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Effects of guanosine tetraphosphate on cell-free synthesis of *Escherichia coli* ribosomal RNA and other gene products

(ppGpp/stringent control/gene regulation *in vitro*)

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ABSTRACT **A cell-free system derived from *E. coli* is described in which mature-sized 16S and 23S ribosomal RNAs (rRNA) are synthesized at a high relative rate, comprising 17–25% of the total transcription. The addition of guanosine tetraphosphate (ppGpp) to this system results in up to a 5-fold selective inhibition of rRNA accumulation. This effect is exerted at the level of synthesis rather than degradation. It is concluded that ppGpp, which is produced in large amounts by *E. coli* during amino-acid deprivation, could mediate the decrease in rRNA synthesis that accompanies such deprivation.**

The expression of other genes has also been investigated. No selective reduction of transfer RNA synthesis by ppGpp is observed. The *trp* and *lac* operons are found to be stimulated at the transcriptional level by the presence of this nucleotide. It is hypothesized that ppGpp interacts with the RNA polymerase in such a manner as to alter the affinity of the enzyme for promoters in an operon-specific fashion.

Guanosine 5′-diphosphate 3′-diphosphate (ppGpp) and pppGpp accumulate when stringent strains of *Escherichia coli* are deprived of amino acids but do not accumulate in similarly deprived relaxed mutant strains (1); such mutants have recently been denoted as *relA* (2). This has led to the proposal that these compounds mediate the amino-acid dependence of RNA accumulation in stringent strains, known as stringent RNA control (3), since this dependence is abolished in *relA* mutant strains (4). The rapid restriction in RNA accumulation involves a severe reduction in the synthesis of rRNA (5, 6) and tRNA (7, 8) and a portion of mRNA species (5, 6).

Evidence obtained *in vivo* strongly supports an inhibitory role for these nucleotides, especially ppGpp, in the regulation of rRNA synthesis (9–11). Cashel has shown that accumulation of ppGpp precedes the reduction of RNA synthesis during amino-acid starvation and that readdition of the deprived amino acid causes the disappearance of ppGpp prior to the resumption of RNA synthesis (9). More recently, Stamminger and Lazzarini demonstrated that in mutants with much slower rates of breakdown of ppGpp, rRNA synthesis does not resume until the ppGpp content falls to basal levels (10). Other evidence suggests that ppGpp levels can be correlated with rRNA content in cells during balanced growth (11), indicating that ppGpp may serve as a normal regulatory effector as well as functioning during conditions of deprivation.

Attempts to demonstrate the regulation of rRNA synthesis uniquely by ppGpp *in vitro* have heretofore been unsuccessful (12–15; see *Results and Discussions*). The attractiveness of a role for ppGpp as an inhibitor of rRNA synthesis in living cells and the lack of clearcut evidence *in vitro* to substantiate such a role have led us to examine the synthesis of rRNA in a modification of the *in vitro* system originally developed for studies of DNA-directed protein synthesis (16).

Abbreviation: SSC, 0.15 M NaCl/0.015 M sodium citrate.

METHODS

Growth of cells and preparation of S-30 extracts was as previously described (16). The S-100 extract was prepared in the same way except that, after preincubation, the extract was centrifuged at 45,000 rpm for 3 hr in a Spinco no. 50 rotor. The decanted supernatant was dialyzed against buffer III (16) and stored in liquid N_2 prior to use.

Bacterial DNA was prepared by a modification of the method of Miura (17). After RNase treatment of the DNA according to Miura (17), 50 μg/ml of Pronase (Calbiochem; predigested for 2 hr at 37°) was added and the mixture was incubated for 1 hr at 37°. The DNA was then extracted twice with phenol/buffer mixture (17), twice more with equal volumes of chloroform/isoamyl alcohol (24:1 v/v), precipitated with 0.54 volume of cold isopropanol, collected on a glass rod, and redissolved in 0.001 × SSC (SSC is 0.15 M NaCl/0.015 M sodium citrate).

