The Study of Guanosine 5′-Diphosphate 3′-Diphosphate-mediated Transcription Regulation in Vitro Using a Coupled Transcription-Translation System*

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The effects of the “alarmone” guanosine 5′-diphosphate 3′-diphosphate (ppGpp) on regulation of the *Salmonella typhimurium* histidine operon and the *Escherichia coli* tRNA*\textsubscript{low}*-dependent transcription-translation system, S-30. The expression of the his\textsubscript{G} promoter is positively regulated by ppGpp, whereas that of the leu\textsubscript{P} promoter (of tRNA*\textsubscript{low}*) is negatively regulated by ppGpp. In an attempt to understand the global regulatory mechanism of ppGpp control, interrelationship between ppGpp-dependent activation and repression of gene expression was examined using these promoters as models. It has been traditionally supposed that the ppGpp-dependent regulation, at least for the activation, is by a passive mode of control: the activation of gene expression by ppGpp is a consequence of the repression of stable RNA gene expression in the condition of RNA polymerase limiting. To test this model, the ppGpp-dependent regulations of both an activable promoter (his\textsubscript{G}) and a repressible promoter (leu\textsubscript{P}) were determined in vitro simultaneously using a mixed template setup. The rationale for this exercise was to see whether the ppGpp-dependent activation and repression are inversely correlated in the in vitro condition in which RNA polymerase is limiting. No correlation was observed. It was concluded that the ppGpp-dependent activation is independent of the repression. Moreover, it was proposed that ppGpp-dependent activation and repression are mediated by titratable factors, each of which operate independently.

After amino acid starvation, bacteria slow down those physiological processes that are less necessary during the adjustment period (*e.g.*, protein synthetic apparatus and cell wall and membrane syntheses) to conserve energy and resources and speed up processes that enable the amino acid deficit to be overcome (*e.g.*, amino acid biosynthesis and protein turnover). The hallmark of the gene expression changes after amino acid starvation is reduction of stable RNA synthesis and increase of amino acid biosynthesis gene expression. This global response is referred to as the “stringent response,” and it is mediated by guanosine 5′-diphosphate 3′-diphosphate (ppGpp) (for review, see Refs. 1–3). The ppGpp is a pleiotropic regulatory effector exerting both negative and positive effects on gene expression in bacteria.

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The abbreviations used are: ppGpp, guanosine 5′-diphosphate 3′-diphosphate; CAT, chloramphenicol acetyltransferase.

Studies of the molecular mechanism by which ppGpp acts to regulate gene expression have resulted in controversial conclusions, although it is generally accepted that the primary regulatory effect of ppGpp is at the level of transcription initiation for both positively and negatively controlled genes (2, 3). In the case of negative control, one hypothesis is that ppGpp inhibits transcription initiation through direct binding to RNA polymerase. This hypothesis is based on analyses of the amino acid prototrophic suppressor mutants arising spontaneously from the strain that was unable to synthesize ppGpp (3–5), as well as RNA polymerase mutants maintaining expression from ribosomal RNA promoter despite elevated ppGpp concentration (6). Also, direct binding of ppGpp to the β subunit of RNA polymerase has been shown by in vitro cross-linking studies with fluorescent-labeled ppGpp (7) or azido-ppGpp (8). Interestingly, however, a negative regulatory effect of ppGpp on the stable RNA promoters has been demonstrated using a minimal in vitro transcription system composed of purified component only with a special condition: a linear DNA template at a high-salt condition (9, 10). The possibility was raised long ago, but not resolved, that a factor might mediate the regulatory effect of ppGpp (1, 11, 12). At least for the stimulatory effect of ppGpp, the activation of lac operon of *Escherichia coli* or his operon (of *Salmonella typhimurium*) gene expression was observed using the in vitro system containing S-30 cell extract in a manner comparable with that observed in vivo but not with purified components (13–15). It was tentatively inferred that a factor missing in the minimal transcription system is required to demonstrate, at least, the stimulatory effect of ppGpp (12, 13).

