Sanger, F., Coulson, A. R., Barrell, B. G., & Smith, A. J. H. (1980) *J. Mol. Biol. 143*, 161–178.
Singh, M., Brooks, G. C., & Srere, P. A. (1970) *J. Biol. Chem. 245*, 4636–4640.
Smith, A. J. H. (1980) *Methods Enzymol. 65*, 560–580.
Srere, P. A. (1975) *Adv. Enzymol. Relat. Areas Mol. Biol. 43*, 57–101.
Staden, R. (1982) *Nucleic Acids Res. 10*, 2951–2961.
Tong, E. K., & Duckworth, H. W. (1975) *Biochemistry 14*, 235–241.
Weitzman, P. D. J. (1966) *Biochim. Biophys. Acta 128*, 213–215.
Weitzman, P. D. J., & Danson, M. J. (1976) *Curr. Top. Cell Regul. 10*, 161–204.

Articles

Effects of Deletions near *Escherichia coli rrn*B Promoter P_2 on Inhibition of in Vitro Transcription by Guanosine Tetraphosphate†

Robert E. Kingston*

ABSTRACT: The regulatory nucleotide guanosine tetraphosphate (ppGpp) inhibits in vitro transcription from the *rrn*B P_2 promoter. In order to locate the sequences responsible for this inhibition, plasmids have been constructed that contain deletions that extend to various points near the *rrn*B P_2 initiation site. Guanosine tetraphosphate has little or no effect on transcription of certain of the altered promoters. The loss of ability of a P_2 promoter to be inhibited by ppGpp correlates with changes around the transcription initiation site, suggesting that these sequences are involved in the interactions that determine sensitivity of a promoter to ppGpp in vitro. RNA polymerase that initiates at *rrn*B promoter P_1 has been shown to pause in the region of P_2 in vitro. The effects of the deletions on these pause sites have been determined. The results strongly support the hypothesis that RNA polymerase at P_2 can cause RNA polymerase that has initiated at promoter P_1 to pause for a substantial length of time.

When growing *Escherichia coli* are deprived of an amino acid or otherwise blocked in the aminoacylation of tRNA, they undergo what has been termed a stringent response [Stent & Brenner, 1961; reviewed by Gallant & Lazzarini (1976)]. The level of expression of numerous operons changes substantially [review by Nierlich (1978) and Gallant (1979)]; in particular, the rate of ribosomal RNA synthesis decreases 10–20-fold. Genetic and physiological studies, as well as in vitro transcription studies, have implicated the nucleotide guanosine tetraphosphate (ppGpp)[1] in these changes (Nierlich, 1978; Gallant, 1979; Travers, 1976a,b; van Ooyen et al., 1976; Travers & Baralle, 1976; Debenham & Travers, 1977; Oostra et al., 1977; Travers et al., 1978; Hamming et al., 1979, 1980). ppGpp specifically inhibits transcription from each of the tandem promoters (P_1, P_2) found in rRNA operons in vitro, though it has no effect on transcription from several phage promoters (Hamming et al., 1979, 1980; Kingston et al., 1981a,b). These results suggest that there are specific areas in promoters involved in the stringent response that render them sensitive to ppGpp. After comparison of the known sequences of promoters involved in the stringent response, Travers proposed that a specific sequence near the transcription initiation site determines sensitivity to ppGpp (Travers, 1980a). A $tRNA^{Tyr}$ promoter altered in this region has been reported to show a changed response to ppGpp (Travers, 1980b). Here the sequences in the *rrn*B P_2 promoter that determine its sensitivity to ppGpp in vitro are defined.

The region around the initiation site of promoter P_2 is of interest not only because of its possible involvement in determining sensitivity to ppGpp but also because this region apparently determines three sites where RNA polymerase pauses during elongation from promoter P_1 in vitro (Kingston & Chamberlin, 1981). These pause sites could potentially play a role in regulating rRNA expression by limiting the maximal rate of transcription of *rrn* operons ("turnstile attenuation"; Kingston & Chamberlin, 1981; Kingston et al., 1981b). Plasmids have been constructed containing deletions extending into the initiation site of promoter P_2 (J. Brosius, unpublished results). The effects of these deletions both on the ability of ppGpp to inhibit transcription from P_2 and on the pausing of RNA polymerase in this region are reported here.

Experimental Procedures

Materials. Plasmids were constructed by and were the gracious gift of Dr. Jürgen Brosius (Columbia University). DNA templates containing P_2 deletions were prepared for transcription by digesting crude plasmid DNA with the appropriate restriction enzyme (pKK34-121, *Pst*I; pKK35-120 and derivatives, *Bam*HI), separating the linearized plasmid DNA from chromosomal DNA and RNA on an agarose gel, and isolating the plasmid DNA by the method of Vogelstein

† From the Department of Biochemistry, University of California, Berkeley, California 94720. *Received January 13, 1983; revised manuscript received June 21, 1983.* This work was supported by Grant GM 12010 from the National Institute of General Medical Sciences (to Dr. Michael Chamberlin) and by a predoctoral National Institutes of Health training grant.

* Address correspondence to this author at the Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139.

[1] Abbreviations: ppGpp, guanosine tetraphosphate; EDTA, ethylenediaminetetraacetic acid.

