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Influence of Amino Acid Starvation on Guanosine 5'-Diphosphate 3'-Diphosphate Basal-Level Synthesis in *Escherichia coli*

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We observed that the synthesis of basal-level guanosine 5'-diphosphate 3'-diphosphate (ppGpp) in both *relA* mutants and *relA*$^+$ *relC* strains of *Escherichia coli* decreased in response to amino acid limitation and that this was accompanied by an increase in ribonucleic acid (RNA) synthesis. Addition of the required amino acid to starved cultures of relaxed bacteria resulted in the resumption of ppGpp synthesis and a concomitant decrease in RNA production. Our results indicate that *relA* mutants retain a stringent factor-independent ribosomal mechanism for basal-level ppGpp synthesis. They also suggest that in *relA*$^+$ bacteria, stringent factor-mediated ppGpp synthesis and the production of basal-level ppGpp are mutually exclusive. These findings substantiate the hypothesis that there are two functionally discrete mechanisms for ppGpp synthesis in *E. coli*. Through these studies we have also obtained new evidence which indicates that ppGpp serves as a modulator of RNA synthesis during balanced growth as well as under conditions of nutritional downshift and starvation.

Bacteria that are capable of coordinating their production of stable RNA with the availability of required amino acids through the synthesis of the nucleotides guanosine 5'-triphosphate 3'-diphosphate (pppGpp) and guanosine 5'-diphosphate 3'-diphosphate (ppGpp) are termed stringent (8, 42). These bacteria produce an ATP:GTP pyrophosphate transferase which is responsible for the production of pppGpp and ppGpp on their ribosomes as a result of the binding of a codon-specific uncharged tRNA during amino acid starvation (12, 27, 28). This protein is the gene product of the *relA* locus and is termed the stringent factor (28).

Bacteria that carry recessive mutations in the *relA* locus are termed relaxed (5, 6, 18) and have been characterized by their failure to produce pppGpp and ppGpp in response to amino acid starvation and by their continued accumulation of RNA during this period (5, 7). These relaxed strains do, however, maintain basal levels of ppGpp (24, 25) and are capable of increasing their intracellular content of this nucleotide from 10- to 20-fold in response to carbon source downshifts or glucose starvation (10, 24, 25, 33, 35). Although relaxed bacteria are capable of producing ppGpp under certain physiological conditions, the involvement of stringent factor in ppGpp synthesis in *relA* mutants has never been conclusively demonstrated (1, 2, 4, 21, 22). Recent work, in fact, has led to the conclusion that the *relA* locus is not essential for cell growth and maintenance (1, 2, 22).

Several types of phenotypically relaxed mutants which are genetically independent of the *relA* locus have also been isolated (16, 23, 34, 36, 37, 44). One of these, initially labeled *relC* (23), produces a functionally active stringent factor protein but contains alterations in ribosomal protein L11 (*rplK*) which is a critical component of the stringent factor reaction complex (21, 23, 37). Thus, although these mutants remain *relA*$^+$, they do not produce ppGpp in response to amino acid starvation and accumulate RNA as though they carried the classic *relA1* mutation (3, 21, 23).

We have discovered, through the use of a recently reported nucleotide extraction procedure (32), that the synthesis of basal-level ppGpp in both *relA* and *relC* (*rplK*) mutants decreases in response to amino acid starvation and that this results in the overproduction of RNA. Our results provide physiological evidence for the existence of a stringent factor-independent mechanism for the synthesis of basal-level ppGpp on the ribosomes of both stringent (*relA*$^+$) strains and *relA* mutants of *Escherichia coli*. In addition, they suggest that this ability to synthesize basal-level ppGpp might be an absolute requirement (17) needed for the control of cell growth through the modulation of RNA production.

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MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1 along with their relevant genotypes.

Media, growth conditions, and radioactive labeling. All bacterial strains were grown in Hershey Tris minimal medium (11) containing 0.33 mM phosphate, 0.3% glucose, thiamine (2 μg/ml), and 50 μg of each required amino acid and nucleoside per ml. Cultures were incubated at 37°C in a PsycroTherm incubator shaker (New Brunswick Scientific Co., Inc.), and growth was monitored turbidimetrically at 720 nm; an optical density at 720 nm (OD_{720}) corresponds to about 10^9 cells/ml. Amino acid starvation was initiated by the addition of 500 μg of valine per ml and was reversed by adding 500 μg of isoleucine per ml. Inhibition of ppGpp synthesis in some experiments was done by adding of 100 μg of chloramphenicol per ml (Sigma Chemical Co.) at the indicated time.

Nucleotides were labeled with carrier-free $H_3{}^{32}PO_4$ (New England Nuclear Corp.), usually at 15 μCi/ml, which was added two generations before sampling. RNA accumulation was measured as the incorporation of [^{14}C]uracil (5 μCi/87.5 μmol per ml) in the presence of 100 μg of unlabeled cytidine per ml (26). To correlate ppGpp synthesis with RNA production, separate cultures, labeled with $H_3{}^{32}PO_4$, were maintained in equal portions of the same medium used for the RNA experiment. Unlabeled uracil (10 μg/ml) and unlabeled cytidine (100 μg/ml) were added to the $H_3{}^{32}PO_4$-labeled cultures at the optical density reading at which the [^{14}C]uracil and unlabeled cytidine were added to the culture used to measure RNA. Subsequent optical density readings were used as a guide to assure parallel growth behavior in both cultures.

