Transcriptional Pausing of RNA Polymerase in the Presence of Guanosine Tetraphosphate Depends on the Promoter and Gene Sequence*

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We have studied the response of the effector molecule guanosine 3',5'-bis(diphosphate) (ppGpp) on RNA polymerase pausing during in vitro transcription elongation. Pausing was followed during single round extension of stalled ternary complexes excluding possible ppGpp effects on initiation. The ppGpp dependences of early pausing sites within different transcription systems controlled by promoters with known response to enhanced ppGpp levels in vivo were quantitatively characterized. Transcription of stable RNAs and mRNA genes were analyzed. In addition, the in vitro pausing behavior of two promoter variants directing the same sequence but differing in their in vivo ppGpp sensitivity were compared. In the presence of ppGpp we noted a slight general enhancement of specific pauses in all transcription systems. However, genes known to be under stringent or growth rate control in vivo revealed a notably stronger pausing enhancement. The sites of pausing are not changed by the presence of ppGpp but appear to be sequence-specific. The effect of ppGpp on the extent of pausing depends on the particular promoter and closely adjacent sequences that the RNA polymerase has passed during initiation. Pausing enhancement requires the presence of ppGpp during elongation but not during initiation. The results underline the importance of pausing for transcription regulation and offer a plausible explanation for inhibition of stable RNA expression under conditions of elevated concentrations of ppGpp.

Bacterial growth is largely determined by two global regulatory networks, stringent control and growth rate regulation. Both phenomena affect transcription but also change many other cellular processes substantially, like replication, translation, proteolysis, or transport (1–3).

Growth rate regulation causes increase in the rate for stable RNAs (rRNA and tRNAs) in proportion to the square of the cell growth rate (4). Stringent control, on the other hand, summarizes a complex pattern of metabolic changes as a consequence of amino acid deprivation. The most dramatic effect of this regulatory network is the immediate repression of stable RNA synthesis concomitant with a rapid increase in the cellular concentration of the effector molecule guanosine 3',5'-bis(diphosphate, ppGpp (2). This hormone-like substance, also termed an “alarmone,” functions as a global transmitter for cellular regulation.

Basically it is unclear today how ppGpp exerts transcriptional regulation. Evidence based on mutational analysis (5–7) as well as on cross-linking results and spectroscopic data (8–10) indicates that the direct target for ppGpp action is the RNA polymerase itself, although unequivocal proof for this notion is still pending.

The effects of ppGpp on both activation and repression of transcription have mainly been related to the initiation step (11–13) in line with the classification of stringent or nonstringent regulated promoters (14, 15). Undoubtedly, at high concentrations of ppGpp (stringent control) different promoters respond in a different way. Outstanding inhibition is observed for ribosomal RNA P1 promoters. Some promoters, like the β-lactamase promoter, are obviously not directly affected, and for several other promoters, like “gearbox” promoters or promoters controlling amino acid biosynthetic operons, de-repression of transcription can be noted at elevated ppGpp concentrations (16–18).

Although the question that structural requirements confer stringent or growth rate dependence has been addressed by numerous investigations, our present knowledge is still incomplete. A GC-rich discriminator motif immediately downstream to the −10 consensus hexamer was shown in vitro and in vivo to be a necessary but not sufficient element for stringent and growth rate-regulated promoters (15, 19–21). Studies with hybrid promoters show that stringent promoters are defined by the complete promoter context (22).

Effects provoked by the stringent or growth rate control are not only restricted to initiation steps of transcription, however; and several studies have demonstrated ppGpp effects during transcription elongation (23–25). Normally, RNA polymerase does not elongate an RNA chain with constant speed but instead moves unevenly, pausing at specific nucleotide positions for distinct times. The reasons for the occurrence of pauses are not completely understood, and several different mechanistic explanations for pausing are discussed (26, 27). Some pauses are believed to play a major role in defining termination and attenuation sites apparently synchronizing transcription and translation (28–30). Often pauses are associated with transcript structure formation or protein binding. Some pauses have been shown to be influenced by ppGpp or other cellular factors (e.g. NusA, template topology). Moreover, pausing has been proposed as a mechanism to regulate heavily transcribed operons (23, 24, 31, 32), and in recent reports growth rate-dependent changes in the RNA elongation rate, probably due to enhanced pausing, was demonstrated (25, 33, 34).

In the subsequent study we have carried out in vitro transcription experiments to answer the following questions. (i)
Does ppGpp alter the RNA polymerase elongation rate via pausing in a general or specific way? (ii) How do different promoters or different transcribed sequences contribute to putative ppGpp effects on RNA polymerase pausing? (iii) At which stage of the transcription cycle may ppGpp exert its effect?

To follow effects exclusively during elongation, we have uncoupled the initiation step by using arrested ternary elongation complexes formed with different templates. Elongation was then resumed in the presence or absence of ppGpp, and pausing of RNA polymerase was accurately quantified by a recently developed analysis procedure (35).