0006-2960/83/0422-5249$01.50/0

& Gillespie (1979). γ-^{32}P-Labeled nucleoside triphosphates were prepared by the method of Johnson & Walseth (1979). All other reagents were prepared as described previously (Kingston & Chamberlin, 1981).

In Vitro Transcription. In vitro transcription reactions to determine the effect of ppGpp on utilization of the *rrn*B P_1 and P_2 promoters were carried out under the reaction conditions of Chamberlin et al. (1979) with the following modifications. T7 DNA (included to provide promoters to compete with the *rrn*B promoters) was present at 16 μg/mL, and plasmid DNA was present at 12 μg/mL (pKK3535) or 7 μg/mL (all others). DNA, salts, and RNA polymerase (12 μg/mL) were mixed on ice, and two equal aliquots were removed: one was added to nucleoside triphosphates (final concentrations 0.4 mM each of GTP, UTP, and ATP; 0.2 mM [α-^{32}P]CTP); the other was added to nucleoside triphosphates and ppGpp (final concentration 0.26 mM). Each reaction (total volume 25 μL) was incubated for 2 min on ice and then for 4 min at 30 °C, at which point heparin was added to 80 μg/mL to prevent further initiation. The reactions were then allowed to proceed for 10 min before the addition of 50 μL of stop solution (1.5 M ammonium acetate/37.5 mM EDTA/45 μg/mL tRNA). This 10-min incubation is well in excess of the time required to complete all P_1 and P_2 chains even in the presence of ppGpp and thus corrects for the effect of ppGpp on the elongation rate of RNA polymerase (Kingston et al., 1981b). Samples were ethanol precipitated and analyzed by polyacrylamide gel electrophoresis (Maniatis et al., 1975).

In vitro transcription to determine RNA polymerase pause sites was carried out exactly as described previously (Kingston & Chamberlin, 1981).

Determination of RNA Polymerase Initiation Sites. Transcription was carried out under the conditions of Chamberlin et al. (1979), except that plasmid DNA was present at 30 μg/mL (pKK3535) or 12 μg/mL (all others). Unlabeled triphosphates were present at 40 μM, and the appropriate γ-^{32}P-labeled triphosphate was present at 10 μM. Reactions were incubated for 30 min at 30 °C. Each template was tested with [γ-^{32}P]ATP, [γ-^{32}P]GTP, and [γ-^{32}P]CTP to determine which nucleotide efficiently labeled the P_2 transcript. The appropriate nucleotide was then used in a preparative reaction, the RNA products were isolated by polyacrylamide gel electrophoresis, and the partial RNA sequence was determined through use of RNase T_1, RNase U_2, and alkali as described (Donis-Keller et al., 1977). The sequence obtained was compared to the known DNA sequence to determine the start sites.

Results

There are seven ribosomal RNA operons in *E. coli* (Kennerly et al., 1977; Kiss et al., 1977). At least six of these have tandem promoters located approximately 115 bases part (deBoer et al., 1979; Young & Steitz, 1979; Csordas-Toth et al., 1979; Brosius et al., 1981a; Shen et al., 1982). The work reported here utilized plasmids containing segments of the *rrn*B operon. To facilitate discussion, sequences have been numbered with +1 being the initiation point for transcription from *rrn*B promoter P_1 [position 1226 of Brosius et al. (1981a)]. Under this nomenclature, *rrn*B promoter P_2 transcription starts at bases 118 and 119 (see below). Deletions have been constructed that extend from the 3′ side of promoter P_2 to varying positions near the initiation site (Figures 1 and 2). These constructions are named to indicate the number of wild-type bases that remain (designated after the hyphen). All constructions contain a wild-type promoter P_1. The DNA sequence around promoter P_2 has been determined for each construction (Figure 4; J. Brosius, unpublished results).

