Characterization of RNA and DNA Synthesis in *Escherichia coli* Strains Devoid of ppGpp*

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The synthesis rates of DNA, rRNA, bulk mRNA, protein, and RNA polymerase β- and β'-subunits were determined as functions of growth rate in a wild-type *Escherichia coli* strain, which produces guanosine tetraphosphate (ppGpp), and in a ΔrelA ΔapoT mutant which does not produce ppGpp. The rate of stable RNA synthesis per amount of protein depends on three factors: RNA polymerase concentration, RNA polymerase activity, and the distribution of active RNA polymerase between stable and mRNA genes, measured as the stable RNA synthesis rate/total RNA synthesis rate, r_s/r_r. In the wild-type strain, all three factors increase with growth rate. In the ppGpp-deficient strains, only RNA polymerase synthesis and activity, but not r_s/r_r, increased with growth rate. Thus, adjustments of r_s/r_r require ppGpp. In the absence of ppGpp, the synthesis of rRNA and bulk mRNA both varied in direct proportion to the concentration of active RNA polymerase, in contrast to the wild-type strain, in which only rRNA synthesis increased with growth rate, while mRNA synthesis remained constant. Thus, a control specific for rRNA is absent in strains lacking ppGpp. In rich media, the ppGpp-deficient strain synthesized up to 4-fold more mRNA than wild-type bacteria, which was associated with a similarly increased RNA polymerase activity. We propose that RNA polymerase is rendered inactive in wild-type bacteria due to ppGpp-dependent transcriptional pausing during the synthesis of mRNA.

Finally, the control of replication initiation was altered in ppGpp-less bacteria, apparently reflecting indirect changes in the cell physiology, rather than a direct effect of ppGpp on replication initiation.

With regard to the synthesis of ribosomal RNA (rRNA) in bacteria, “stringent control” has been distinguished from “growth rate control.” The former refers to the reduction in rRNA synthesis in response to amino acid starvation (Stent and Brenner, 1961), the latter refers to adjustments of rRNA synthesis in response to changes in the nutritional quality of the growth medium (Schaechter et al., 1958). Both kinds of control are associated with changes in the cytoplasmic concentration of guanosine tetraphosphate (ppGpp) in a manner which has suggested that ppGpp is a negative effector in the control of rRNA synthesis (Lazzarini et al., 1971; Ryals et al., 1982a, 1982b, 1982c; Baracchini and Bremer, 1988). Stringent control is absent in bacteria with a mutational defect in the relA gene (Stent and Brenner, 1961), which encodes a ribosome-associated guanosine tetraphosphate synthetase (PSI, for ppGpp synthetase I). PSI is present on about 1% of ribosomes and is activated by uncharged tRNA during amino acid starvation (Haseltine et al., 1972; Haseltine and Block, 1973). Overproduction of PSI from a cloned relA gene, so that a large fraction of ribosomes bears PSI (RelA), causes a rapid accumulation of ppGpp and subsequent inhibition of rRNA synthesis in the absence of amino acid starvation (Schreiber et al., 1991; Tedin and Bremer, 1982). These observations are consistent with the proposal that ppGpp is a negative regulator of rRNA synthesis. Furthermore, in vitro studies indicate that ppGpp preferentially inhibits transcription initiation from rRNA promoters (van Ooyen et al., 1975, 1976; Travers, 1976; Kajitani and Ishihama, 1984; Ohlsen and Gralla, 1992), and enhances RNA polymerase pausing at specific sites during the elongation of RNA chains (Kingston and Chamberlin, 1981; Kingston et al., 1981). Whereas these studies have established ppGpp as an inhibitor of rRNA synthesis at high concentrations, the significance of lower ppGpp levels acting as a regulator for the growth rate control of rRNA synthesis has remained controversial (Gaal and Gourse, 1990; and reviews by Lindahl and Zengel, 1986; Cashel and Rudd, 1987; Jinks-Robertson and Nomura, 1987; Bremer and Dennis, 1987).

Generally the control of rRNA synthesis has been considered together with the control of (bulk) tRNA synthesis since both classes of RNA are essentially coregulated (Dennis, 1972; Shen and Bremer, 1977a). Thus, for the following considerations, we divide total bacterial RNA into stable RNA (rRNA + tRNA; synthesis rate r_s) and mRNA fractions (synthesis rate r_m); the sum of stable RNA and mRNA synthesis rates is the total or “instantaneous” rate of RNA synthesis (r = r_s + r_m). In previous work from different laboratories, stable RNA synthesis rates have been measured per cell (r_s/cell), per genome equivalent of DNA (r_s/genome), per rRNA gene (r_s/gene), per amount of protein (r_s/P), per amount of ribosomal RNA (r_s/R), or per total RNA synthesis rate (r_s/r_r). For most genetic systems it is not crucial which reference unit is used, since changes in the synthesis rate of a specific enzyme do not significantly alter the macromolecular composition of the cell. However, when the synthesis of rRNA changes, the reference unit may also be altered, so that an increase in the rate with one unit may show no effect or even a decrease with another (Baracchini and Bremer, 1991). This may be one of the reasons why contradictory conclusions about the growth rate control of rRNA synthesis, and about the role of ppGpp in this control, have appeared in the literature.

The rate of stable RNA synthesis (e.g. r_s/P) is the product
of several factors which include the RNA polymerase concentration and activity, the RNA chain elongation rate, and the distribution of RNA polymerase over stable RNA and mRNA promoters. Each of these factors is subject to its own control which may or may not involve ppGpp. We assume changes in the RNA chain elongation rates, or in the RNA polymerase concentration and activity, affect the rates of stable and mRNA synthesis alike, and are therefore not specific for stable RNA (Ryals et al., 1982a, 1982b). The fraction of the total RNA synthesis rate that is stable RNA (rs/r) is a parameter which we have singled out as more suited than others to characterize the control of stable RNA synthesis since it represents the specific control of stable RNA synthesis relative to all other transcriptional activities. Changes in rs/r were found to be strictly correlated with changes in the cytoplasmic level of ppGpp (Ryals et al., 1982b; Baracchini and Bremer, 1988; Hernandez and Bremer, 1990; Tedin and Bremer, 1992). For example, when a culture is subjected to a temperature upshift, there is a substantial accumulation of ppGpp, but the rate of rRNA accumulation is not reduced; therefore, it was originally concluded that ppGpp is not essential for the control of rRNA synthesis (Gallant et al., 1977; Gallant, 1979). However, subsequent measurements showed that rs/r was indeed reduced under these conditions, but this reduction in rs/r was compensated, and thus obscured, by an increased RNA chain elongation rate due to the higher temperature (Ryals et al., 1982c).

During exponential growth, basal levels of ppGpp in Escherichia coli are synthesized by a second guanosine tetraphosphate synthetase (PSII, for ppGpp synthetase II; i.e. the spoT gene product (Hernandez and Bremer, 1991; Xiao et al., 1991)). PSII is apparently inactivated, rather than activated (as is PSI), during amino acid starvation. It has been proposed that the activity of PSI responds to the balance between the supply and consumption of amino acids (Hernandez and Bremer, 1990). In this way the concentration of active ribosomes could control PSI activity by affecting amino acid consumption. This would provide a feedback that adjusts ppGpp levels, which in turn adjust ribosome synthesis, until changes in the RNA chain elongation rate due to the higher temperature upshift, there is a substantial accumulation of active ribosomes that may or may not involve ppGpp. We assume a repressor role of free or translating ribosomes, either directly or via their activity, without involving a control of PSI activity and ppGpp synthesis (Jinks-Robertson and Nomura, 1987; Cole et al., 1987). This model was originally derived from observations with bacteria carrying extra copies of intact or defective rrn genes on plasmids (Jinks-Robertson et al., 1983). No evidence has been reported yet which identifies a ribosome-related repressor or effector for this control, or a repressor binding site, i.e. rrn operator (Jinks-Robertson and Nomura, 1987). Furthermore, the interpretation of the original observations obtained with artificially increased rrn gene dosage has been put into serious question (Baracchini and Bremer, 1991).