**EXPERIMENTAL PROCEDURES**

**Materials—**RNA polymerase was isolated according to published procedures (36, 37) from *Escherichia coli* MRE600 cells as well as from strain KT87 known to be partially resistant to ppGpp due to an rpoB mutation and the parent strain KT86 (7). Enzyme preparations were functionally characterized by a quantitative assay as described (38). Nucleoside triphosphates and 3'-deoxynucleoside triphosphates were purchased from Pharmacia Biotech, Inc. Guanosine tetraphosphate (ppGpp) was prepared and purified as described recently (39). [α-32P]CTP and [α-32P]UTP were purchased from Hartmann Analytic.

**Buffers—**Transcription buffer, 44 mM Tris-HCl, pH 8.0, 14 mM MgCl₂, 14 mM β-mercaptoethanol, 100 mM KCl, 2% (v/v) glycerol, 40 mM acetylated bovine serum albumin. Buffer K, 20 mM Tris-HCl, pH 8.0, 5% (v/v) glycerol, 100 mM KCl, 1 mM MgCl₂, 100 mM EDTA, 100 μM dithiothreitol. Electrophoresis buffer (1 x TBE), 89 mM Tris borate, pH 8.3, 2.5 mM EDTA.

**DNA Templates—**The plasmid pMK-1 was derived from pKKS-1 (41) by a 2729-bp *PvuI/SalI* deletion. It contains the gene coding for ampicillin resistance (βla), the RNA I gene, and a truncated *rrnB* operon with the complete *rrnB* leader sequence including the ribosomal promoters *rrnB* P1 and P2 up to the 5' end of the 16 S RNA gene. pMK-1 was derived from pMK-1 by a 453-bp *StuI/BsiBI* deletion, which removes the ribosomal promoters P1 and P2. pMK-7 is a pMK-1 derivative with the 51-bp multicloning site from pUC18 (42) substituted for the 3360-bp *EcoRl/HindIII* fragment. In the *SmaI/SalI* opened multicloning site a 350-bp *SalI/SmaI* fragment from pTucW (21) containing the promoter DNA ligated. pMK-8 was constructed from pMK-1 by replacing the 389-bp *BstBI/HindIII* *rrnB* P1 promoter fragment by a 411-bp *SmaI/HindIII* T7 promoter fragment from *pLT7 P1* (43). The plasmid p2P and p2P2 have been described recently (21). They contain promoter fusions with the *rrnB* P2 promoter attached to the CAT gene at position +14, with respect to the P2 start. The promoter P2P2 differs from P2 by a single point mutation at position +5. In contrast to P2, promoter P2P2 confers growth rate regulation and response to the stringent control in vivo. In pMK-10 the βla gene was brought under the control of the *rrnB* P1 promoter. It was constructed by replacing the 1238-bp *SspI/BstBI* fragment from pMK-5 with a 152-bp *BstBI/MspI* fragment from pMK-1, thereby fusing the P1 transcription sequence at position +37 to the *bla* transcription sequence at position −11.

**Preparation of Teritary Complexes—**Tertiary complex reactions wereobtained and purified as described (26). The preparation involves the formation of stable elongation complexes stalled at a defined template site. Specific initiation of transcription was achieved using a di- or trinucleotide as primer and a limited subset of NTP substrates. The ternary complexes, consisting of RNA polymerase, the stalled transcript, and the superhelical template were separated from nucleotides and excess polymerase by Sepharose 6B column chromatography with buffer K. Generally, 20–30 nm RNA polymerase was incubated at 30 °C with 20–30 nm superhelical plasmid DNA and the appropriate nucleotide mixtures for 3–5 min. The nucleotide compositions for formation of ternary complexes at the different promoters were as follows: (a) *U1* RNA I complex, 3 μM pMK DNA; 5 μM each ATP, GTP, UTP, and [α-32P]CTP (50 μCi/μM), 50 μM ApC as initiating dinucleotide; incubation for 5 min; (b) *U1*-β-lactamase complexes, 30 nm pMK DNA; 3 μM each ATP, GTP, UTP, and [α-32P]UTP (50 μCi/μM); incubation for 3 min; (c) *G4428-rrnB* P1 complexes, 20 nm pMK-1 DNA; 3 μM each ATP, CTP, GTP, and [α-32P]CTP (50 μCi/μM); 25 μM ApCpU as initiating dinucleotide; incubation for 5 min; (d) *G4428-rrnB* P2 complexes, 30 nm pRK-8 DNA; 1.25 μM each CTP, GTP, and [α-32P]CTP (50 μCi/μM); 60 μM CppC as initiating dinucleotide; incubation for 3 min; (f) *C10/11-P2* and *C10/11-P2F* complexes, same conditions as e. The total reaction volume for ternary complex formation was 150 μl in each case. Precise adherence to the above conditions was important for the correct formation of ternary complexes, and changing the nucleotide concentrations or the incubation times leads to heterogeneous read through complexes or shorter abortive products.