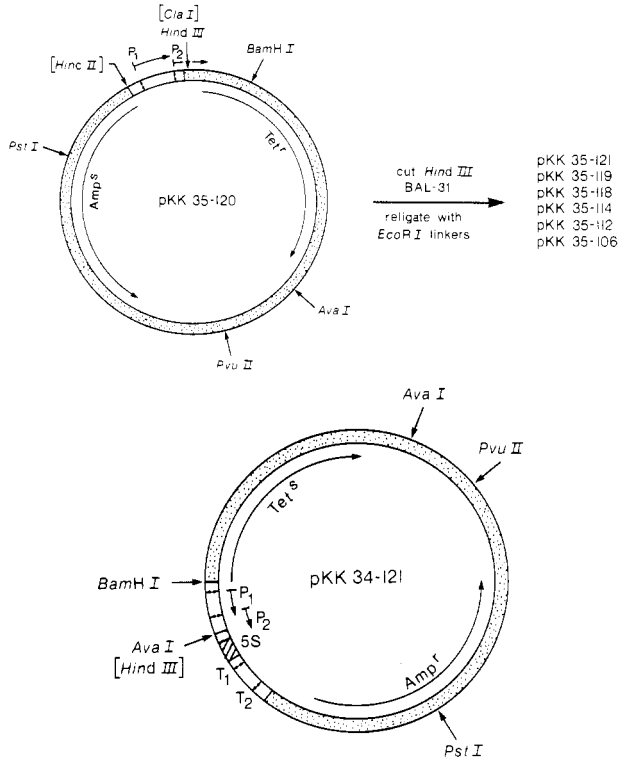

FIGURE 1: Plasmids were constructed by and were the generous gift of Dr. Jürgen Brosius. Hatched areas indicate pBR322 sequences; open boxes represent sequences of the *rrn*B operon. Deletions were created at the *Hin*dIII site of pKK35-120 as indicated to produce the remainder of the pKK35 series. In pKK34-121, the promoter region was fused to the 5S rRNA coding region and *rrn*B terminators T_1 and T_2 as shown. The sequence surrounding promoter P_2 in each construction has been determined (J. Brosius, unpublished results; see Figure 4). The junction between pBR322 sequences and the *rrn*B sequence proximal to promoter P_1 in each construction is at base −65 of P_1 [base 1162 of Brosius et al. (1981a)]. pKK35-120 was constructed as follows: pKT21 (Talmadge & Gilbert, 1980) was cleaved with *Cla*I and *Hin*cII, and the two overhanging nucleotides of the *Cla*I site were filled in. The large fragment was isolated and ligated to the 185 base pair *Fnu*DII rRNA promoter fragment (positions 1162–1346; Brosius et al., 1981a).

Deletion Mapping of the rrnB P_2 Promoter Sequences That Determine Sensitivity to ppGpp. ppGpp specifically inhibits the production of RNA chains from both the P_1 and P_2 promoters of the *rrn*B operon in vitro. This inhibition apparently occurs through an impaired ability of RNA polymerase to bind to the *rrn*B promoters in the presence of ppGpp (Kingston et al., 1981a). The deletion mutants described above have been used to determine the sequences in promoter P_2 that are responsible for the sensitivity of transcription to ppGpp. DNA templates were digested with the indicated restriction enzymes so that in vitro transcription would produce short (200–400 nucleotides) "run-off" transcripts from promoters P_1 and P_2. Plasmid pKK34-121 is an exception: the major transcripts are the result of termination at the *rrn*B terminator T_1 contained on this plasmid. The short P_1 and P_2 transcripts produced can be easily separated on a polyacrylamide gel, allowing independent determination of the effects of ppGpp on transcription from each promoter.

The site(s) of transcriptional initiation from the wild-type and altered P_2 promoters was (were) determined according to Donis-Keller et al. (1977) by RNase U_2 and RNase T_1 digestion of the 5′ end labeled transcript (arrows, Figure 2). Transcription initiates at two of a string of four C residues on the wild-type promoter. In three deletions, a purine results at one of these four positions. In each case, transcription will

Plasmid	Sequence	% Inhibition by ppGpp		Pause strength		
		P_1	P_2	90/91	119	126
	115 125					
pKK3535	*TATTATG*CACACCCCGCGCCGCTGAGAA	58	48	+++	+	+
pKK34-121	*TATTATG*CACACCCCGAGCTTGGCTGTT	63	59	+++	+	-
pKK35-121	*TATTATG*CACACCCCGGAATTCCTAGTT	63	43	nd	nd	nd
pKK35-120	*TATTATG*CACACCCCCGATAAGCTTTAA	40	27	+++	++	-
pKK35-119	*TATTATG*CACACCCGGAATTCCCAGGCA	49	24	++	+	-
pKK35-118	*TATTATG*CACACCGGAATTCCTAAATTG	66	8	-	+	-
pKK35-114	*TATTATG*CAGGAATTCCGTAGTTTATCA	60	0	+	-	-
pKK35-112	*TATTATG*GGAATTCCCACCGTGTATGAA	52	4	nd	nd	nd
pKK35-106	*T*GGAATTCCGTAGTTTATCACAGTTAAA	nd	-	(+)*	-	-

FIGURE 2: Effects of deletions near promoter P_2 on inhibition by ppGpp and on pausing. The DNA sequence of the antisense strand around the P_2 initiation site of the plasmids is shown (J. Brosius, unpublished results). The −10 region (Pribnow, 1975) is italicized. The arrows indicate the points of initiation of transcription determined as described under Experimental Procedures. Transcription initiates at two of the three bases 118, 119, and 120 on plasmid pKK35-120. The amount of inhibition of transcription from promoters P_1 and P_2 by ppGpp is shown for each plasmid. The strength of the P_2 promoter on each plasmid was calculated by comparing the transcription level to that of the P_1 promoter and then correcting for the lengths of the two transcripts. The values obtained for the ratio P_2/P_1 are as follows: pKK3535, 1.0; pKK34-121, 1.8; pKK35-121, 0.9; pKK35-120, 1.1; pKK35-119, 1.0; pKK35-118, 1.3; pKK35-114, 1.1; pKK35-112, 1.3. The levels of transcription for these determinations were determined by densitometer scanning of the appropriate regions of the autoradiograms shown in Figure 3 and those from similar experiments. The effects of the deletions on pausing at the 90- and 91-, 119-, and 126-base pause sites are also shown. The strength of the pauses was estimated by visual examination of the experiments shown in Figure 4. (*) The 90- and 91-base pause on pKK35-106 is shifted to approximately base 85 (see Figure 3); nd, not determined. The first base after the *Eco*RI linker GGAATTCC is pBR322 nucleotide number [numbering from Sutcliffe (1978)] 43 (pKK35-121), 74 (pKK35-119), 57 (pKK35-118), 42 (pKK35-114), 78 (pKK35-112), and 42 (pKK35-106), respectively.

