Essential Steps in the ppGpp-dependent Regulation of Bacterial Ribosomal RNA Promoters Can Be Explained by Substrate Competition*

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Transcription of stable RNA genes is known to be dramatically reduced in the presence of guanosine tetraphosphate (ppGpp), the mediator of the stringent response. Using in vitro transcription systems with ribosomal RNA P1 promoters, we have analyzed which step of the initiation cycle is inhibited by the effector ppGpp. We show that formation of the ternary transcription initiation complex consisting of RNA polymerase holoenzyme, the promoter DNA, and the first initiating nucleotide triphosphate is the major step at which ppGpp exerts its regulation. Neither primary binding of RNA polymerase to the promoter nor isomerization to the open binary complexes or the subsequent promoter clearance steps contributes notably to the observed inhibition. The effect of ppGpp-dependent inhibition in the formation of the ternary transcription initiation complex could be mimicked by nucleotide derivatives known to bind to the RNA polymerase active center. Using these model compounds, almost identical inhibition characteristics were observed as seen with ppGpp. The results support the previously published model, which suggests that ppGpp-dependent inhibition is based on competition between the inhibitor molecules and NTP substrates for access to the active center of RNA polymerase.

Bacterial cells adapt their growth very efficiently in response to changes in the environmental conditions. The cellular growth is generally determined by the capacity for protein synthesis, which itself is tightly linked to the number of ribosomes. During amino acid deprivation bacterial cells react, among other metabolic adaptations, by an immediate transcriptional shut-down of ribosomal RNAs (rRNAs), the key molecules for the constitution of the protein synthesis apparatus. This global regulation is triggered by the rapid accumulation of the effector molecule guanosine tetraphosphate (ppGpp) and termed the stringent response (1). Although gradual progress in understanding this remarkable network has been made in recent years, the molecular details of the regulatory mechanism(s) are still obscure (2–10). The observations that some promoters (e.g. amino acid biosynthesis operons) are activated during the stringent response and that in vivo and in vitro studies have often yielded contradictory results have further aggravated our understanding of the mechanism of ppGpp-dependent regulation (11). Moreover, ppGpp-dependent regulation is not restricted to the steps of transcription initiation but has also been shown to act during elongation where the pausing properties of transcribing RNA polymerase are specifically changed (6, 12).

There is consensus today that RNA polymerase is the obvious target for the effector molecule ppGpp (7, 8, 13). Binding has been shown to occur close to the active center formed modularly by both the β and β′ subunits. The presence of ppGpp renders the activity of the enzyme in such a way that transcription from stringent promoters (e.g. rRNA and tRNA promoters) is strongly repressed. However, mutations in the RNA polymerase sigma subunits have also been reported which apparently obstruct σ70-core polymerase interaction and thereby cause altered initiation properties of RNA polymerase at elevated ppGpp concentrations (14). This phenomenon has been observed for other sigma factors like σ24, σ38, and σ54 as well (9, 15).

Whether a gene is stringently regulated or not is clearly encoded by its promoter structure. It is known for some time that promoters under negative stringent regulation share a common GC-rich sequence element downstream of the −10 promoter recognition element (15). This sequence is termed the discriminator, and it has been shown to be a necessary, although not sufficient, structural element for stringent regulation. It is also clear, however, that other properties of a promoter, in addition to the discriminator sequence, are involved in defining whether a transcription unit is under stringent control or not (5). Studies in the past have revealed that promoters under negative ppGpp-dependent control are characterized by a striking instability of their binary open complexes. For instance, the stringently regulated rRNA promoters will only form stable open complexes in presence of their corresponding starting nucleotides (3, 4, 17, 18). In the absence of starting nucleotides, the lifetime of open binary complexes is very short and such complexes have a high tendency to dissociate. Hence, at low NTP concentrations, formation of the ternary complexes will be the critical, rate-limiting step for productive transcription initiation. How, and at which step of the initiation pathway, can ppGpp affect transcription from such promoters? To answer this question we have systematically analyzed the effect of ppGpp on the individual steps leading to productive transcription initiation at several rRNA P1 promoters. Studies were performed in vitro, and the unregulated PtacI promoter served as a control. We show here that ppGpp affects the negatively regulated rRNA promoters at the transition from the binary open complexes to the ternary initiation com-

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1 The abbreviations used are: rRNA, ribosomal RNA; R, RNA polymerase; P, promoter; RP, closed complex; RPn, binary open complex; RPoc, ternary open complex or initiating complex that contains at least one bound substrate NTP; EC, elongating complex.
plexes. The concentration of the starting substrate NTPs play a crucial role in this inhibition, suggesting that competition between the substrate NTPs and ppGpp for the catalytic site is involved. This conclusion is supported by almost identical inhibition characteristics when ppGpp is replaced by NTP substrate analogues known to bind to the active site of RNA polymerase.