Nucleotide extraction and quantitation. Nucleotide extracts were prepared according to procedures detailed previously (32) except that all volumes were reduced in half. Briefly, 5-ml culture samples were collected in Corex tubes (previously chilled in a dry ice-acetone bath) and centrifuged at 23,500 × *g*. The supernatants were carefully removed, and 100 μl of lysozyme (1 mg/ml) in 0.01 M Tris-hydrochloride (pH 7.8) and 0.015 M magnesium acetate was added to the cell pellets. After three cycles of freezing in a dry ice-acetone bath and thawing in ice water at 0°C with intermittent blending, 10 μl of a 10% deoxycholate solution was added to each lysozyme extract. All samples were again centrifuged, and the supernatant solutions were saved for chromatography. It is critical that the cells and subsequent extracts stay as cold as possible and that the chromatography be carried out immediately because gradual degradation of the ppGpp in these extracts was found to occur upon storage. One molar formic acid (8) was used to extract the nucleotides shown in Fig. 1 and was used only for the purpose of comparison.

Nucleotides were separated by two-dimensional thin-layer chromatography on commercially prepared polyethyleneimine-cellulose sheets (Brinkmann Instruments Inc.); 1.5 M LiCl and 2 M HCOOH were used as the solvent in the first dimension, and 1.5 M KH_2PO_4, pH 3.4, was used in the second dimension (26). Radioactive compounds were located by radioautography using Kodak X-Omat R film, cut from the polyethyleneimine-cellulose sheets, and counted in a Nuclear-Chicago liquid scintillation counter. The nucleotide levels, expressed as picomoles per OD_{720}, were adjusted to compensate for the slight dilution differences which arose during the course of the experiments and during the extraction procedures (32).

RNA determination. RNA production was quantitated by mixing 50 μl of [^{14}C]uracil-labeled culture samples with 1.5 ml of ice-cold 10% trichloroacetic acid. The precipitates were collected by filtration and were washed with a total of 15 ml of cold 10% trichloroacetic acid. Radioactivities were determined by using Formula 949 (New England Nuclear Corp.), a toluene-based scintillation fluid.

RESULTS

ppGpp recovery from *relA*$^+$ and *relA* strains. The amount of ppGpp which can be recovered from stringent (*relA*$^+$) strains of *E. coli* has been shown to be dependent upon the pH of the reagents used during its extraction (32) due to the presence of acid phosphatases within the cell (39, 43). Through the use of an

TABLE 1. *E. coli K-12 strains used*[a]

Strain	Markers and properties	Origin
NF859	F$^-$, *metB argA relA*$^+$ *spoT*$^+$	(21)
Hfr3000	Hfr PO1 *thi-1 relA1 spoT1*	(3)
KL99	Hfr PO42 *thi-1 relA1 spoT1*	(3)
PL10	F$^-$, *metB relA1 spoT*$^+$ *rplK*$^+$	Interrupted Hfr mating between KL99 and PL5(NF859 Str^r)
PL92	*his argH pyrE thi relA1 spoT*$^+$ *rplK*$^+$	NF1083 × P1(NF859)*thr*$^+$ *leu*$^+$ and then × P1(NF1086)*fuc*$^+$ (21)
PL101	*his argH pyrE thi relA1 spoT1 rplK*$^+$	NF1086 × P1(NF859)*thr*$^+$ *leu*$^+$ (21)
PL106	*his argH thiA relA*$^+$ *rplK spoT*$^+$	JF418 × P1(NF859)*thr*$^+$ *leu*$^+$ from J. D. Friesen
PL111	*his argH thiA relA*$^+$ *rplK*$^+$ *spoT*$^+$ Nal^r	JF478 × P1(NF859)*thr*$^+$ *leu*$^+$ (37)
PL116	*his argH*$^+$ *thiA relA*$^+$ *rplK1 spoT*$^+$ Nal^r	JF484 × P1(NF859)*thr*$^+$ *leu*$^+$ (37)
PL121	*his argH*$^+$ *thiA relA*$^+$ *rplK*$^+$ *spoT*$^+$ Nal^r	JF501 × P1(NF859)*thr*$^+$ *leu*$^+$ (37)

[a] We have found that the presence of the *leu* and the *thr* markers in *E. coli* K-12 strains distorts their response to valine-induced isoleucine starvation.

enzymatic nucleotide extraction procedure, which involves freezing and thawing of the cells in the presence of lysozyme at a neutral pH followed by treatment with deoxycholate (see Materials and Methods), we have been able to recover substantially more ppGpp from both stringent and relaxed bacteria than had previously been thought to exist in these strains (32; this study). A comparison between this technique and the most widely used formic acid extraction procedure with the stringent strain NF859 (*relA*$^+$ *spoT*$^+$) is presented in Fig. 1A. Note that similar fluctuations in the ppGpp level occurred throughout the course of amino acid starvation with both extraction procedures. The extent of these changes, however, was significantly amplified when this nucleotide was extracted enzymatically at a neutral pH. There were approximately threefold differences between the amounts of basal-level ppGpp recovered from both PL10 (*relA1 spoT*$^+$) and Hfr3000 (*relA1 spoT1*) when the lysozyme freeze-thaw technique was used instead of the formic acid procedure (Fig. 1B,C). Thus, the absolute levels of ppGpp which can be recovered from both stringent and relaxed strains of *E. coli* are directly influenced by the reagents used during nucleotide extraction. We have also found this to be true for pppGpp (unpublished data).