In an attempt to explain the global effect of stringent regulation, especially for positive effect, various passive regulation models have been introduced. The model currently proposed by Zhou and Jin (16) is based on the assumption that there are two classes of promoters with different rate-limiting steps: RNA polymerase binding for amino acid biosynthesis promoters (positively regulated by ppGpp) and an isomerization step for stable RNA promoters (negatively regulated by ppGpp). In the absence of ppGpp, during rapid growth, most RNA polymerases are engaged in the transcription of stable RNA genes but not in that of amino acid biosynthesis genes. During stringent response, an elevated level of ppGpp destabilizes the open promoter complex formation at the stable RNA promoters (5, 6), which results in an increase of free RNA polymerase inside the cell. Consequently, amino acid biosynthesis genes are transcribed. Thus, stimulation of amino acid biosynthesis genes on stringent response is a consequence of reduced transcription of stable RNA genes in the condition of RNA polymerase limiting. In contrast, Jensen and colleagues claimed that stable RNA promoters have relatively low affinity for RNA polymerase and high V\textsubscript{max} for open complex formation and promoter clearance (17, 18). The major effect of ppGpp was suggested to be on RNA polymerization such that ppGpp reduces the rate of transcri-
tion elongation, which subsequently limits the quantity of free RNA polymerase able to initiate transcription. (4, 17–19). A reduced pool of free RNA polymerase would then reduce the transcription initiation more drastically with those stable RNA promoters that have low affinity for RNA polymerase than with amino acid biosynthesis promoters, thereby achieving a differential regulatory effect on gene expression. It should be noted that these models evolved from the passive regulation model originally proposed by Maaloe (21–23) to account for the growth rate control of ribosome synthesis: those genes encoding ribosomal components were constitutively derepressed, not under any active control. This idea was further developed into an "RNA polymerase partitioning model" by Baracchini and Bremer (24) to account for ppGpp-dependent differential gene regulation: RNA polymerases exist in two forms with different promoter specificity, and the relative amounts of the two forms are dependent on intracellular concentration of ppGpp.

To understand the molecular mechanism of ppGpp-mediated regulation, especially that of gene activation, a "mixed template" in vitro experiment using a cell-free transcription-translation system was devised in this study (25, 26). More specifically, the passive model of differential gene regulation by ppGpp was tested in vitro by varying those crucial elements of regulation: the ppGpp concentration, gene dosage (DNA template concentration), and RNA polymerase concentration. This systematic in vitro analysis was expected to provide a more direct measurement of ppGpp effects without the possible complications faced when carried out in vivo. The DNA templates directing expression of either the hisG promoter (his operon), regulated positively by ppGpp (15, 25, 27), or the leuV promoter (tRNAleu operon), regulated negatively by ppGpp (28, 29), were tested. Both promoters have been demonstrated to be regulated by a physiological concentration of ppGpp using the crude in vitro system (25). The main idea of this exercise was to observe the response of these promoters to ppGpp in a mixed template condition in which RNA polymerase is limiting. It was concluded from this study that ppGpp-mediated gene activation is independent of repression, and both activation and repression are mediated by different titratable factors, each of which operates independently.

MATERIALS AND METHODS

Construction and Isolation of DNA Templates—The construction of pAZ2 directing expression of his promoters of S. typhimurium has been described (16). Briefly, pAZ2 had the fragment extending from the hisG promoter/regulatory region to the amino terminus of the third structural gene of the his operon, hisC, fused in-frame to the lacZ gene of a translational fusion vector, pJES35 (30). The presence of an operon-specific attenuator in these plasmids has been shown to have little effect on ppGpp regulation (16). pAZ16 is identical to pAZ2, except that it carried TATAAT at the -10 hexamer of hisGp. The pLC76 and pLeuS plasmids directing expression from the wild-type and the mutant tRNAleu promoter (leuVp) of E. coli, respectively, have been described elsewhere (28, 29). Briefly, plasmid pLC76 carried the leuVp promoter sequence from -45 to +8 of leuVp (synthesized in vitro) cloned into the multiple cloning site, upstream of the promoterless gene of chloramphenicol acetyltransferase (CAT) in a transcriptional fusion vector, pSL100 (31). Plasmid pLeuS was identical to the pLC76, except that the discriminator sequence between -1 and -4 of leuVp was altered.

To propagate plasmids an E. coli strain, JM103, Δ(lac-pro) supE thi strA sbcB15 endA hapR4 [F' traD36 proAB lacI (LacF') j lacZ M15], was used (32). For the preparation of plasmid DNA template, pAZ plasmids were amplified with chloramphenicol (0.17 mg/ml) and plasmids pLC76 and pLeuS were amplified with spectinomycin (0.3 mg/ml); these plasmids were isolated by the cleared lysate protocol and purified with two cycles of CsC1 gradient centrifugation (33).