**EXPERIMENTAL PROCEDURES**

**Materials**—RNA polymerase holoenzyme was purified from *Escherichia coli* DG156 cells (19) as described previously (20, 21), and the activity of the enzyme was assessed by a quantitative transcription assay (22). Templates for RNA polymerase binding and *in vitro* transcription were isolated by restriction hydrolysis of plasmid DNAs. The following fragments were used for *in vitro* transcription: a 256-bp *rrnB* P1 promoter fragment (positions −183 to +63, relative to the transcription start site) obtained from plasmid pUC18-1 (5), a 575-bp *rrnD* P1 promoter fragment (positions −295 to +316, relative to the transcription start site) obtained from pHD11, and a 403-bp *Ptac* P1 promoter fragment (positions −223 to +180, relative to the transcription start site) from PtacW (5). Overhanging DNA ends were filled in by a Klenow reaction. Fragments employed for gel retardation were labeled by Klenow reaction and [α-32P]dATP.

ppGpp was prepared and purified as described previously (6). Care was taken to keep residual LiCl concentrations in the final reaction mixture below 2 nm. Ultrapure NTPs (Amersham Biosciences, HPLC-purified) were used for *in vitro* transcription. Dinucleotides (ApA, ApC, ApU) were purchased from Sigma.

**Multiple-round in Vitro Transcription**—Multiple-round *in vitro* transcription reactions were performed at 30 °C with linear templates (1 nm) as described recently (4, 23) in 50 μl of transcription buffer (50 mm Tris acetate, pH 8.0, 80 mm potassium glutamate, 10 mm MgAc, 1 mm dithiothreitol, 0.1 mm EDTA, 10 μg/ml acetylated bovine serum albumin). The reactions were incubated for 8 min after the addition of RNA polymerase (3 nm) and incubation was continued for another 15 min. ATP and radioactively labeled CTP (5 μCi), were added and incubation continued for an additional 15 min. Reactions were chased in the presence of all four NTPs (433 μM each) and 433 μg/ml heparin for 6 min at 30 °C. The transcripts obtained were precipitated with ethanol, resolved, and denatured in formamide sample buffer. Products were identified by autoradiography after separation of transcripts on denaturing 15% polyacrylamide gels.

**Single-round in Vitro Transcription**—Reactions were performed as described (23). Briefly, RNA polymerase (3 nm) was incubated with template DNA (1 nm) in 20 μl of transcription buffer for 8 min at 30 °C. 5 μl of a NTP solution containing a final concentration of 500 or 50 μM ATP and radioactively labeled CTP (5 μCi, 5 μM) was incubated for 15 min. Reactions were then chased for 8 min in the presence of 433 μg/ml heparin and 433 μM ATP and radioactively labeled CTP (5 μCi, 5 μM) was incubated for 15 min. Reactions were stopped by addition of formamide sample buffer and separated as described above.

**Formation of Ternary Initiation Complexes**—RNA polymerase (3 nm) together with template DNA (1 nm) was incubated in the presence of the first two initiation nucleotides (each 50 μM) in 20 μl of transcription buffer for 8 min at 30 °C to form ternary initiation complexes. Heparin was then added (final concentration: 200 μg/ml) to prevent reinitiation. For the elongation reaction the lacking NTPs (each 50 μM), one of which was radioactively labeled (5 μCi), were added and incubated for 15 min. Reactions were chased for 8 min and stopped by addition of formamide sample buffer prior to gel electrophoresis.

**Determination of Ternary Complex Stabilities**—The time-dependent decrease in the concentration of preformed ternary complexes was determined by single-round *in vitro* transcription reactions under conditions that precluded reinitiation. Reactions consisted of 5 nm template DNA with the *rrnB* P1 promoter and 15 nm RNA polymerase in transcription buffer. Samples were incubated in the absence or presence of 300 μM ppGpp for 8 min at 30 °C. For ternary complex formation, the starting nucleotides ATP (50 μM) and 20 μCi of [α-32P]CTP (5 μM) were added and incubation was continued for another 30 min. Heparin was then added (final concentration: 200 μg/ml) to prevent reinitiation. From this mixture aliquots were withdrawn at defined time intervals and subjected to a chase reaction for 8 min. Reactions were stopped by the addition of formamide sample buffer and analyzed by gel electrophoresis.

**KMnO4 Footprinting**—The structure of the transcription bubble in

A. Hillebrand and R. Wagner, unpublished data.

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**Fig. 1.** A, the sequences of the core promoter structures *rrnB* P1, *rrnD* P1, and *Ptac* are presented. The −35 and −10 recognition sequences are boxed, and the transcription start site is denoted by +1. The starting nucleotide is given in bold type, and nucleotides matching the discriminator consensus are underlined. B, kinetic scheme indicating the four main steps of transcription initiation. I indicates the first binding of RNA polymerase (R) to a promoter (P) forming a closed binary RNA polymerase-promoter complex (RP). II indicates isomerization to the binary open complex (RPα), and III formation of a ternary initiating complex after binding of the first NTPs (RPβ). Finally formation of an elongating complex (EC) under release of the promoter is shown schematically. The latter step involves the loss of the sigma subunit (σ), which may be reused for the next initiation cycle. Before the elongating complex is formed and productive transcription is started, the initiating complex may undergo multiple rounds of abortive cycling upon which short transcripts (Abortive products) are repetitively released.

**ternary initiation complexes was tested by KMnO4 footprinting** (23, 24). The ternary initiation complexes were preformed by incubation of 15 nm RNAP, 5 nm *rrnB* P1 DNA, 50 μM ATP, and 5 μM CTP in transcription buffer and in the absence of ppGpp (300 μM) for 30 min at 30 °C. Heparin was added (final concentration: 200 μg/ml) to prevent reinititation, and samples were treated with 37 nm KMnO4 for 1 min at 30 °C. To this mixture β-mercaptoethanol was added at a final concentration of 10%. The modified DNA was ethanol-precipitated, resolved in 50 μl of TE-buffer, and purified using the Qiagen PCR purification kit (Qiagen, Germany) according to the protocols from the manufacturer. KMnO4-modified nucleotides were identified by primer extension as described (23).