The components of the incubation mixture have been described (16) except that the polyethylene glycol was omitted and [^{3}H]UTP (1 Ci/mmol) was reduced to 0.1 mM. Mg^{++} and Ca^{++} were present at final concentrations of 13 mM and 6.5 mM, respectively. For synthesis of rRNA, 45 μg of *E. coli* DNA and synthesis mix were incubated at 30° for 3 min. Then either 3.25 mg of S-30 extract protein or an equivalent volume of S-100 extract was added to give a final volume of 0.5 ml and synthesis was done at 30° for 20 min unless otherwise specified. To terminate transcription, 10 μg of DNase were added and incubation was continued for 10 min. Aliquots were removed for determination of incorporation of radioactivity; then 0.5 ml of 6 × SSC was added and the resultant mixture was extracted three times with 3 × SSC-saturated phenol. The resultant aqueous phase was used directly for hybridization. If the RNA was to be electrophoresed, it was dialyzed successively against 200 volumes each of 0.01 M EDTA, 1 mM EDTA, and H_2O two times, then lyophilized and redissolved in the buffer described below.

Unlabeled or ^{14}C-labeled rRNA was prepared by the method of Lazzarini and Dahlberg (6) from strain HZ-1 (an A-19 derivative obtained from Howard Zalkin).

Hybridization was carried out by the sequential hybridization competition method of Murooka and Lazzarini (14) in 3 × SSC/50% formamide. In this method, DNA-containing filters were incubated for 20 hr at 37°, some in the pres-

Table 1. Estimation of rRNA content by RNA·DNA hybridization

Synthesis conditions	[^{14}C]rRNA cpm		[^{3}H]RNA cpm			
	Input	Hybrids	Input	Uncompeted hybrids	Competed hybrids	% rRNA
(*1*) No ppGpp	815	352	11,584	1016	170	17.2
(*2*) 200 μM ppGpp	815	285	12,212	439	154	6.7

ence of excess *E. coli* rRNA to presaturate the complementary sites on the DNA. The filters were then washed, transferred to vials containing [^{3}H]RNA synthesized *in vitro* and a trace of [^{14}C]rRNA as an internal standard, and incubated an additional 20 hr at 37°. The method of estimating rRNA content of the transcript from hybridization data is shown in Table 1. RNA synthesized *in vitro* was sequentially hybridized (14) to nitrocellulose filters containing 55–60 μg of *Proteus mirabilis* DNA in 0.5 ml of 3 × SSC/50% formamide. In sample *1*, of 11,584 cpm of RNA synthesized *in vitro* (about 10 μl), 1016 cpm is bound by duplicate filters, which normally differ by less than 10%. Prehybridization of filters to 10 μg of *E. coli* rRNA reduces the hybridizable cpm to 170. The difference, 846 cpm, is taken to represent rRNA in the transcript. [^{14}C]rRNA (0.1 μg) added as an internal standard hybridizes with an efficiency of 43% (352/815) under these conditions. Adjusting the [^{3}H]rRNA cpm hybridized for this efficiency, we obtain a value of 17.2% rRNA in the transcript. As illustrated by sample 2, the presence of ppGpp during synthesis of [^{3}H]RNA reduces the amount of hybridizable cpm and therefore the percentage of rRNA. Each synthesis was repeated independently at least twice. Variations in rRNA content detected by hybridization were 10% or less between experiments.

Polyacrylamide gel electrophoresis was carried out on 5 mm × 10 cm cylindrical gels (3.0% acrylamide/0.15% methylenebisacrylamide) containing 0.5% agarose in the Tris/borate/EDTA buffer system of Peacock and Dingman (18), containing 10% glycerol. Sodium dodecyl sulfate (0.2%) was added to the buffer reservoirs, but was not present in the gels during polymerization. RNA samples were dissolved in Tris/borate/EDTA buffer containing 20% glycerol, 0.2% sodium dodecyl sulfate, and 0.005% bromphenol blue and heated to 65° for 5 min. Twenty-five-microliter samples containing about 50,000 cpm and 25 μg of RNA were layered on the gels and run at 3 mA per gel at room temperature for about 3 hr.

Gels were cut into 1-mm slices, dried, and dissolved in 0.3 ml of 30% H_2O_2. One milliliter of 0.1% NaI/0.1% LiOH was added to reduce excess H_2O_2, then 2 drops of 30% Na_2SO_3 were added and swirled. Ten milliliters of scintillation fluid (16.5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene in 1 liter of Triton X-100 and 2 liters of toluene) were added, and the radioactivity in the vials was determined in a Beckman LS-223 scintillation counter. Recovery of radioactivity was over 90%.

About 10 μg of unlabeled *E. coli* rRNA were electrophoresed on parallel gels. After completion of the electrophoresis, the gel was washed for 1 hr in 1 liter of distilled H_2O, stained for 20 min with 0.2% methylene blue in 0.4 M acetic acid/0.4 M sodium acetate, and destained in H_2O with shaking.

ppGpp was prepared as described (19).

