The RNA Chain Elongation Rate of the λ Late mRNA Is Unaffected by High Levels of ppGpp in the Absence of Amino Acid Starvation*

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Karsten Tедин‡ and Udo Bläsi
From the Institute for Microbiology and Genetics, The University of Vienna, Biocenter, Dr. Bohr-Gasse 9, A-1030 Vienna, Austria

In this study, the effects of high levels of guanosine tetraphosphate (ppGpp) on the decay and RNA chain elongation kinetics of the bacteriophage λ late transcript in Escherichia coli were examined in the absence of amino acid starvation. The accumulation, mRNA decay kinetics, and RNA chain elongation rate of the λ late mRNA were determined after heat induction of acl857 lysogens in the presence of high levels of ppGpp induced from a RelA fragment-overproducing plasmid. The accumulation kinetics and elongation rate determinations of the late mRNA were made at long times after induction to allow a new steady state of transcriptional activities under conditions of elevated intracellular levels of ppGpp. The results indicate no prolonged or significant effect on either mRNA decay or the RNA chain elongation rate of the late mRNA as a result of elevated ppGpp levels. Surprisingly, the RNA chain elongation rate determinations indicate an RNA polymerase processivity of approximately 90-100 nucleotides/s for the λ late transcript despite the presence of high levels of ppGpp. The results are discussed in terms of various models for regulation of stable and messenger RNA synthesis in E. coli.

Guanosine tetraphosphate (ppGpp) was first recognized as a potential regulator of transcriptional activities in Escherichia coli during amino acid starvation, where levels of this nucleotide accumulate rapidly to very high levels, correlating with the immediate and preferential cessation of stable ribosomal and transfer RNA synthesis (rRNA, tRNA), an effect known as the stringent response to amino acid starvation (Stent and Brenner, 1961; Cashel, 1969; Cashel and Gallant, 1969). The ribosome-associated enzyme responsible for synthesis of ppGpp during amino acid starvation is encoded by the relA gene (Alföldi et al., 1962; Haseltine et al., 1972; Cashel, 1975). The enzyme responsible for the basal level synthesis of ppGpp therefore determines the relative fraction of RNA polymerase and indirectly reducing stable RNA synthesis. The effects of ppGpp on the transcription of mRNA genes are quite variable, showing activation and inhibition of some gene promoters while others remain indifferent (Reines et al., 1975; Stephens et al., 1975; Primakoff and Arzt, 1979; Primakoff, 1981; Mizushima-Sugano et al., 1983; Riggs et al., 1986; Kajitani and Ishihama, 1984; Mizushima-Sugano and Kaziro, 1985).

Two models that accept ppGpp as a regulator of RNA synthesis have been proposed. The RNA polymerase partitioning model of Bremer and co-workers (Ryals et al., 1982; Bremer et al., 1988; Baracchini and Bremer, 1988) proposes that ppGpp binds RNA polymerase and "partitions" it into two forms, an unbound form that initiates transcription preferentially at stable RNA promoters and a ppGpp-bound form that initiates transcription at mRNA promoters. The intracellular concentration of ppGpp therefore determines the relative fraction of transcriptional activities devoted to either stable or messenger RNA synthesis. In addition, Hernandez and Bremer (1993) and Hernandez and Bremer and Ehrenberg (1995) have proposed that the low, basal levels of ppGpp present during normal exponential growth inhibit mRNA synthesis, possibly by causing pausing or "queuing" of RNA polymerase at or near mRNA promoters and thereby indirectly stimulating initiation at stable RNA promoters as unengaged or "free" RNA polymerase concentrations increase as mRNA promoters become blocked. This hypothesis was proposed to explain the 3- to 4-fold increase in mRNA synthesis in strains deleted for both the relA and spoT genes, which contain no measurable ppGpp levels, and the apparently large fraction (70- to 80%) of RNA polymerase in E. coli which is not actively engaged in transcription (Dalbow, 1973; Matzura et al., 1973; Shepherd et al., 1980a, 1980b; Bremer and Dennis, 1987).

The RNA chain elongation model of Jensen and Pedersen (1990) proposes that ppGpp functions by slowing transcription elongation of mRNA synthesis, thereby sequestering RNA polymerase and indirectly reducing stable RNA synthesis. According to this model, the stable RNA promoters are assumed...
to be more sensitive to the concentration of free RNA polymerase, with mRNA promoters being at or close to saturation with RNA polymerase. The concentration of RNA polymerase available for transcription of stable RNA could be controlled, therefore, through alterations in the mRNA chain elongation rates by such reductions or pausing during the elongation phase of transcription. The work of Vogel et al. (1992) and Vogel and Jensen (1994a, 1994b) measuring the mRNA chain elongation rates in vivo of RNA transcription of lacZ or infB constructs during partial amino acid starvation or during exponential growth on different carbon sources has been taken as support for the idea that the concentration of RNA polymerase applies only to mRNA synthesis.

One of the major problems that has made studies on the effects of ppGpp on gene expression difficult to assess has been the necessity for the use of either amino acid or carbon source starvation protocols to provoke accumulation of ppGpp in E. coli, situations that have pleiotropic effects on gene expression. The recent cloning and overexpression of the relA gene under transcriptional control of inducible and repressible promoters has allowed for the first time the separation of the effects of increased intracellular levels of ppGpp from the secondary effects of amino acid starvation (Schreiber et al., 1991; Tedin and Bremer, 1992). The use of the plasmid-encoded relA gene product and ribosome-independent derivatives permits the accumulation of concentrations of ppGpp equivalent to the levels of ppGpp which accumulate during amino acid starvation, but without the use of amino acid or carbon source starvation. In this study, the effects of high levels of ppGpp after overexpression of the ribosome-independent relA gene fragment on the transcription elongation rate of a translated mRNA have been examined. Accumulation of the bacteriophage λ late mRNA after heat induction of acl 857 lysogens in the presence of high levels of ppGpp is shown to be delayed relative to control cultures due to an inhibition of DNA replication, but the elongation rate of initiated RNA chains is unchanged. In addition, the elongation rate determinations indicate an RNA chain elongation rate of approximately 100 nucleotides/s, a value close to that determined previously for the rrn operon RNA chain elongation in E. coli.