**Gel Retardation Assays**—The kinetics of ternary complex formation was analyzed by gel retardation (23). Briefly, RNA polymerase (3 nm) and radioactively labeled DNA promoter fragments (1 nm) were incubated in transcription buffer for 8 min at 30 °C. Complex formation was started by addition of the first two nucleotides necessary for initiation at the concentrations indicated. At different time intervals, aliquots (10 μl) were withdrawn and complex formation was stopped by transferring samples to fresh tubes containing heparin (final concentration: 200 μg/ml). Initiation complexes were separated from free DNA by native gel electrophoresis after the addition of glycerol (5%).

**RESULTS**

**Effect of ppGpp on Overall Transcription from Different Promoters**—To compare the inhibitory effect of ppGpp on different promoters, we performed multiple-round *in vitro* transcription reactions with DNA fragments containing either one of the following promoters: *rrnB* P1, *rrnD* P1, or, as unregulated control promoter, *Ptac* (see Fig. 1A). Standard reactions were
performed with 3 nM active E. coli RNA polymerase holoenzyme and 1 nM DNA template with increasing ppGpp concentrations (0–1 mM) as described under "Experimental Procedures." RNA polymerase, template DNA, and ppGpp, when present, were preincubated for 8 min before the reaction was started by adding the NTP mixture (50 μM each ATP, GTP, CTP; 0.85 nM (2.5 μCi) [α-32P]UTP) for the rrnB P1 and Ptacl promoters. For the rrnD P1 promoter, the reaction mixture contained 50 μM each ATP, GTP, and UTP and 0.85 nM (2.5 μCi) [α-32P]CTP. The reactions were stopped after 15 min by addition of heparin (433 mg/ml) followed by a 6-min chase with 433 μM amounts of all four NTPs. For the quantitative analysis of transcription products, a radioactive DNA fragment that differed in size from the expected transcripts was added as recovery standard and samples were precipitated with EtOH prior to loading on a sequencing gel. Fig. 2 shows the autoradiogram of a typical inhibition experiment after gel electrophoretic separation. The quantitative evaluation of the transcription products according to densitometry is presented as an inset (Fig. 2D). Strong inhibition can be seen for the rrnB (92%) and rrnD (82%) P1 promoters, reaching a maximum at ppGpp concentrations above 300 μM. As expected, only a weak and nonspecific inhibition is apparent for the Ptacl promoter under the same conditions. This multiple-round in vitro transcription experiment provides information on the overall inhibition, including the various steps of the initiation cycle, as well as promoter clearance and elongation. To address the question at which defined step(s) of the transcription pathway ppGpp exerts its inhibitory effect, it was necessary to dissect the individual steps of the transcription reaction.

Effect of ppGpp on the Different Stages of Transcription—The different steps of the transcription cycle may formally be broken down into initiation, elongation, and termination. Initiation itself is a multistep process, and a simplified description of the reactions from the first encounter of RNA polymerase with a promoter to the elongation complex is outlined in Fig. 1B. The schematic pathway is divided in four substeps (I–IV), which lead to characteristic intermediates on the way to productive transcription. Those intermediates have been characterized as the closed complex (RPc), the binary open complex (RPo), the ternary open complex or initiating complex that contains at least one bound substrate NTP (RPinit), and finally the elongating complex (EC), which has moved away from the promoter and generally lost the sigma subunit. In principle, the formation of each of the complexes might be rate-limiting and thus represent a target for ppGpp-dependent regulation.

To determine whether ppGpp exerts its effect before or after formation of the ternary initiation complexes (RPinit), we took advantage of the fact that open binary rRNA P1 promoter complexes (RPo) are notoriously unstable and decompose with a very short half-life if not stabilized by the addition of the initiating NTPs (17, 18, 25). Hence, to find out whether ppGpp acts before or after formation of the ternary initiating complexes, we measured the inhibitory effect when ppGpp was either present from the start of the reaction (before substrate NTPs were added) or only after the initiating nucleotide sub-
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Fig. 3. Effects of ppGpp addition at different steps during the transcription cycle. Results from single-round in vitro transcription reactions with the rrnB P1, rrnD P1, and PtacI promoters are summarized with ppGpp present before (left panel) or after (right panel) ternary initiation complex formation. Relative transcription yields in the absence (−) of ppGpp (gray bars) are normalized to 1. Transcripts in the presence of ppGpp (+) are indicated by black bars.