initiate at the purine, even when one or both of the normal initiation nucleotides are present (plasmids pKK35-119, pKK35-118, pKK35-114; Figure 2). The strength of the altered P_2 promoters was determined by comparing the level of transcription to that from the wild-type P_1 promoter contained on each construction (see legend to Figure 2). By this comparison, the P_2 promoter of pKK34-121, which retains a C start, is strengthened approximately 80% with respect to the wild-type P_2 promoter. Changing the start site to a purine only slightly increases the strength of the P_2 promoter with respect to the wild type (pKK35-118, pKK35-114, pKK35-112).

In order to determine the effect of ppGpp on transcription from the *rrn*B promoters, a reaction protocol was used that corrects for the effect of ppGpp on the elongation rate of RNA polymerase [see Experimental Procedures; also see Kingston et al. (1981a)]. Transcription of plasmid DNA was carried out in the presence and absence of ppGpp, and the resultant RNA products were separated on a polyacrylamide gel (Figure 3). The amount of label incorporated into the P_1 and P_2 bands in the presence and absence of ppGpp was determined by densitometer scanning of the appropriate regions of the autoradiograms (Figure 2). When wild-type DNA (pKK3535) is used, ppGpp inhibits transcription from both P_1 and P_2 by approximately 50%. The presence of an unaltered P_1 promoter on all of the constructions allows the determination of the effect of ppGpp on a wild-type promoter in each reaction. Transcription from promoter P_1 is inhibited 40–70% by ppGpp on the various constructions. Transcription from promoter P_2 is inhibited in a similar manner on plasmids containing the wild-type sequence through base 120. Transcription from promoter P_2 on plasmid pKK35-119 is less inhibited than transcription from promoter P_1, while on plasmids pKK35-118, pKK35-114, and pKK35-112 there is very little effect of ppGpp on transcription from P_2. Sequences necessary for the full inhibitory effect of ppGpp on this promoter have therefore been deleted in the latter constructions.

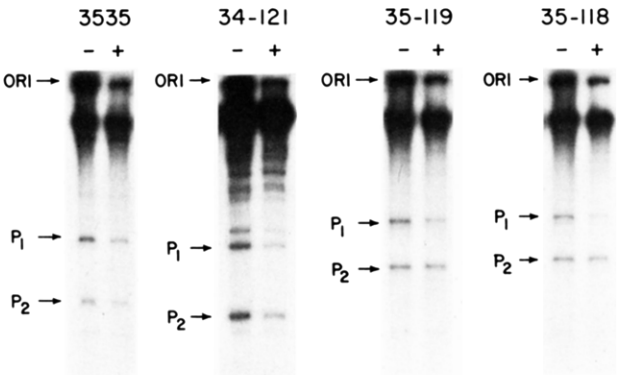

FIGURE 3: Sensitivity of altered P_2 promoters to inhibition by ppGpp. Assays were performed as described under Experimental Procedures. (−) No ppGpp present; (+) 0.26 mM ppGpp present. Plasmid DNAs were digested with the following restriction enzymes prior to transcription: pKK3535, *Hin*dIII; pKK34-121, *Pst*I; pKK35-119 and pKK35-118, *Bam*HI. The RNA samples were analyzed on a 5% polyacrylamide gel [acrylamide:bis(acrylamide) ratio 30:0.8] containing 7 M urea (Maniatis et al., 1975). The dark band near the top of each track is the result of transcription from T7 DNA (included to provide competing promoters). The following amounts of [α-^{32}P]CMP were incorporated in each reaction: plasmid pKK3535, −ppGpp 0.30 nmol, +ppGpp 0.13 nmol; plasmid pKK34-121, (−) 0.14 nmol, (+) 0.07 nmol; plasmid pKK35-119, (−) 0.23 nmol, (+) 0.10 nmol; plasmid pKK35-118, (−) 0.23 nmol, (+) 0.12 nmol.

Deletions at Promoter P_2 Alter Pausing at Bases 90, 91, 119, and 126. RNA polymerase that has initiated transcription at promoter P_1 pauses at several sites in the *rrn*B leader region in vitro; among these are sites at 90 and 91 bases, 119 bases, and 126 bases (Kingston & Chamberlin, 1981). In order to determine the effects of the deletions on the location and strength of RNA polymerase pause sites, a reaction protocol