Conditions for Protein Synthesis in Vitro—The preparation of S-30 cell extract and the in vitro reaction mixtures was essentially as described by Choy (25) using a glutamate substitution modification. S. typhimurium strain TA705 (hisD(GCBH253 hisT1504 relA1) (34) was used to prepare an S-30 cell extract. The preparation and procedure for the mixed template experiment were the same as for the single-template experiment.

Enzyme Assays—Assay conditions and procedures for β-galactosidase enzyme were essentially as described by Lesley (26). The assay condition and procedure for CAT enzyme were described by Choy (25).

RESULTS

Titration of DNA Templates Directing Expression of hisGp or leuVp—The expression from hisGp of the his operon in S. typhimurium (15, 25) and leuVp, tRNAleu promoter of E. coli (25, 28, 29) was determined as a function of the DNA template concentrations in vitro using a coupled transcription and translation system containing an S-30 cell extract of S. typhimurium (Figs. 1 and 2). To examine the regulatory effect of ppGpp, the DNA template titration was carried out in both the absence and presence of ppGpp. The DNA templates directing expression of these promoters were from supercoiled plasmids pAZ2 (hisGp) and pLC76 (leuVp).

Fig. 1 shows the hisGp DNA (pAZ2) titration by measuring β-galactosidase activity as a function of DNA concentration. The β-galactosidase activity from the chimeric hisC-lacZ protein represents the hisG promoter activity. Approximately 70 nm of DNA template saturated the crude in vitro system in the presence or absence of ppGpp; hisGp expression in the presence of 0.1 mM ppGpp was elevated 10-fold over the range of saturating DNA concentrations (>70 nm). Note that the saturating DNA concentrations in the presence or absence of ppGpp were about the same. The concentration of 0.1 mM ppGpp used in this experiment was within the range of concentrations that stimulated hisGp expression maximally in the presence of a saturating concentration of DNA template (15, 25, 27).

In the case of the leuVp DNA (pLC76) titration, the CAT activity was determined as a function of DNA concentration (Fig. 2). The CAT activity from the leuV-cat fusion represents the leuV promoter activity. Approximately 40 nm of DNA template saturated the crude in vitro system in the absence of ppGpp. However, in the presence of increasing concentrations of ppGpp, the leuVp DNA (pLC76) titration curve shifted to the higher DNA concentrations. Concentrations of 0.2 and 0.4 mM ppGpp inhibited leuVp expression 50 and 80%, respectively, when 40 nm DNA was used (see Fig. 4 in Ref. 25). It should be noticed that despite the shift of curves to the higher concentrations of DNA in the presence of ppGpp, activities at the satu-
The concentration of DNA template and began to reach saturation imminently (25). The expression of hisG DNA—(see below). An inhibitory effect of ppGpp on stable RNA gene expression is relieved by increasing DNA concentration.

**Mixed Template Experiments**—In an attempt to examine the pleiotropic regulatory effect of ppGpp in a physiologically more relevant condition, we designed a mixed template experiment in which the activities of activable and repressible promoters could be determined simultaneously. This was feasible because the two promoters were fused to different reporter genes. The intent was to determine whether the stimulation and inhibition of protein synthesis by ppGpp are interrelated under the condition in which RNA polymerase is limiting. An in vitro condition in which RNA polymerase is limiting was created by the use of a saturating concentration of DNA template (see below).