E. coli strains have been constructed in which the genes required for both PSI and PSII activity, relA and spoT, respectively, are deleted (Xiao et al., 1991). Since these strains are devoid of ppGpp (Xiao et al., 1991; Gaal and Gourse, 1990, they provide a further opportunity to assess the role of ppGpp. A previous study of such ppGpp-less strains has suggested that ppGpp is not required for the growth rate control of rRNA synthesis (Gaal and Gourse, 1990). The study of ppGpp-less strains presented here confirms that, for a given growth rate, the stable RNA synthesis rate (rs/rP) is the same in wild-type and ppGpp-deficient bacteria. However, it is shown that the control of stable RNA synthesis (rs) cannot be expected to change rs/rP at a given growth rate unless it changes average ribosome activity, and at high growth rates ribosome activity is unaffected by the absence of ppGpp. On the other hand, we found that growth rate-dependent adjustments of rs/rP do require ppGpp. In addition, we report several new in vivo effects of ppGpp that occur only at very low levels of ppGpp. Most striking among them is an inhibition of bulk mRNA synthesis that indirectly affects rs and might be caused by ppGpp-dependent transcriptional pausing.

Finally, it has been suggested that ppGpp plays an important role in the control of initiation of chromosome replication and its adjustment to the rate of bacterial growth (Zyskind and Smith, 1992). Our results with ppGpp-less bacteria indicate that ppGpp may play only an ancillary role in this regulation.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Growth Conditions**—Bacterial strains used and constructed here are listed in Table I. The strains CF1648 (MG1655, prototype E. coli K-12 wild-type strain; B. Bachman), CF1693 ( ΔrelA251 ΔspoT207), and CF1678 ( ΔrelA251 ΔspoT209) were obtained from M. Casnell (National Institutes of Health). Different growth rates were obtained (see below) using Luria-Bertani (LB) medium with 0.2% glucose or minimal Medium C (Helmstetter, 1967) with either 0.2% glucose (Glc), glycerol, or succinate (Suc) and supplemented with the nucleic acid bases adenine, cytosine, uracil, and uracil at 50 μg/ml each; 20 amino acids (aa) at 50 μg/ml each; and serine (Ser) at 500 μg/ml. The average growth rates in doublings/h (± 5% variation) achieved in various media CF1648 (wild-type) were: μ = 2.85 in LB Glc; μ = 2.31 in Glc asaSer + adenosine, cytosine, xanthine, uracil; μ = 1.95 in Gla asaSer; μ = 1.71 in Gla as; μ = 1.58 in glycerol asaSer; μ = 1.32 in glycerol as; μ = 1.25 in Suc as; μ = 1.00 in Glc, and μ = 0.67 in glycerol. The ppGpp-less strains CF1693, CF1678, VH2734, and VH2735 (Table I) were always grown in the presence of 20 amino acids. The strain VH2733 (Table I) when grown in minimal medium was also supplemented with arginine at 50 μg/ml. Overnight cultures were diluted 1000–2000-fold into fresh media for physiological experiments. All strains were grown at 37 °C in Erlenmeyer flasks with an initial flask-to-culture volume ratio of 1:5 and aeration provided by rotation at 175 revolutions/min. Growth of cultures was determined turbidometrically at 460 nm (1-cm light path), and samples were taken at an optical density (OD600) between 0.6 and 1.0.

**RNA, DNA, and Protein Determinations**—RNA, DNA, and protein determinations of bacterial cultures were performed as previously described (Bruscchedel et al., 1977) with changes specified below. For the acid precipitation of cells from cultures grown in LB medium, cells were initially collected by filtration on glass fiber filters (Whatman GF/F), washed three times with 5 ml of Medium C (see above),

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then treated with 5 ml of 0.5 M perchloric acid, which was allowed to drip through with no filtration pressure. This was done to wash away ribo- and deoxyribonucleotide oligomers which are present in LB medium and prevent their inclusion upon perchloric acid precipitation. Cultures in other media were likewise treated for consistency. Duplicate 5-ml samples were assayed for RNA/protein ratio determination. Following alkali hydrolysis, perchloric acid-soluble RNA nucleotides were quantitated spectrophotometrically at 260 nm assuming one A_{260} unit at pH 2 corresponds to 93 nmol of ribonucleotides. Protein concentrations were measured by the Lowry method (Miller, 1972; Hernandez and Bremer, 1990). Specific activity of the strain VH2733 was transduced to cat+ with a P1 phage lysate grown on CF1678. The presence of the AspoT209::cat allele was confirmed by amplification of the chromosomal spo region by the Taq DNA polymerase chain reaction method and checking for the correct amplification and restriction fragment length polymorphisms by agarose gel electrophoresis.

**Determination of the Relative Rate of Stable RNA Synthesis—**The rRNA synthesis rate per amount of protein was calculated as (drRNA/dt)/P, where RIP may represent any extensive property of the culture and T is the culture doubling time. The derivations employed simple mathematical manipulations such as substitutions, expansion, differentiation, resolution for a different parameter, etc. These formulas follow from an appropriate definition of the parameters used (Bremer, 1982) and are thus based on a single assumption for their application: that the cultures used are in steady-state exponential growth. Since the time constant for changes in the macromolecular composition due to changes in growth conditions is the generation time of the bacteria, it is necessary that cultures have grown for at least four to five generations under steady-state conditions before their properties can be described by the formulas below. Routinely, fresh overnight cultures were diluted at least 1000-fold, so that they had grown at least 100-fold before any samples were taken. References for the formulas used are given (reviewed by Bremer and Dennis, 1987). The RNA synthesis rate per amount of protein was calculated as the number of RNA transcripts/min/amino acid residue in total cell protein from the amount of RNA per protein (R/P; Fig. 2, A and B) using the formula (see "Discussion").

\[
(\text{d}r\text{RNA/d}t)/P = 0.84(R/P)/4568 \times \ln 2/r
\]

where \(R/P\) is RNA nucleotides/amino acid residue; 0.84 = fraction of

**TABLE I**

**Bacterial strains**

| Strain          | Genotype                              | Reference/source |
|-----------------|---------------------------------------|------------------|
| CF1648 (= MG1655) | K-12 wild-type, F-                     | B. Bachmann/M. Cashel |
| CF1693*         | MG1655, ΔrelA251::kan, ΔspoT207::cat (ΔspoT) | Xiao et al., 1991 |
| CF1678*         | MG1655, ΔrelA251::kan, ΔspoT209::cat (ΔspoSTU) | Xiao et al., 1991 |
| MC1400          | F', araD139, Δ(argF-lacI)169, rpsL150, relA1, thrB5301, deoC1, ptsF25, rpsL | Casadaban, 1976 |
| VH2733          | MC4103, ΔrelA251::kan, argA, malE, malE'–rrnB P1–X174 E'– lacZ–kan– malK | This study |
| VH2735*         | VH2733, ΔspoT207·cat                  | Hernandez and Bremer, 1991 |
| VH2735*         | VH2733, ΔspoT209·cat                  | Hernandez and Bremer, 1991 |

*ΔrelA251::kan = deletion of 90% of relA (N terminus) replaced with kan; ΔspoT207::cat = deletion of 78% of spoT (N terminus) replaced with cat.