Basal-level ppGpp content of relA mutants. Our results indicate that all *relA* mutants maintain significant basal levels of ppGpp during balanced growth. A comparison of the lysozyme-extracted samples from the relaxed strains in Fig. 1B and 1C, however, revealed that the amount of ppGpp in *relA* mutants during balanced growth appeared to be under the control of the *spoT* locus, which has been shown to be responsible for ppGpp catabolism (29, 30, 41). Figure 2 provides proof for this observation through the use of a pair of *spoT*$^+$/*spoT* isogenic *relA1* strains. During balanced growth and under the same experimental conditions, PL101

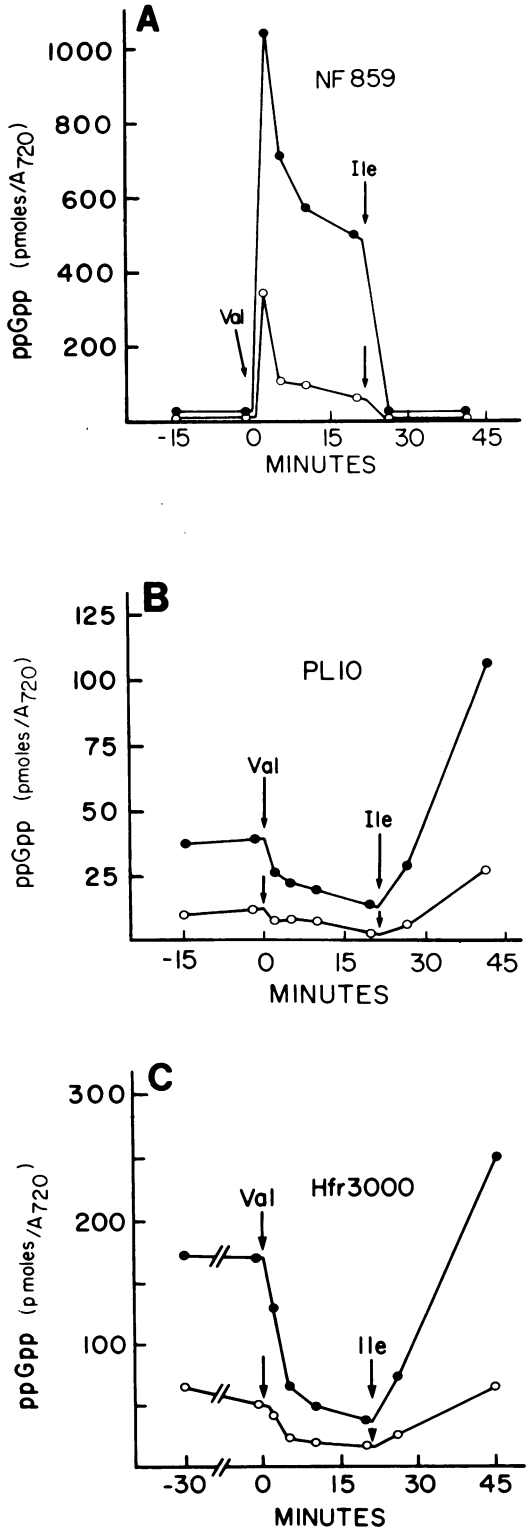

FIG. 1. *Influence of extraction conditions on ppGpp recovery. Cultures of NF859 (relA*$^+$ *spoT*$^+$*), PL10 (relA1 spoT*$^+$*), and Hfr3000 (relA1 spoT1) were each grown to midlog phase, and their nucleotides were labeled with carrier-free* $H_3{}^{32}PO_4$ *which was added two generations before the first sample was taken. Amino acid starvation was initiated at time zero by the addition of 500 μg of valine per ml and was relieved 20 min later by the addition of 500 μg of isoleucine per ml. Duplicate cultures samples were removed at each time point and processed according to the extraction procedures outlined in Materials and Methods. Symbols: ●, lysozyme-deoxycholate; ○, 1 M formic acid.*

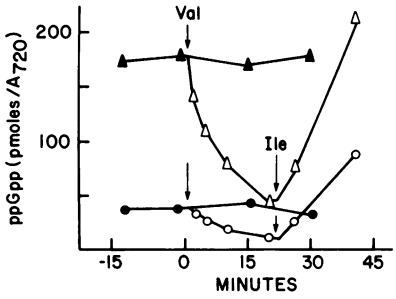

FIG. 2. *Influence of the spoT locus on basal-level ppGpp content in relA mutants. Cultures of the spoT⁺/spoT isogenic relA1 pair PL92 (relA1 spoT⁺) and PL101 (relA1 spoT1) were each grown on samples of the same minimal medium and their nucleotides were labeled with carrier-free $H_3{}^{32}PO_4$ as indicated in Fig. 1. At −10 min, each culture was split into two parts with one maintained as an unstarved control. Valine-induced isoleucine starvation was carried out on the other sample as stated in Fig. 1. Symbols: ●, Unstarved PL92; ○, starved and resupplemented PL92; ▲, unstarved PL101; △, starved and resupplemented PL101.*

(*relA1 spoT1*) maintained a ppGpp basal level which was approximately four times higher than the basal level of PL92 (*relA1 spoT⁺*). Similar results were also obtained with all other *relA spoT⁺* and *relA spoT* strains studied (data not shown).

