) indicating that the accumulation of radioactivity in the precursor rRNA areas of the slab gels from KL99 and PL10 whole cell extracts during amino acid starvation is not an artifact of the extraction procedure.

Fate of the RNA Produced in Relaxed Mutants during Amino Acid Starvation—Polyacrylamide-agarose gel electrophoresis of amino acid-starved whole cell extracts from relA mutants indicates that the content of precursor ribosomal RNA begins to increase at the onset of starvation and that this accumulation persists throughout the course of the amino

1 Technical difficulties with this type of gel electrophoresis system do not permit accurate reproducible separation of extremely large RNA molecules derived from unfraccionated cell extracts so all radioactive molecules larger than 23 S were always quantitated as a single unit.
RNA Synthesis and Basal Level ppGpp

The discovery that relA spoT mutants accumulate twice as much ppGpp during recovery from amino acid limitation as they produce during balanced growth and that this increase can reduce the initial rates of RNA synthesis in these strains by 60% revealed the existence of an unique situation in which the cell has the capability of synthesizing protein at the same time that it is accumulating substantial amounts of ppGpp. The influence of ppGpp content on the initial rates of protein synthesis in KL99 was therefore examined during this period in order to test the hypothesized inverse relationship between these two parameters under non-starvation conditions. The results presented in Fig. 5 indicate that, within 3 min of the onset of amino acid starvation, the rate of protein synthesis in this strain decreased to a level which represents a 70% reduction from its rate during logarithmic growth and which it maintains throughout the entire period of starvation. The synthesis of basal level ppGpp also drops in response to amino acid starvation as demonstrated previously.

Immediately after isoleucine has been added back to the isoleucine-starved culture, the initial rate of protein synthesis increases dramatically, and within 3 min, it shows a 5-fold increase over its residual starvation rate (representing a 60% increase from its prestarvation rate). This is not an unexpected result of amino acid repletion (Fig. 1, bottom panel) as well as the elevated levels of both mature 23 S rRNA and 16 S rRNA which result from its subsequent processing eventually decline until they reach their prestarvation basal levels. These results indicate that after processing the excess RNA which accumulated during amino acid starvation, relaxed bacteria degrade it and therefore are not likely to significantly increase their ribosome content beyond the amount dictated by their current growth potential.

The Influence of ppGpp on the Initial Rates of Protein Synthesis in KL99—The maintenance of normal translational fidelity in relA+ bacteria during periods of limited availability of a specific aminoacyl-tRNA species has been attributed to the large increase in ppGpp content which accompanies amino acid starvation (23). The hypothesis which has been put forward to explain these results rests on the premise that translational activity is directly influenced by ppGpp such that when the ppGpp level of a culture is increased either the rate of binding the cognate aminoacyl-tRNA-EF-Tu-GTP ternary complex to the A-site of the ribosome (28) or the rate of peptide chain elongation (29) decreases. The simple prediction made by this hypothesis is that a direct inverse relationship exists between ppGpp content and the rate of protein biosynthesis. The results used to substantiate this claim, however, were obtained solely through comparative studies of relA+ bacteria and relA mutants during amino acid starvation (23).

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acid limitation (Fig. 3; Fig. 4, top panel). Fig. 3 also reveals that the precursor rRNA molecules contained in the 17.5 S fraction are apparently processed into mature 16 S ribosomal RNA species after a time lag of about 5 min following resupplementation. These results also indicate that there is no apparent change in the 23 S and larger RNA fraction (which contains 30 S and 25 S precursor rRNA in addition to 23 S rRNA) during this same period of time. The apparent maintenance of the elevated levels of 23 S and larger rRNA found in the whole cell extracts after amino acid resupplementation (Fig. 3), however, can be explained as arising from the continued, although progressively diminished, elevation in the level of the rates of RNA synthesis (Fig. 2). This would result in the continued accumulation of total cellular RNA in excess of the basal level amount for the initial 20-min period following isoleucine addition (between 40 and 60 min). The increased amounts of total cellular RNA accumulated during amino acid starvation of KL99 (Fig. 1, bottom panel) as well as the elevated levels of both mature 23 S rRNA and 16 S rRNA which result from its subsequent processing eventually decline until they reach their prestarvation basal levels. These results indicate that after processing the excess RNA which accumulated during amino acid starvation, relaxed bacteria degrade it and therefore are not likely to significantly increase their ribosome content beyond the amount dictated by their current growth potential.

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result in as much as a significant proportion of the RNA which was synthesized during starvation must have been messenger RNA. This large increase in the rate of protein synthesis is maintained for about 25 min (41 to 65 min) and corresponds approximately to the time period required for ppGpp to accumulate to its prestarvation basal amount. Within the next 60 min, however, while the ppGpp content of the culture is at its highest, the initial rates of protein synthesis return to the level of their prestarvation rate and maintain this position despite an initial doubling of the basal amount of ppGpp followed by a 50% decline in its content. The lack of correlation between ppGpp content and the initial rates of protein synthesis revealed by these results indicates that ppGpp cannot be directly involved in the control of the rate of protein synthesis.