states had been added. Reactions were performed under single-round in vitro transcription conditions with preformed initiation complexes in the presence of only two NTPs (ATP and CTP for rrnB P1; GTP and UTP for rrnD P1). Samples were prepared in duplicate with either ppGpp present before or after the addition of the initiating NTPs. After 8 min at 30 °C, heparin was added (final concentration: 200 μg/ml) to prevent reinitiation and elongation was allowed for 15 min after addition of the remaining NTPs (50 μM each, including 5 μCi of [α-32P]UTP for rrnB P1 and 5 μCi of [α-32P]CTP for rrnD P1). The reaction was stopped after an 8-min chase, and samples were mixed with loading buffer prior to separation on a denaturing polyacrylamide gel. For each promoter-specific template, a control reaction was performed in exactly the same way, except that ppGpp was omitted during the complete procedure. Transcription products were quantitated by densitometry, and the results are presented in Fig. 3. From this experiment it can be concluded that both rRNA promoters (rrnB and rrnD P1) are inhibited by ppGpp before the stable ternary initiation complexes were formed. The degree of inhibition roughly matches the overall inhibition presented in Fig. 2, with a somewhat stronger inhibition for the rrnB compared with the rrnD P1 promoter. Most remarkably, no significant inhibition can be seen for both rRNA promoters for the reactions analyzed after the addition of the initiating NTPs which enable stable ternary complex formation. As expected, the PtacI promoter is not specifically affected before or after ternary complex formation. Note that formation of the open binary initiation complex for PtacI does not require the presence of starting NTPs for stabilization. From the results it can be concluded that the major effect of ppGpp-dependent inhibition must occur before RNA polymerase forms the ternary transcription initiation complex. Taken together the results demonstrate that ppGpp-dependent inhibition occurs during the early initiation phase, before ternary initiation complexes are formed, and steps after formation of the stable ternary complex (non-productive, abortive transcripts, promoter clearance, and elongation) do not contribute appreciably to overall inhibition.

Effects of the Starting NTP Concentration on ppGpp-dependent Inhibition—Because the presence of the starting NTPs appears to be necessary for rRNA promoters to form stable initiation complexes, we wished to understand the role of the initiating nucleotides for the ppGpp-dependent inhibition for this step of transcription. Therefore, we performed transcription experiments with the rrnB P1 promoter with different concentrations of the starting nucleotide ATP in the absence and presence of ppGpp.

According to earlier studies it has been reported that the first nucleotide of rRNA promoters has a very high apparent K₀, which limits formation of ternary complexes at rRNA P1 promoters. This step has therefore been considered as rate-limiting during transcription initiation (see, e.g., Ref. 26). To test the ppGpp-dependent effect on this step of rrnB P1 initiation, we used multiple-round in vitro transcription experiments with increasing concentrations of the starting nucleotide ATP in absence or presence of 300 μM ppGpp. The range of ATP concentrations was varied between 0.1 μM and 1 mM. The results are summarized in Fig. 4. It can be seen that ppGpp inhibits transcription at all ATP concentrations, however, with different efficiencies. Inhibition is maximal within a medium concentration range between 10 and 100 μM ATP. At ATP concentrations below or above this range, the ppGpp-dependent inhibition is less pronounced. The results indicate that the inhibitory effect of ppGpp on ternary complex formation can be released at high initiating NTP concentrations, suggesting competition between the inhibitor and the substrate NTPs, whereas at very low substrate NTP concentrations transcription is too inefficient to allow accurate determination of the effects on inhibition.

ppGpp Effects on Promoter Escape—It is known for several promoters that the slowest step in transcription initiation is not represented by RNA polymerase binding or open initiation complex formation but occurs during passage of RNA polymerase through the initial transcribed region. Those promoters are considered to be limited at the promoter escape or promoter clearance step (27). Usually, before RNA polymerase escapes from the promoter, a characteristic set of short RNA products (between 2 and 12 nucleotides in length) is synthesized and released as abortive transcripts. During abortive initiation RNA polymerase cycles repeatedly at the promoter without dissociation. At some point during abortive cycling, triggered by an unknown mechanism, a decision is made in favor of productive transcription and RNA polymerase changes into elongation mode. We wished to know whether this step is affected by ppGpp. The question could in principle be answered by a quantitative assessment of the accumulation of abortive products after formation of the initiation complexes (28). Hence we quantitated the abortive products derived from rrnB P1 ini-
Effect of ppGpp on the Structure and Stability of Ternary Initiation Complexes—It is known that promoters under negative stringent control generally do not form stable open binary complexes (see, however, Ref. 29). Although the lifetime of binary open complexes is affected by ppGpp at almost any promoter, the specific inhibitory effect of ppGpp has been attributed in a recent study to the intrinsically short lifetime of sensitive promoters (2, 3). Here we asked whether ppGpp has any effect on the structure, stability, and lifetime of the ternary initiation complexes. Therefore we analyzed the structure of ternary complexes of the rrrB P1 promoter by footprinting, monitoring the accessible nucleotides of the template and non-template strand within the single-stranded region of the initiation complex. Footprint modification was performed with KMnO₄ as described (23, 30), and the patterns were compared for complexes that had been formed in the presence or absence of ppGpp. Modified positions were identified by primer extension analysis after KMnO₄ modification of the ternary transcription complex at the rrrB P1 promoter is shown. Products for the template and the non-template strand were separated by gel electrophoresis. Samples were run next to nucleotide-specific sequencing ladders (A, C, G, T). The relevant sequence given at the margin. K⁻ and K⁺ denote protein-free DNA controls with (+) and without (−) KMnO₄ modification, respectively. Lanes marked with 0 or 300 indicate the absence or presence of 300 μM ppGpp. Numbers at the right margin indicate the modified nucleotide positions in the open complex. A, time-dependent decrease of transcription-competent ternary complexes formed at the rrrB P1 promoter. The decrease in complex concentration was determined by assessing the relative amounts of transcripts that could be formed after addition of heparin. Reactions were followed in the absence (dark squares) and presence of (300 μM) ppGpp (open squares).