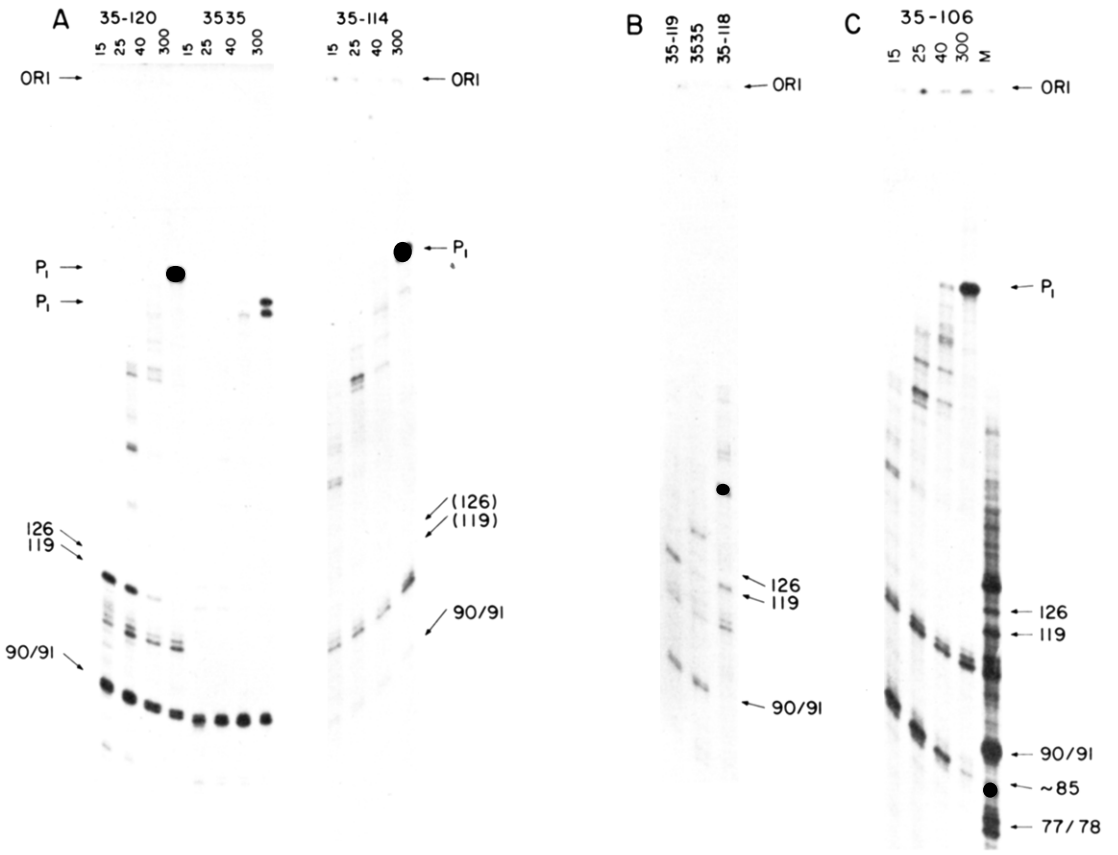

FIGURE 4: Pausing of RNA polymerase in the *rrn*B leader region. RNA polymerase pause sites in the wild-type and deleted *rrn*B leader region were determined as described under Experimental Procedures. The template used is shown above each experiment. The RNA samples were analyzed on a 10% polyacrylamide gel [acrylamide:bis(acrylamide) ratio 30:0.8] containing 7 M urea (Maniatis et al., 1975). In (A) and (C), the numbers above each track refer to the amount of time in seconds that transcription was allowed to proceed after initiation. In (B), only the 15-s time points are shown from the experiments with the three indicated templates. In (A), a 1.3-kilobase (kb) *Pst*I/*Hin*dIII fragment of pKK3535 that contains only the *rrn*B P_1 and P_2 promoters (Kingston & Chamberlin, 1981) was used as template in the indicated reaction (3535). The triplet band below the 119-base pause seen in all but the 3535 tracks is the result of transcription from pBR322 sequences contained on the plasmids. The lane marked M contains markers made after 15 s of transcription using pKK3535 as template. RNA polymerase was present at 25 μg/mL (1.3-kb pKK3535 fragment, pKK35-120) or 12.5 μg/mL (all others). DNA concentration was 4 μg/mL (1.3-kb pKK3535 fragment), 16 μg/mL (pKK35-120), or 10 μg/mL (all others). The following amounts of [γ-^{32}P]ATP were incorporated in each reaction: 3535, 0.02 pmol; 35-120, 0.01 pmol; 35-114, 0.01 pmol; 35-119, 0901 pmol; 35-118, 0.02 pmol; 35-106, 0.01 pmol.

was used that results in synchronous initiation of transcription from the P_1 promoter (Kingston & Chamberlin, 1981), and samples were removed 15, 25, 40, and 300 s after initiation. [γ-^{32}P]ATP was used that the other transcripts from the plasmids were not labeled. When the above time points are analyzed on a polyacrylamide gel, an RNA polymerase pause site appears as a transcript of a discrete length that is converted to a full-length transcript with time.

The strongest pause site in the *rrn*B leader region occurs at bases 90 and 91. The amount of RNA polymerase that pauses at the site varies with the enzyme to DNA ratio in a manner which suggests that pausing at this site is caused by RNA polymerase at P_2 blocking the elongation of RNA polymerase that has initiated at promoter P_1 (Kingston & Chamberlin, 1981). This hypothesis predicts that alterations at promoter P_2 should alter pausing at this site. This is the observed result (Figure 4B,C). The 90/91 pause site is present when wild-type (pKK3535) DNA is used as template and is also present with deletions extending to base 119 [note that the pause has a $t_{1/2}$ of approximately 4 min in vitro (Kingston & Chamberlin, 1981) and therefore is not fully converted to a full-length transcript in the experiments in Figure 4]. The paused transcript is not seen on pKK35-118, is weakly present on pKK35-114, and changes its duration and position, to approximately base 85, on a plasmid (pKK35-106) where the −10 region of P_2 is deleted.