**EXPERIMENTAL PROCEDURES**

Materials—Restriction endonucleases and other modifying enzymes were purchased from either Boehringer Mannheim, Promega, Stratagene, or New England Biolabs and used according to the manufacturer's instructions. Antibiotics and IPTG were purchased from Boehringer (Hamburg, Germany). Oligonucleotides were synthesized using an Applied Biosystems DNA synthesizer.

**TABLE I**

| Strains               | Genotype* | Source or reference |
|-----------------------|-----------|---------------------|
| MG1655                | Wild type E. coli K-12 F⁻ | H. Bremer           |
| KT1015                | MG1655(lacB57) F⁻ | This study          |
| KT1021                | KT1015 (F' lacZAM15 proAB⁺ tet) | This study          |
| KT1033                | KT1021(pKT31) | This study          |
| KT1035                | KT1021(pKT40) | This study          |
| KT1180                | KT1021(CEQ3, pKT38) | This study          |
| KT1182                | KT1021(CEQ3, pKT41) | This study          |
| KT1374                | MG1655(lcl 857Sam7) F⁻ | This study          |
| KT1376                | KT1374(F' lacZAM15 proAB⁺ tet) | This study          |
| KT1384                | KT1376(pKT31) | This study          |
| KT1386                | KT1376(pKT40) | This study          |
| XL1-Blue              | endA1 gyrA96 hsdR17 lac reA1 relA1 supE44 thi1 | Stratagene          |

Plasmids

| λd                    | Laboratory stock |
|-----------------------|-------------------|
| λd B57(Ts)            | Laboratory stock |
| λd B57(Ts)Sam7        | Laboratory stock |

* Brackets denote bacteriophage lysogens; parentheses denote episomes. For details of construction of strains and plasmids, see “Experimental Procedures.”
Strain Constructions—Bacteriophage λ d357 and λ d3575am7 lysogens of E. coli K-12 strain MG1655 were constructed by infection with lysates essentially as described by Miller (1972) and Arber et al. (1983). For introduction of F’ factors containing the lacI gene for Lac repression, the lysogenic recipient strains KT1015 and KT1374 (Table I) were mated with the donor strain XL1-Blue (Strategene) as described in Miller (1972). The donor DNA was harvested from samples of overnight cultures grown in LB medium with aeration at 28°C and containing IPTG at a final concentration of 1.0 mM. Lysates were then prepared by boiling in 2 volumes of 1.0% SDS, with 50 µg of proteinase K (Boehringer) and incubation at 37°C for 45 min. After chilling on ice, the lysates were centrifuged and the supernatants were used for transformation into the recipient strain.

Plasmid Constructions—Plasmids used in this study are listed in Table I. Plasmid pKT31 was constructed by subcloning an EcoRI-Xhol fragment encoding the reAα fragment from plasmid pKT24 (Tedin and Bremer, 1992) into the EcoRI and Xhol sites of pHue21-2, which contains the bacteriophage T7 A1 promoter and two lac operator sequences.

DNA samples were collected by centrifugation, washed twice with 70% ethanol, dried under vacuum, and resuspended in 0.2 ml of diethyl pyrocarbonate-treated deionized water. One half of the total nuclear acids was then made 0.1 M sodium acetate and 5.0 mM MgCl2, and treated with 15 units of RNase free DNase (Boehringer Mannheim) at 37°C for 4 h. The remaining half of the samples was treated with RNase A at a final concentration of 10.0 µg/ml and also incubated at 37°C for 4 h. Mitochondrial DNA was extracted from 40% samples with 0.3 M sodium acetate, and precipitated by the addition of 2.5 volumes of 100% ethanol. After centrifugation, the resulting pellets were again washed twice with 70% ethanol and resuspended in 0.1 ml of 0.1 M EDTA, and the concentration was determined spectrophotometrically at a wavelength of 260 nm. Verification of the absence of either DNA for the RNA samples or RNA for the DNA samples was performed by both agarose (0.8%) and polyacrylamide (6.0%) gel electrophoresis with overloaded samples.

Hybridization Probe Synthesis—Polymerase chain reaction-generated (α-32P)CMP-labeled DNA hybridization probes corresponding to either the a gene at the 5’ or to the λ ρ genes at the 3’ end of the late mRNA were synthesized using 100 ng of purified λ d357am751 DNA (New England Biolabs) as template and the primers CS5 (5’-GCCACGTCTGTCGTTGTG-3’) and P3 (5’-GCGCATATTATCTGCGCGGCGG-3’) for the 5’ probe and the primers Z6 (5’-GGAATACCCCGTCGTTCATCAG-3’) and C7 (5’-GTTCCGTTGATTTAGTGATGCGTG-3’) for the 3’ probe, each at 100 ng/reaction. The deoxyribonucleotides present in 250 µM dATP, dCTP, dGTP, and dTTP were each added to 25 µM of the specific probe. The DNA polymerase (Stratagene). Both probes were synthesized using a polymerase chain reaction program consisting of a temperature cycle of 92°C, 1 min; 50°C, 1.5 min; and 74°C for 1 min for a total of 35 cycles. Hybridization probes were phenol extracted twice and precipitated with 0.3 M sodium acetate and 2.5 volumes of 100% ethanol. Purity of the samples was verified by polyacrylamide gel electrophoresis.