Additional physiological factors, besides the gene product of the *spoT* locus, also appeared to influence basal-level ppGpp content (data not shown). In the course of this study, however, we never found a relaxed *spoT⁺* strain with a higher basal level of ppGpp than a *relA spoT* strain. These findings are also consistent with the phenotypic expression of the *spoT* locus in stringent bacteria (30, 31, 41). Thus the ppGpp which is being produced in relaxed strains appears to be under the same degradative control that is known to operate in stringent bacteria (13, 15, 30, 41, 45).

Response Of relA mutants to amino acid starvation. The importance of our ability to extract significant amounts of ppGpp from relaxed strains of *E. coli* during balanced growth was shown when these bacteria were subjected to amino acid starvation. Lysozyme nucleotide extraction of *relA* mutants has revealed that these strains exhibit an unusual response in their ppGpp levels during amino acid limitation which is not clearly discernible in nucleotide extracts prepared with formic acid. There was very little fluctuation in the ppGpp level of strain PL10 (*relA1 spoT⁺*) during amino acid starvation when the culture samples were extracted with formic acid (Fig. 1B). In the samples extracted with lysozyme, however, a clearly visible decrease of the ppGpp content from 40 to 25 pmol/OD_{720} could be seen within 2 min of the onset of amino acid starvation. The level of ppGpp remained low throughout the starvation period, dropping to 15 pmoles/OD_{720} by 20 min. Upon addition of the required amino acid, the amount of ppGpp rose dramatically and eventually surpassed its original basal level. This same pattern of decline in the ppGpp level in response to amino acid starvation and its subsequent rise as a result of resupplementation was also evident in the lysozyme-extracted samples taken from the *spoT* mutant Hfr3000 (*relA1 spoT1*) (Fig. 1C). Starting from a basal level of 180 pmol/OD_{720}, the ppGpp content of this strain dropped to less than 40 pmol/OD_{720} within the 20-min starvation period. In the 25 min after the addition of isoleucine to the starved culture, the amount of ppGpp recovered also exceeded its original basal level.

This response of relaxed bacteria to amino acid limitation has been observed in every *relA* mutant studied (*relA1* [3]; *relA2* [19]; *relA2 relX* [NF1035] [36]; and the nonsense and insertion *relA* mutants of Friesen et al. [22]), irrespective of the genotype of its *spoT* locus, and was found to be independent of the amino acid withheld (unpublished data).

Relaxed control of ppGpp production during amino acid starvation. During balanced growth, chloramphenicol, which blocks ppGpp synthesis (14, 25), mimicks the effects of amino acid starvation on the ppGpp levels of both *spoT⁺* and *spoT* derivatives of *relA* mutants. Addition of chloramphenicol to cultures of PL10 (*relA1 spoT⁺*) and KL99 (*relA1 spoT1*) reduced the amounts of ppGpp in them to nearly the same levels as did valine-induced isoleucine starvation (Fig. 3). The kinetics of this response were analyzed to determine whether the decline in the ppGpp level of *relA* mutants during amino acid starvation was due to an increase in its rate of degradation or to a decrease in its rate of synthesis. Amino acid starvation of strain KL99 elicited an immediate first-order decay rate constant of about 0.2 min^{-1} (Fig. 4). This value is virtually indistinguishable from both the rate constant of a parallel amino acid-starved culture which was also simultaneously subjected to chloramphenicol treatment (Fig. 4) and the published rate constant for ppGpp decay in chloramphenicol-inhibited *relA1 spoT1* bacteria (25). These results indicate that the decline in the level of ppGpp in *relA* mutants during the initial phase of their response to amino acid starvation is due to a halt of ribosome-mediated ppGpp synthesis and not to an increase in its rate of degradation in vivo.

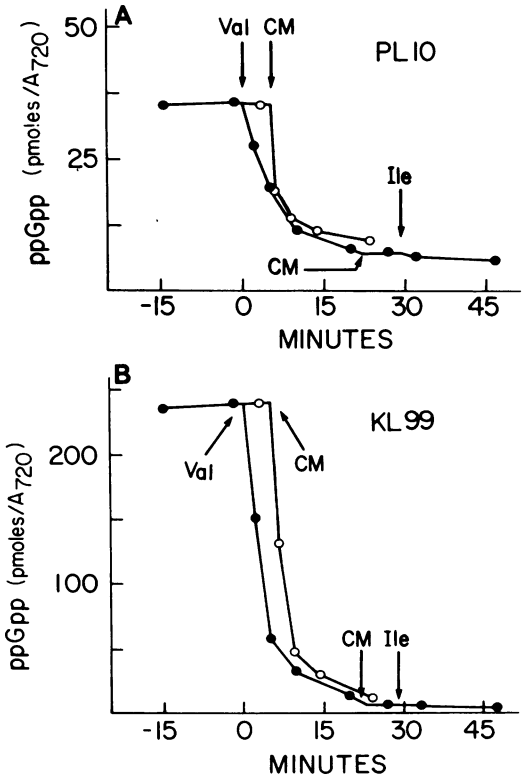

FIG. 3. *Influence of chloramphenicol and amino acid starvation on ppGpp production in relA mutants. Growth conditions for PL10 (relA1 spoT⁺) and KL99 (relA1 spoT1) were the same as described in Fig. 1. At −10 min the cultures were split into two equal parts. One part was subjected to amino acid starvation at time zero by the addition of 500 μg of valine per ml ●, and the other was treated with 100 μg of chloramphenicol per ml (○). After 20 min 100 μg of chloramphenicol per ml was added to the amino acid starved cultures, and 7 min later 500 μg of isoleucine per ml was added.*

The second phase in the response of *relA* mutants to amino acid starvation is the maintenance of the diminished levels of ppGpp. The ppGpp content of several different *relA1* strains was maintained at greatly reduced levels throughout the course of amino acid starvation (Fig. 1B,C and 2). The slight increases in the amounts of ppGpp observed during the latter stages of prolonged starvation (60 min or more; data not shown) can be attributed to gradual protein turnover which would supply limited quantities of the required amino acid, thus circumventing the constraints of amino acid limitation. The overall response in *relA* mutants, however, appeared to be very stable throughout the entire period of starvation.