RNA polymerase pauses for a shorter duration at both bases 119 and 126. All of the deletions eliminate the 126-base pause (Figure 4). The 119-base pause is enhanced on plasmid pKK35-120, is present on templates with deletions extending to base 118, and is absent on plasmids pKK35-114 and pKK35-106 (Figure 4, summarized in Figure 2).

Discussion

Guanosine tetraphosphate specifically inhibits transcription from rRNA promoters in vitro (van Ooyen et al., 1976; Travers, 1976a,b; Oostra et al., 1977; Kingston et al., 1981a). In contrast, the production of RNA from promoters of bacteriophages λ and T7 is not inhibited (Kingston et al., 1981a,b). This suggests that rRNA promoters contain specific sequences that allow response to ppGpp. In order to locate these sequences, deletions have been constructed that extend to various points surrounding the initiation site for *rrn*B promoter P_2 (Figure 2). An unaltered *rrn*B P_1 promoter contained on these constructions serves as an internal control for the inhibitory effect of ppGpp.

Transcription initiation from promoter P_2 in vitro usually occurs at the middle two of a stretch of four C's (bases 118, and 119, bases are numbered with 1 assigned as the initiation site for *rrn*B P_1; see Figure 2). One of the deletions (pKK35-119) alters the start nucleotide to a purine, even when the wild-type initiation nucleotides are still present. These data suggest that nucleotide sequences near or at the initiation site play a role in determining the exact nucleotide for the start of transcription. The strength of the promoters that use a purine as a start nucleotide is not significantly altered from the wild type, suggesting that in all cases the nucleotide concentrations used are above the apparent K_s for initiation.

Transcription from *rrn*B P_2 is inhibited approximately 50% by ppGpp in the wild-type construction. A similar inhibition by ppGpp is observed with deletions that extend to bases 121 or 120 (Figures 2 and 3). Loss of normal sensitivity of P_2 to ppGpp occurs as deletions extend into the initiation site (pKK35-119, pKK35-118). These data imply that the sequences that determine the sensitivity of the P_2 promoter to ppGpp in vitro lie promoter-proximal to base 120 and that sequences immediately surrounding the point of transcription initiation are necessary for this inhibition. Examination of the sequences of the promoters that are inhibited during the stringent response reveals conserved elements near the initiation site [see also Travers (1980a)]. Although no strict sequence is conserved, three elements occur at high frequency: (1) a C after the last base of the −10 region; (2) a C at −4, where +1 is defined as the start nucleotide; (3) two pyrimidines, usually C's, at −1 and +1.

By what mechanism does ppGpp specifically inhibit transcription from the *rrn*B promoters in vitro? Polymerase must open the DNA duplex surrounding the start nucleotide to allow initiation. The localization of sequences necessary for inhibition by ppGpp to this area suggests that ppGpp may inhibit this melting process.

Regulation of rrnB P_2 in Vivo. This work demonstrates that the sequences around the initiation site are necessary for the inhibition of transcription from *rrn*B promoter P_2 by ppGpp in vitro. It must be stressed that there are probably factors that effect the utilization of rRNA promoters in vivo that are missing from our purified in vitro system. RNA polymerase initiates transcription on the rRNA operons at a rate of approximately 1 s^{-1} in vivo (Maaloe & Kjeldgaard, 1966), much above the observed in vitro rate. Additionally, ppGpp elicits a 2-fold reduction of rRNA transcription in vitro, as compared to the 10–20-fold reduction seen in the stringent response in vivo (Gallant & Lazzarini, 1976). These considerations make it unlikely that the sequence determinants reported here are totally responsible for the inhibition observed during the stringent response.

Indeed, recent studies report that the level of transcription from the *rrn*A P_2 promoter is mildly inhibited under stringent growth conditions in both stringent and relaxed strains of *E. coli* (Sarmientos et al., 1983). Although this result suggests that accumulation of ppGpp does not result in inhibition of *rrn*A P_2 transcription, a 2-fold effect cannot be ruled out due to the complexity of measuring in vivo transcription rates. Interpretation of these studies is complicated by the close proximity, as well as the potential interaction through the tertiary DNA structure, of the *rrn* P_1 and P_2 promoters (Shen et al., 1982). Since transcription of P_1 and P_2 is probably linked in vivo, it is difficult to precisely determine the effect of a change in the cellular environment on the avidity of an individual *rrn* promoter for RNA polymerase.

Two considerations argue that the observed inhibition of rRNA transcription by ppGpp in vitro is relevant to in vivo regulation: the inhibition is specific to rRNA promoters and other stringently controlled promoters; the sequences responsible for the inhibition show conserved elements among these promoters.