RESULTS AND DISCUSSIONS

The cell-free system is capable of producing RNAs of the size of the mature 16S and 23S polymers at a high relative rate

The system used for cell-free synthesis of RNA contains a crude cell-free extract known as an S-30, a source of DNA, and the added salts and substrates necessary for DNA-directed RNA and protein synthesis (see *Methods*). *E. coli* DNA has been used as the source of ribosomal genes. In most studies the ribosomes have been removed from the S-30 extract (yielding an S-100) prior to the synthesis step. The purpose of this modification is to avoid the dilution of the radioactively labeled RNA synthesized in the cell-free system by unlabeled rRNA. [^{3}H]UTP is incorporated into the synthesis mixture to insure labeling of the RNA product. The resulting radioactive RNA is isolated by a phenol method (see *Methods*). Gross RNA synthesis, measured as cold trichloroacetic acid-precipitable radioactivity, proceeds at an approximately linear rate for at least 1 hr (Fig. 1a). The radioactive rRNA present in the total product has been esti-

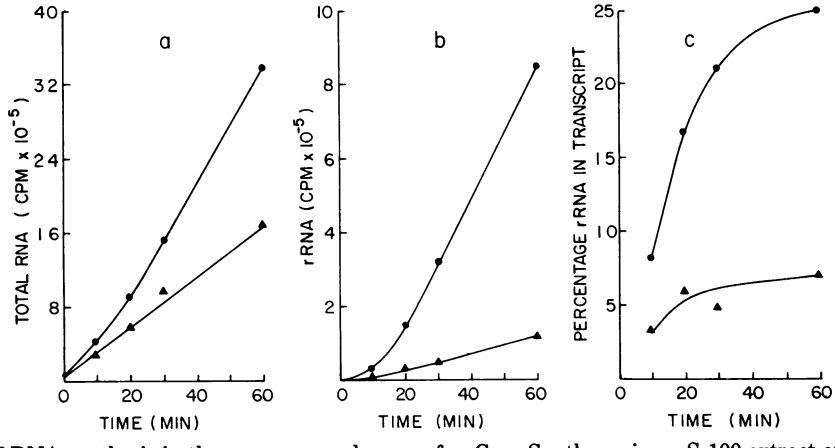

FIG. 1. Time course of RNA synthesis in the presence or absence of ppGpp. Syntheses in an S-100 extract of *E. coli* strain Z19i^{q} (38) directed by *E. coli* DNA were carried out at 30° for the indicated times prior to termination of transcription by addition of DNase. Hybridization analysis of the product was performed as described in *Methods*. Total RNA synthesis was determined by measurement of cold 5% trichloroacetic acid-precipitable radioactivity in duplicate 5-μl aliquots. rRNA synthesis was estimated by hybridization, and the percentage of rRNA calculated from the ratio of rRNA to total RNA. ppGpp concentrations present during synthesis were: (●) none; (▲) 200 μM.

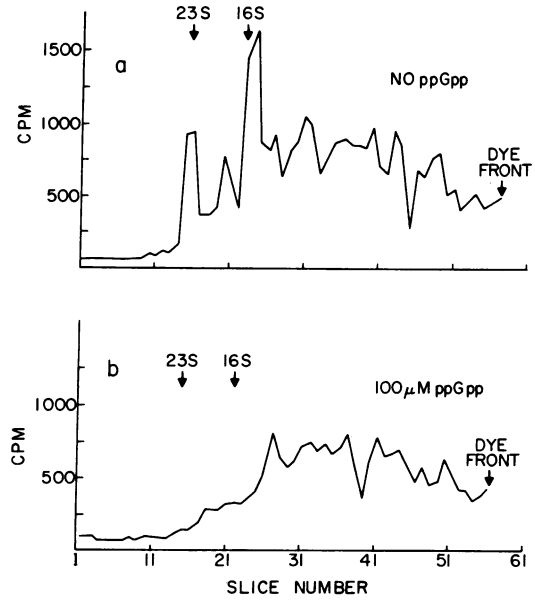

FIG. 2. Electrophoresis of RNA synthesized *in vitro* on polyacrylamide gels. RNA was synthesized for 20 min at 30° in the presence of the indicated concentrations of ppGpp in an S-100 extract of *E. coli* strain Z19i^q. Processing of the RNA after synthesis and conditions for electrophoresis and processing of gels are described in *Methods*. The arrows indicate the positions of marker *E. coli* rRNAs electrophoresed on parallel gels.

mated and characterized by two methods: (*i*) a selective hybridization procedure which detects sequences that compete with rRNA synthesized *in vivo;* (*ii*) an electrophoresis procedure which yields radioactive peaks with characteristic mobilities that may be compared with normal 16S and 23S rRNAs.