Addition of the required amino acid to starved *relA* cultures resulted in increases in their ppGpp content which eventually exceeded their original basal levels (Fig. 1B,C and 2). If chloramphenicol was added to cultures of *relA* mutants during amino acid starvation, however, these increases in the ppGpp content after resupplementation could be totally abolished. The inhibitory effects of chloramphenicol, added during valine-induced isoleucine starvation of strains PL10 and KL99, could not be reversed by the addition of isoleucine. These results indicate that the increase in the ppGpp levels observed in relaxed mutants during their recovery from amino acid starvation occurs, at least in part, through the resumption of ppGpp synthesis on their ribosomes.

Amino acid starvation of *relA*⁺ *relC* strains. To determine whether the response of *relA* mutants to amino acid starvation reflects the existence of a previously uncharacterized mechanism for basal-level ppGpp production or is an artifact resulting from the *relA* mutation itself, we examined the influence of amino acid deprivation on ppGpp synthesis in several *relA*⁺ *relC* (*spoT*⁺) strains. These strains produce functionally active stringent factor but contain alterations in ribosomal protein L11 which render them phenotypically relaxed in response to amino acid starvation (21, 23, 37). When the *relA*⁺ *relC* (*spoT*⁺) strains PL106 and PL116 were subjected to short-term valine-induced isoleucine starvation, they responded by decreasing their production of basal-level ppGpp in a manner similar to the response seen in *relA* mutants. (For the purpose of comparison, Fig. 5B shows that both the *relA*⁺ *relC*⁺ parental strain [PL111] and the spontaneous *relC*⁺ revertant

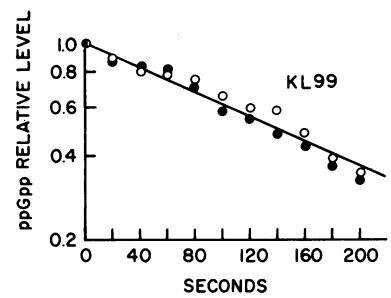

FIG. 4. *Kinetics of ppGpp degradation in KL99. Growth conditions for KL99 (relA1 spoT1) were the same as described in Fig. 1. At −10 min the culture was split into two equal parts. One part was subjected to amino acid starvation at time zero by the addition of 500 μg of valine per ml (○), and the other was treated with valine (500 μg/ml) and chloramphenicol (100 μg/ml) simultaneously (●). The amount of ppGpp recovered in both cases is expressed as a percentage of the ppGpp content present in each culture before starvation.*

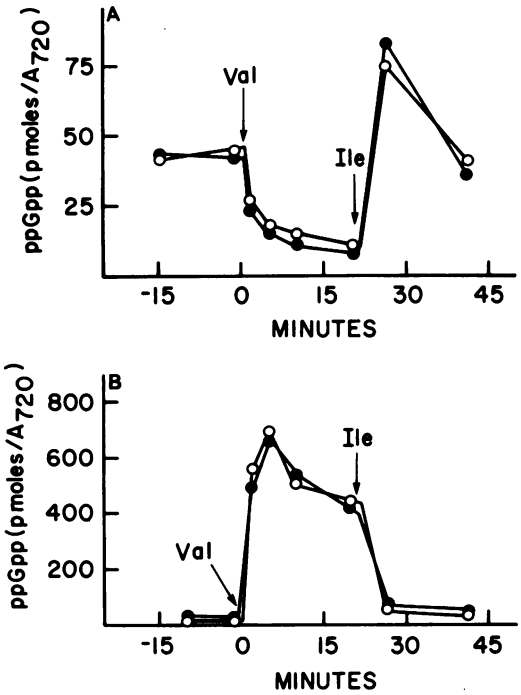

FIG. 5. *Influence of amino acid starvation on the ppGpp levels of relA⁺ relC spoT⁺ and relA⁺ relC⁺ spoT⁺ bacteria. All cultures were grown in Hershey Tris minimal medium and labeled with $H_3{}^{32}PO_4$ as indicated in Fig. 1. Valine (500 μg/ml) was added to each culture at time zero, and isoleucine (500 μg/ml) was added 20 min later. (A) ●, PL106 (relA⁺ relC spoT⁺); ○, PL116 (relA⁺ relC spoT⁺). (B) ●, PL111 (relA⁺ relC⁺ spoT⁺); ○, PL121 (relA⁺ relC⁺ spoT⁺). Note the difference in the scale of the ordinate in the bottom panel.*

[PL121] of strain PL116 exhibited the characteristic stringent response during this same period of amino acid limitation.)