Fig. 6. KMnO₄ modification of ternary transcription complexes. A, a primer extension analysis after KMnO₄ modification of the ternary transcription complex at the rrrB P1 promoter is shown. Products for the template and the non-template strand were separated by gel electrophoresis. Samples were run next to nucleotide-specific sequencing ladders (A, C, G, T). The relevant sequence given at the margin. K⁻ and K⁺ denote protein-free DNA controls with (+) and without (−) KMnO₄ modification, respectively. Lanes marked with 0 or 300 indicate the absence or presence of 300 μM ppGpp. Numbers at the right margin indicate the modified nucleotide positions in the open complex. B, time-dependent decrease of transcription-competent ternary complexes formed at the rrrB P1 promoter. The decrease in complex concentration was determined by assessing the relative amounts of transcripts that could be formed after addition of heparin. Reactions were followed in the absence (dark squares) and presence of (300 μM) ppGpp (open squares).
suitable to determine the stability of preformed ternary complexes. First, we measured the time-dependent decrease in the capacity of the complexes for RNA product formation after challenging preformed ternary complexes with heparin (200 μg/ml). For this purpose aliquots of ternary complexes that were formed either in the presence or absence of ppGpp were subjected to conditions for run-off transcription by adding a mixture of all four substrate NTPs including [α-32P]CTP. The run-off products were then quantified after gel electrophoresis by densitometry. A summary of the results is presented in Fig. 6B. Consistent with the high stability of ternary initiation complexes, only a small reduction of the transcription products can be observed within the time course of the experiment. Moreover, it is evident that the presence of ppGpp has only a slight effect on the complex stability. Although in the absence of ppGpp approximately 5% of the starting complexes were inactivated within 30 min, this inhibition increased only by a factor of 2 (10%) in the presence of ppGpp. Very similar results were obtained when the lifetime of the ternary complexes was analyzed in a similar way as described for the footprint analysis, namely by the KMnO4 reactivity of single-stranded T nucleotides in the template strand of the transcription bubble (data not shown). The small decrease in stability of the ternary initiating complexes is clearly not sufficient to explain the measured inhibition of transcription in the presence of ppGpp. We conclude from this results that the observed inhibition mediated by ppGpp cannot be explained by destabilization of the ternary initiation complexes.

**Effect of ppGpp on the Rate of Ternary Complex Formation**—It is known that the binary open complexes of rrr promoters (RP0, Fig. 1B) are very unstable and are hardly accessible for a quantitative analysis (17). We therefore measured the formation of the stable ternary complexes (RPinit) after the addition of the appropriate starting NTPs to RNA polymerase, which had been preincubated with promoter DNA in the presence or absence of ppGpp. The rate of RPinit formation was followed in different ways. In one approach the time-dependent formation of ternary complexes was analyzed directly by gel retardation as described previously (23). In this experiment we either employed 32P-labeled promoter DNA or non-labeled DNA, which was complexed in the presence of 32P-labeled starting NTPs. Separation of free DNA and RNA polymerase complexes was performed on 5% nondenaturing polyacrylamide gels. The respective amounts of complexes formed were determined by autoradiography. In an alternative approach, we determined the increase of KMnO4 modification of position −10 in the template strand (see above), and finally we measured the time-dependent increase in synthesized RNA transcription products. Measuring the RNA products has the potential advantage that only productive ternary complexes capable of elongating transcription are evaluated and transcription-incompetent complexes, dead-end complexes, or moribund complexes (31) will remain unconsidered. All methods gave almost identical results, and therefore only findings from the latter experiments are presented in Fig. 7. It can be assumed that at the initial phase of the reaction the rate of RNA products formed should be directly proportional to the concentration of the ternary initiation complexes. As shown in Fig. 7, comparison of the initial rates of ternary complex formation yields notable differences in the absence and presence of ppGpp. Interestingly, this difference depends on the concentration of the starting nucleotides. Although there is approximately a 10-fold difference in the initial product rates at low ATP (50 μM) concentrations, inhibition is significantly reduced at higher amounts of the starting NTP (500 μM), and now only approximately a 1.2-fold reduction is apparent. It can also be seen by the plateau values presented in Fig. 7 that the final yield of RNA transcripts is not significantly different at high versus low ATP concentrations. The results are consistent with the conclusion that the presence of ppGpp reduces the rate of RPinit formation. This inhibition can be overcome largely by increasing the concentration of starting nucleotides. We take this finding as an indication that competition for the substrates might be involved in the ppGpp-dependent inhibition mechanism.