**In Vitro Elongation of Tertiary Complexes—**Tertiary complexes were elongated in the presence of 140 μM each ATP, CTP, GTP, and UTP in transcription buffer with or without ppGpp (final concentration, 350 μM) for various reaction times. To resolve the short time intervals, we have connected a repeat pipette with the event marker input of an LKB recorder. 8 or 12 slots of a standard microtiter dish were filled with 8 μl of four NTP mix (1 mM each) and each reaction was started by adding 50 μl of the respective ternary complexes with the repeat pipette. Thus, the start of every single reaction was marked by the recorder. Following incubation the reactions were stopped with an 8- or 12-channel pipette by adding simultaneously 100 μl of ice-cold stop solution (1.5 mM NaHAc, 97.5 mM EDTA, 50 μg/ml yeast RNA) to all elongation reactions. This event was also recorded to indicate the extensions of single reactions. Samples were extracted with phenol/chloroform, ethanol-precipitated, lyophilized, and redisolved in 5 μl of loading buffer containing 7 M urea. Separation of the transcripts was carried out on denaturing polyacrylamide gels (15% polyacrylamide, 7 M urea, 1 x TBE buffer at a constant 75 watts). Gels were exposed for autoradiography films at -70 °C for 7 days.

**RNA Sequencing—**Purification of transcripts from the gel corrected for all termination events preceding site i (including the fraction of starting ternary complexes that is not chased into longer products). *h* = correction factor; this factor corrects for differences in the amounts of reaction products applied to individual gel lanes corresponding to different elongation from the stalled ternary complexes that had been initiated from only one promoter. The amounts of transcription products according to autoradiographic bands on x-ray films are calculated from densitometric integration according to Equation 1:

$$I_{env} = I_n - \frac{I_m}{\sum I_m} \cdot h$$

(Eq. 1)

1 The abbreviations used are: bp, base pair(s); CAT, chloramphenicol acetyltransferase.
Transcriptional Pausing of RNA Polymerase

RESULTS

Effect of ppGpp on RNA Polymerases Elongating Different Transcription Units—For these investigations we have chosen the following transcription units that are known to respond differentially to changes in the ppGpp concentration. (i) RNA I gene that negatively regulates ColE1 plasmid replication (45). Transcription of this gene is considered to be growth rate-dependent but not under stringent control (46, 47, 54–56). (ii) The \( \beta \)-lactamase gene that is expressed constitutively and not under stringent control (48). (iii) Transcription units that are known to respond differentially to changes in the ppGpp concentration. (iv) The \( \beta \)-lactamase gene. Specifically stalled transcription elongation complexes were preformed with the above constructs and used for subsequent elongation kinetics in the presence or absence of ppGpp. Fig. 1 shows a schematic summary of the different templates.

The RNA I Transcription System—Ternary complexes stalled at position \( \mathbf{1} \) were prepared for the \textit{in vitro} transcription elongation of the \( \mathbf{P} \), \( \mathbf{C} \), and \( \mathbf{G} \) \( \mathbf{P} \)-directed gene employing plasmid \( \mathbf{pM} \). Supercoiled plasmid DNA instead of DNA fragments was used throughout this study to better match the template topology within the cell and to minimize artifacts. In addition, we used high NTP concentrations to approach the \textit{in vivo} RNA polymerase elongation rate. Fig. 2 shows the results of \textit{in vitro} transcription reactions in the presence (left panel) and absence of ppGpp (right panel). Characteristic RNA polymerase pausing positions are marked within the 108 nucleotide RNA I transcript. Pauses can be recognized by the characteristic time-dependent appearance and decay of gel bands. In the presence of ppGpp no new pauses were introduced, but at some positions the time course of band appearance is changed. Because band intensities are influenced by upstream pausing events and the time intervals of the kinetics presented in Fig. 2 are not identical for the plus and minus ppGpp experiment, it is difficult to recognize pausing differences by mere visual inspection. Therefore, exact pausing strength values \( \tau \) have been determined for the nucleotide positions \( \mathbf{G} \), \( \mathbf{U} \), and \( \mathbf{C} \) as described under “Experimental Procedures.” In Fig. 3 the relative occupancies at these pausing positions are plotted over time. Integration of the corresponding peak areas yields the pausing strength values \( \tau \) for these pause sites. The ratio of these numbers obtained in the presence and absence of ppGpp are given in Table I as pausing factors. It can be seen that some pauses, like \( \mathbf{G} \) and \( \mathbf{C} \) are unaltered by ppGpp while others, like \( \mathbf{U} \) and \( \mathbf{A} \), are notably enhanced. The effects are relatively small but clearly significant and reproducible.

To rule out possible termination effects on the pause strength values \( \tau \) we have routinely made a “long time” reaction. Examples are presented in Fig. 2, in which the transcrip-
tion reactions were performed for 5 min. During that time all active ternary complexes have reached the transcription termination site, and bands between the ternary complex (U14) and the termination site must be considered as dead-end complexes or premature terminations. Apart from a small amount of U14 complexes that resisted elongation, only a weak band between positions U23 and G24 can be observed in all lanes indicative of a dead-end complex or a premature termination event. However, this band is already visible in the original ternary complex before elongation was resumed. Therefore, the observed pauses were not influenced by premature termination events.