*ΔspoT209 = deletion of spoS, spoT, and 26% of spoU (N terminus) replaced with cat.

*The presence of the ΔspoT207·cat allele was confirmed by amplification of the chromosomal spo region by the Taq DNA polymerase chain reaction method and checking for the correct amplification and restriction fragment length polymorphisms by agarose gel electrophoresis.

*The strain VH2733 was transduced to cat+ with a P1 phage lysate grown on CF1678. The presence of the ΔspoT209·cat allele was confirmed by polymerase chain reaction as described above (footnote c).
total RNA which is rRNA; 4566 = RNA nucleotides/ribosome; and τ = culture doubling time in minutes.

To calculate \( r/P \) (rate of stable RNA synthesis per amount of protein) in nucleotides/min per microgram of RNA polymerase (\( P \)), we use the formula,

\[
r/P = 1.2 \times 0.98 \times (R/P) \times \ln 2/\tau
\]

where the factor 1.2 corrects for the degradation of unstable spacers in the stable RNA precursors, assumed to correspond to 20% of precursor transcript lengths, and 0.98 is the fraction of total RNA that is rRNA and tRNA.

The mRNA synthesis rate/protein (\( r_{\text{mRNA}}/P \)) was calculated as mRNA nucleotides/min/aa residue in total cell protein from \( r/P \) and \( r/\tau \) as follows,

\[
r_{\text{mRNA}}/P = r_{\text{mRNA}}/\tau = r/P \times \left[ \frac{1}{(r/\tau)} \right] - 1
\]

To express this value in mRNA transcripts/min/aa, it was divided by 1000, assuming average mRNAs are 1000 nucleotides long.

The number of active RNA polymerases (\( N_p \)) was determined by calculating the number of growing RNA chains/\( \mu \text{g} \) protein from the ratio RNA/protein (\( R/P \)) and from \( r/\tau \), using the following formula (Shepherd et al., 1980),

\[
N_p/P = \frac{[0.98 \times 1.2 \times 10^{10} \times \ln 2]}{108 \times 10^6 \times 60]} \times (R/P) \times (1/[1+(r/\tau)] - 1)/500/\tau
\]

where 0.98 is the fraction of total RNA that is stable RNA; 1.2 is correction for unstable rRNA spacers; 6 \( \times 10^{10} \) = Avogadro’s number; 108 = average molecular weight of an amino acid residue in \( E. \text{coli} \) protein; 105 = \( \mu \text{g} / \text{g} \) of dry cell weight; 60 = stable RNA chain elongation rate in nucleotides/s; 108 = calculated from Moin (1976); Shen and Bremer, 1977; Ryals and Bremer, 1982; Ryals et al., 1982a); 50 = mRNA chain elongation rate in nucleotides/s (Bremer and Yuan, 1968).

The RNA chain elongation rate value of 85 nucleotides/s has been questioned (Gotta et al., 1991). Using electron microscopic visualization of the active rrr genes at different times after rifampicin treatment, these authors conclude that the RNA chain elongation rate is only 42 nucleotides/s at a growth rate of 2.4 doublings/h. However in the same report, the average distance between RNA polymerase molecules on the rDNA template was observed to be 83 bp. At a growth rate of 2.4 doublings/h, RNA chains are initiated at a rate of about one chain/s/rrn gene (Bremer and Dennis, 1987; Fig. 10a below). This initiation rate, together with the distance of RNA polymerase molecules on DNA, implies an RNA chain elongation rate of 85 nucleotides/s, close to the value of 85 used here. If both the value of 42 nucleotides/s and the distance of 85 bp were correct, then the rate of chain elongation would be one chain every 2 s, in contradiction to all reported initiation rates (reviewed by Bremer and Dennis, 1987), as well as to the independently determined initiation rate obtained in this work (Fig. 10a; Equation 8, below).

The RNA polymerase activity (fraction \( \beta_p \)) was calculated by dividing the number of active RNA polymerase molecules/\( \mu \text{g} \) protein (\( N_p/P \)) cf. above (Equation 3), by the observed number of total RNA polymerase molecules/\( \mu \text{g} \) protein.

The chromosomal replication time (C-period) was determined by standard methods for comparison with previously published data, as described (Pritchard and Zaritsky, 1970; Churchward and Bremer, 1977) from the ratio (\( \Delta G \)) of the amount of DNA which accumulates after inhibition of initiation of rounds of DNA replication and the amount of DNA prior to inhibition. The time required for completion of a single round of DNA replication (C in min) was calculated relative to the culture doubling time (\( \tau \)) from \( \Delta G \) (oriC/genome) using the following formula (Pritchard and Zaritsky, 1970; Bremer et al., 1979).

\[
\Delta G = \ln 2 (C/\tau) + (1 - 2^{-C/\tau})
\]

The amount of DNA present after completion of ongoing rounds of replication, measured in genome equivalents, gives directly the number of functional replication origins present at the time of inhibition of initiation, as has been confirmed by flow cytometry (Skarstad et al., 1986). In contrast, hybridization methods with an oriC probe give only relative, but not absolute, values because of uncertainties in the hybridization efficiency, specific radioactivity, efficiency of counting radioactivity, recovery of oriC sequences, etc. The average replication velocity (DNA nucleotides/s per replication fork) was obtained by dividing the number of base pairs/genome (3500 kbp) by 2C (two replication forks; \( C \) expressed in s).

The initiation mass (\( P_i \)) in amino acid residues/oriC was calculated from \( \Delta G \) (oriC/genome) and the DNA concentration (\( G/P = \) genomes/ \( \mu \text{g} \) of DNA) as follows.

\[
P_i = 1/[\Delta G \times (G/P)]
\]

The average number of RNA genes/genome (\( N_{\text{mRNA}}/G \)) in actively replicating chromosomes at a particular culture doubling time (\( \tau \)) was calculated as follows,

\[
N_{\text{mRNA}}/G = \frac{\Delta G}{2 \pi \tau \sqrt{\pi \tau}}
\]

where \( m' \) = map location of the rrr genes (A, 86.5; B, 88.7; C, 84.5; D, 72.1; E, 90.4; G, 56.5, and H, 5.1) relative to oriC (see Bremer and Dennis (1987) for conversion of map location into \( m' \) values).

The rrr gene activity (\( i_{\text{mRNA}} \), initiation frequency) was then calculated as transcripts/min per rrr gene from \( r/P \), DNA concentration (\( G/P \)), and \( N_{\text{mRNA}}/G \) using the following formula:

\[
i_{\text{mRNA}} = \frac{\left[(\text{dRNA/dt})/\pi \times (G/P)\right]}{1000}
\]

assuming that mRNA genes are equally distributed throughout the chromosome and an average mRNA transcript length of 1000 nucleotides. The calculations assume a chromosome branching pattern, which, although different for every individual cell, is accurately described for the whole cell population as long as replication forks move at constant speeds (Bremer et al., 1979). For chromosome replication from oriC, a constant replication velocity has been shown by marker frequency measurements under various growth conditions (Bird et al., 1972).
FIG. 1. Medium-dependent relative growth rates of ppGpp-less strains. The wild-type parent CF1648, and the ppGpp-less strains CF1683 (ΔrelAΔ251 Δsp0T207 = ΔΔsp0T207) and CF1678 (ΔrelAΔ251 Δsp0T209 = ΔΔsp0T209), were grown in different media, see "Experimental Procedures." In the following five media: 1) LB Gic; 2) Gic aaSer + adenine, xanthine, cytosine, uracil; 3) Gic aaSer; 4) glycerol aa; and 5) Suc aa, the growth rates of the wild-type strain were 2.85, 2.31, 1.95, 1.32, and 1.25 doublings/h, respectively. The two highest growth rate values are based on the averages of three different cultures (±5%), and the three lower growth rate values are based on the averages of at least five different cultures (±5%). The ratio of the growth rate (μ) of the ΔrelA Δsp0T strains (ΔΔ) to that of the wild-type parent (wt) is plotted as a function of the growth rate of the wild-type strain in the same medium. O, ΔΔsp0T207; Δ, ΔΔsp0T209.