**hisGp DNA Titration in the Presence of Saturating leuVp DNA**—The hisGp DNA (pAZ2) was titrated by determining the expression from hisGp in the presence or absence of 0.1 mM ppGpp in a mixed template condition in which a fixed, saturating concentration of leuVp DNA (pLC76) was included. In this experiment, the changes in the magnitude of ppGpp inhibition on leuVp expression in the presence of an increasing concentration of an activable promoter, hisGp, were examined. The 0.1 mM ppGpp concentration used in this experiment inhibits the expression from leuVp DNA ~30%, yet it is within the range of concentrations that stimulate hisGp expression maximally (25). The expression of hisGp increased with increasing concentration of DNA template and began to reach saturation at ~65 nM in the presence or absence of 0.1 mM ppGpp (Fig. 2A). The stimulation of hisGp expression by ppGpp was similar to that in the single-template condition (Fig. 1), but the magnitude was somewhat enhanced in the presence of leuVp DNA: 19- versus 10-fold. The expression from leuVp, in the same reaction tubes (the relative activity shown in Fig. 2B) declined progressively with increasing hisGp DNA, but the decline was similar in both the presence and absence of ppGpp (Fig. 2B). Thus, the relative inhibition of leuVp expression (Fig. 2B, square symbols) by 0.1 mM ppGpp was altered little: 32% in the absence of hisGp DNA and 42% in the presence of 77 nM hisGp DNA. Apparently, the stimulation of hisGp expression by ppGpp has relatively little impact on the inhibition of leuVp expression.

**To verify that the limitation imposed in these mixed template conditions was RNA polymerase, RNA polymerase was added in the presence of 65 nM hisGp and 46 nM LeuVp in the absence of ppGpp (Fig. 3C). In this mixed template condition, hisGp activity was reduced ~30% and leuVp activity was reduced ~50% compared with those in the single-template condition. The addition of RNA polymerase increased the expression from both promoters, indicating that the RNA polymerase, but no other component in this DNA-dependent protein expression system, was limiting. Thus, any change in either promoter activity should be reflected by corresponding changes in the competing promoter activity in this condition of RNA polymerase limiting.**

**leuVp DNA Titration in the Presence of Saturating hisGp DNA**—This was the converse of the experiments described above (Fig. 3). The leuVp DNA (pLC76) was titrated in the presence or absence of 0.1 mM ppGpp in a mixed template condition in which a fixed, saturating concentration of hisGp DNA (pAZ2) was included (Fig. 4). Thus, in this experiment the changes in stimulatory effect on hisGp expression by ppGpp in the presence of increasing concentrations of a repressible promoter, leuVp, were determined.

The expression from leuVp increased with increasing concentrations of DNA template and saturated the in vitro system at ~30 to ~40 nM in the absence of ppGpp (Fig. 4A). The presence of ppGpp shifted the titration curve to higher concentrations of DNA similar to those observed in the single-template condition.
ppGpp-dependent Transcription Regulation

Wild-type hisGp DNA—The mutant leuVp DNA (pLeuS) contains GCGTTTT in place of the wild-type sequence, GCGCCTC, at the discriminator (29). This base pair substitution mutation rendered the leuVp insensitive to ppGpp-mediated inhibition (Figs. 5 and 6). The stimulatory effect of ppGpp on the expression from hisGp in the same reaction tubes declined more drastically in the absence of ppGpp (Fig. 4B). This resulted in a greater stimulation of hisGp by ppGpp in the presence of leuVp expression: ~10-fold in the absence of leuVp DNA (pLC76) and ~53-fold with 0.1 mM ppGpp at the highest leuVp concentration (58 nM; Fig. 4B, square symbols). Thus, the stimulatory effect of ppGpp on hisGp expression was not reduced but enhanced in the presence of a repressible promoter, leuVp.

A Reciprocal Titration of the Mutant leuVp DNA and the Wild-type hisGp DNA—The mutant leuVp DNA (pLeuS) contains GCGTTTT in place of the wild-type sequence, GCGCCTC, at the discriminator (29). This base pair substitution mutation rendered the leuVp insensitive to ppGpp-mediated inhibition (Figs. 5 and 6). The stimulatory effect of ppGpp (0.1 mM) on hisGp expression was examined in the presence of this neutral leuVp promoter. Fig. 5 shows a titration of hisGp DNA (pAZ2) in the presence or absence of 0.1 mM ppGpp in a mixed template setup containing a fixed saturating concentration of the discriminator mutant leuVp DNA (pLeuS, 35 nM). Thus, this is comparable with the experiment carried out with the wild-type leuVp DNA shown in Fig. 3. An almost identical result was obtained, except that expression from the discriminator mutant leuVp was about the same in the presence or absence of 0.1 mM ppGpp.