To be sure that effects on pausing are due to the specific influence of the effector molecule ppGpp and not caused by an increase in the concentration of general guanosine nucleotides, we have performed control elongation reactions where ppGpp was replaced by 5'-guanosine diphosphate. No change in pausing factors for any nucleotide position could be detected in these control experiments ruling out an unspecific guanosine nucleotide effect, for instance at the GTP substrate site (data not shown).

Since it is known that in vitro transcription reactions are strongly influenced by changes in the ionic conditions, we have determined the contaminating salt concentration of the ppGpp preparation (39). Mock experiments with adequate LiCl concentrations were performed but did not affect pausing strength in a measurable way (data not shown). Hence, we can rule out the influence of contaminating salt on the results obtained here. The same controls have been performed with all transcription systems.

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**TABLE I**

| Nucleotide position | Pausing factors |
|---------------------|-----------------|
| G24                 | 1.14 (± 0.06)   |
| U53                 | 1.73 (± 0.11)   |
| A66                 | 1.46 (± 0.03)   |
| C69                 | 1.11 (± 0.14)   |

**FIG. 2.** Analysis of RNA I promoter-directed transcriptional pauses. The gel electrophoretic separation of transcription products obtained in the presence and absence of ppGpp is shown. Elongation reactions were started from U14 ternary complexes exclusively initiated at the RNA I promoter. Lanes a–d, defined aliquots of the ternary U14 complex were separated for calibration. U, G, C, and A are the respective chain termination sequencing lanes. Numbers above the lanes indicate transcription elongation times in seconds. The lanes on the left side of the sequencing tracts were performed in the presence of ppGpp. On the right margin representative pausing site positions and the position of the starting U14 complex are indicated.

**FIG. 3.** Relative occupancies of pause positions G34, U53, A66, and C69 of the RNA I transcription as functions of time. \(I_{pa}\) values given on the ordinate are defined under "Experimental Procedures" and were obtained from the band intensities of respective pausing sites. The different values for the pausing strength \(\tau\) are represented by the areas under the curves, e.g. G24: 17.5 vs 15.8 s; U53: 34.2 vs 20.7 s; A66: 33.1 vs 22.3 s; C69: 33.5 vs 33.2 s in the presence or absence of ppGpp, respectively. The resulting pausing factors are presented in Table I. Open and closed symbols represent reactions in the absence or presence of ppGpp, respectively.
Transcription of the β-Lactamase Gene—Transcription of the β-lactamase gene (bla) can be regarded as unaffected during changes in growth rate or under stringent control (48). We were interested, therefore, how ppGpp would influence pausing in this apparently unregulated transcription system. Employing ternary US complexes obtained with the plasmid pMKΔ, we analyzed the Pbla-directed transcription unit as described for the RNA I system (Fig. 4). Elongation times ranging from 3 to 33 s. The pausing factors for the early transcribed region of the Pbla-directed transcription unit are summarized in Table II. It can be seen that ppGpp has a differential influence on individual pauses. Pauses at positions U38 and C41 are not affected or even show a slightly reduced pausing factor in case of C41. In contrast, pauses G26 and C30 are slightly enhanced, and A37 and U44 are clearly enhanced. Evaluation of the pausing strength at position G51 is subject to a large error because it turns into a termination site.

The pausing factors noticed here seem to indicate a slight general inhibition of RNA polymerase activity by reducing the transcription rate due to overall enhanced pausing. The observation that β-lactamase transcripts in the cell are apparently independent from changes in the ppGpp level may be explained by the relatively small number of pauses and the fact that at low initiation frequency small pauses will not contribute to notable repression.

Ribosomal RNA P1 Promoter-directed Transcription—The archetype genes known to be under growth rate and stringent control are directed by ribosomal RNA P1 promoters. Expression from rrrB P1, for instance, is inversely correlated to the cellular ppGpp concentration (50, 53, 57) and has been shown to be under stringent and growth rate control in vivo and in vitro (2, 3, 21). P1 promoter upstream sequences, which are important for factor-dependent and independent regulation, are not required for both types of control (51). However, the core promoter including the sequences immediately flanking the transcription start are known to be crucial for ppGpp

![Image](http://www.jbc.org/)

**Fig. 4. Analysis of transcriptional pauses employing the bla transcription system.** Elongation reactions were started from US ternary complexes exclusively initiated at the bla promoter. Lanes a–c, defined aliquots of the ternary complexes were separated for calibration. A, C, G, and U are the respective chain termination sequencing lanes. In the samples on the right side of the sequencing tract elongation reactions were performed in the presence and on the left side in the absence of ppGpp, respectively. Numbers from 1 to 8 refer to different elongation times (seconds). Left side: 1, 3.1; 2, 6.0; 3, 9.1; 4, 12.0; 5, 15.0; 6, 17.7; 7, 20.6; and 8, 31.8. Right side: 1, 3.4; 2, 6.3; 3, 9.5; 4, 12.4; 5, 15.2; 6, 17.8; 7, 20.8; 8, 33.0. On the right margin representative pausing site positions and the position of the starting US complex are indicated.

**Table II**

| Nucleotide positions | Pausing factors |
|---------------------|-----------------|
| G26                 | 1.24            |
| C30                 | 1.27            |
| A37                 | 1.58            |
| U38                 | 0.9             |
| C41                 | 0.66            |
| U44                 | 1.56            |
| G51                 | 1.82*           |

*This number is prone to a large error because of a simultaneous termination at this position.