In Fig. 1b is shown a plot of the accumulated rRNA synthesized as a function of time using the hybridization method of detection. The proportion of rRNA in the transcript (Fig. 1c) appears to increase with time to a plateau of about 25% of the RNA synthesized after 1 hr. Since only 0.3–0.4% of the *E. coli* DNA encodes rRNA cistrons (20), this system clearly supports preferential synthesis of rRNA as is the case *in vivo* (5, 6). Why the percentage drops off with shorter times of synthesis *in vitro* has not been resolved. We suspect that the uncompleted chains present during the early period of synthesis hybridize less efficiently than the larger RNA molecules which accumulate with time, resulting in an underestimation of the fraction of rRNA in the transcript at the shorter times of synthesis.

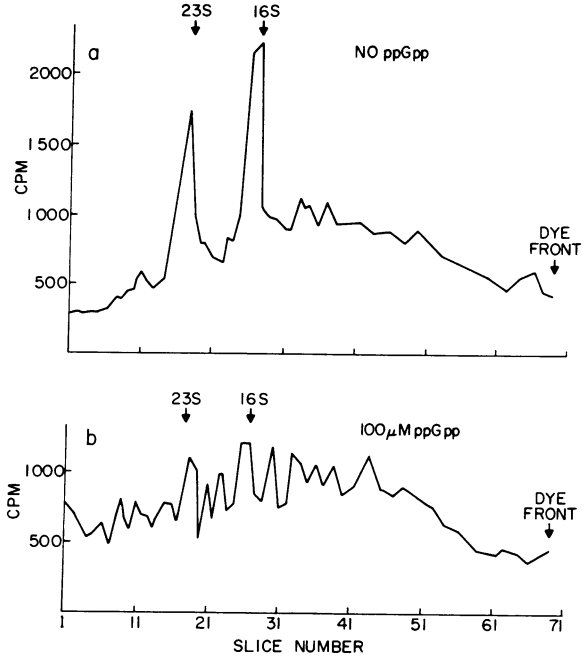

FIG. 3. Electrophoresis of RNA synthesized in an S-30 extract *in vitro* on polyacrylamide gels. RNA was synthesized for 30 min at 30° in the presence of the indicated concentrations of ppGpp in an S-30 extract of *E. coli* strain Z19i^q. Conditions for processing and electrophoresis of RNA were as for Fig. 2. Arrows indicate the positions of marker *E. coli* rRNAs electrophoresed on parallel gels.

When rRNA synthesized *in vitro* is analyzed by gel electrophoresis (Figs. 2a and 3a), peaks coincident with the 23S and 16S peaks of mature rRNAs are obtained. Similar results are obtained with S-100 or S-30 extracts. The synthesis of rRNA is completely inhibited by 2 μg/ml of rifampicin. While these results do not prove that the rRNA genes are properly initiated and terminated during the transcription process, they are a strong indication that the rRNA is synthesized in a form that allows it to be recognized and processed to the proper size by the appropriate enzymes (21, 22). The high yields and characteristics of the rRNA indicate that the synthesis of rRNA *in vitro* retains many features characteristic of the process *in vivo*.

Guanosine 5′-diphosphate 3′-diphosphate (ppGpp) selectively inhibits rRNA synthesis

In the cell-free system a progressive inhibition of rRNA synthesis accompanies ppGpp addition (Fig. 4a and b). In the presence of the highest concentration of ppGpp tested (1000