Nuclease Acid Hybridization—Preparation of buffers and hybridization solutions were performed according to Sambrook et al. (1989). A volume of total RNA or DNA corresponding to either 5 or 10 µg of nuclear acid was denatured by addition of 1/3 volume of a solution containing 70% deionized formamide, 10% formic acid, and 1 × SSC buffer, heating at 65°C for 10 min, and then on ice. Samples were loaded into the slots of a Schleicher & Schuell SRC 072/0 MiniSlot II slot-blot apparatus fitted with BA85 nitrocellulose sheets prewet and preweighed with 1.0 ml of 10 × SSC. Each sample was washed with 1.0 ml of 10 × SSC, dried for 5 min under slight vacuum, and then removed and air dried for 30 min. Filters were baked for 2 h at 80°C and placed in hybridization flasks. Filters were prehybridized for 2 h in 20.0 ml of a solution containing 50% formamide, 5 × SSPE buffer, 2 × Denhardt’s solution, and 0.1% SDS at 45°C as described in Sambrook et al. (1989). Hybridization probes were denatured for 5 min in 0.2× NaOH, 2.0 ml EDTA at 37°C and then added directly to the prehybridization solution. The filters were washed at 45°C overnight in a solution containing 5 × SSC, 0.1% SDS at 25°C for 30 min and three washes of 1 × SSC, 0.1% SDS at 45°C each for 30 min. After drying and exposure of an x-ray film, the individual slots were either excised and hybridized radioactivity determined by liquid scintillation counting or quantitated using a Molecular Dynamics PhosphorImager and accompanying software. A background radioactivity signal was used to indicate the presence of non-specific binding. Samples of total E. coli RNA from a non-lysogen or plasmid DNA were equivalent to slots containing no RNA. The maximal bound radioactivity for late time samples was approximately 1,000–2,000 cpm. As a measure for the hybridization efficiencies and to assure that each probe used was in excess, duplicate samples of different amounts (5, 10, 50 and 100 ng) of purified late mRNA was denatured and the DNA content of the solution of the nitrocellulose to be probed with either the 5’ or 3’ probes as a measure of the hybridization efficiencies for each probe. From a standard curve constructed from the hybridization of a DNA, a small correction factor was calculated (approximately 9%) for plotting of the hybridization signals for the RNA or DNA hybridization curves.

RESULTS

RNA and Protein Accumulation in the Presence of High Levels of ppGpp—A reduction in total RNA and protein accumulation in the absence of amino acid starvation has been observed after overexpression of ppGpp in strains harboring inducible relA- containing plasmids (Schreiber et al., 1991; Tedin and Bremer, 1992; Svitil et al., 1993). Consistent with these observations, IPTG induction of ppGpp synthesis in

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strain KT1033 harboring plasmid pKT31 (Fig. 1) results in a rapid cessation of both RNA and protein accumulation (Fig. 2, B and D), correlating with the accumulation of high levels of ppGpp (Fig. 3B). However, what is also apparent is that the rate of protein synthesis in the presence of high levels of ppGpp only mirrors the decline in the reduction of cell mass as measured by optical density (Fig. 2F), whereas total RNA accumulation, the bulk of which is stable rRNA and tRNA (Bremer and Dennis, 1987), ceases immediately. Since protein synthesis continues under these conditions, although at a reduced rate, these results imply that mRNA synthesis is less inhibited than stable RNA synthesis. In contrast, IPTG induction of the control strain, KT1035, which does not accumulate ppGpp after induction of strain KT1033 and control strain KT1035. As can be seen from the growth curve for strain KT1033, after up to 3.5 h after induction there is only approximately a 50% decrease in optical density, indicating either an extremely delayed or incomplete lysis, whereas the control strain KT1035 showed normal lysis kinetics. Addition of IPTG at times prior to or within 5 min after the heat shift showed similar effects, whereas IPTG addition after approximately 10 min showed essentially no effect on the outcome of heat induction (data not shown), suggesting that ppGpp affected events occurring only within the first few minutes after induction.

The two genes required for host lysis by bacteriophage λ are the S and R genes present at the extreme 5' end of the λ late region, with the S gene the most 5'-proximal and followed directly by the R and Rz genes, respectively. The S gene is absolutely required for lysis and encodes two protein species responsible for introducing a nonspecific lesion in the inner membrane of the host to allow passage of the R gene product, an endolysin, to the periplasm where its murein substrate is then degraded, resulting in cell lysis (for review, see Young (1992)). The failure of strain KT1033 to lyse after heat/IPTG induction could be the result of a failure to initiate transcription of the late message, for example if the pr' promoter were
negatively regulated by ppGpp, and therefore results in no synthesis of either the S or R gene products. The absence of lysis, however, does not always provide information about late mRNA transcription, since lysis is only a measure of S protein synthesis which is not required for either prophage induction or phage assembly, e.g. in the case of λSam phage (Goldberg and Howe, 1969), which accumulate higher than normal levels of progeny phage because lysis is prevented. It is known that the appearance of the R gene product begins between 15 and 20 min after induction, with about a 10-fold increased activity at 30 min (Echols, 1971). A simple test for the presence of the R gene product encoding the endolysin.

Fig. 3. Accumulation of ppGpp in strains KT1033 and KT1035 after IPTG induction. Samples correspond to the experiment shown in Fig. 2. Preinduction samples are indicated by open circles (○), postinduction samples by filled circles (●). Panel A, accumulation of ppGpp in the control strain KT1035; panel B, strain KT1033. The values shown are the averages of triplicate determinations.