Our goal was to determine whether this well documented dependence would also be reflected in the strength of specific transcriptional pauses, and whether there is a qualitative and/or quantitative difference compared with the pausing behavior observed for the RNA I and β-lactamase genes described above. Fig. 5 shows an example of the in vitro transcription elongation analysis employing specific rrnB P1-G3443 ternary complexes obtained with plasmid pMK-1. The pausing pattern derived from the rrnB P1 initiated transcript shows an exceptional large number of pauses in the region between U44 and C58. The quantitative evaluation of ppGpp-dependent pauses factors for the prominent pauses within this region (listed in Table III) reveals that almost all of them are considerably enhanced. The pausing factors obtained are notably higher than those determined for the unstable RNA transcription units (RNA I and bla genes), where the highest factors observed (1.7, Tables I and II) are only in the range of the lowest factors measured for P1-directed stable RNA transcription. Note that pausing effects are additive and amplified by high initiation frequency, known for rRNA genes. A single pause in the range of a few seconds may, therefore, repress the overall transcription of that gene dramatically (25). Hence, pausing strength parameters determined under single round in vitro
conditions have to be correlated to the frequency by which RNA polymerase initiates at the respective promoter in order to understand the \textit{in vivo} implications (see “Discussion”).

Ribosomal RNA P2 Promoter-directed Transcription—Complicated by the fact that transcription from the close-by upstream promoter P1 impedes unambiguous analysis \textit{in vivo}, the effects of growth rate and stringent control on the activity of \textit{rrn} P2 promoters are discussed controversially (21, 31, 49, 50). It was more or less accepted that P2 promoters respond only weakly, if at all, to stringent control or changes in growth rate. However, recent reports have provided clear \textit{in vivo} evidence that the isolated P2 promoters also respond to changes in the ppGpp level (52, 53).

We asked, therefore, whether rRNA transcription directed by the \textit{rrnB} P2 promoter would give rise to similar small ppGpp-dependent pausing factors as observed for unstable RNA transcription (\textit{bla} or RNA I genes) or comparable with those of \textit{rrnB} P1-directed transcripts.

Specific ternary \textit{rrnB} P2C1021 complexes were prepared with plasmid pMK-1. Although the stalled transcript is rather short, these complexes were sufficiently stable and readily resumed elongation after adding NTP mixtures without noticeable lag. They must be considered as true ternary elongation complexes. When a T to G mutation was introduced at position +12 of the \textit{rrnB} P2 sequence, which leads to a ternary elongation complex with a 32-nucleotide stalled transcript, the same transcription behavior as with the wild-type C1021 complex was observed (data not shown). Fig. 6 shows the \textit{in vitro} elongation kinetics of the C1021 complex with elongation times from 3 to 33 s. The corresponding pausing factors for the P2-directed rRNA transcription are listed in Table IV. They range from 1.3 to 2, indicating that pausing factors for the \textit{rrnB} P2-controlled transcription can be classified as “intermediate” between those

\begin{table}[h]
\centering
\begin{tabular}{|c|c|}
\hline
Nucleotide position & Pausing factors \\
\hline
U44 & 2.45 (±0.12) \\
U50 & 2.37 (±0.06) \\
G53 & 0.93 (±0.04) \\
C62 & 1.76 (±0.4) \\
G92 & 1.64 (±0.4) \\
C96 & 1.76 (±0.9) \\
\hline
\end{tabular}
\caption{ppGpp-dependent pausing factors of \textit{rrnB} P1 transcription}
\end{table}
of RNA I or P$_{\text{Blu}}$ and the rrnB P1-directed transcription, respectively. Remarkably, P$_2$-directed rRNA transcription reveals only a few distinct pauses in the early transcribed region compared with the P1-directed reaction. Since the sequence directed by P$_2$ is also present in P1-initiated transcripts, the same pausing sites can be detected in the late pausing pattern of P1-directed transcription (see Fig. 5).

The strong bands denoted as $t_L$ (Fig. 6) are identical to the previously described ppGpp- and NusA-dependent pause/termination site (24). At this position RNA polymerase stalls for several minutes with frequent termination. The conditions used in our in vitro system are optimized for the analysis of short pauses. Therefore, long pauses, as in case of $t_L$, are not within the scope of our measurements. Obviously, the $t_L$ structure is of special interest with respect to the folding kinetics of the ribosomal leader RNA. The region is known to be involved in the structure formation and assembly of the small ribosomal subunit (43, 58). Since transcription of this part of the sequence also depends on antitermination factors, we feel that pausing at $t_L$ may have a very special function, most likely related to the phenomenon investigated here.