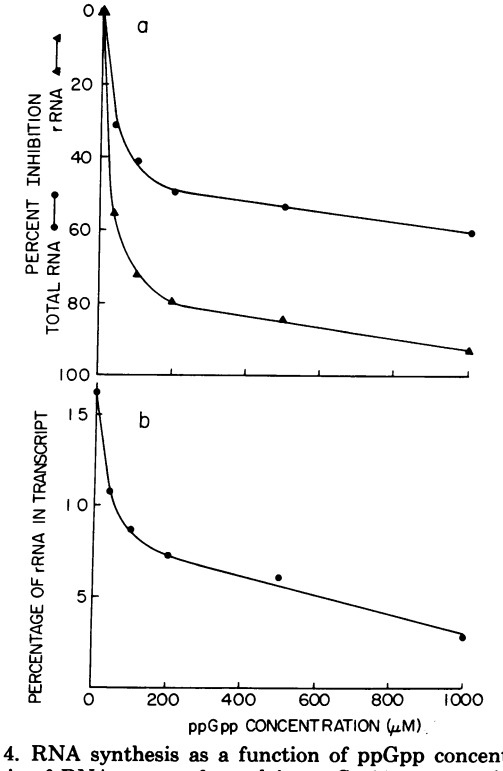

FIG. 4. RNA synthesis as a function of ppGpp concentration. Synthesis of RNA was performed in an S-100 extract of *E. coli* strain Z19i^q directed by *E. coli* DNA for 20 min at 30°. ppGpp was present at the indicated concentrations. Total RNA, rRNA, and percentage of rRNA in the transcript were determined as in the legend to Fig. 1. Approximately 800 pmol of [^{3}H]UTP are incorporated into total RNA in the absence of ppGpp.

μM), rRNA accounts for less than 3% of the total transcript. These data were obtained with S-100 extracts derived from a *relA* relaxed strain, but similar results were obtained with extracts of stringent cells. Although the relaxed strain has a defective apparatus for ppGpp synthesis (23), it retains a similar sensitivity to the regulatory effects of ppGpp. The overall pattern of RNA transcription *in vitro* in the presence of added ppGpp is consistent with patterns observed in stringent cells accumulating ppGpp during amino-acid deprivation (5, 6). A reduction in gross RNA accumulation is observed together with a more severe reduction of rRNA.

The time courses of RNA synthesis in the presence and absence of 200 μM ppGpp are compared in Fig. 1. Total incorporation of radioactivity is nearly linear and is moderately (55%) inhibited by the presence of 200 μM ppGpp. In contrast, 200 μM ppGpp severely reduces the percentage of rRNA in the transcript (Fig. 1a and b). Without added ppGpp the proportion of rRNA in the transcript increases with time to a plateau of about 25% of RNA synthesized after 1 hr. With 200 μM ppGpp present, rRNA plateaus at 7% of the total RNA synthesized.

The reduction in rRNA accumulation could be due to a decreased rate of synthesis or to an increased rate of degradation (24). The latter possibility was considered in one set of experiments in which RNA synthesis was terminated by addition of 20 μg/ml of DNase, and incubation was then continued for an additional 20 min. The same percentage of rRNA was obtained when 200 μM ppGpp was added after termination of transcription by DNase as when no ppGpp was present during the incubation. Thus, degradation of preexisting rRNA is not stimulated by ppGpp in this system, although it cannot be ruled out that an unstable precursor to rRNA is preferentially degraded in the presence of ppGpp (25).

The effects of ppGpp on rRNA synthesis were also examined by gel electrophoresis as described above. The presence of 100 μM ppGpp results in almost total elimination of 16S and 23S peaks, which would otherwise be prominent (compare Fig. 2a and b). In order to ascertain whether the presence of ribosomes would affect the response of the system to ppGpp, RNA was synthesized in an S-30 extract and analyzed by gel electrophoresis (Fig. 3). Again, prominent 16S and 23S peaks are obtained in the control, whereas the presence of 100 μM ppGpp results in a substantial diminution of these peaks relative to the other species being synthesized. It is concluded that the addition of ppGpp selectively inhibits rRNA synthesis in this system, as judged both by hybridization and gel electrophoresis.

Other *in vitro* systems have also shown preferential synthesis of rRNA (12–14), but contrary to the system studied here do not show selective inhibition of rRNA synthesis by ppGpp. Using permeabilized cells, Lazzarini and Johnson (12) and Atherly (13) have obtained high relative rates of rRNA synthesis, comparable to those obtained *in vivo* (5, 6). Addition of ppGpp to these preparations, however, resulted in no preferential reduction in rRNA synthesis. Further, Murooka and Lazzarini have isolated a cell-free DNA-protein complex which gives a high preferential rate of initiation and synthesis of rRNA, but which is also not specifically reduced in the presence of ppGpp (14). The effects of ppGpp on more purified systems have also been investigated. Haseltine has shown that, in contrast to an earlier report (26), as much as 10% of the transcript in a purified transcriptional system was rRNA in the absence of factors other than RNA polymerase (15). He found that protein elongation factor TuTs increased, and ppGpp decreased, the total amount of RNA synthesized in his system, but neither altered the fraction of the transcript that was rRNA. This suggested that positive control factors were not necessary for selective expression of the rRNA cistrons. More recently, Travers has found that use of low temperature (34°) and high ionic strength (0.1 M KCl) caused inefficient transcription of rRNA genes in a purified *in vitro* system (27). Under these conditions, the addition of TuTs increased the percentage of rRNA synthesized by 5-fold, to 12%, and the presence of 500 μM ppGpp, or GDP, blocked this stimulation (27, 28). *In vivo* the levels of GDP, unlike those of ppGpp, are not correlated with the stringent response (1). In our hands, GDP does not duplicate either the specific inhibitory effect of ppGpp on rRNA synthesis or the effects on DNA-dependent enzyme synthesis (29). These findings, together with the apparent difficulty (30) of reproducing the stimulation by TuTs of tRNA synthesis reported by Travers (27), leads one to question the relevance of the TuTs-stimulated system to conditions in living cells.