Fig. 6 shows the converse experiment, in which discriminator mutant leuVp DNA (pLeuS) was titrated in the presence of a fixed saturating concentration of wild-type hisGp DNA (pAZ2, 65 nM). Again, ppGpp had little effect on the expression from the discriminator mutant leuVp promoter. The discriminator mutant leuVp DNA titration in the presence of wild-type hisGp DNA (Fig. 6A) was almost the same as that in the single template condition (data not shown). The expression from hisGp declined with increasing concentration of the discriminator mutant leuVp DNA (pLeuS) in the presence or absence of 0.1 mM ppGpp (Fig. 6B) and was similar to that in the presence of increasing wild-type leuVp DNA (pLC76), as shown in Fig. 4B. The stimulation by ppGpp on hisGp expression was enhanced: ~10-fold in the absence and ~45-fold at the highest concentration of the discriminator mutant leuVp DNA (58 nM) (Fig. 6B, square symbols). Thus, stimulation of hisGp expression by ppGpp in the presence of the neutral mutant promoter was similar to that in the presence of the repressible wild-type promoter.

A Reciprocal Titration of the Mutant hisGp DNA and the Wild-type leuVp DNA—The mutant allele of hisGp, hisGp3400, has the consensus sequence of E. coli70 promoter at the -10 region, which relieves the requirement for ppGpp for maximal expression (15, 35). The DNA template directing expression of the mutant hisGp (pAZ16) has a plasmid construction identical to that of the wild-type hisGp (pAZ2). In an attempt to analyze the ppGpp-mediated regulation of wild-type leuVp expression in the presence of a neutral promoter, hisGp3400, the DNAs were titrated in the presence of each (Fig. 7, A and B). Fig. 7A shows a titration of the mutant hisGp DNA (pAZ16) in the presence of a fixed concentration of wild-type leuVp DNA (pLC76, 46 nM). As the concentration of the mutant hisGp DNA (pAZ16) increased, the expression of hisGp increased, and this was accompanied by a drastic reduction of leuVp expression. This result established a definite pattern of competition between the two templates. Fig. 7B shows the converse experimen...
ppGpp-dependent Transcription Regulation

In this study, a crude in vitro system was used to test the passive model of ppGpp-mediated gene expression regulation, namely whether ppGpp-mediated activation and repression were inversely related. A mixed template experiment was designed to measure the stimulation and inhibition simultaneously in a single reaction tube. A major advantage of the in vitro studies, compared with in vivo physiology, is that the possible influence of global regulatory systems can be isolated and studied without the wide-ranging indirect effects caused in vivo by cascades of events. Although similar mixed template experiments have been carried out previously with highly purified components in a minimal in vitro transcription system (13, 36, 37), this crude system using supercoiled DNA templates instead of DNA fragments could be far more relevant for a quantitative assay of gene expression (25). The S-30 extract is considered a reasonably reliable source for most cytoplasmic components (25, 38). The passive control model assumes that the rate of total transcription initiation is limited by the availability of RNA polymerase (39). Thus, the crude in vitro system needed to be adjusted to create a condition in which repressible and activatable promoters would compete for the limiting RNA polymerase. This was met by the use of a saturating DNA template concentration as shown in the RNA polymerase titration experiment (Fig. 3C).

The Stimulation and Inhibition by ppGpp Are Independent Processes—The results obtained from the mixed template experiments provide strong evidence against the passive control model. In the mixed template condition, the patterns of ppGpp-mediated activation (hisGp) and repression (leuVp) appeared not to be interdependent.

The experiment presented in this study provides an in vitro test of the RNA polymerase partitioning model. The main theme of this passive control model was ppGpp-dependent partitioning of RNA polymerase into two forms with different promoter selectivity. That is, in the absence of ppGpp, "RNA polymerase is assumed to be in a form which initiates RNA chains exclusively at stable RNA promoters," whereas in the presence of ppGpp, ppGpp "converts the enzyme into a form which prefers mRNA promoter" (24). Therefore, transcription initiation from mRNA and stable RNA promoters are inversely related in the condition of RNA polymerase limiting (24). The experiment of leuVp titration in the presence of a saturating concentration of hisGp, shown in Fig. 4, was a setup analogous to the in vivo gene dosage experiment of Baracchini and Bremer (24), which provided the basis of the RNA partitioning model. It was shown in this manuscript that the increase in the gene dosage of leuVp restored the expression from leuVp in the presence of ppGpp almost to the level of that in the absence of ppGpp. The titratable effect is apparently maintained in the mixed template experiment as well as in the single-template experiment (Fig. 2). This result is comparable with the in vivo measurement: the addition of extra rrn genes reduced the extent of the stringent response (24). Thus, if the same interpretation developed for the in vivo gene dosage experiment is applied to these in vitro experimental results, i.e. that the RNA polymerase modified with ppGpp is sequestered by the addi-
The decline in expression from the mutant polymerase available to initiate transcription from discriminator mutant B (Fig. 3) ppGpp. The rate (slope) of reciprocal decline of consequence of inhibition. Therefore, concluded that the stimulation by ppGpp is not the range of 10^4:1), the simplest explanation is that the inhibitory activity in the presence of ppGpp is reduced a little (−50%), but certainly not down to the level seen in the absence of ppGpp. This is in contrast to the partitioning model: in the presence of extra leuVp DNA there should be little ppGpp-modified RNA polymerase available to initiate transcription from hisGp. It is, therefore, concluded that the stimulation by ppGpp is not the consequence of inhibition.