Pausing of RNA Polymerase in the rrnB Leader Region. RNA polymerase does not proceed smoothly when elongating an RNA chain but instead moves unevenly, pausing at specific bases for varying periods of time. Such pausing is believed to play a major role in determining termination and attenuation sites (Yanofsky, 1981; Platt, 1981) and has been proposed as a mechanism to regulate heavily transcribed operons (Kingston & Chamberlin, 1981). Pausing of RNA polymerase has been theorized to result from hairpin formation in the RNA or from G-C-rich regions immediately preceding the pause sites (Gilbert, 1976; Yanofsky, 1981). The pause sites investigated here, located at 90/91, 119, and 126 bases of the *rrn*B operon, are apparently not caused by either of these mechanisms.

The pause site at bases 90 and 91 is a very strong pause site ($t_{1/2}$ of approximately 4 min in vitro; Kingston & Chamberlin, 1981). Previous work suggested that pausing at this site was the result of RNA polymerase bound at *rrn*B promoter P_2 blocking elongation of RNA polymerase that had initiated at promoter P_1 (Kingston & Chamberlin, 1981). This hypothesis would predict that changes in the ability of RNA polymerase to bind to promoter P_2 would alter the strength of this pause site. Under the in vitro conditions used (prebinding of RNA polymerase in the presence of ATP and CTP, followed by incubation with heparin), RNA polymerase would not be expected to form a stable complex at promoter P_2 if the start sequence were altered. When the two C residues are eliminated, pausing at 90/91 is substantially reduced or eliminated (pKK35-118, pKK35-114). Furthermore, deletion of the P_2 −10 region (pKK35-106) eliminates this pause, and a new pause site is seen at approximately 85 bases. These data strongly support the hypothesis that pausing at 90/91 is triggered by RNA polymerase at promoter P_2. It is not clear whether the length of this pause reflects the length of time polymerase is blocking at P_2, or instead reflects interaction with sequences near bases 90 and 91. A similar pause at this site in vivo would drastically reduce *rrn*B transcription.

Acknowledgments

I am grateful to Dr. Jürgen Brosius for donation of the plasmids used in this study and for many helpful discussions. I thank Dr. Michael J. Chamberlin, in whose laboratory this work was carried out, for his support and advice.

Registry No. Guanosine tetraphosphate, 32452-17-8; RNA polymerase, 9014-24-8.

References

Brosius, J., Dull, T. J., Sleeter, D. D., & Noller, H. F. (1981a) *J. Mol. Biol. 148*, 107–127.

Brosius, J., Ullrich, A., Raker, M. A., Gray, A., Dull, T. J., Gutell, R. R., & Noller, H. F. (1981b) *Plasmid 6*, 112–118.

Chamberlin, M. J., Nierman, W. C., Wiggs, J., & Neff, N. (1979) *J. Biol. Chem. 254*, 10061–10069.

Csordás-Tóth, E., Kiss, I., & Venetianer, P. (1979) *Nucleic Acids Res. 7*, 2189–2197.

Debenham, R. G., & Travers, A. (1977) *Eur. J. Biochem. 72*, 515–523.

deBoer, H. A., Gilbert, S. F., & Nomura, M. (1979) *Cell (Cambridge, Mass.) 17*, 201–209.

Donis-Keller, H., Maxam, A., & Gilbert, W. (1977) *Nucleic Acids Res. 4*, 2527–2531.

Gallant, J. A. (1979) *Annu. Rev. Genet. 13*, 393–415.
Gallant, J., & Lazzarini, R. A. (1976) in *Protein Synthesis: A Series of Advances* (McCartney, E. H., Ed.) Vol. 2, pp 309–359, Marcel Dekker, New York.
Gilbert, W. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M. J., Eds.) pp 193–205, Cold Spring Harbor Press, Cold Spring Harbor, NY.
Hamming, J., Gruber, M., & Ab, G. (1979) *Nucleic Acids Res. 7*, 1019–1033.
Hamming, J., Ab, G., & Gruber, M. (1980) *Nucleic Acids Res. 8*, 3947–3963.
Johnson, R. A., & Walseth, T. F. (1979) in *Advances in Cyclic Nucleotide Research* (Brooks, G., Greengard, P., & Robison, G. A., Eds.) pp 135–167, Raven Press, New York.
Kennerly, M. E., Morgan, E. A., Post, L., Lindahl, L., & Nomura, M. (1977) *J. Bacteriol. 132*, 932–949.
Kingston, R. E., & Chamberlin, M. J. (1981) *Cell (Cambridge, Mass.) 27*, 523–531.
Kingston, R. E., Gutell, R. R., Taylor, A. R., & Chamberlin, M. J. (1981a) *J. Mol. Biol. 146*, 433–449.
Kingston, R. E., Nierman, W. C., & Chamberlin, M. J. (1981b) *J. Biol. Chem. 256*, 2787–2797.
Kiss, A., Sain, B., & Venetianer, P. (1977) *FEBS Lett. 79*, 77–79.
Maaloe, O., & Kjeldgaard, N. O. (1966) *Control of Macromolecular Synthesis*, pp 94–96, W. A. Benjamin, New York.
Maniatis, T., Jeffrey, A., & van de Sande, H. (1975) *Biochemistry 14*, 3787–3794.
Nierlich, D. P. (1978) *Annu. Rev. Microbiol. 32*, 393–432.
Oostra, B. A., van Ooyen, A. J. J., & Gruber, M. (1977) *Mol. Gen. Genet. 152*, 1–6.
Platt, T. (1981) *Cell (Cambridge, Mass.) 24*, 10–23.
Pribnow, D. (1975) *J. Mol. Biol. 99*, 419–443.
Sarmientos, P., Sylvester, J. E., Contente, E., & Cashel, M. (1983) *Cell (Cambridge, Mass.) 32*, 1337–1346.
Shen, W.-F., Squires, C., & Squires, C. L. (1982) *Nucleic Acids Res. 10*, 3303–3313.
Stent, G., & Brenner, S. (1961) *Proc. Natl. Acad. Sci. U.S.A. 47*, 2005–2014.
Sutcliffe, J. G. (1978) *Cold Spring Harbor Symp. Quant. Biol. 43*, 77–90.
Talmadge, K., & Gilbert, W. (1980) *Gene 12*, 235–241.
Travers, A. (1976a) *Mol. Gen. Genet. 147*, 225–232.
Travers, A. (1976b) *Nature (London) 263*, 641–646.
Travers, A., & Baralle, F. E. (1976) *Alfred Benzon Symp. 9*, 241–251.
Travers, A., Buckland, R., Goman, M., LeGrice, S. S. G., & Scaife, J. G. (1978) *Nature (London) 273*, 354–358.
Travers, A. A. (1980a) *J. Bacteriol. 141*, 973–976.
Travers, A. A. (1980b) *J. Mol. Biol. 141*, 91–97.
van Ooyen, A. J. J., Gruber, M., & Jorgenson, P. (1976) *Cell (Cambridge, Mass.) 8*, 123–128.
Vogelstein, B., & Gillespie, D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 615–619.
Yanofsky, C. (1981) *Nature (London) 289*, 751–758.
Young, R. A., & Steitz, J. A. (1979) *Cell (Cambridge, Mass.) 17*, 225–234.