Fig. 4. Heat induction of λ1857 in the presence of high levels of ppGpp. At time t = 0 min, cultures growing in LB medium at 28 °C were shifted to 44 °C for inactivation of temperature-sensitive λ repressor with simultaneous addition of IPTG to a final concentration of 1.0 mM. At time t = 30 min, the cultures were shifted to 37 °C and the cultures monitored for lysis by the decrease in optical density. Panel A, strains KT1033 (●) and KT1035 (○); panel B, strains KT1180 (●) and KT1182 (○) (see Table I). The arrow above the OD curve for strain KT1033 indicates the time of addition of chloroform to test for the expression of the λ R gene product encoding the endolysin.

as 30 min after heat/IPTG induction was found to show the same immediate effect of lysis (data not shown).

Heat/IPTG Induction of KT1033 with Q Overexpression—A second possibility explaining the failure of heat/IPTG induction of strain KT1033 to result in lysis might be an increase in the frequency of premature terminations of initiated RNA chains in the presence of elevated levels of ppGpp. The chloroform lysis test might not necessarily discriminate against this possibility because of the rapid accumulation of the R gene product, which might still occur despite an increased frequency of premature terminations of the late transcript. In an attempt to discriminate between an effect on transcription initiation at pR' or increased premature terminations, the effects of high levels of ppGpp concomitantly with overexpression of the λ Q protein were examined.

The 8–10-min delay in appearance of the late mRNA after induction of λ lysogens is ascribed to the time required to synthesize the product of the Q gene of λ. The Q protein is the product of one of the pR'-initiated delayed-early genes, which is required for antitermination through the terminators proximal to the 5' end of the late mRNA coding sequences (Roberts, 1975; Forbes and Herskowitz, 1982; Luk and Szybalski, 1983). Two possibilities for the delay in RNA synthesis from pR' might be either a reduced affinity of the RNA polymerase for the pR'-promoter caused by ppGpp, despite the presence of Q, or that it is the Q protein itself that is limiting. In the former case, additional Q protein might not be expected to increase transcription from pR', whereas in the latter case increased levels of Q protein might overcome the reduction in mRNA synthesis and result in a rescue of the observed lysis inhibition. To test the hypothesis that it is the Q protein that is limiting rather than a direct effect by ppGpp on the initiation of transcription at pR', strain KT1021 was transformed with plasmids pKT38 or pKT41 and pCEQ3 (Petrenko et al., 1989; see Table I), the latter plasmid encoding the Q antiterminator protein. As can be seen in Fig. 4B, heat induction and lysis of cultures harboring λ prophage concomitantly with IPTG induction of ppGpp synthesis are only partially restored in the presence of extra Q protein (compare Fig. 4A), showing only a slight increase in the onset of lysis approximately 30–60 min earlier. In other experiments, induction of Q synthesis from plasmid pCEQ3 was found to be capable of trans-activation of both λ wild type and λimm434 lysogens, indicating that the plasmid-encoded Q protein was both inducible and functional (data not shown). The presence of plasmid pCEQ3 encoding additional Q protein was not sufficient to overcome completely the inhibition of λ1857 induction by ppGpp, suggesting either that (i) limiting Q protein and hence premature termination of transcription were not one of the major effects of ppGpp on λ induction, and the inhibition reflects some other effect of ppGpp e.g. inhibition of transcription initiation at pR'; or (ii) in the presence of ppGpp, Q synthesis, both phage λ- and plasmid pCEQ3-encoded, was inhibited. There is some evidence suggesting that the latter situation might be the case since in both cases Q synthesis is under transcriptional control of the pR' promoter, which has been suggested to be under stringent control (Wegrzyn et al., 1991; Szalewska-Palasz et al., 1994). Despite the lack of a significant difference on λ induction in the presence of the Q-overproducing plasmid, sufficient Q protein must have been present in the presence of ppGpp to allow transcription initiation at the pR' promoter to allow synthesis of the R gene product, the presence of which was demonstrable by chloroform addition. These results suggested that the late mRNA was capable of being transcribed under these conditions and that at least the pR' promoter itself was not directly inhibited by
ppGpp, an important prerequisite for determination of the effects of ppGpp on transcription of the λ late mRNA.

Determination of λ Late mRNA Accumulation Kinetics after Heat Shift and ppGpp Induction—From the chloroform lysis test for R synthesis, it was apparent that the λ late mRNA was being initiated and at least the 5' proximal portion transcribed and translated. However, this method could not provide an answer to the question of whether the RNA chain elongation or premature termination of transcription caused by ppGpp at some point distal to the R gene, although accumulation of infective phage particles to within 30% of that of the control strain at long times after the induction suggested the latter was not the case (data not shown). To make these distinctions, the accumulation kinetics and elongation rate of transcription of the late mRNA were determined. Cultures of strains KT1035 and KT1035 were grown to an OD600 of approximately 0.2, at which OD the cultures were simultaneously shifted to 44°C and made 1.0 mM IPTG. After 20 min at 44°C the cultures were returned to 37°C. Samples were removed at different times before and after induction for determination of the relative amounts of λ late mRNA, phage DNA, and ppGpp determinations. Samples collected in duplicate for RNA and DNA were bound to nitrocellulose membranes for hybridization to either a 5' probe specific for the λ gene or to a 3' probe specific to the extreme distal J and lom genes.

Because it was of importance that both the 5' and 3' determinations be made with as close to the same conditions as possible, the filters were not stripped and then reprobed; rather, duplicate samples were blotted onto the same filter, which was later cut into strips and probed separately with either the 5' or 3' probes. Likewise, care was taken that the hybridization probes themselves were of approximately the same length and GC content and exactly the same molar content of the labeled deoxynucleotide to avoid hybridization artifacts that would affect the appearance of the elongation kinetics when plotted. Fig. 5 shows representative autoradiograms of slot blots of material hybridizing to either the 5' or 3' probes for the late mRNA (Fig. 5A and B) or DNA (Fig. 5C and D), respectively. Qualitatively, a slower accumulation of hybridizable material is apparent between samples from strain KT1035 (panels B and D) relative to strain KT1035 (panels A and C).