**ppGpp-dependent Inhibition Is Related to Substrate Inhibition**—Together the experiments described above all point to a mechanism of ppGpp-dependent inhibition consistent with competition of the effector for the step of ternary initiation complex formation. For this step it is essential that the substrate NTP representing the first nucleotide in the growing chain of the transcript is recognized by sequence complementarity to the template position +1 and bound into the catalytic site of the RNA polymerase, where it must be arranged such that its 3′ hydroxyl group is ready for the acceptance of a phosphodiester bond with the 5′ phosphate of the incoming second NTP. If the mechanism of ppGpp-dependent inhibition is related to substrate competition, then any potential other substrate able to reach the active center of RNA polymerase but not suitable to produce productive RNA chains should principally show a similar inhibition characteristic as ppGpp. We therefore tested a set of substances known to be accepted in the active site of the RNA polymerase but unable to produce transcriptionally competent ternary complexes. The dinucleotides ApA, ApC, or CpC, for instance, are such potential substrates. It is known that they are accepted in the active center of RNA polymerase, where they have been shown to act as
acceptors for growing RNA chains, e.g. 5' primers for RNA transcription. On the other hand, because of the lack of a 5' triphosphate group, they cannot be incorporated as substrates for 3' chain elongation via phosphodiester bond formation and pyrophosphate release. Their dinucleotide character might otherwise be essential for a retarded passage through the nucleotide entrance channel formed by a cavity of the RNA polymerase β' β' subunits (32). Hence, we used these compounds as potential analogs that might mimic the inhibitory effect of ppGpp. To avoid problems resulting from interference as RNA primers, we selected different dinucleotide phosphates that did not match the start sequence of the different promoters analyzed (see Fig. 1). For the control Ptac promoter, we chose CpC and ApC. For the rrnD promoter ApA, and for the rrnB promoter ApA and ApU, were tested as potential effectors for transcription. The chosen compounds were then analyzed in the same way as shown for the effector compound ppGpp before. First we determined the effect on overall transcription in multiple-round in vitro transcription reactions. Experiments were performed as described before, except that these dinucleotides were added as effectors instead of ppGpp. As shown in Fig. 8 (A–C), it turned out that the effector compounds resulted in a very similar overall inhibition of the transcription reactions. A very strong inhibition, essentially comparable with the effect induced by ppGpp, is apparent when ApA is used as a competitor with both rRNA promoters rrnB and rrnD P1 (Fig. 8, A and B). Inhibition of the rrnB P1 promoter with ApU is significant, although markedly reduced in comparison with ApA (Fig. 8A). Moreover, when the dinucleotide CpC was used as competitor (Fig. 8C), the Ptac promoter showed only a very weak and apparently nonspecific response that had already been observed with ppGpp (compare with Fig. 2). Note that the same dinucleotide ApA could not be analyzed with the Ptac promoter because it serves as starting primer. ApU could also not be used for this promoter because it provoked a different start site (data not shown). It is interesting to note that inhibition by the dinucleotide competitors occurred at a concentration range similar to the range that has been found for ppGpp-mediated inhibition.

To characterize at which step(s) the dinucleotide competitors exert their inhibition, we performed single-round transcription initiation and elongation experiments in presence or absence of the competitor compounds as described before when ppGpp served as inhibitor (compare with Fig. 3). The results obtained were again remarkably similar to those in the experiments where ppGpp acted as inhibitor. A summary of the data is presented in Fig. 9A. Both rrn promoters, rrnB and rrnD P1, are significantly inhibited when the dinucleotide effectors (300 μM) were present before ternary complex formation. The activity of the non-regulated Ptac promoter is again not affected. As shown with ppGpp before, addition of the competitor substances after the ternary complexes had been formed did not cause any inhibition for all promoters tested. Fully consistent with the observation that ppGpp does not specifically inhibit the Ptac promoter, we found a slight increase in transcription efficiency for this promoter in presence of ApC both before and after ternary complex formation. The results demonstrate that the dinucleotide competitors behave very similar to ppGpp. They show the same promoter specificity, and specific inhibition occurs at a concentration range similar to the range that has been determined for ppGpp.

Next, we wished to know whether the inhibitory effect of the dinucleotides could also be ascribed to the change of the rate at which ternary transcription initiation complexes are formed (see Fig. 7). To answer this question, we analyzed the rrnB P1 promoter and used ApA as inhibitor substance at a concentration of 150 μM. In addition we tested, in the same way as we did before with ppGpp as inhibitor substance, whether the presence of increasing concentrations of the starting nucleotide triphosphates (ATP) would reduce or overcome the extend of inhibition. The results summarized in Fig. 9B demonstrate that the rate of ternary complex formation at the rrnB P1 promoter is clearly reduced in the presence of moderate concentrations of the dinucleotide ApA. As shown for the regulator ppGpp, this inhibition is reduced or partly counteracted when higher concentrations (500 μM instead of 50 μM) of the starting NTP are present during the reaction. We infer from this result that the mechanism of inhibition must be very similar for ppGpp and the competitor dinucleotides tested. Moreover, the results further support the notion that the inhibition can most easily be described as substrate competition during the formation of the stable ternary transcription initiation complexes.

**DISCUSSION**

In this study we have attempted to solve some of the questions that are still pending with respect to the mechanism of
the stringent control. This type of regulation is mediated by the small effector molecule ppGpp, which affects transcription of many genes, in particular stable RNA genes. The concentration of this global regulator changes rapidly in response to a variety of environmental signals, for which amino acid starvation is the most notable example. Despite numerous attempts to understand the mechanism of this fundamental regulatory process, bacterial physiology, we still lack important details. Many conclusions from former studies are controversial, although some findings are consistent and in general agreement. There is consensus, for instance, that RNA polymerase is the target for ppGpp (7, 8, 13), and, although not all the criteria are completely understood, it is known that the respective promoter structures determine whether a gene is under stringent control or not (5, 33–35). Moreover, both activation and repression of transcription are mediated by ppGpp, yet activation may not be a direct effect of the regulator. For instance, ribosomal RNA promoters represent the most outstanding examples for negative stringent control, whereas for example the universal stress protein UspA or amino acid biosynthesis proteins are known to be induced at elevated ppGpp concentrations. Much of the recent data suggest that the latter group of genes is probably regulated by passive mechanisms (2), although direct effects have also been reported (36–38).