The conclusion drawn above is strengthened with the observation that the extent of promoter occupancy for the limiting RNA polymerase by two kinds of promoters was unaffected by ppGpp. The rate (slope) of reciprocal decline of leuVp expression (Fig. 3B) accompanying the increased expression from hisGp (Fig. 3A) was about the same in the absence or presence of ppGpp. More obviously, this trend is reproduced with the discriminator mutant leuVp, a promoter insensitive to the ppGpp regulation.2 The decline in expression from the mutant leuVp was the same in the presence or absence of ppGpp (Fig. 5). If the stimulation of the hisGp is sequestering more RNA polymerase, as the partitioning model would predict, then the decline of the wild-type or discriminator mutant leuVp expression should have been far more drastic in the presence of ppGpp than in the absence of ppGpp; the modified form of RNA polymerase initiates RNA chain synthesis in preference to mRNA promoters, and thus the increasing gene dosage of mRNA promoters should reduce the expression from stable RNA promoters in the presence of ppGpp but have little effect in the absence of ppGpp. Therefore, it is unlikely that ppGpp-mediated activation is the result of sequestering the RNA polymerase. This is also in contrast to all the other models of ppGpp-dependent differential gene regulation, which propose passive control (16–19).

Moreover, accumulated evidence suggests that positive control operates independently of negative control. Although 25 μM ppGpp is enough for the half-maximal stimulation of hisGp in vitro (16, 25, 27), ~200 μM ppGpp is needed for the half-maximal repression of leuVp (Fig. 2 and Ref. 25), which is in the vicinity of the concentrations of ppGpp reported to achieve half-maximal repression of stable RNA synthesis in vivo (24). It is conceivable that bacteria have evolved to cope with an onset of mild amino acid starvation by activating the amino acid biosynthetic operons without shutting off those processes related to bacterial growth: negatively controlled genes. The hierarchy of regulation would be determined by the sensitivity of individual promoters for ppGpp based on their needs for participation, depending on the severity of amino acid starvation.

ppGpp Regulation Is an Active Mode of Control—Although more direct evidence is awaited, the results in this work are most easily explained with an active control model. It is speculated that the ppGpp-mediated repression involves the participation of a titratable factor. The repressive effect of ppGpp on the expression of leuVp was relieved by increasing DNA concentrations (Fig. 2). Because ppGpp itself cannot be titrated out (the molar ratio of ppGpp to DNA template was in the range of 10^5:1), the simplest explanation is that the inhibitory effect of ppGpp is mediated by a titratable factor. Presumably, a ppGpp-factor complex represses gene expression by binding to the DNA template. Thus the repressive effect could be sequestered with an expenditure of the DNA template.