**DISCUSSION**

The ability of relaxed bacteria to greatly reduce their synthesis of basal level ppGpp in response to amino acid starvation has provided an opportunity to re-examine the influence of ppGpp synthesis on RNA production. The reductions in basal level ppGpp which accompany amino acid starvation of relaxed bacteria were found to be coordinated with increases in both their initial rates of RNA synthesis (Fig. 2) and, consequently, with total RNA accumulation (Fig. 1). Slab gel electrophoresis performed on RNA extracts of relaxed bacteria during amino acid starvation indicate that a large majority of the RNA which accumulates at this time is in the form of precursor ribosomal RNA species (Figs. 3 and 4) (16, 24, 30, 31). The fact that these precursor ribosomal RNA molecules are apparently stable throughout the course of the starvation period indicates that proper rRNA processing requires continued protein synthesis (30, 31) and suggests that the cell does not randomly destroy potentially useful RNA species during periods of metabolic imbalance. Following resupplementation with the required amino acid, however, relaxed cells begin to process the accumulated precursor rRNA species (Fig. 3). During this same period of time (41 to 60 min), the levels of ppGpp in these strains begin to increase, causing gradual reductions in the accumulation of new RNA due to a decrease in the rate of RNA synthesis. The consequence of processing and subsequent degradation of accumulated rRNA precursor molecules (Fig. 3) and the reduced accumulation of new RNA (Fig. 1) is reflected in a net loss of RNA from the bacterial culture (90 to 90 min) until the prestarved basal level content of RNA is reached. These results indicate that relaxed bacteria are not likely to increase their ribosome number beyond some limit dictated by their current growth potential.

The eventual overaccumulation of ppGpp in relaxed strains during recovery from amino acid starvation was found to be accompanied by a temporary decline in the rate of RNA synthesis below the level characteristic of balanced growth (Fig. 2). Conversely, following a transient surge in the initial rates of protein synthesis, the prestarvation rate of polypeptide production was resumed while the ppGpp content was still much higher than its basal level (Fig. 5; 70 to 120 min). The fluctuations in the initial rates of RNA synthesis, however, were found to be inversely correlated with the ppGpp content of the culture throughout the entire period of logarithmic growth, amino acid starvation, and recovery following resupplementation (Fig. 2). It appears, therefore, that the accumulation of excess RNA which occurs during starvation and the modulation of protein synthesis which accompanies this response both result from changes in the rate of RNA synthesis brought about by fluctuations in the ppGpp content of the cell. This inverse relationship between ppGpp levels and the initial rates of RNA synthesis in relaxed bacteria over extended periods of time provides new evidence to support the hypothesis that ppGpp is the control element of RNA synthesis during periods of metabolic imbalance (3, 4, 8, 10, 11, 15, 16, 32-35) as well as during balanced growth (5, 7).

(Recently, E. coli mutants (relS), which lack basal level ppGpp, have been reported (36). The ppGpp levels in these strains were determined using the conventional formic acid extraction procedure which is known to cause ppGpp degradation (21). We have observed that relS mutants do maintain small but measurable basal levels of ppGpp when this nucleotide is extracted by the lysozyme-deoxycholate procedure.)

**A Model for the Involvement of ppGpp as a Control Element during All Phases of Cell Growth—**The inverse correlation between the ppGpp content of relA mutants and their rates of RNA synthesis suggest the possible existence of a mechanism for the autogenous regulation of RNA production in all strains of bacteria which produce ppGpp. As stated previously, the decline of the ppGpp basal level of a relaxed mutant in response to amino acid starvation causes an increase in its rate of RNA production. Due to a lack of sufficient protein synthesis (Fig. 5) (4, 23, 37-39), however, the majority of the RNA which is produced during this period cannot be immediately utilized and therefore begins to accumulate (Figs. 3 and 4) (3, 4, 11, 15, 33). When the required amino acid is added back to a starved culture the capacity to synthesize protein returns (Fig. 5) and is accompanied by the resumption of ppGpp synthesis on the ribosome (7). The paradox which immediately presents itself, however, is that during this period of recovery from amino acid starvation, while the culture is growing, it is accumulating large amounts of ppGpp and gradually reducing its synthesis of new RNA (Fig. 2). (This situation had not been broached previously in the literature due to the lack of information about the response of basal level ppGpp synthesis in amino acid-starved relA mutants.) The simplest way to account for this would be to postulate a mechanism which would be sensitive to some consequence of excess RNA production and which could interact with the genetic product of the spoT locus causing it to shut off degradation of basal level ppGpp in a manner similar to the response observed when both stringent and relaxed spoT + and spoT strains are subjected to a carbon source downshift (7). This would allow ppGpp to accumulate through normal basal level production and, at the same time, would not interfere with protein synthesis (Fig. 5). The cell would therefore have the ability to determine the amount of RNA which is contained and to modulate its rate of RNA synthesis until it could properly dispose of any excess. This proposed mechanism may also be operative in unstarved bacteria (Fig. 1). The fact that relA mutants must be used to demonstrate the possible existence of this mechanism should not be interpreted as an indication that they have evolved a special physiologic distinct from wild type bacterium. The relA locus controls the production of stringent factor (34, 42-45) which is a component of this same control mechanism. The ability to observe the functions of this postulated mechanism in a relA background results solely from the capacity of these mutants to survive after removal of this component of the system.

Several attempts were made to verify the presence of ppGp (MS III) (46) in the nucleotide extracts used to quantitate the ppGpp measured during the course of this study. We were not able to reproducibly recover measurable amounts of ppGp from relA mutants using the lysozyme-deoxycholate extraction procedure (21) and under no circumstance was a corre-

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1. P. A. Lagosky, unpublished work.
2. C. C. Pao, personal communication.
lation between ppGpp content and the initial rates of RNA synthesis observed in these strains. In order to obtain measurable amounts of ppGpp, the lysozyme nucleotide extracts required re-extraction with formic acid. This also resulted, however, in an almost complete disappearance of ppGpp from the resulting extracts (17, 21). The closely coupled inverse correlation between ppGpp content and the initial rates of RNA synthesis demonstrated in this report, together with the above-mentioned inconclusive results, argues strongly in favor of a direct role for ppGpp in the control of RNA synthesis.

REFERENCES