The results presented so far have shown heterogeneous effects of ppGpp on RNA polymerase pausing. Most of the investigated pauses are enhanced in the presence of ppGpp, enhancements ranging from 10% to more than two-fold. There are also some pauses that seem to be unchanged, and in several cases we have determined pausing factors indicating a reduced pausing strength (P$_{\text{Blu}}$, C$^{37}$, rrnB P2, U$^{77}$, P1-Blu, A$^{45}$). Since it is known that transcriptional pausing can be caused by several different mechanisms, heterogeneous effects mediated by ppGpp within one round of transcription are not surprising. Overall, the different effects on pausing strength correlate nicely with what is known about the growth rate or stringent dependence of the individual transcription systems analyzed here. Therefore, they provide first evidence for a variable response of transcriptional pausing triggered by ppGpp for different genes. However, the question still remains whether the effect of elevated ppGpp concentrations on pausing is due to a general inhibition of the RNA polymerase transcription rate or whether there are major promoter-specific elements.

**Transcriptional Pausing Is Dependent on the Promoter Region**—In line with the general assumption that transcription regulation occurs mainly during initiation, the early findings of ppGpp-dependent regulation have all been considered to be caused by distinct promoter structures. This was confirmed by studies demonstrating that a GC-rich discriminator sequence close to the −10 region, in addition to unknown structural features of the complete promoter context, were shown to be necessary for ppGpp-controlled gene expression (15, 20, 21). Promoters sensitive to ppGpp were believed to be of low RNA polymerase affinity or slow in the transition toward productive transcription. Regulation during elongation requires different mechanisms. If ppGpp exerts its effects during transcription elongation, a mechanism modifying RNA polymerase activity over time and distance must exist. Furthermore, the question arises at what stage modification of the polymerases might occur. Again, the promoter structures appear to be suitable distinction elements at which either selection or programming of the RNA polymerases for the respective transcription unit might take place.

To address these questions we have performed pausing analyses of identical transcription units under the control of two promoters known to respond differentially under conditions of enhanced ppGpp levels in vivo. For these studies the ribosomal rrnB P2 and the corresponding variant P2F were chosen (21, 22). P2F differs from P2 only in a single A to G transition that introduces a perfect discriminator motif. Both promoters have been fused at position +15 to the chloramphenicol acetyltransferase gene (CAT) and control identical sequences (see Fig. 1).

For the in vitro analysis we have prepared C$^{10}$T$_{11}$ ternary complexes with both promoters. The natural transcription start point has not been changed by fusion to the CAT gene. The two promoters initiate at either one of two cytosines, 6 or 7 nucleotides downstream of the −10 region. This is reflected by double bands in Fig. 7 where the pausing analysis for P2F is exemplified. Regardless whether transcription was started from P2 or from P2F the same pausing positions within the CAT gene were obtained. However, some of the pause strength values determined in the presence and absence of ppGpp are significantly different for both systems. This can be seen in Table V, where the quantitative assessment of pausing factors is presented. As observed for the pausing analysis of RNA I, P$_{\text{Blu}}$, rrnB P1, or rrnB P2 controlled transcription units, some pauses are not altered by ppGpp (see Table V, G$^{26}$, A$^{33}$, C$^{57}$, and A$^{62}$). Most notably, however, pauses C$^{25}$, U$^{30}$, C$^{32}$, and U$^{46}$ are enhanced between 30 to 65% for P2F-controlled transcription. The experiment reveals the striking fact that the promoter variant P2F that confers growth rate and stringent control in vivo also enhances ppGpp-dependent transcriptional pausing in vitro over that observed for P2, leading to the same large pausing factors as observed for rrnB P1.

In a second system the rRNA P1 promoter and its early transcription sequence were fused to the bla gene. Transcripts from these constructs are initiated at the strongly regulated P1 promoter and its discriminator sequence, but ternary complexes are elongated into the bla sequence where only minor ppGpp-dependent pauses have been shown. At least for the identical sequences this system should allow a comparison of the promoter effects on pausing. Hence, ternary complexes performed with plasmid pMK-10 were subjected to a pausing analysis. Results are presented in Fig. 8 and Table VI. Specifically, pauses G$^{26}$, C$^{37}$, and A$^{46}$ can be identified as the analogous positions G$^{26}$, C$^{50}$, and A$^{37}$ of the P$_{\text{Blu}}$-directed transcript. While the pause at position G$^{26}$ is not or only marginally increased, relative to the pause G$^{26}$ in the P$_{\text{Blu}}$ transcription system, pause A$^{37}$ (according to the bla promoter) is measurable, and pause C$^{37}$ (pause C$^{30}$, according to the bla promoter) is significantly enhanced. These findings are consistent with the results obtained with the P2/P2F promoter system and corroborate the conclusion that the promoter structure can indeed influence the strength of some distal pauses.

Interestingly, as observed in several cases the pause at position A$^{46}$ (not present in the genuine bla transcript) is clearly reduced in the presence of ppGpp, underlining the observation that enhancement of pausing by ppGpp is a specific effect and not due to a generally reduced transcription rate.