To explain the mechanism of the stringent control, various steps of the transcription cycle have been proposed to be crucial for negative regulation. For instance, formation of the open promoter complex has been suggested as the relevant kinetic step for the negative control of rrn promoters (39, 40). A different conclusion, namely trapping in the non-productive closed complex followed by efficient inhibition at promoter clearance, was reached by a kinetic analysis of stringently and non-stringently controlled rrnB P2 promoter variants (4). The model is consistent with the partition model, proposed earlier, which suggests that RNA polymerase can exist in two interconvertible forms with different properties. Interconversion of the enzyme is triggered by bound ppGpp. RNA polymerase without ppGpp preferentially transcribes stable RNA promoters, whereas the enzyme with bound ppGpp prefers mRNA promoters (41). Based on the observation that stable RNA promoters form intrinsically unstable open complexes, models for both positive and negative stringent control have been proposed recently (3, 42). According to those models ppGpp has a direct effect only on promoters under negative stringent control (e.g. stable RNA promoters), whereas positive stringent regulation is explained as passive effect of increasing RNA polymerase concentration, which results from transcriptional shut-down of the stable RNA genes. Recent studies describe clear evidence in vivo and in vitro for transcriptional inhibition by ppGpp at the lambda P_R promoter (29, 43). Interestingly, the P_R promoter forms rather stable open complexes indicating that ppGpp-dependent regulation is apparently not restricted to promoters with short-lived open complexes. Moreover, based on earlier studies, several passive models for stringent control have been proposed previously (44, 45). The many different models, and the fact that none by itself can explain all the existing data, may reflect that there is probably not one single mechanism for the stringent control. This view is further strengthened by the finding that ppGpp is able to affect steps during transcription initiation but also during elongation (6, 12, 46).

In this study we have performed experiments in vitro, designed to define which step(s) of the initiation pathway are primarily affected by ppGpp and to explore the molecular mechanism by which ppGpp exerts it regulatory function. We selected the ribosomal RNA P1 promoters from the rrnB and rrnD operons, which differ in their starting nucleotide (ATP for rrnB and GTP for rrnD), bearing in mind the NTP-sensing model, which proposes that the starting NTP concentrations in the cell determines the sensitivity of stringent regulated promoters (18). For comparison we employed the synthetic P_tacI promoter, which does not contain a stringent discriminator sequence and which is known not to be affected by ppGpp. Consistent with the expectation, we found only a very small inhibition in multiple-round transcription experiments. In contrast, the two stable RNA promoters were strongly inhibited under the same conditions. At the same ppGpp concentration, inhibition of the two rrn promoters was not identical, however, and the rrnB P1 promoter exhibited a stronger inhibition (92% compared with 82% for rrnD). Whether this slight difference is caused by the minor structural deviations of the core promoter sequences (rrnB P1 deviates in only one, and rrnD P1 in three positions from the ideal consensus sequence; see Fig. 1A) or

### Figure 9

**Effects of competitor dinucleotides on the different steps of transcription initiation.** A, diagram showing the effects of the dinucleotides ApA and CpC, respectively, on the relative amount of transcription from the rrnB P1, rrnD P1, and PtacI promoters when present before or after ternary transcription initiation complexes were formed. Relative amounts of transcripts in the absence of competitors are normalized to 1. B, kinetics of ternary transcription complex formation in the presence and absence of the dinucleotide competitor substance ApA. Relative amount of transcripts is shown for reactions performed at low (50 μM, squares) and high (500 μM, circles) concentrations of the starting nucleotide ATP in presence (open symbols) or absence (filled symbols) of 150 μM ApA.
whether the difference reflects the alternative starting nucleotides (ATP versus GTP) remains unclear.

Our study clearly demonstrates that the major effect of ppGpp is exerted before or during the formation of the ternary transcription initiation complexes. This can be concluded from the following findings. 1) Strong inhibition is only observed when ppGpp is added before or at the formation of the ternary complex (see Fig. 3). 2) Effects later during the initiation cycle (promoter clearance, abortive initiation or pausing) do not contribute significantly to overall inhibition (see Fig. 5). In addition we can show that the concentration of initiating nucleotides is crucial for the extent of inhibition. High starting NTP concentrations abrogate ppGpp-dependent inhibition (see Fig. 4). We could further demonstrate that the DNA structure within the open complex is very likely not altered in presence of ppGpp. We take this as indication that the conformation of ternary transcription complexes are not changed dramatically by ppGpp (see Fig. 6A). Consistent with previous studies (e.g., Ref. 3), we obtained evidence that the difference in stability of the ternary complexes in presence or absence of ppGpp is insufficient to explain the degree of inhibition (see Fig. 6B).

Most interestingly, we find that the rate of ternary complex formation is reduced in presence of ppGpp. This effect can be compensated by an increase in the concentration of the initiating NTPs (see Fig. 7).