### Table I

Medium-dependent RNA accumulation and synthesis in the absence of ppGpp

| Medium* | Strain     | μ    | RNA/protein | a  | r/r1 |
|---------|------------|------|-------------|----|-----|
|         |            | doublings/h |        |    |     |
| LB Gic  | wt         | 2.85  | 0.193       | 15.7 | ND |
|         | Δsp0T207   | 2.61  | 0.175       | 15.6 | ND |
|         | Δsp0T209   | 2.31  | 0.154       | 16.0 | ND |
| Glu aaSer + bases  | wt         | 2.31  | 0.156       | 15.1 | 0.90* |
|         | Δsp0T207   | 2.00  | 0.140       | 15.0 | 0.66 |
|         | Δsp0T209   | 1.50  | 0.120       | 13.1 | 0.52 |
| Glu aaSer | wt         | 1.95  | 0.130       | 15.7 | 0.82 |
|         | Δsp0T207   | 1.64  | 0.120       | 13.2 | 0.65 |
|         | Δsp0T209   | 1.27  | 0.109       | 12.1 | 0.54 |
| Gly aa  | wt         | 1.32  | 0.090       | 15.4 | 0.61 |
|         | Δsp0T207   | 1.00  | 0.090       | 11.6 | 0.56 |
|         | Δsp0T209   | 0.88  | 0.080       | 11.5 | 0.42 |

*LB Gic = Luria-Bertani medium with 0.2% glucose; Glu aaSer = Medium C with 0.2% glucose, 19 amino acids (50 μg/ml each), serine (50 μg/ml); adenine, xanthine, cytosine, uracil (50 μg/ml each); Gly aa = Medium C with 0.2% glycerol, 20 amino acids (50 μg/ml each).

†Ribonucleotide residues in total RNA/amino acid residues in total protein.

Riboasyme activity in amino acid residues/s per average ribosome (see Equation 16 under "Discussion").

ND, not determined (radioactive labeling in LB medium is too inefficient).

> Value of 0.90 was calculated based on ppGpp concentration extrapolated from the culture doubling time and the ppGpp concentration (K) at which RNA polymerase is equiportioned between ppGpp bound and unbound forms, K = 5 pmol/OD600 unit of culture, determined from previous data for E. coli K-12 (Baracchini et al., 1988; Hernandez and Bremer, 1990).

‡Adenine, xanthine, cytosine, and uracil.

Riboasymes in the ppGpp-less and wild-type strains, increasing in proportion to the growth rate (Fig. 2, A and B) as previously reported (Gaal and Gourse, 1990). However, in a given medium (producing different growth rates for ppGpp-less and wild-type strains), R/P was reduced in the ppGpp-less strains at all except the lowest growth rates (Table II).
an rDNA probe. This ratio measures the distribution of transcriptional activities between stable RNA and mRNA genes. In the wild-type strain, $r_s/r_t$ increased continuously from 0.4 to 0.9 as the growth rate increased from 0.6 to 1.8 doublings/h (Fig. 4, filled circles), in agreement with previous values obtained in E. coli B/r and K-12 strains (Ryals et al., 1982a; Baracchini et al., 1988). In contrast, in the ppGpp-deficient strains $r_s/r_t$ was nearly constant, about 0.6 in $\Delta\Delta$spoT207 and 0.5 in $\Delta\Delta$spoT209, at all growth rates (Fig. 4, open symbols). However, in a given medium $r_s/r_t$ was reduced in the ppGpp-less strains relative to the wild-type strain (Table II).

Expression of lacZ From an rRNA P1 Promoter—The $\Delta$relA and $\Delta$spoT alleles of strains CF1693 and CF1673 were recombined into a “ppGpp reporter” strain bearing a single chromosomal copy of an rRNA P1-lacZ fusion (Table I; Hernandez and Bremer, 1990, 1991). In the $\Delta$relA spoT strain, VH2733, $\beta$-galactosidase specific activity increased with growth rate, as previously observed in a relA background (Hernandez and Bremer, 1990). However, in the ppGpp-deficient strains, the specific activity remained nearly constant (Fig. 5), consistent with the growth rate invariance of $r_s/r_t$ in ppGpp-less strains (Fig. 4). When the specific activity was determined per amount of protein rather than per cell mass, the same results were obtained (data not shown).

Synthesis Rate of mRNA—The mRNA synthesis rate/protein, calculated as described (Equation 3 under “Experimental Procedures”), was nearly constant in the wild-type strain, but it increased dramatically with growth rate for the $\Delta\Delta$spoT207 strain so that in rich media the ppGpp-less strain synthesized 4-fold more mRNA/protein than the wild-type (Fig. 6). This suggests that mRNA synthesis is severely inhibited at very low, basal levels of ppGpp.

RNA Polymerase Concentration and Activity

The RNA polymerase concentration, determined as $\beta'$-subunits/total protein, increased with growth rate in the $\Delta\Delta$spoT207 ppGpp-less strain, but less than in the wild-type strain (Fig. 7A, uppermost curves). On the other hand, the number of active RNA polymerase molecules, determined as described (Equation 4 under “Experimental Procedures”), was greater in the $\Delta\Delta$spoT207 than in the wild-type strain (Fig. 7A, lower curves).

The fraction of total RNA polymerase engaged in transcription at any one time (RNA polymerase activity) increased with growth rate from 15 to 30% in the wild-type strain (Fig. 7B), as previously reported (Shepherd et al., 1980a). In the $\Delta\Delta$spoT207 strain, RNA polymerase activity increased twice as much in this range of growth rates, from 20 to 60% (Fig. 7B). Thus, the increased RNA polymerase activity in rich media more than compensates for the decreased total RNA polymerase concentration in the $\Delta\Delta$spoT207 strain.

Chromosome Replication and Gene Activities

To evaluate the possibility that $rnn$ genes might be limiting for transcription in the ppGpp-less strain, thus forcing RNA polymerase to overexpress mRNA genes, the DNA replication time and $rnn$ gene dosage at different growth rates were determined in the ppGpp-less $\Delta\Delta$spoT207 strain.

DNA Replication Time—In the presence of rifampicin, initiation of replication ceases, but ongoing rounds of replication proceed to completion (Skarstad et al., 1986). The increase in DNA after cessation of initiation, $AG$ (Fig. 8, A and B), corresponds to the average number of oriC sites per genome equivalent of DNA, oriC/genome (Pritchard and Zaritsky, 1970). The number of oriC sites per genome is a measure for
ppGpp-less E. coli

Fig. 7. RNA polymerase synthesis and activity. A, (upper curves) number of RNA polymerase molecules/µg protein, determined by quantitation of β'-subunits of RNA polymerase on polyacrylamide gels. The dotted line shows the fit of the values obtained here (filled symbols) with values previously reported for E. coli B/r (Bremer and Dennis, 1987). A, (lower curves) number of active RNA polymerase molecules/µg protein, calculated from RNA/protein ratio (Fig. 2) and r/f (Fig. 4; Table II) as described under "Experimental Procedures." The exact time after addition of rifampicin at which initiation of replication stops was determined from the kinetics of DNA accumulation after rifampicin addition (Bremer and Churchward, 1977) as illustrated graphically in Fig. 8, C and D. The time delay in rifampicin action occurred at all growth rates in the wild-type and was negligible for the ΔΔspoT strain.