For quantitation of 5' and 3' mRNA accumulation kinetics, the individual slots for each time point shown in Fig. 5A and B, were excised and counted directly for hybridizable material is apparent between samples from strain KT1035 (panels B and D) relative to strain KT1035 (panels A and C). For quantitation of 5' and 3' mRNA accumulation kinetics, the individual slots for each time point shown in Fig. 5A and B, were excised and counted directly for hybridizable material corresponding to the late mRNA. The induction kinetics shown in Fig. 5A for the control culture is consistent with previously reported accumulation kinetics for the λ late mRNA, with approximately the same fold-increase in hybridizable material with time (Dove, 1966; Champoux, 1971). The variability in RNA hybridization for the control strain KT1035 at later time points (Fig. 6A) presumably results from the onset of lysis, which made cell recovery difficult. The horizontal distance between the two RNA accumulation curves shown in Fig. 6 (panels A and B) represents the time delay between the accumulation of the same amount of hybridizable material for a probe for either the 5' or 3' end, i.e. the RNA chain elongation rate. Fig. 6B shows the induction kinetics for the late mRNA in the presence of high levels of ppGpp for strain KT1033. The accumulation kinetics of ppGpp for strains KT1035 and KT1033 is shown in Fig. 6C and 6D, respectively. Although the initial appearance and accumulation kinetics of the late mRNA are obviously delayed in the presence of high levels of ppGpp, the distance between the two curves remains approximately the same as seen in the RNA accumulation curve for the control strain KT1035 shown in Fig. 6A, indicating no significant effect on the elongation rate. This is seen more clearly in a linear regression analysis of the accumulation curves shown in Fig. 7. Panel A of Fig. 7 shows the analysis of points for control strain KT1035, and panel B shows that for strain KT1033. Based on a known length between the two probes of 22,639 base pairs and a shift in the time required for accumulation of equivalent amounts of 5' and 3' hybridizable material of approximately 4 min (4.4 and 4.5 min for strains KT1035 and KT1033, respectively) the elongation rate for the λ late mRNA can therefore be estimated to be between 90 and 100 nucleotides/sec, a value much higher than that reported for other translated mRNAs but close to that reported for RNA chain elongation rates for the rrr operons in E. coli (Molin, 1976; Ryals et al., 1982; Condon et al., 1993; Vogel and Jensen, 1994b, 1995).

ppGpp Does Not Increase Premature Terminations of the Late Transcript—An increase in the ratio of 5' to 3' mRNA hybridization signals shown in Fig. 6 would indicate if the RNA polymerase were to terminate prematurely at some point distal to the hybridization site of the 5' probe. A plot of the ratios of accumulation of the 5' to 3' ends of the late mRNA is shown in Fig. 8 (panels A and B). Although the 5' to 3' ratio is approximately 40% higher in strain KT1035 than KT1033, respectively, the ratio falls quickly back to the control strain levels within the following 5 min, i.e. no significant or prolonged increase in the ratios of 5' to 3' ends is apparent for strain KT1033, despite the high intracellular levels of ppGpp. In addition, the heat induction alone increases the intracellular ppGpp concentration approximately 5-fold even in the control strain KT1035, which is not IPTG-inducible for ppGpp synthesis (see panel E of Fig. 6), without increasing the 5' to 3' late mRNA ratios (Fig. 8A). These results therefore suggest that increased premature termination during transcription of the late mRNA is not one of the effects of ppGpp. This observation also supports the interpretation from experiments with concomitant overexpression of the λ Q protein with ppGpp induction (Fig. 4B; see also "Discussion").

Determination of λ DNA Accumulation after Heat Shift and
ppGpp Induction—A portion of the samples for total nucleic acids used for late mRNA determinations was also treated with RNase A and hybridized at the same time for determination of λ DNA accumulation. As can be seen in panels C and D of Fig. 6, λ DNA accumulation in strain KT1033 is also delayed relative to the control strain KT1035. This suggests that the major cause for the reduction in late mRNA accumulation in strain KT1033 is a delay or inhibition of λ DNA replication or events prior to DNA replication. Because the late mRNA continues to accumulate, however (panels A and B, Fig. 6), this implies the pr and pr' promoters are not inhibited by ppGpp. It appears, therefore, that the reason for the delay in late mRNA accumulation in the presence of high levels of ppGpp occurs at some early step in the induction pathway, possibly by interfering with events also involved in λ DNA replication. It is known that transcription is required for λ DNA replication, not only for synthesis of the λ O and P proteins, but also for activation of the λ origin of replication (Dove et al., 1971).
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Matsubara, 1981; Hase et al., 1989). In addition, Wegrzyn et al., (1991) and Szalewska-Palasz et al. (1994) have implicated ppGpp in an inhibition of λ O protein synthesis resulting from an inhibition of transcription initiation by RNA polymerase at the pR promoter. However, although DNA replication is apparently slowed, the amount of the late transcript increases steadily, concomitantly with the increase in DNA accumulation. This is shown in the comparison of the ratios of late mRNA to DNA (Fig. B, C and D). The control strain KT1035 shows a 3–4-fold increase in the mRNA to DNA ratio shortly before onset of lysis (Fig. 8C), whereas the strain KT1033 begins a slight increase only at later times (Fig. 8D). Therefore, although there is a reduction in DNA replication, transcription initiations and the continued high rates of RNA chain elongation of the late mRNA are unaffected.