Effects of ppGpp on other genes and operons are varied: A general model to explain these effects emerges

In light of the results with rRNA, and in order to analyze further the mechanism by which ppGpp is functioning *in vitro*, studies on other genes were conducted. A reexamination of tRNA synthesis in an S-100 extract, directed by either *E. coli* or transducing phage $\phi 80psu^{+}_{III}$ DNA and analyzed by gel electrophoresis, has yielded results consistent with earlier studies (31). That is, ppGpp at levels up to 1000 μM results in only a moderate (25–50%) decrease in tRNA synthesis when either $tRNA^{Tyr}$ ($\phi 80psu^{+}_{III}$ DNA-directed) or bulk tRNA (*E. coli* DNA-directed) are examined. This result was surprising since these tRNA species are known to be subject to stringent control *in vivo* (7, 8). However, since the control of tRNA and rRNA synthesis *in vivo* is not strictly coordinate (32), it may be that some additional tRNA-specific component has been inactivated in this system or that ppGpp only indirectly regulates tRNA synthesis.

Operon-specific effects of ppGpp on DNA-dependent protein-synthesizing systems have previously been observed (29). Studies on the expression of several bacterial operons in DNA-directed cell-free systems reveal 2 to 3-fold stimulation of the *lac*, *trp*, and *ara* operons by ppGpp as well as severe inhibition of the *argE* gene (29). Recently, N. Kelker in our laboratory has found that *argH* gene expression is stimulated rather than inhibited by ppGpp (unpublished results). Direct analysis by RNA·DNA hybridization of the RNA produced in the DNA-directed protein-synthesizing system indicates that 100 μM ppGpp causes about a 2-fold increase in the amount of *lac* operon-specific mRNA which is produced.

Further studies were carried out which support the view that ppGpp is acting at the transcription level rather than the translation level. In a *lac* mRNA-directed translation system ppGpp does not stimulate *lac* operon expression (33). Finally, use was made of the uncoupled protein-synthesizing system developed by Schumacher and Ehring (34). In this system, amino acids are initially omitted from the DNA-directed synthesizing system, in which case transcription, but not translation, occurs. Transcription is then terminated by addition of DNase followed by addition of amino acids in order to permit translation of the cell-free synthesized mRNA. Both the *lac* and *trp* operons are stimulated by ad-

dition of 200 μM ppGpp during the transcriptional stage, but not by the addition of ppGpp after DNase, i.e., during the translational stage. Similar findings have been made by Kung *et al.* (35).

The concept of the function of ppGpp that emerges from these studies is that of a general transcriptional effector which exerts its influence in a gene-specific manner. The precise mechanism that is involved will probably remain unsettled until appropriate mutants, which affect ppGpp function, can be isolated and analyzed *in vitro*. Because our studies have been conducted in crude cell-free extracts, we are not in a position to say if ppGpp affects transcription by acting directly on RNA polymerase or indirectly by acting in conjunction with other factors present in the crude S-30 system. Indeed, others have suggested that additional factors may be involved both in the inhibition of rRNA synthesis (15, 27) and in the stimulation of *lac* messenger (36) synthesis. On the other hand, it has been shown that ppGpp can affect initiation specificity of the RNA polymerase acting alone (37). Although this matter is not settled, we prefer the simpler hypothesis which explains the diverse effects via a direct interaction of ppGpp with RNA polymerase. Two predictions follow from this simplest model. First, it should be possible to reproduce the ppGpp effects in a purified transcriptional system that contains only those factors required for transcription. Second, it should be possible to isolate mutants of the relaxed phenotype that have a defect in the polymerase receptor site for ppGpp.

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