The extent of chromosome branching due to overlapping rounds of replication and allows determination of the DNA replication time (C-period (Pritchard and Zaritsky, 1970; Churchward and Bremer, 1977)).

In the ΔΔspoT strain ΔG (oric/genome) was 10–20% greater than in the wild-type strain (Fig. 9B). This indicates more chromosome branching resulting from a 20–30% longer replication time, corresponding to a lower average replication velocity in the absence of ppGpp (Fig. 9C). At growth rates between 1.0 and 2.5 doublings/h, the average replication velocity in the wild-type strain increased with growth rate from 600 to 800 bp/s per replication fork, in good agreement with values reported for E. coli B/r (Churchward and Bremer, 1977). In the ΔΔspoT strain, the average replication velocity was lower and increased from 500 to 600 bp/s/fork over the same range of growth rates. The longer C-period in the ppGpp-less strain was associated with a reduced initiation mass (Fig. 9D, see below). Previously, the same correlation has been observed after overproduction of DnaA protein and was thought to be due to stalled replication forks when initiation has occurred prematurely (Lobner-Olesen et al., 1989).

DNA Concentration—In the wild-type strain the DNA concentration (DNA/protein; Fig. 9A) decreased at higher growth rates as previously reported (Brunschede et al., 1977; Bremer and Dennis, 1987). Conversely, in the ΔΔspoT strain, the DNA concentration increased with growth rate and was higher than in the wild-type strain at high growth rates.

Initiation Mass—The initiation mass (cell mass per replication origin at initiation of replication (Donachie, 1988)) is given by the amount of protein per oric site (Fig. 9D), and was obtained from the amount of protein/genome (Fig. 9A) and oric/genome (Fig. 9B). In the wild-type strain, the initiation mass was fairly constant, in agreement with previously reported observations, equal to about 5 x10^8 amino acid residues/oric (Donachie, 1968; Bremer and Dennis, 1987). In the ppGpp-less strain ΔΔspoT, the initiation mass decreased from 6 x10^8 at 1.0 doubling/h to 3.5 x10^8 amino acid residues/oric at 2.5 doublings/h (Fig. 9D).

Ribosomal RNA Gene Activities—The rrr gene activity was calculated as transcriptional initiations/min per rrr gene (Fig. 10A) by combining rRNA synthesis rates (Fig. 4A) and DNA concentrations (Fig. 9A), and taking into account the replication velocity (Fig. 9C) and chromosomal map locations of the seven rrr genes (using Equation 7 of "Experimental Procedures"). The rrr gene activity increased parabolically with growth rate in the wild-type strain from 10 to about 75 transcripts/min per gene between 1.0 and 2.5 doublings/h (Fig. 10A). The latter value corresponds to 1.25 transcripts/s per rrr gene, which approaches physical saturation of rrr genes with RNA polymerase. In the ppGpp-less strain, rrr gene activity increased less, from 20 to 60 transcripts/min per gene (Fig. 10A). Relative to the wild-type strain, this rate was reduced above, and increased below, a growth rate of 1.25 doublings/h, which is consistent with the differences in r/f.
ppGpp-less E. coli

Fig. 9. DNA concentration, replication velocity, and initiation mass. Different growth rates were obtained using the media described in Fig. 2. A, DNA concentration (genome equivalents/amino acid residue in protein). B, oriC sites/genome equivalent (ΔG, obtained as in Fig. 8). C, replication velocity (base pairs/s/replication fork) calculated from ΔG. D, initiation mass (protein/oriC) calculated from the DNA concentration, and oriC/genome (see “Experimental Procedures” for formulas used in calculations). Filled symbols, wild-type strain; open symbols, ΔΔapoT207.

values observed in these strains by independent measurements (Fig. 4).

Messenger RNA Gene Activities—Assuming that mRNA genes are evenly distributed throughout the chromosome, a relative value for the average mRNA gene activity was calculated from mRNA synthesis rates (Fig. 6) and DNA concentrations (Fig. 9A; Equation 9, “Experimental Procedures”). In the wild-type strain mRNA gene activity decreased 30% from 750 to 500 initiations/min per genome between 1.0 and 2.5 doublings/h, whereas in the ΔΔapoT207 strain it increased almost 4-fold from 550 to 2000 initiations/min per genome over the same growth rate range (Fig. 10A), in parallel to both the rRNA gene activity (Fig. 10A) and the RNA polymerase activity (Fig. 7B).

DISCUSSION

E. coli K-12 strains that do not produce ppGpp were found to differ from wild-type strains with regard to growth rate regulation of RNA synthesis (r/r'), RNA polymerase synthesis, and DNA synthesis, but only slightly with respect to protein synthesis (i.e., ribosome activity). To assess the role of ppGpp from these results, a relationship between bacterial growth rate and ribosome concentration, first reported by Schleif (1967), will be again derived and evaluated here for clarity.

Ribosomal RNA Synthesis at Different Growth Rates

Relationship between Growth Rate and Ribosome Concentration—Bacterial growth may be defined as increase in protein, \( P \), of an exponential culture with the doubling time \( \tau \) (in min) as follows.

\[
P = P_0 \times 2^{\tau t}\tag{Eq. 10}
\]

The rate of protein accumulation is obtained from this expression by differentiation:

\[
dP/dt = P \times \ln 2/\tau\tag{Eq. 11}
\]

Under most conditions bulk protein turnover is negligible, so that the rate of synthesis equals the rate of accumulation of protein. Substituting the culture growth rate \( \mu \) (in doublings/h; \( \mu = 60 \text{ min}/\tau \)), setting \( k = \ln 2/(60 \text{ min}/\tau) \), and rearranging to make \( \mu \) the dependent variable, gives the growth rate in terms of the rate of protein synthesis as follows.

\[
\mu = (dP/dt)/P\tag{Eq. 12}
\]

Expanding this expression by the number of ribosomes, \( N_r \), gives,

\[
\mu = (dP/dt)/N_r \times [N_r/P]\tag{Eq. 13}
\]

where \([N_r/P]\) is the number of ribosomes per amount of protein, i.e., the ribosome concentration, and \((dP/dt)/N_r\) is the protein synthesis rate (amino acids polymerized/min) per average ribosome, or ribosome activity, \( \alpha \), as follows.

\[
\mu = [N_r/P] \times \alpha\tag{Eq. 14}
\]

This relationship (Equation 14) defines the bacterial growth rate as the product of ribosome concentration and activity. (Ribosome activity, defined as protein synthesis rate divided by the number of ribosomes, is to be distinguished from peptide chain elongation rate, defined as protein synthesis rate divided by the number of active ribosomes.)