Kinetics of Calcium Uptake by Isolated Sarcoplasmic Reticulum Vesicles Using Flash Photolysis of Caged Adenosine 5′-Triphosphate†

Dorothy H. Pierce,* Antonio Scarpa, Michael R. Topp, and J. Kent Blasie

ABSTRACT: The kinetics of ATP-induced Ca^{2+} uptake by vesicular dispersions of sarcoplasmic reticulum were determined with a time resolution of about 10 ms, depending on the temperature. Ca^{2+} uptake was initiated by the addition of ATP through the flash photolysis of P^3-1-(2-nitrophenyl)-ethyl adenosine 5′-triphosphate utilizing a frequency-doubled ruby laser and measured with two different detector systems that followed the absorbance changes of the metallochromic indicator arsenazo III sensitive to changes in the extravesicular [Ca^{2+}]. The temperature range investigated was −2 to 26 °C. The Ca^{2+} ionophore A23187 was used to distinguish those features of the Ca^{2+} uptake kinetics associated with the formation of a transmembrane Ca^{2+} gradient. The acid-stable phosphorylated enzyme intermediate, E∼P, was determined independently with a quenched-flow technique. Ca^{2+} uptake is characterized by at least two phases, a fast initial phase and a slow phase. The fast phase exhibits pseudo-first-order kinetics with a specific rate constant of 64 ± 10 s^{-1} at 23–26 °C, an activation energy of 16 ± 1 kcal mol^{-1}, and a ΔS^* of ∼5 cal deg^{-1} mol^{-1}, is insensitive to the presence of a Ca^{2+} ionophore, and occurs simultaneously with the formation of the phosphorylated enzyme, E∼P, with a stoichiometry of ∼2 mol of Ca^{2+}/mol of phosphorylated enzyme intermediate. The slow phase also exhibits pseudo-first-order kinetics with a specific rate constant of 0.60 ± 0.09 s^{-1} at 25–26 °C, an activation energy of 22 ± 1 kcal mol^{-1}, and a ΔS^* of ∼16 cal deg^{-1} mol^{-1}, is inhibited by the presence of a Ca^{2+} ionophore, and has a stoichiometry of ∼2 mol of Ca^{2+}/mol of ATP hydrolyzed.

The sarcoplasmic reticulum (SR) membrane has an important role in the control of the cytoplasmic [Ca^{2+}] in muscle contraction and relaxation. In relaxation, calcium is removed from the cytoplasm across the SR membrane into the sarcotubular system (Ebashi et al., 1969; Hasselbach & Waas, 1982). The ATP-induced Ca^{2+} uptake by SR occurs against the Ca^{2+} concentration gradient, through a process mediated by the Ca^{2+}-ATPase in a cyclic, reversible mechanism of many steps (deMeis & Vianna, 1979; Chaloub & deMeis, 1980; Takakuwa & Kanazawa, 1981).

ATP-induced Ca^{2+} uptake by SR vesicles seems to consist of more than one phase. In quenched-flow experiments using radioactive Ca^{2+}, Inesi and co-workers obtained Ca^{2+} uptake

† From the Departments of Chemistry (D.H.P., M.R.T., and J.K.B.), and of Biochemistry and Biophysics (A.S. and J.K.B.), University of Pennsylvania, Philadelphia, Pennsylvania 19104. *Received April 12, 1983.* This work was supported by National Institutes of Health Grants HL-18708 and AA 05662 to J.K.B. and A.S.

0006-2960/83/0422-5254$01.50/0