Fig. 9. Determination of λ late mRNA and DNA accumulation in λcI857sam7 lysogenic strains KT1384 and KT1386. Panels A, C, and E indicate results for control strain KT1386; panels B, D, and F, strain KT1384. Panels A and B indicate growth of cultures after heat/IPTG induction. Open circles in panels C–F (○) represent hybridization signals using the proximal 5′ probe; filled circles in panels C–F (●), hybridization signals using the distal 3′ probe. Panels C and D, hybridization results for λ late mRNA accumulation; panels E and F, for λ DNA accumulation. The experiment plotted in this figure was repeated twice, independently including culture growth and sampling, with the same results.

A formal possibility for the unchanged appearance in the accumulation kinetics of the λ late mRNA for both the S+ and Sam7 lysogens is the presence of high levels of ppGpp relative to their isogenic counterparts are depicted (Fig. 12, A and B, respectively), even with high intracellular levels of ppGpp the late mRNA accumulates at longer times to within 80% the level of the control.

A plot of the ratios of 5′ to 3′ hybridization signals for the two strains, KT1384 and KT1386, shows a transient higher level of 5′ ends during the first 15–20 min after the induction (Fig. 11, panels A and B). In both strains, however, the ratio of 5′ to 3′ ends returns to approximately the same level after 15–20 min after the induction, indicating again no significant differences that would suggest premature termination as a major effect as the result of the elevated levels of ppGpp. Likewise, the λ late mRNA to λ DNA ratios were also approximately the same (Fig. 11, panels C and D), implying that transcription initiations of the λ late mRNA were not affected by high levels of ppGpp. Indeed, despite the more rapid accumulation of the late mRNA in the control strains, when the ratios of the late mRNA for both the S+ and Sam7 lysogens in the presence of high levels of ppGpp relative to their isogenic counterparts are depicted (Fig. 12, A and B, respectively), even with high intracellular levels of ppGpp the late mRNA accumulates at longer times to within 80% the level of the control.
signals for the late mRNA.

proximal 5

through the ends due to the slowing of RNA polymerase transcribing would then compensate for the reduced accumulation of the 3' ends. To discriminate against this possibility, in addition to samples for determination of the RNA chain elongation rates, at the times indicated in Fig. 9, A and B, rifampicin was added to a portion of the shifted culture to inhibit any new transcription initiations, and samples were removed for measurement of the 5' and 3' region decay kinetics. As shown in Fig. 13, the 5' mRNA decay kinetics for both strain KT1384 and control strain KT1386 are approximately the same, with exponential decay of the 5' region of the late mRNA in both strains beginning after a lag of between 1.9 and 2.2 min, corresponding to half-lives of 2.2 and 2.1 min, respectively, average for E. coli mRNAs (Pedersen et al., 1978; Baracchini and Bremer, 1987). Decay of the 3' regions begins after a much more extended lag for the reasons mentioned below, with the onset of decay beginning 5.2 and 5.9 min after the addition of rifampicin. The corresponding half-lives for the 3' regions of the late mRNA in strains KT1384 and KT1386 can therefore be calculated to be 1.9 and 2.0 min, respectively. What is clear from these results is that at least the rates of decay for both the 5' and 3' regions are essentially identical between the two strains, despite the reduced absolute amounts of the late message in strain KT1384 and elevated levels of ppGpp.

Determination of the decay kinetics of the late mRNA also allows another, independent means of measuring the RNA chain elongation rates. The addition of rifampicin prevents new transcription initiations; however, those RNA polymerase molecules that have already initiated would require additional time to traverse the template to the 3' end of the late region and shift the decay curve for the 3' hybridizable material until all RNA polymerase molecules have finished their transcripts, at which time the 3' region would also begin to be degraded. This lag difference therefore becomes an additional means of measuring the RNA chain elongation rate. The time delay in the decay curves between the 5' and 3' regions of the late mRNA corresponds to 3.3 and 3.8 min for strains KT1384 and KT1386, respectively, which is slightly faster than the rate determinations from the accumulation curves. This slight increase might represent an expansion of the nucleotide pools after the addition of rifampicin, as has been suggested previously (Jensen and Pedersen, 1990). As before, using the distance between the two coding regions examined, it can be calculated that the RNA chain elongation rate corresponds to approximately 115 nucleotides/s in both strains (114 and 116 nucleotides/s for KT1386 and KT1384, respectively) and in
addition, verifies that the RNA chain elongation rate of the \( \lambda \) late mRNA is not affected by the high intracellular levels of ppGpp.

**DISCUSSION**

In this work, we have determined the effects of high levels of ppGpp in the absence of amino acid starvation on the transcription, decay kinetics, and RNA chain elongation rate of the bacteriophage \( \lambda \) late mRNA. The late transcript of bacteriophage \( \lambda \) was chosen as a model system for this study because its extreme length lends itself to determinations of the elongation rate, and the gene products encoded within the late transcript allow relatively straightforward assays for levels of protein synthesis as another measure for the functional levels of mRNA being transcribed. In addition, since the majority of the early, transcriptionally active, circular \( \lambda \) DNA replication forms cease accumulating after approximately the first 15–20 min after induction or infection (Furth and Wickner, 1983), only the steady-state levels of transcription at longer times are examined without the additional complications of increasing copy number. For the mRNA accumulation and degradation kinetics determinations, the 5′-proximal S and 3′-distal J-lom coding regions were chosen because of their extreme positions within the late mRNA and similar levels of translation.