Since 84% of the total amount of E. coli RNA which accumulates is rRNA (2% is mRNA (Baracchini and Bremer, 1987); and 14% is tRNA (Bremer and Dennis, 1987)), and each 70 S ribosome contains collectively 4566 nucleotide residues in its 16 S, 23 S, and 5 S rRNAs, the ribosome...
concentration, \([N/P]\), is found from the ratio RNA/protein, \(R/P\) (RNA nucleotides/amino acid residue) as follows.

\[
[N/P] = (0.84/4566) \times R/P \tag{Eq. 15}
\]

Substituting this expression into Equation 14 and setting the numerical factor \(c = k(4566/0.84) = 62.8\), gives \(c_P\) as the product of \(R/P\) and \(a_c\).

\[
c_P = R/P \times a_c. \tag{Eq. 16}
\]

The results in Fig. 2, A and B, show that \(R/P\) is nearly proportional to \(\mu\) for both wild-type and ppGpp-less strains. According to Equation 16, this means that the ribosome activity, \(a_c\), is approximately constant. Using Equation 16, \(a_c\) was calculated from \(R/P\) and \(\mu\) (Fig. 2, C and D) and was found to increase from about 12 amino acid residues polymerized/s (aa/s) at 0.6 doublings/h to a plateau of 15 aa/s at growth rates above 1.5 doublings/h, in agreement with previous estimates (Schleif, 1967; Dennis and Bremer, 1974).

The ppGpp independence of \(a_c\) at growth rates above 1.5 doublings/h (Fig. 2, C and D) is consistent with previous observations that the ribosome activity is not altered in spoT strains which have elevated basal levels of ppGpp (Hernandez and Bremer, 1990). At low growth rates the ppGpp-less strains did have somewhat lower ribosome activity than the wild-type strain due to a small overproduction of ribosomes (Fig. 2, A and B). Thus, the reduction in growth rates of ppGpp-less strains in a given medium (Fig. 1) was mainly due to decreased ribosome concentration at high growth rates and to decreased ribosome activity (up to 30%) at low growth rates (Table II).

From the observation that the growth rate dependence of \(R/P\) is not altered by the absence of ppGpp, Gaal and Gourse (1990) concluded that ppGpp is not required for the growth rate-dependent control of rRNA synthesis. This interpretation is not justified, because the control of the rRNA synthesis rate cannot change \(R/P\) at a given growth rate unless it also changes ribosome activity (Equation 16).

Based on the assumption that ppGpp inhibits ribosome biogenesis, one might have expected that ppGpp-less bacteria would overproduce ribosomes at all but the fastest growth rates. The excess of ribosomes, evident as an elevated \(R/P\) value at a given growth rate, might then deplete the supply of substrates for protein synthesis and lead to a reduced ribosome activity, as is observed when extra copies of plasmid-borne \(rRNA\) genes are present (Baracchini and Bremer, 1991). To a limited extent, our observations agree with this expectation: at low growth rates, ribosomes were 10–20% overproduced in the ppGpp-less strains, seen as a 10–20% greater \(R/P\) at a given growth rate, which resulted in a 10–20% reduced ribosome activity. However, one might have expected a much greater effect, such as a constant high \(R/P\) value in the absence of ppGpp at all growth rates. In this case the growth rate could only vary as a result of changes in ribosome activity. This is not observed for a variety of reasons. First, a greater overproduction of ribosomes would be expected to waste sufficient energy to lower the growth rate and thus cause a number of secondary changes in global transcription control. Second, the rate of stable RNA synthesis is regulated not only by ppGpp, but also by changes in the RNA polymerase concentration and activity (see below). Third, the expectation that ppGpp only inhibits ribosome synthesis (so that the absence of ppGpp should stimulate ribosome synthesis) must be modified on the basis of observations reported here. Unexpectedly, it was found that there is a significant difference between zero and absolute zero levels of ppGpp in the global control of transcription. At very low levels, ppGpp preferentially inhibits mRNA synthesis, so that the total absence of ppGpp leads to a stimulation of mRNA synthesis in rich media at the expense of stable RNA synthesis. These phenomena are discussed below.

That a reduction in the level of ppGpp stimulates rRNA synthesis in a given medium without amino acid starvation has been shown previously by the isolation of PSII-deficient mutants upon selection for increased expression from an rRNA promoter (Hernandez and Bremer, 1991). Conversely, increasing the level of ppGpp by variable induction of relA from PlacUV5 suffices to reduce both \(r_P\) and \(r_r\) (Tedin and Bremer, 1992).

Expression of lacZ from an rRNA Promoter—We have previously reported the construction of a relA1 ΔlacZ strain carrying an \(rrnB\) P1-lacZ fusion; this fusion was recombined into the chromosome at a position close to the normal map location of \(rrnB\) and oriented so that the direction of transcription coincides with the direction of replication, which simulates the natural disposition of the \(rrn\) genes. Under conditions of reduced ppGpp concentration, e.g. in rich media or during the relaxed response, \(\beta\)-galactosidase specific activity expressed from \(rrnB\) P1-lacZ is increased, reflecting increased rRNA gene activity (Hernandez and Bremer, 1980). Here the relA1 allele of this strain was replaced with a relA deletion. Again, \(\beta\)-galactosidase specific activity (activity per cell mass) increased with growth rate (Fig. 5, filled symbols). Following deletion of the spoT gene as well, \(\beta\)-galactosidase specific activity became growth rate-independent (Fig. 5, open symbols).

The specific activity of \(\beta\)-galactosidase (enzyme per total protein) is proportional to the ratio of the synthesis rates, lacZ-mRNA/total mRNA, and when lacZ is expressed from a stable RNA promoter, \(\beta\)-galactosidase specific activity is expressed from \(rrnB\) P1 promoter to the total mRNA synthesis rate (Hernandez and Bremer, 1991). Since \(r_P/r_r\) is constant in ppGpp-less strains (Fig. 4), then \(r_P/r_r\) must also be constant (because \(r_r = r_P/r_P\)). Thus, a constant \(\beta\)-galactosidase activity in the absence of ppGpp (Fig. 5) is expected from the direct measurements of \(r_P\) and \(r_r\).

The \(\beta\)-galactosidase activity expressed from a stable RNA promoter might be expected to increase with growth rate like \(R/P\) or \(r_P\). The comparison of Figs. 2, A and B, and 5 shows that this is evidently not the case. The increases in \(R/P\) and \(r_P\) in the ppGpp-less strain reflect the increasing concentration of active RNA polymerase, which increases \(r_P\) and \(r_r\) equally (Figs. 3 and 6), so that the ratio \(r_P/r_r\) does not change. Differences between \(\beta\)-galactosidase/\(P\) (corresponding to \(r_P/r_P\)) and \(P/P\) or \(r_P/r_P\), respectively, are due to the fact that the total rate of protein synthesis is not limited by mRNA, but by the number of ribosomes (Bremer and Dennis, 1987). That is, overproduction of bulk mRNA reduces the fraction of mRNAs which represent lacZ-mRNA produced from an \(rrnB\) P1 promoter, and since ribosomes are limiting for protein synthesis, this reduces the translation of lacZ-mRNAs. \(R/P\) and \(r_P\) are not affected in this case because the rate of total protein synthesis remains the same.