It seems that the titratable factor and RNA polymerase competitively interact with the DNA template. This is shown in the RNA polymerase titration experiment (Fig. 7): the addition of exogenous RNA polymerase abolished the repressive effect of ppGpp. Taken together, these results suggest the following molecular mechanism. The titratable factor activated in the presence of ppGpp interacts with the repressible promoter, presumably in a specific sequence with the discriminator (see below), and this prevents RNA polymerase from initiating transcription. Thus, increasing RNA polymerase concentration results in the abolition of the negative regulatory effect. However, one cannot exclude the alternative possibility that the titratable factor could be the RNA polymerase modified by ppGpp either directly or in conjunction with an unknown factor. This is certainly consistent with the results of gene dosage (Fig. 2) and RNA polymerase titration (Fig. 7) experiments. These could lead to the explanation that ppGpp-modified RNA polymerases were sequestered by the addition of more DNA-bearing repressible promoters or competed out by the addition of exogenous RNA polymerase. Recent studies demonstrating the repression of stable RNA promoters by ppGpp in vitro using purified components, although in a special condition (9, 10, 11), indicate the possibility that the titratable factor for negative regulation is the modified RNA polymerase itself. However, the mixed template experiment results (Figs. 3–6) suggest that even if RNA polymerase is directly modified by ppGpp and exerts negative control, it is not a part of the activation pathway. This is because when the additional leuVp DNA abolished the inhibitory effect of ppGpp in the mixed template condition, when the titratable factor was mostly sequestered, the stimulatory effect of ppGpp on the expression of hisGp was not impaired but enhanced. If the same ppGpp-modified RNA polymerase mediates activation as well as repression, the stimulation should have been impaired. Thus, these experiments lead to the conclusion that the activation by ppGpp is an active process independent of the repression mediated probably by a different titratable factor. Bartlet and colleagues (5) have proposed an active control model for ppGpp-dependent activation of amino acid biosynthetic promoters: the activation is at the promoter clearance step. By contrast, the study using hisGpΔ400, the up-promoter mutation that alleviated the ppGpp requirement for maximal expression, proposed that ppGpp action is at a step related to the function of ~10 hexamer but unlikely to be at the promoter clearance step (15, 35). Further speculation on the mechanism underlying ppGpp-mediated transcription regulation awaits the identification of such factor(s).

cis-acting Element of ppGpp Regulation—What are the cis-acting element(s) on promoters that determine the response of promoters to elevated ppGpp, positively or negatively? It has been proposed that the sequence between the −10 region and the transcription start site of the promoter, called the “discriminator” (40, 41), plays an important role in the ppGpp regulation, although the discriminator region of promoters is under ppGpp control; promoters that are negatively regulated by ppGpp tend to have G/C-rich sequences with a consensus sequence of GCCCCNC, whereas the positively regulated promoters have relatively A/T-rich sequences. Although the molecular mechanism involved in the function of the discriminator is still unclear, the importance of the discriminator has been substantiated with experiments showing that the base pair substitutions in this region of repressible promoters, tyrT (of tRNA^A^C^; Refs. 42, 43) and tufB (of EF-TU and four tRNAs; Ref. 44), abolished the ppGpp dependent inhibition in vivo and in vitro. Because the discriminator is located within the region of

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DNA that is melted on open complex formation, the G/C content rather than the specific nucleotide sequence of this region was considered significant (5, 16, 42, 45). However, it has been reported that the specific nucleotide sequence, and not merely the G/C content of the discriminator, is the significant element in stringent regulation, thereby suggesting that the discriminator sequence might be the site of interaction with an unknown regulatory protein involved in the stringent regulation (28, 35). Here, base pair substitutions at the discriminator region, which render the leuV promoter insensitive to ppGpp, were presented (Figs. 5 and 6). The same discriminator mutation of pLeuS has been shown to abolish ppGpp-dependent regulation of the leuV promoter in vivo (29). Because the discriminator sequence seems important for regulation during the stringent response, this sequence might provide cis-acting elements for interactions with titratable factors participating in ppGpp-dependent regulation.