The addition of the required amino acid to starved cultures of PL106 and PL116 resulted in their rapid accumulation of ppGpp to amounts that temporarily exceeded their prestarvation basal levels (Fig. 5A). If chloramphenicol was added before resupplementation, these increases could be totally abolished (data not shown), indicating that recovery from amino acid starvation in *relA⁺ relC* (*spoT⁺*) strains was similar to the response previously described in *relA* mutants (Fig. 2 and 3). There appeared to be significant differences, however, between the time required for these *relA⁺ relC* (*spoT⁺*) strains to overshoot their prestarvation basal levels of ppGpp in the aftermath of amino acid starvation as opposed to the time needed for the *relA relC⁺ spoT⁺* mutants PL10 (Fig. 1B) and PL92 (Fig. 2). This difference in recovery times was approximately fourfold and might have resulted from the presence of either functionally active stringent factor or the defective ribosomal protein L11 in the *relA⁺ relC* (*spoT⁺*) strains. We are currently trying to obtain a *relA relC* (*spoT⁺*) double mutant in order to test these possibilities.

The influence of amino acid limitation on the ppGpp levels of the *relA⁺ relC* (*spoT⁺*) strains PL106 and PL116 has been found to be similar to the response we have observed in *relA relC⁺* (*spoT⁺*) mutants. This indicates that the decline of ppGpp basal-level synthesis in response to amino acid starvation is not an artifact due to the presence of the *relA* mutation but rather a general characteristic of both *relA⁺* and *relA* mutant strains of *E. coli*.

Relaxed control of RNA synthesis. The correlation between ppGpp content and RNA synthesis was examined in *relA* mutants to determine the physiological implications of their response to amino acid starvation. As the ppGpp levels of PL10 (*relA1 spoT⁺*) and Hfr3000 (*relA1 spoT1*) declined in response to amino acid starvation, their rates of RNA accumulation increased (Fig. 6). The magnitude of these increases was directly correlated with the differences between the basal levels of ppGpp before starvation and the amount of ppGpp present at the time of isoleucine addition. Thus, the decline of the ppGpp level in PL10 by about 35 pmol/OD_{720} over the course of starvation was accompanied by only a 1.09-fold increase in the rate of RNA accumulation. In Hfr3000, however, the ppGpp level decreased by 185 pmol/OD_{720} and resulted in a 1.5-fold increase in the rate of RNA accumulation. These results indicate that the *spoT* locus, which controls ppGpp catabolism and therefore basal-level ppGpp content, might influence the growth rate of the cell by regulating the rate of RNA accumulation via the degradation of ppGpp.

Addition of the required amino acid to starved cultures of *relA* mutants caused the ppGpp levels of these strains to increase. This was accompanied by subsequent decreases in their rates of RNA accumulation. The increases in the ppGpp levels of *relA* mutants in the aftermath of amino acid starvation, however, always resulted in the overshooting of their prestarvation basal levels. The fact that RNA accumulation correspondingly decreased during this period, especially in the *spoT* mutant Hfr3000, indicates a very close coupling between ppGpp content and RNA synthesis in *relA* mutants.

Influence of amino acid starvation on subsequent RNA accumulation. The *E. coli* strain KL99 (*relA1 spoT1*) was used to deter-