The use of \(r_P/r_r\) to characterize the control of \(r_r\) has been considered inappropriate (Nomura et al., 1984; Jinks-Robertson and Nomura, 1987). Because of the close relationship between \(r_P/r_r\) and \(r/r_r\), this criticism would equally apply to measurements of \(\beta\)-galactosidase expressed from an rRNA promoter. These authors themselves, however, have used such measurements as a function of \(\mu\) as indicators of \(rrn\) promoter activity (e.g. Gourse et al., 1986), assuming \(\beta\)-galactosidase/\(P\) expressed from an rRNA promoter as a measure for \(R/P\) or \(r_P/P\).
Using the same ΔTrpA and ΔpO-T alleles in combination with a different rrn B1-P1-lacZ fusion (containing about 1 kbp of trp sequences in the lacZ mRNA leader), Gao and Gourse (1990) found no difference in the growth rate-dependence of β-galactosidase specific activity between ppGpp-deficient and wild-type strains. Their fusion gene was inserted at a different location on the chromosome (i.e. at the katt site, since it is imbedded within a transcribing phase) and in an orientation opposing the direction of replication fork movement. We do not know if either of these differences might be responsible for the discrepancy between their and our results. The inhibition of rrn P1 promoter activity by ppGpp in vitro can be modulated by small changes in superhelical density of the DNA templates. High negative superhelical density causes maximal rrn P1 promoter activity and insensitivity to ppGpp; upon relaxation of the DNA template up to 80% inhibition by ppGpp is observed (Olshen and Gralla, 1992). In addition, it has been shown that RNA polymerase pausing within the rrnB leader is sensitive to the superhelical density of the template (Krohn et al., 1992). Therefore, differences in chromosome location of the rrnB P1-lacZ fusion might affect their control. Besides potential effects of DNA superhelicity on the rrn promoter activity, the expression of lacZ is subject to a number of effects unrelated to promoter activity; these include mRNA stability, intracistronic polarity, and translational efficiency of different mRNAs (Hernandez and Bremer, 1990), which may differ for different lacZ fusions.

Rate of rRNA Synthesis/Protein—At a given growth rate, the rRNA synthesis rate/protein (rRNA transcripts/min per amino acid residue) depends only on the ribosome activity. This is seen by substitution of Equation 16 above into Equation 1 (“Experimental Procedures”), which yields,

\[
\frac{dfRNA}{dt}/P = \left(\frac{f}{a_0}\right) \times \mu \tag{Eq. 17}
\]

where \( f = 4655k^2 = 0.62 \) (this value of \( f \) assumes \( a_0 \) in amino acids polymerized/min per ribosome). Equation 17 shows, as long as ppGpp does not alter \( a_0 \) (see above), it cannot affect the rate of rRNA synthesis/protein as a function of \( \mu \), as observed (Fig. 3). The parabolic shape of the curve in Fig. 3 agrees with the expectation that the rate of rRNA synthesis/protein is proportional to \( \mu^2 \) and implies a constant (or nearly constant) ribosome activity.

Maaloe (1969) stated that the rate of rRNA synthesis per genome equivalent of DNA increases with \( \mu^2 \). The rates of rRNA synthesis per protein and per genome can both increase with \( \mu^2 \) only if DNA/protein remains growth rate-invariant. In reality this condition is met neither in wild-type nor in ppGpp-deficient strains (Fig. 9A and Brunschede et al., 1977; Churchward et al., 1982). Thus, only the rate of rRNA synthesis per protein, but not per genome, increases with \( \mu^2 \).

Growth Rate- and ppGpp Dependence of Stable RNA Synthesis—Transcriptional activities like \( r_\text{s} \) are generally expressed either per amount of protein (\( r_\text{s}/P \)), or per amount of total RNA (\( r_\text{s}/R \)), or per total RNA synthesis rate (\( r_\text{s}/r_\text{s} \)). Whereas \( r_\text{s}/P \) increases with \( \mu^2 \) and is independent of ppGpp (see above), \( r_\text{s}/R \) increases in direct proportion to \( \mu \) (seen by substituting \( R \) for \( P \) in Equation 11, above), and is also independent of ppGpp; \( r_\text{s}/r_\text{s} \) may increase with \( \mu \) or remain constant (Fig. 4) and is strictly dependent on the concentration of ppGpp (Ryals et al., 1982b; Fig. 4). These differences in the growth rate and ppGpp dependence are due to the control of the reference unit. Depending on the parameter used to express the stable RNA synthesis rate different properties of \( r_\text{s} \) are being measured. At a given growth rate, \( r_\text{s}/P \) reflects the ribosome activity (see preceding section); \( r_\text{s}/R \) reflects the stability of stable RNA (Baracchini and Bremer, 1991); and \( r_\text{s}/r_\text{s} \) reflects the proportions of mRNA and stable RNA synthesis. Evidently, there is no “clean” measure to define \( r_\text{s} \), which obscures the role of ppGpp in its control.

Growth Medium Control of Stable RNA Synthesis—Since \( r_\text{s}/P \) is fixed at a given growth rate so long as ribosome activity remains constant, neither ppGpp nor any other factor can alter it. Only if \( r_\text{s}/P \) is compared in the wild-type and ppGpp-less mutant for a given growth medium (i.e. not for a given growth rate), can it be seen that the absence of ppGpp does affect \( r_\text{s}/P \). Maaloe and coworkers suggested that only the growth rate is important, but not the composition of a particular growth medium used to achieve that growth rate (Schaechter et al., 1958). Therefore, physiological parameters in bacteria are commonly presented as functions of \( \mu \), which assumes \( \mu \) as an independent variable. Actually, only the growth medium can be freely chosen, the composition of which ultimately determines the growth rate. If only wild-type bacteria are considered, there is no need to distinguish between “growth rate control” and “growth medium control” of \( r_\text{s} \). However, for mutants with altered control of ribosome synthesis this distinction must be made. That the absence of ppGpp alters the growth medium-dependence of \( r_\text{s}/P \) is evident from the data in Table II. In a graph, growth medium-dependent effects of ppGpp can be visualized by using the growth rate of wild-type bacteria to represent the growth medium, as in Fig. 1.

Factors Determining the Rate of Stable RNA Synthesis/Protein—Even for a given growth medium, the absence or presence of ppGpp does not strongly affect \( r_\text{s}/P \) (5-20%; Table II). The reason for this small effect is that \( r_\text{s}/P \) is the product of four factors (Bremer, 1975) with different ppGpp dependencies which partly compensate one another as follows.

\[
\frac{r_\text{s}}{P} = \left[\text{RNAP}\right] \times \phi_\text{s} \times \psi_\text{s} \times c_\text{s} \tag{Eq. 18}
\]

In this expression [RNAP] is the RNA polymerase concentration, given as the number of core RNA polymerase molecules/protein (Fig. 7A, upper curves), \( \phi_\text{s} \) is the fraction of RNA polymerase that is active at any instant (Fig. 7B), \( \psi_\text{s} \) is the fraction of active RNA polymerase synthesizing stable RNA at any instant (a function of \( r_\text{s}/r_\text{s} \)), and \( c_\text{s} \) is the stable RNA chain elongation rate (nucleotides/min per growing chain). With the exception of \( c_\text{s} \), all other parameters in this relationship have been determined here in strains with and without ppGpp. Since the stable RNA chain elongation rate is the same during both the relaxed and stringent response, i.e. at very low and high levels of ppGpp (Shen and Bremer, 1977; Ryals and Bremer, 1982), we have assumed \( c_\text{s} \) to be independent of ppGpp (see “Experimental Procedures,” Equation 4). In the wild-type strain, [RNAP] (Fig. 7A), \( \phi_\text{s} \) (Fig. 7B), and \( \psi_\text{s} \) (derived from \( r_\text{s}/r_\text{s} \), Fig. 4) all increased with growth rate. The combined increases in these factors causes the parabolic increase with \( \mu^2 \) in \( r_\text{s}/P \) (Fig. 3). In the ppGpp-deficient strain, \( \phi_\text{s} \) (i.e. \( r_\text{s}/r_\text{s} \) was constant (Fig. 4) and \( r_\text{s}/P \) was adjusted at different growth rates entirely as a result of changes in RNA polymerase synthesis and activity (Fig. 7). Thus, in the absence of ppGpp and in rich media, the loss of specific control by ppGpp (i.e. \( r_\text{s}/r_\text{s} \)) is compensated by a nonspecific increase in global transcription that equally affects both stable and mRNA synthesis. In poor media, rRNA is overproduced in ppGpp-deficient bacteria, which is partly compensated by a reduced ribosome activity (Table III).