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REFERENCES
1. Gallant, J. A. (1979) Annu. Rev. Genet. 13, 393–415
2. Cashel, M., and Rudd, K. E. (1987) in Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology (Neidhardt, F. C., ed), Vol. 2, pp. 1410–1438, American Society for Microbiology, Washington, D.C.
3. Cashel, M., Gentry, D., Hernandez, V. J., and Vinella, D. (1996) in Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology (Neidhardt, F. C., ed), vol. 2, pp. 1458–1468, American Society for Microbiology, Washington, D.C.
4. Hernandez, V. J., and Cashel, M. (1995) J. Mol. Biol. 252, 536–549
5. Bartlett, M. M., Gaal, T., Ross, W., Gourse, R. L. (1998) J. Mol. Biol. 279, 331–345
6. Tedin, K., and Bremer, H. (1992) J. Biol. Chem. 267, 2337–2344
7. Reddy, P. S., Raghavan, A., and Chatterji, D. (1995) Mol. Microbiol. 15, 255–256
8. Chatterji, D., Fujita, N., and Ishihama, A. (1998) Genes Cells 3, 279–287
9. Kajitani, M., and Ishihama, A. (1984) J. Biol. Chem. 259, 1951–1957
10. Ohles, K. I., and Grulla, J. D. (1992) Mol. Microbiol. 6, 2243–2251
11. Aboud, M., and Pastan, I. (1973) J. Biol. Chem. 248, 3356–3358
12. Aboud, M., and Pastan, I. (1975) J. Biol. Chem. 250, 2189–2195
13. De Crombrugghe, B., Chen, B., Andersen, W., Niesley, P., Gottesman, M., Pastan, I., and Perlman, R. (1971) Nat. New Biol. 231, 139–142
14. Frimakoff, P., and Artz, S. W. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 1726–1730
15. Riggs, D. L., Mueller, R. D., Kwan, H. S., and Artz, S. W. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 9333–9337
16. Zhou, Y. N., and Jin, D. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2908–2913
17. Jensen, K. F., and Pederson, S. (1990) Microbiol. Rev. 54, 89–100
18. Vogel, U., Sorenson, M., Pederson, S., Jensen, K. F., and Kilestrup, M. (1992) Mol. Microbiol. 6, 2191–2200
19. Sorenson M. A., Jensen, K. F., and Pederson, S. (1994) J. Mol. Biol. 238, 441–454
20. Little, R., Ryals, J., and Bremer, H. (1983) J. Bacteriol. 155, 1162–1170
21. Maaloe, O. (1968) in Biochemistry of Ribosomes and Messenger-RNA (Lindigkildt, R., Langen, P., and Richter, J., eds), pp. 231–255, Symposium, Castle Reinhardsbrunn
22. Maaloe, O. (1969) Dev. Biol. 3, (suppl.) 33–58
23. Maaloe, O. (1979) in Biological Regulation and Development, Gene Expression, Vol. 1, pp. 487–542, Plenum, New York
24. Baracchini, E., and Bremer, H. (1988) J. Biol. Chem. 263, 2597–2602
25. Choy, H. (1997) Biochem. Biophys. Acta 1353, 61–68
26. Lesley S. A. (1995) in Methods in Molecular Biology: In vitro Transcription and Translation Protocols, (Tymm, M. J., ed), Vol. 37, pp. 265–277, Humana Press, Totowa, NJ
27. Stephens, J. C., Artz, S. W., and Ames, B. N. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 4389–4393
28. Bauer, B. F., Kar, E. G., Elford R. M., and Holmes, W. M. (1988) Gene (Amst.) 63, 123–134
29. Rowley, K. B., Elford, R. M., Roberts, I., and Holmes, W. M. (1993) J. Bacteriol. 175, 1309–1325
30. Hirschman, J., Wong, P. K., Sei, K., Keener, J., and Kustu, S. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 7525–7529
31. Li, S. C., Squires, C. L., and Squires, C. (1984) Cell 38, 851–860
32. Messing, J. (1983) Methods Enzymol. 101, 20–78
33. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
34. Martin, R. G. (1968) J. Mol. Biol. 127–134
35. Da Costa, Z., and Artz, S. W. (1997) J. Bacteriol. 179, 5211–5217
36. Kajitani, M., and Ishihama, A. (1983) Nucleic Acids Res. 11, 671–686
37. Kajitani, M., and Ishihama, A. (1983) Nucleic Acids Res. 11, 3873–3889
38. Zuhay, G. (1973) Annu. Rev. Genet. 7, 267–287
39. Churchward, G., Bremer, H., and Young, R. (1982) J. Bacteriol. 150, 572–581
40. Travers, A. A. (1980) J. Bacteriol. 141, 973–976
41. Travers, A. A. (1984) Nucleic Acids Res. 12, 2605–2618
42. Travers, A. A. (1980) J. Mol. Biol. 141, 91–97
43. Lamond, A. I., and Travers, A. A. (1985) Cell 40, 319–326
44. Mizushima-Sugano, J., and Kaziro, Y. (1985) EMBO J. 4, 1053–1058
45. Figueroa-Bossi, N., Guerin, M., Rahmouni, R., Leng, M., and Bossi, L. (1998) EMBO J. 17, 2359–2367