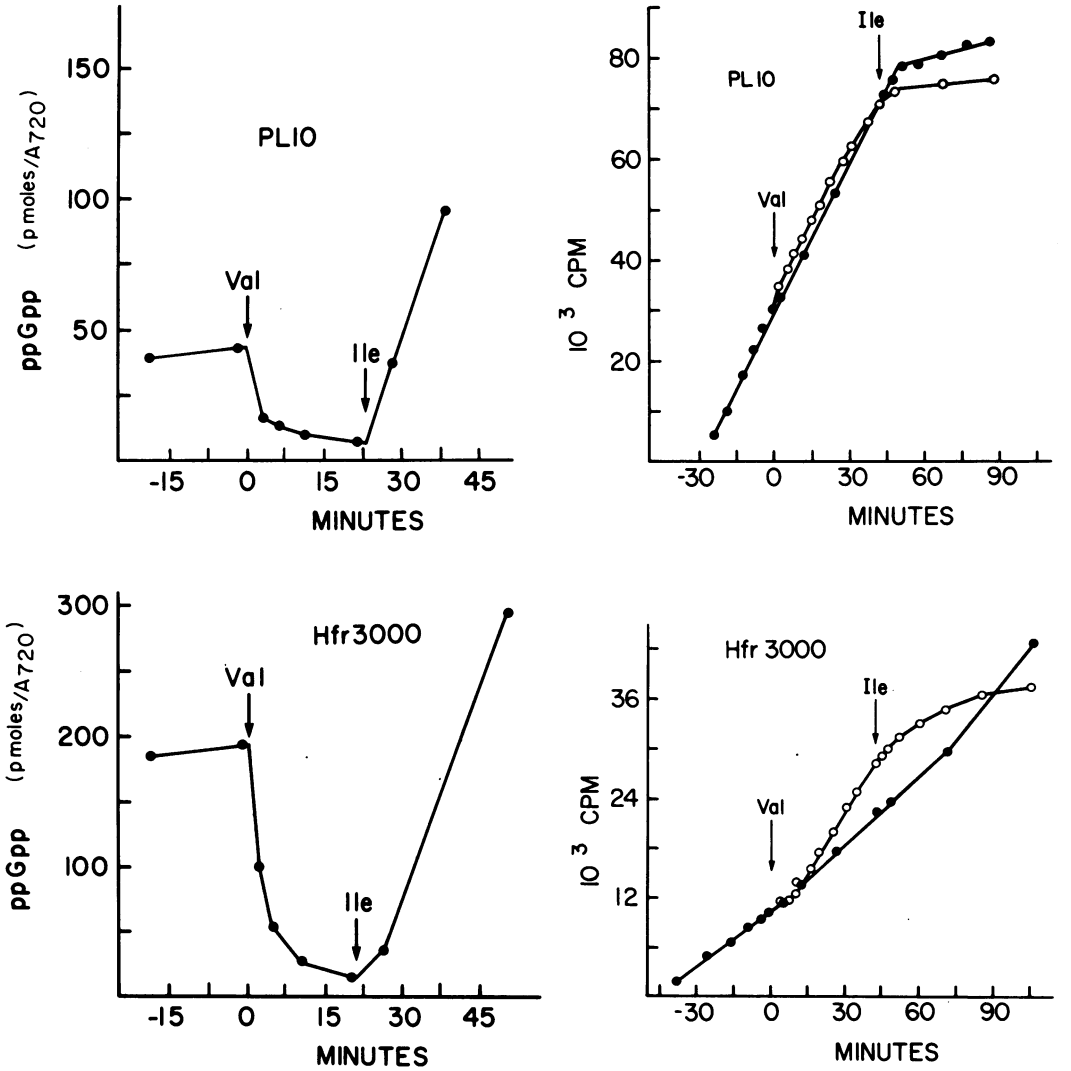

FIG. 6. *Effects of amino acid starvation on ppGpp synthesis and RNA accumulation in relA mutants. Two cultures of PL10 (relA1 spoT⁺) were grown in equal portions of the same Hershey Tris minimal medium with 0.33 mM phosphate as were two cultures of Hfr3000 (relA1 spoT1). Amino acid starvation and resupplementation in all four cultures were carried out as described in Fig. 1. For the ppGpp experiments (left panels), $H_3{}^{32}PO_4$ (15 μCi/ml) was added to one culture of each strain two generations before sampling. Unlabeled uracil (10 μg/ml) and unlabeled cytidine (100 μg/ml) were added at −30 min. To measure RNA accumulation (right panels), [^{14}C]uracil (5 μCi/87.5 μmol per ml) along with 100 μg of unlabeled cytidine per ml was added to the other culture of each strain at −30 min. These cultures were split at −10 min, and one half of each was maintained as an unstarved control. Symbols: ●, Unstarved; ○, amino acid starved and resupplemented.*

mine the long-term effects of amino acid starvation on subsequent RNA accumulation because it maintains the highest basal level of ppGpp we have found in a relaxed strain and because it grows slowly. The decrease in the amount of ppGpp in this strain at the onset of amino acid starvation was accompanied by a dramatic 2.6-fold increase in its rate of RNA accumulation (Fig. 7). As starvation proceeded, ppGpp was maintained at a greatly diminished level and RNA synthesis continued at a very high rate. When the required amino acid was added to the starved culture, the level of ppGpp increased and surpassed its prestarvation basal level. At the same time, RNA accumulation slowed down and virtually stopped. This situa-

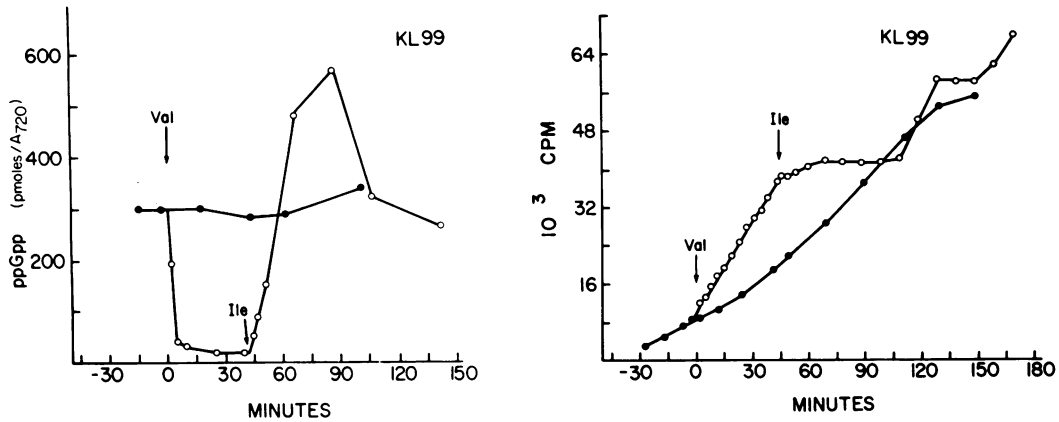

FIG. 7. *Influence of amino acid starvation on subsequent RNA production in a relA mutant. Two separate cultures of KL99 (relA1 spoT1) were subjected to amino acid starvation and resupplementation, and the production of ppGpp in one and RNA in the other were determined as detailed in Fig. 6. Both cultures were split before amino acid starvation, and one half of each was maintained as an unstarved control. Symbols: ●, unstarved; ○, amino acid starved.*

tion was maintained for about 40 min and corresponded to the time period when the ppGpp level in this relaxed strain was at its highest. Eventually, the level of ppGpp decreased and the accumulation of RNA once again resumed. The fact that the inverse relationship between ppGpp levels and RNA synthesis was maintained throughout the recovery period provides new evidence to substantiate the role of ppGpp as a control element of DNA transcription and suggests that ppGpp serves as a modulator of RNA synthesis during normal growth as well as under nutritionally unfavorable conditions.