Increased Synthesis of mRNA in the Absence of ppGpp

The rate of mRNA synthesis was up to 4-fold higher in ppGpp-deficient bacteria than in wild-type bacteria (Fig. 6). The difference increased with growth rate and was absent at
growth rates below 1.25 doublings/h. This increased mRNA synthesis was associated with an increased RNA polymerase activity relative to the wild-type strain (Fig. 7), as if RNA polymerase were inactivated during the synthesis of mRNA in ppGpp-deficient mutants. Independent of whether these effects are caused directly or indirectly by ppGpp, one would expect the synthesis to diminish with increasing growth rate when the ppGpp concentration in wild-type bacteria approaches zero. However, contrary to this expectation the effects were found to be maximal during fast growth (Figs. 5 and 7). We suggest this paradox may be attributable to ppGpp-dependent transcriptional pausing of RNA polymerase during the synthesis of mRNA. If gene expression were inhibited by transcriptional pausing, it would depend on the fraction of RNA polymerase that pauses, the duration of the pause, the distance of the pause site from the promoter, and most importantly on the time intervals between successive transcription initiations. If these initiation intervals are longer than the pause time, then pausing would not affect the rate of transcription initiation, nor how large the fraction of polymerase that pauses. Therefore, transcriptional pausing should have a minimal effect on the rate of gene expression during slow growth when RNA polymerase concentrations are low and the number of derepressed mRNA genes competing for RNA polymerase are high; i.e. the ratio of free RNA polymerase to available promoters is low. Only at high growth rates, when RNA polymerase concentrations are high and the number of remaining derepressed mRNA genes are low, could ppGpp-induced pausing significantly reduce the rate of transcription and transiently inactivate RNA polymerase. Enhanced transcriptional pausing at specific sites in the presence of ppGpp has been observed in vitro (Kingston et al., 1981; Kingston and Chamberlin, 1981). Thus, the growth rate-dependent differences in mRNA synthesis and RNA polymerase activity observed here between wild-type and ppGpp-deficient bacteria (Figs. 6 and 7) suggest that ppGpp-enhanced transcriptional pausing could also be occurring in vivo. In addition, this would explain the unexpected observation that at high growth rates r-f ratio is lower in ppGpp-deficient than in wild-type bacteria (Fig. 4); i.e., since r is increased. The presence of antitermination sites in the stable RNA genes might explain why the effect appears to be specific for mRNA synthesis.

The lower r-f ratio values in fast growing ppGpp-less bacteria indicates that stable RNA synthesis rates can also be controlled indirectly, via the control of the rate of mRNA synthesis. This reflects the fact that in any transcription system that is not limited by the concentration of DNA, promoters compete for RNA polymerase. It has been shown in vivo, that transcription in E. coli is not limited by DNA (Churchward et al., 1992). Thus, if two classes of promoters are considered, i.e., mRNA and stable RNA promoters, then the distribution of RNA polymerase over these two classes of genes can be changed by either activating one group, or inhibiting the other group of promoters. For example, an increase in stable RNA synthesis can be achieved by activating stable RNA promoters by inactivating mRNA promoters. Therefore, the control of which mRNA synthesis is inseparable from the control of stable RNA synthesis.

**Modulation of RNA Polymerase Synthesis and Activity by ppGpp**

In wild-type bacteria, changes in RNA polymerase concentration and activity accompany changes in ppGpp levels and contribute to the control of stable RNA synthesis (see above).
ppGpp-less E. coli

Hernandez, V. J., and Bremer, H. (1991) J. Biol. Chem. 266, 5991–5999
Jensen, K. F., and Pedersen, S. (1990) Microbiol. Rev. 54, 89–100
Jinks-Robertson, S., and Nomura, M. (1987) in Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology (Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M., and Umbarger, H. E., eds) pp. 1558–1565, American Society for Microbiology, Wash. D.C.
Jinks-Robertson, S., Gourse, R. L., and Nomura, M. (1983) Cell 33, 865–876
Kajitani, M., and Ishihama, A. (1984) J. Bwl. Chem. 259, 1951–1957
Kingston, R. E., and Chamberlin, M. J. (1981) Cell 27, 523–531
Krohn, M., Pardon, B., and Wagner, R. (1992) Mol. Microbiol. 6, 581–589
Lazzarini, R. A., Cashel, M., and Gallant, J. (1971) J. Biol. Chem. 246, 4381–4385
Maaloe, O. (1969) Dec. Biol. Suppl. 3, 33–38
Miller, J. H. (1972) in Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, pp. 352–355
Molin, S. (1976) Alfred Benzon Symp. IX, 333–339
Nomura, M., Gourse, R. L., and Baughman, G. (1984) Annu. Rev. Biochem. 53, 75–117
Ohlsen, K. L., and Gralla, J. D. (1992) Mol. Microbiol. 6, 2243–2255
van Ooyen, A. J. J., deBoer, H. A., Ab, G., and Gruber, M. (1975) Nature 254, 530–531
van Ooyen, A. J. J., Gruber, M., and Jorgenson, P. (1976) Cell 8, 123–128
Ritchard, R. H., and Zazisky, A. (1970) Nature 226, 126–131
Rokhsaz, L. A., and Zyskind, J. F. (1986) Cell 44, 765–771
Ryals, J., and Bremer, H. (1982) J. Bacteriol. 150, 168–179
Ryals, J., Little, R., and Bremer, H. (1982a) J. Bacteriol. 151, 879–887
Ryals, J., Little, R., and Bremer, H. (1982b) J. Bacteriol. 151, 1261–1266
Ryals, J., Little, R., and Bremer, H. (1982c) J. Bacteriol. 151, 1425–1432
Schaechter, M., Maaloe, O., and Kjeldgaard, N. O. (1958) J. Gen. Microbiol. 19, 592–606
Schleif, R. (1967) J. Mol. Biol. 27, 41–58
Shepherd, N. S., Churchward, G., and Bremer, H. (1980) J. Bacteriol. 141, 1179–1184
Shay, J. W., Pierce, D. J., and Werbin, H. (1990) J. Biol. Chem. 265, 14802–14807
Shen, V., and Bremer, H. (1977) J. Bacteriol. 130, 1109–1116
Schreiber, G., Metzger, S., Aizenman, E., Roza, S., Cashel, M., and Glaser, G. (1991) J. Biol. Chem. 266, 3760–3767
Shay, J. W., Pierce, D. J., and Werbin, H. (1990) J. Biol. Chem. 265, 14802–14807
Stent, G. S., and Brenner, S. (1961) Proc. Natl. Acad. Sci. U. S. A. 47, 2005–2014
Tedin, K., and Bremer, H. (1992) J. Biol. Chem. 267, 2337–2344
Travers, A. (1976) Mol. Gen. Genet. 147, 229–232
Xiao, H., Kalman, M., Ichihara, K., Zemel, S., Glaser, G., and Cashel, M. (1991) J. Biol. Chem. 266, 5980–5990
Zyskind, J., and Smith, D. W. (1990) Cell 69, 5–8