The question whether ppGpp binding is necessary for RNA polymerase prior to promoter binding in order to be discriminated (shown enhanced pausing) can clearly be answered with no. In the experiments described here no ppGpp has been added to the system during initiation complex formation. If, on the other hand, ppGpp was present only during ternary com-

| Nucleotide position | Pausing factors |
|---------------------|-----------------|
| U$^{37}$             | 2.21 (±0.17)    |
| U$^{38}$             | 2.02 (±0.14)    |
| U$^{77}$             | 1.50 (±0.2)     |
| G$^{22}$             | 1.35 (±0.02)    |
| U$^{10}$             | 1.37 (±0.08)    |
| U$^{70}$             | 0.43 (±0.07)    |
plex formation no measurable effect on pausing could be detected (data not shown). Consequently, in order to discriminate or modify RNA polymerase pausing behavior no ppGpp but a defined promoter structure is required during initiation. However, the strength of the respective pauses are influenced by the presence of ppGpp during the elongation cycle.

It can be concluded from these findings that RNA polymerase can be preadjusted or selected by the promoter flanking discriminator region in the absence of ppGpp. It also means that the discriminator sequence has a vital function in programming transcriptional pausing. The pausing sites, however, seem to be defined by the particular sequence of the gene distal to the promoter and may depend on the DNA sequence (conformation) or the structure of the nascent transcript. The length of some of these pauses are clearly influenced by ppGpp provided that RNA polymerase has passed a certain sequence context at or close to the promoter site.

Whether ppGpp-dependent pausing is mediated by stable binding of ppGpp to the RNA polymerase during elongation or by a transient conformational change of the RNA polymerase while binding to the promoter that is stabilized in the presence of ppGpp remains to be investigated. So far no satisfying results have been obtained that clearly prove a direct binding of ppGpp to the RNA polymerase under transcription conditions.

In previous in vivo studies a mutant E. coli strain was characterized that had acquired partial resistance to enhanced levels of ppGpp. The respective strain was shown to harbor mutations in the rpoB gene coding for the β-subunit of RNA polymerase (7). In general one should expect that RNA polymerase preparations from such strains might yield different effects on transcription in the presence of ppGpp under in vitro conditions. Therefore, we have purified RNA polymerase from strains KT87 (partially ppGpp-resistant) and the isogenic wild-type KT86. Using these two polymerase preparations we have performed in vitro control experiments under multiple round conditions as described (59) with 15 nM template, 5–30 nM RNA polymerase, 140 μM each of the four NTPs, and reaction times between 5 and 10 min. Transcription products directed from rrnB P1 and P2 promoters, the RNA I promoter, or transcripts controlled by the tac promoter present on plasmid pMK-7 were quantified. We were unable to detect a selective difference in transcription products in the presence of various concentra-

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**TABLE V**

Comparison of the pausing factors of P2 and P2F transcription

| Nucleotide position | Pausing factors, P2 | Pausing factors, P2F |
|---------------------|---------------------|----------------------|
| C25                 | 1.68 (±0.14)        | 2.31 (±0.16)*        |
| G26                 | 0.99 (±0.01)        | 1.09 (±0.01)         |
| U30                 | 1.55 (±0.1)         | 2.12 (±0.07)         |
| C32                 | 1.62 (±0.26)        | 2.67 (±0.18)         |
| A33                 | 1.14 (±0.12)        | 0.91                 |
| U36                 | 1.36 (±0.08)        | 1.91 (±0.18)         |
| C57                 | 0.90 (±0.02)        | 1.03 (±0.03)         |
| A62                 | 1.13 (±0.08)        | 1.11 (±0.06)         |

* Boldface numbers indicate significant pausing enhancement.
different degrees for different genes in the presence of ppGpp. Our analysis of the constitutive bla gene and the RNA I gene revealed a small but significant enhancement of several pauses within the early transcribed region. We like to interpret these enhanced pausing factors as the result of a general ppGpp effect on the RNA polymerase pausing behavior. It is likely that this enhanced pausing is related to the recently published observation (34) that the RNA elongation rate for lacZ transcription decreases at increased ppGpp levels. As suggested by the authors enhanced pausing caused by ppGpp generally reduces the RNA chain elongation rate.

Pauses that appear to be unchanged or even reduced under our assay conditions may result from alternative pausing mechanisms with different or no response to ppGpp-modified RNA polymerases.

It is unclear how ppGpp can mediate its effects differentially for various promoters and during transcription elongation of different genes. A feasible explanation may be provided by the observation that RNA polymerase, which is almost certainly the target for ppGpp, can form transcription complexes of distinct conformations during initiation or elongation depending on the individual sequence context (30, 60–62).

The analysis of stable RNA genes reveals that transcription from rRNA promoter P1 known for its clear ppGpp dependence in vivo shows the strongest pausing enhancements, significantly above the effects noted for pausing of unstable RNA genes. P2 promoters known for their reduced but measurable response toward ppGpp yielded pausing factors in between the basal enhancements observed for the bla or the RNA I genes and the strong rRNA P1 effect. Although an extensive comparative analysis has not been performed, the characteristic examples studied here suggest that the pausing factors determined in vitro reflect the in vivo variation in the expression of the respective genes at altered ppGpp levels.

In the course of our study we did not see a reduction in the general chain elongation rate due to an enhancement of the average step time within the transcription cycle. Clearly, the dominant effect in the reduction of the elongation rate is caused by pausing at defined positions. In no case were new pauses provoked by the presence of ppGpp. We conclude, therefore, that ppGpp does not create pauses but alters some pauses that are inherent to the transcription of a particular gene.