DISCUSSION

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 observe that both *relA* mutants and *relA*$^+$ *relC* strains exhibit a unique response to amino acid limitation (Fig. 1B,C and 5A). Our results have shown that at the onset of amino acid starvation, both *relA spoT*$^+$ and *relA spoT* mutants display an immediate and sustained decline in their ppGpp basal levels. These diminished levels of ppGpp are maintained throughout the course of the starvation, increase rapidly upon addition of the required amino acid, and eventually surpass their prestarvation basal levels. Strains carrying the *relC* (*rplK*) mutation in a *relA*$^+$ *spoT*$^+$ genetic background were also found to exhibit a similar response. Previous failure to report this unusual response in phenotypically relaxed bacteria might be attributed to the use of low-pH reagents, especially formic acid, in the preparation of nucleotide extracts, since ppGpp degradation has been shown to occur during its extraction under low-pH conditions (32, 39, 43).

The relationship between cellular growth rates and the basal-level ppGpp content of various bacterial strains suggests that these two factors are in some way interrelated (7, 9, 20, 24, 25, 33, 40). We have found that the amount of basal-level ppGpp which relaxed bacteria maintain is primarily under the control of the gene product of the *spoT* locus (Fig. 2), although other factors also seem to be involved (unpublished data). Our results have shown, however, that *relA spoT* mutants consistently maintain higher basal levels of ppGpp during balanced growth than do *relA spoT*$^+$ strains (Fig. 1B,C and 2).

Isogenic pairs of *relA*$^+$ and *relA* strains have been shown to maintain similar basal levels of ppGpp, in accordance with their *spoT* allele, when they are grown on the same carbon source (24, 25). Since the presence of stringent factor has been shown to be expendable in *relA* mutants (1, 2, 22), these results suggest that stringent factor is not necessary for the synthesis of basal-level ppGpp (24, 25). The ability of both stringent and relaxed strains to maintain equivalent basal levels of ppGpp during balanced growth and the similar response of both *relA relC*$^+$ and *relA*$^+$ *relC* mutants to amino acid starvation reported here suggest the existence of a stringent factor-independent system for basal-level ppGpp synthesis. If there were such a mechanism, it would have to meet at least two obvious criteria. First, it would have to be under the control of an independent genetic locus; sec-

ond, it would have to be operative in both wild-type strains and *relA* mutants and be under the same physiological control in both. With regard to the first criterion, recent work has implicated both the *relX* locus (36) and the *relS* mutation (17) as possible genetic sites which may be responsible for basal-level ppGpp synthesis. With regard to the second criterion, we have found that the in vivo basal-level synthesis of ppGpp in both *relA spoT*$^+$ and *relA spoT* bacteria can be blocked, to virtually the same extent, through the action of either amino acid starvation or chloramphenicol inhibition (Fig. 3 and 4; unpublished data). The ability of *relA*$^+$ *relC* (*spoT*$^+$) strains (which produce functionally active stringent factor) to also halt basal-level ppGpp synthesis in response to amino acid starvation indicates that this phenomenon is not an artifact of the *relA* mutation itself. We would not expect to be able to experimentally demonstrate this response, however, in low-relaxed *relA* mutants (4, 19, 21) and *relA*$^+$ *relC spoT* strains (21) because these bacteria have been shown to be capable of synthesizing or accumulating ppGpp in response to amino acid starvation (4, 18, 19, 21). Nevertheless, these results suggest that the halt of basal-level ppGpp synthesis in response to amino acid starvation is a characteristic of all *relA*$^+$ and *relA* strains of *E. coli*.

The signal which triggers basal-level ppGpp synthesis has not yet been determined, but it appears to involve the ribosome and at least several aspects of normal protein synthesis. We have shown that chloramphenicol, which inhibits peptidyltransferase activity (14, 25), and amino acid starvation of *relA* mutants and *relA*$^+$ *relC* strains, which results in the binding of uncharged tRNA species to the ribosomal A site (27), both cause a similar reduction in the synthesis of basal-level ppGpp (Fig. 2–4 and 5A; unpublished data). We have also found that erythromycin, which blocks the translocation step during protein biosynthesis (38), can inhibit basal-level ppGpp synthesis in both stringent and relaxed strains (unpublished data). These results provide physiological evidence for the existence of a ribosome-mediated, stringent factor-independent mechanism for basal-level ppGpp synthesis in *E. coli* which is inhibited by amino acid starvation as well as by agents that interfere with protein synthesis (14, 25; unpublished data).

The ability of relaxed bacteria to greatly reduce their synthesis of ppGpp in response to amino acid starvation has provided us with the unique opportunity to reexamine 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 their rates of RNA accumulation (Fig. 6). A comparison of the results obtained with PL10, Hfr3000, and KL99 also reveals that the greater the decline in the ppGpp level of a *relA* mutant after starvation, the larger the increase in its rate of RNA accumulation (Fig. 6 and 7). Resupplementation of amino acid-starved *relA* mutants caused their levels of ppGpp to increase and their rates of RNA synthesis to decline. The eventual overaccumulation of ppGpp in these strains during recovery from amino acid starvation was found to be accompanied by a temporary halt in RNA production which prevailed until the ppGpp content returned to its prestarvation basal level (Fig. 7). This inverse relationship between ppGpp levels and rates of RNA accumulation in relaxed bacteria over extended periods of time argues strongly in favor of the hypothesis that ppGpp is the control element of RNA synthesis during periods of metabolic imbalance (9, 10, 20, 21, 29, 30, 40, 41) and also suggests that basal-level ppGpp is intimately involved in the control of RNA production during balanced growth.

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