Together these results are consistent with a previously published model, suggesting that ppGpp competes with the initiating NTP substrates (25). The model makes the assumption that the obligatory path for substrate NTPs to enter RNA polymerase active center is the secondary channel, a structure formed by elements of the β' domains F and G (32). The diameter of this nucleotide entry channel is broad enough to allow passage of only one NTP at a time but also to accommodate ppGpp. It is feasible, therefore, that at high ppGpp concentrations free movement of substrate NTPs to the active site is restricted. This does not matter much if the $K_m$ values for substrate binding are low and the respective complexes are stable. If, however, the requirements for substrate NTPs are high (high apparent $K_m$ values), as is the case for initiation complexes at stable RNA promoters, then the competition situation will take effect. Stringent sensitivity can thus be explained by the promoter structure, which through its conformation and specific interaction with RNA polymerase, determines the stability of the initiating complex and the apparent $K_m$ for the first substrate NTP. The effector molecule ppGpp, which accumulates to millimolar concentrations during the stringent control, may cause a competition situation. According to the simplest explanation, ppGpp effectively competes with the substrate NTPs for access to the active center of RNA polymerase. As a consequence less or no productive initiation complexes are formed.

Our conclusions are in an apparent contrast to a previously published study (51) demonstrating that ppGpp lowers the overall elongation rate of RNA polymerase by enhanced pausing of the transcription elongation complex. The authors, by performing classical kinetic analyses, concluded that ppGpp-dependent inhibition did not appear to be competitive with the ribonucleoside triphosphate substrates. There are several possible explanations for this discrepancy. First, the mechanism of ppGpp-dependent inhibition on elongation may be substantially different from that on ternary initiation complex formation where the promoter structure is certainly involved. The latter studies, however, were performed at elongation with transcription complexes initiated at the non-stringently controlled T7 A1 promoter. Moreover, because overall elongation rates and not individual pauses were studied, it is not completely clear whether the kinetic formalism correctly applies to the rather complex situation with individual pauses dependent on a sequence-specific context. The observed overall inhibition may be the result of more than one inhibition mechanism, and competitive inhibition may be masked under such a complex situation.

Whether substrate competition is actually involved in the mechanism of ppGpp-dependent inhibition was further investigated by substituting the effector molecule ppGpp with nucleotide analogues. We selected dinucleotides (ApA, ApU, and CpC) that are known to be accepted as primers and thus are able to enter the active center of RNA polymerase. As can be seen in Figs. 8 and 9, these competitor substances not only inhibited transcription of the stable RNA promoters but showed almost the same characteristics of inhibition and were effective at the same concentration range as ppGpp. Moreover, in all cases the PtacI promoter was not inhibited. As shown for ppGpp, the dinucleotide competitors acted exclusively before or at the step leading to formation of the ternary initiation complex. Moreover, they exhibited the same dependence on the concentration of the starting NTPs as observed with the regulator ppGpp; finally, they reduced the rate at which the ternary complexes are formed. We believe that all these observations are certainly not an accidental coincidence. Therefore, we take these findings as a strong indication that substrate competition plays a major role in the mechanism of ppGpp-mediated stringent control.

Of course there is also the possibility that dinucleotide substrates might mimic other mechanisms of ppGpp-dependent inhibition. For instance, such substrates might directly interact with a putative ppGpp binding site and invoke allosteric effects in much the same way as might be the case for ppGpp. In a recent in vitro transcription initiation analysis employing the dinucleotide ApU at the lambda PR promoter, it was shown that ppGpp-mediated inhibition was abrogated (29). As the simplest interpretation of this finding, the authors concluded that ppGpp inhibits formation of the first phosphodiester bond and this step is by-passed by the dinucleotide. It was thus concluded that the effect of ppGpp is less drastic in the presence of the dinucleotide ApU. The alternative explanation as outlined above might, however, also apply to this observation. To further test the model and to explore the exact type of inhibition or competition, more experiments with competitor analogues are under way.

There is independent evidence for the regulation of NTP access to the active site of RNA polymerase, and blocking the secondary channel for free passage of NTP substrates has been proposed recently in quite a different context. For the structure that is central for the secondary channel, different conformations have been described. Within the RNA polymerase, the bridging helix formed by β' domains F and G (alternatively bent or straight) are believed to provide a mechanism for RNA polymerase translocation. The bent β' subunit F bridge involves a clash competing with the incoming nucleotides at the active site of RNA polymerase. The alternating movement pushes template bases from the $i + 1$ site (translocation) and concomitantly blocks the entry for nucleotides into the active site. This switch mechanism, termed “swing-gate” by the authors, explains translocation of the transcript and simultaneously regulates the entry of substrate nucleotides (47).

The proposed mechanism of competition outlined above does not rest on a static RNA polymerase structure. To the contrary, more complex scenarios can be envisaged involving dynamic changes of the transcription complex. Many observations are in favor of the view that RNA polymerase activity can be regulated by allosteric mechanisms, as in the case of the swing-gate
mechanism, where the conformation of the F helix is modulated by factors acting elsewhere in the molecule binding to stringent promoters or the interaction with an effector molecule like ppGpp might change the architecture of distant domains within the multi-subunit enzyme. Allosteric mechanisms could principally explain a different phenomenon of the stringent control, which appears to be independent from substrate competition. For instance, several reports have provided evidence that ppGpp affects the relative competitiveness of sigma factor interaction with core RNA polymerase (9, 15, 48). In this case ppGpp might change the architecture of distant domains promoters or the interaction with an effector molecule like

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Essential Steps in the ppGpp-dependent Regulation of Bacterial Ribosomal RNA Promoters Can Be Explained by Substrate Competition
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