ppGpp Does Not Affect the Late mRNA Decay Kinetics or RNA Chain Elongation Rate—The rate of mRNA chain elongation in \( E. coli \) at 37 °C has been determined as approximately 40–50 nucleotides/s (Mueller and Bremer, 1968; Bremer and Yuan, 1968; Manor et al., 1969; Molin, 1976; Vogel and Jensen, 1994a, 1994b), at which rate it would take the RNA polymerase between 8 and 10 min to extend the late transcript from the 5′ to 3′ end. The RNA chain elongation rate determinations presented in this work, however, indicate that the \( \lambda \) late mRNA is extended at a much higher rate of approximately 90–100 nucleotides/s at 37 °C, which is quite close to the values determined previously for \( \lambda \) mRNA operons (Nyland et al., 1982; Condon et al., 1993; Vogel and Jensen, 1994a, 1995). This high RNA chain elongation rate is also consistent with the observations of Ray and Pearson (1975) who measured the decay kinetics and functional stability of \( \chi \) \( \lambda \) late mRNA after the addition of rifampicin. A comparison of the lag periods observed by these authors before the beginning of exponential decay of the coding capacity of genes C (5′-proximal) and J (3′-distal) indicated a delay of approximately 2 min between the start of decay of C relative to J (see Fig. 4, A and B; Ray and Pearson, 1975). These authors expressed surprise that the inactivation of the gene coding segment was so rapid; however, they had assumed an RNA chain elongation rate of 55 nucleotides/s, and the delay in inactivation of the intervening genes might be explained by the high levels of translation of these coding regions, which could protect them from RNase inactivation. With this consideration in mind and a distance of about 12,000 base pairs between the C and J genes, their results also indicate an RNA chain elongation rate of approximately 100 nucleotides/s. Although the rate determinations in the wild type S+ gene lysogens (strains KT1033 and KT1035) were complicated by the scatter present at late times in the control strain (KT1035), the RNA chain elongation rates correlate well with those that can be extrapolated from the work of Ray and Pearson and from the accumulation kinetics and time delay in the 5′ and 3′ mRNA decay curves in the \( \chi \) \( \lambda \) lysogens where lysis does not occur (strains KT1384 and KT1386), indicating in all cases the same high RNA chain elongation rates despite the presence of high intracellular levels of ppGpp.

Determination of the mRNA decay kinetics, in addition to verifying the absence of a significant difference in decay rates in the presence of ppGpp of the late mRNA, also provided an additional, independent means of measuring the RNA chain elongation rates. A difference in the decay rates might have been expected, since the major effect of ppGpp is correlated to a cessation in ribosome synthesis (see the Introduction). Assuming that RNA stability is in part related to the efficiency of ribosome loading on a given mRNA, one might have expected the late mRNA to show altered decay rates in the presence of high levels of ppGpp, where de novo ribosome synthesis is drastically reduced. Our interpretation for the absence of such an effect in the case of the \( \lambda \) late mRNA is the concomitant reduction in accumulation of the late mRNA because of delayed \( \lambda \) DNA replication, which compensates for the reduction in continued ribosome synthesis.