A clear answer as to whether the promoter structure influences ppGpp-dependent pauses was provided by the comparative analysis of the two promoter variants P2 and P2F. These two promoters differ only at one nucleotide position between the transcription start site and the −10 region (discriminator sequence). Furthermore, the sequences they control are identical. However, in line with their different in vitro activity the two promoters respond distinctly differently in their ppGpp-dependent in vitro pausing pattern. P2F shows almost the same strong pausing enhancements as rRNA promoter P1. It is evident, therefore, that the discriminator sequence has an immediate effect on the pausing properties of RNA polymerases that have passed this sequence. Hence, strong or weak ppGpp dependence of pausing must at least in part be encoded at or close to the promoter core sequence. An attractive assumption would be that ppGpp either by direct binding and/or subsequently changing the conformation of RNA polymerase represents a kind of "chemical memory" for altered transcription efficiency.

The analysis with the P1-Bla fusion system is largely consistent with this observation, although the situation is generally more complicated by fusion of two different genes, and a small part of the sequence is not present in the analyzed P_Bla transcript (A309 to G45). Nevertheless, transcription initiation at

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**Table VI**

Comparison of the pausing factors for transcripts initiated at P1-Bla and P_Bla.

| Nucleotide position (P1 start) | Pausing factors (P1-Bla, start) | Nucleotide position (P_Bla, start) | Pausing factors (P_Bla, start) |
|-------------------------------|---------------------------------|---------------------------------|--------------------------------|
| A35                           | 0.63 (±0.07)                    | A-5                             | ND*                             |
| U70                           | 1.50 (±0.13)                    | U21                             | ND                              |
| G75                           | 1.36 (±0.26)                    | G26                             | 1.24                            |
| C79                           | 2.09 (±0.1)                     | C30                             | 1.27                            |
| A66                           | 1.73 (±0.04)                    | A37                             | 1.58                            |
| U111                          | >1.75                           | U62                             | ND                              |

*ND, not determined.

*Within the time scale analyzed this number could not be determined accurately.

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2 Heinemann and Wagner, unpublished results.

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**Fig. 8. Analysis of transcriptional pauses employing the P1-Bla transcription system.** Elongation reactions were started from G24/A29 ternary complexes initiated at the rnb B P1 promoter. Products on the left side were obtained in the absence and on the right side in the presence of ppGpp, respectively. Numbers 1–11 refer to elongation times (seconds). Left lanes: 1, 2.5; 2, 3.8; 3, 5.9; 4, 7.6; 5, 9.2; 6, 11.6; 7, 15.7; 8, 19.5; 9, 23.7; 10, 27.6; and 11, 31.4. Right lanes: 1, 2.1; 2, 3.9; 3, 5.4; 4, 7.7; 5, 9.3; 6, 11.3; 7, 15.6; 8, 19.3; 9, 23.1; 10, 27.4; and 11, 30.1.

Representative pause positions and the starting A39 complex are indicated at the right margin. Pause positions G26, C79, and A37 of the P_Bla-directed transcript, respectively (see Fig. 4). A, C, G, and U denote the respective sequencing lanes.

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**DISCUSSION**

Within the complex and densely linked scenario of ppGpp-mediated effects on gene expression, we have addressed a few specific questions. Our analysis was deliberately restricted to the elongation cycle of transcription in order to avoid complications due to different promoter affinities. A crucial question to answer was if transcriptional pausing, which seems to be an intrinsic property of almost any gene, would be influenced to
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In vitro transcription elongation rates under different growth rates and under isoleucine starvation have been determined. Generally, our results agree with those findings. There are, however, some notable differences. In the above in vivo studies transcription elongation of infB and lacZ mRNAs are reduced at slow growth rates and under conditions of isoleucine starvation. In contrast, a hybrid construction with stable RNA sequences was only altered at different growth rates. The authors could show that the in vivo rates for rRNA transcription are faster than for mRNA and that a functional antiterminator boxA sequence influences the elongation rates. In their studies deletion of the boxA sequence reduces transcription rates insensitive to the ppGpp pool. We do not know the exact answer for this apparent discrepancy. It should be noted, however, that all the in vivo measurements were performed with constructs containing the same inducible hybrid promoter. Promoter-specific effects, such as the one observed here must, therefore, remain undetected.

The high ppGpp concentrations (350 μM) employed in this in vitro study correspond to stringent rather than growth rate conditions in the cell. This does not imply that the pausing effects observed here are preferably related to stringent control. It can rather be assumed that the consequences of pausing may range from a moderate reduction to a severe shut-down of transcription and therefore affect both growth rate regulation and stringent control.

In summary, the in vitro observed increased pausing in the presence of ppGpp for transcripts directed by stringent or growth rate-regulated promoters shows good correlation to what is known about the in vivo expression of the corresponding genes. However, it should be noted that in vitro experiments cannot always completely describe the actual situation in vivo. Therefore, the correctness of the assumptions drawn here has to be verified by future in vivo studies.

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