Implications for Growth Rate and Stringent Control of RNA Synthesis by ppGpp—As mentioned in the Introduction, there are currently two models that are based on the predicate that ppGpp is the major regulator of RNA synthesis in \( E. coli \). The RNA polymerase partitioning model of Bremer and co-workers proposes a direct and preferential inhibition of transcription initiation at stable RNA promoters by ppGpp, the fraction of ppGpp-modified RNA polymerase thereby determining the amount of transcriptional activity dedicated to either stable RNA or mRNA synthesis. The RNA chain elongation model of Jensen and Pedersen proposes that stable RNA promoters preferentially lose activity because of competition with mRNA promoters as more RNA polymerase becomes trapped due to the reduced transcription elongation rate of mRNA synthesis caused by ppGpp. The results presented here indicate that a slowing of RNA chain elongation is apparently not an effect exerted by ppGpp during synthesis of the \( \lambda \) late mRNA. These observations confirm the observations of Vogel and Jensen (1995) who found that the leader regions of \( rrr \) transcripts which contain \( \chi \) nut-like (boxA) sequences conferred higher elongation rates to both lacZ and inB4 mRNA's in artificial constructs. The observed high elongation rates are therefore most likely conferred by the \( \chi \) late mRNA put sequence, similar to the \( \lambda \) boxA regions, allowing the RNA polymerase to escape transcriptional pausing or termination sites (for review, see Morgan, 1986; Yager and von Hippel, 1987; Condon et al., 1995). These results also explain the previously observed differences in RNA chain elongation rates for mRNA synthesis (40–50 nucleotides/s) and rRNA synthesis (80–90 nucleotides/s) and imply that a reduction in the RNA chain elongation rate is not a general effect of ppGpp, but rather depends upon either the type of RNA examined (antiterminated) or whether...
the RNA polymerase is complexed with other factors. In addition, the observations that the presence of the rrrB boxA sequence in either rrr or lacZ and infB constructs showed unaltered RNA chain elongation rates during the stringent response (Vogel and Jensen, 1995) and the absence of an effect in this study of ppGpp on the RNA chain elongation rate of the λ late mRNA suggest that assembly of an antitermination complex could either alter or block the binding site for ppGpp on the RNA polymerase (Owens et al., 1987; Reddy et al., 1995), rendering transcription elongation of an RNA immune to ppGpp-mediated pausing effects. With regard to stable RNA synthesis, since the RNA chain elongation rates of initiated chains are not affected by ppGpp (postinitiation), these observations underscore the idea that the effect of ppGpp on stable RNA synthesis is on the promoter selectivity (initiation) of RNA polymerase, an idea supported by a wealth of evidence including in vitro experiments with purified components (see the Introduction) and the isolation of mutants in RNA polymerase subunits with altered responses (Nomura, 1987; for review see Condon et al., 1995). For effects on the RNA chain elongation rate by ppGpp to function as a regulatory mechanism for stable RNA synthesis, the RNA chain elongation model assumes that the stable RNA promoters require higher levels of RNA polymerase for maximal activities, making them particularly sensitive to the concentration of free RNA polymerase. This model proposes that mRNA promoters are at or close to saturation with RNA polymerase, with stable RNA synthesis therefore dependent upon the limiting pool of free RNA polymerase. Two observations speak against limiting RNA polymerase and effects of ppGpp on RNA chain elongation with regard to stable RNA regulation. It has been shown previously that transcription of stable RNA occurs at the expense of mRNA synthesis, e.g. during nutritional shift-ups (Shepherd et al., 1980b) and during artificial limitations of RNA polymerase cloned under transcriptional control of inducible promoters (Bedwell and Nomura, 1986; Nomura et al., 1987). In the latter experiments, at limiting concentrations of inducer, stable RNA synthesis remained unchanged while mRNA synthesis decreased, implying that the stable RNA promoters compete effectively for RNA polymerase. This result does not imply, however, that RNA polymerase is not limiting in the cell. The fact that mRNA synthesis decreased under these limiting conditions indicated that RNA polymerase was limiting (as it was supposed to be), but the converse experiment with elevated RNA polymerase synthesis resulted in increased mRNA synthesis while stable RNA synthesis remained unchanged. Second, from the work of Hernandez and BREMER (1993) using the ΔrelAΔsspT (ppGpp−) strains, which lack ppGpp, it is also clear that mRNA promoters are not saturated with RNA polymerase, since in the absence of ppGpp, mRNA synthesis is 3–4-fold overrepresented at fast growth rates, matching the increased synthesis and activity of RNA polymerase, parameters that were also determined in these experiments. These results imply that (i) in general, RNA polymerase is limiting in the cell, but only for mRNA synthesis; and (ii) there is a separate mechanism that regulates the ability of stable RNA promoters to compete for limiting RNA polymerase. This second mechanism most likely involves ppGpp. In a recent model developed by BREMER and ERENBERG (1995) to explain effects observed in the ppGpp− strains, ppGpp could affect transcription of mRNA genes during the early phase of transcription through pausing effects at or near the promoters but without affecting the RNA chain elongation rate of RNA polymerase molecules that have escaped this early, ppGpp-mediated pause site. This is a subtle difference with regard to the model proposed by JENSEN and PEDERSEN (1990) and VOGEL AND JENSEN (1994a, 1994b) and has different predictions with respect to regulation of gene expression by ppGpp. The model of JENSEN AND PEDERSEN (1990) and VOGEL AND JENSEN (1994a, 1994b) would predict that the λ late mRNA transcription, like stable RNA synthesis, should also become limited by RNA polymerase as more RNA polymerase is trapped by a reduction in the RNA chain elongation rate during mRNA synthesis. From the results presented in this study, it is clear that transcription of the λ late mRNA shows no signs of depletion of RNA polymerase even after long times after the induction in the presence of high levels of ppGpp, up to 4 h in the case of the ΔΔ857sam7 lysogens (compare panels C and D, Figs. 8 and 11), suggesting that RNA polymerase is not limiting for transcription of the late mRNA. Our interpretation of the observations with the λ late mRNA is therefore more compatible with the model proposed by BREMER AND ERENBERG (1995), where a direct inhibition of transcription initiation at stable RNA genes would free a large pool of RNA polymerase for transcription of other genes, including the λ late genes, and pausing or queuing effects at or close to the promoters of mRNA genes would not be expected to affect significantly the pools of free RNA polymerase available for transcription at other mRNA genes not subject to ppGpp-mediated inhibition of transcription initiation or RNA chain elongation. It appears that the bacteriophage λ late mRNA represents a different strategy, incorporating an antitermination system to prevent premature termination and slowed RNA chain elongation of initiated chains such as the E. coli rrr operons, but including a promoter (pR') which is apparently not negatively regulated by ppGpp, in contrast to the rrr P1 promoters. The close similarities between the bacteriophage λ and the E. coli rrr antitermination systems with regard to ppGpp-mediated effects on the RNA chain elongation, the presence of as yet unidentified antitermination factors (Squires et al., 1993) and other possible nut-like mRNA sequences (Almond et al., 1989), suggest that the roles of antitermination and ppGpp in regulation of RNA synthesis warrant further investigation.

We have considered here only those models that involve ppGpp as a regulator of stable and messenger RNA synthesis; however, another model for growth rate regulation of stable RNA synthesis present in the literature also requires some comment. Although the role of ppGpp in the regulation of RNA synthesis during the stringent response has been more or less accepted, there is currently no consensus with regard to the mechanism of growth rate regulation of stable RNA synthesis. The ribosome- or translational-feedback model of Nomura and co-workers proposes that it is the translational activity of ribosomes which generates a signal responsible for the up- or down-regulation of ribosome synthesis and that ppGpp is only a result of this regulation, and not the cause. Links-Robertson et al., 1983; Jinks-Robertson and Nomura, 1987; Cole et al., 1987, 1988; GAAL and Gourse, 1990; for review, see CONDON et al., 1995). A reasonable resolution of these differences, we would point out, is that the proposed signal is ppGpp, generated in response to changes in amino acid or energy consumption and availability to mediate the regulation of stable RNA synthesis. However, regardless of the state of consensus among models for growth rate regulation of stable RNA synthesis, the well-documented effects of ppGpp on transcription of both stable and messenger RNA synthesis in vitro and in vivo (see the Introduction) and the equal compatibility of observations taken as supporting feedback regulation with models involving ppGpp (discussed in BARACCHINI and BREMER, 1988, 1991; HERNANDEZ AND BREMER, 1990, 1991, 1993) certainly support the latter as a justified model to examine the regulation of gene expression in general and RNA synthesis in particular.
