The EMBO Journal vol.4 no.4 pp.1053–1058, 1985

Regulation of the expression of the *tufB* operon: DNA sequences directly involved in the stringent control

Junko Mizushima-Sugano[1] and Yoshito Kaziro

Institute of Medical Science, University of Tokyo, Takanawa, Minato-ku, Tokyo 108, Japan

[1]Present address: Rockefeller University, New York, NY 10021, USA

Communicated by P.Chapeville

We have located the DNA sequence involved in the stringent control of the *Escherichia coli tufB* operon. Various deletion and insertion mutants of the promoter locus were constructed by *in vitro* mutagenesis, and their response to guanosine-5′-diphosphate-3′-diphosphate (ppGpp) was examined in a cell-free transcription system consisting of purified RNA polymerase holoenzyme. The nucleotide sequence (GpCpGpC) from positions −7 to −4 (designating the initiation site of mRNA as position +1) is responsible for the selective inhibition by ppGpp of *tufB* transcription. Point mutations were then constructed in which each one of the above four nucleotides was replaced by an A or T residue and tested for their response to ppGpp in the *in vitro* transcription system. The results indicated that the alteration of any nucleotide in the GpCpGpC sequence leads to the loss of the stringent response.

Key words: guanosine tetraphosphate/*in vitro* transcription/site-directed mutagenesis/stringent control/*tufB* operon

Introduction

When *Escherichia coli* cells growing in an amino acid-rich medium are shifted to an amino acid-poor medium, the synthesis of macromolecules such as rRNAs, tRNAs and ribosomal proteins is severely and coordinately depressed (for a review, see Gallant, 1979). This regulation, designated as stringent control, has been extensively studied for >30 years; however, its precise mechanism still remains unknown.

Guanosine-5′-diphosphate-3′-diphosphate (ppGpp), which accumulates within the amino acid-starved $relA^+$ cells (Cashel and Gallant, 1969), is considered to be a 'chemical mediator' for the stringent control. Numerous *in vivo* and *in vitro* studies show the inhibitory effect of ppGpp on transcription; however, the precise mechanism by which ppGpp selectively inhibits the transcription of the stringently-controlled promoters has not yet been elucidated. Travers (1980a, 1984) compared the sequences of promoters involved in the stringent control and suggested that the sequences rich in G and C residues located immediately upstream of the transcription initiation site may be responsible for the stringent control. However, no direct evidence has yet been provided for this assumption.

The synthesis of elongation factor Tu (EF-Tu) *in vivo* is under stringent control (Miyajima and Kaziro, 1978), and the synthesis of EF-Tu or its mRNA directed by *tufB* DNA in a crude cell-free system is inhibited by ppGpp (Shibuya and Kaziro, 1979). In previous reports (Miyajima *et al.*, 1981; Mizushima-Sugano *et al.*, 1983), we examined in detail the effect of ppGpp on the transcription of the *tufB* and *recA* genes in a cell-free system consisting of purified RNA polymerase holoenzyme. Transcription of *tufB* was markedly inhibited by 0.2 mM ppGpp, whereas that of *recA* was little affected. Furthermore, it was shown that ppGpp inhibited initiation of the transcription of *tufB*, while the rate of RNA chain elongation was not much affected by ppGpp (Mizushima-Sugano *et al.*, 1983).

We have now constructed various deletion, insertion and point mutations in the *tufB* promoter locus by *in vitro* mutagenesis to locate the DNA sequences involved in the stringent control. The results indicate clearly that the short nucleotide sequence GpCpGpC which is located at positions −7 to −4 is responsible for the stringent control.

Results

Cloning of a DNA restriction fragment carrying the tufB promoter

The *tufB* operon has been cloned on a ColE1 derivative plasmid to yield pTUB1 and pTUB2 (Miyajima *et al.*, 1979). *tufB* is co-transcribed with the four adjacent tRNA genes (*thrU, tyrU, glyT* and *thrT*) upstream of the coding region of the *tufB* structural gene (Miyajima *et al.*, 1981; Lee *et al.*, 1981; Hudson *et al.*, 1981). pTUB2, a spontaneous deletion mutant carrying a *thrU-thrT* hybrid gene instead of four tRNA genes, did not show any alteration in the expression of *tufB* either *in vivo* or *in vitro* (Miyajima *et al.*, 1983). Therefore, we utilized plasmid pTUB2 as a source of the *tufB* template rather than pTUB1 because of its structural simplicity.

To obtain the smallest DNA fragment containing the *tufB* promoter which shows the stringent response to ppGpp in the *in vitro* transcription system, we first digested pTUB2 with *Eco*RI and *Sma*I. When the 6.4-kb *Eco*RI-*Sma*I fragment was used as a template, transcription of *tufB* was markedly inhibited by 0.5 mM ppGpp. Smaller DNA restriction fragments containing the *tufB* promoter were prepared to test their ppGpp sensitivity in the *in vitro* transcription system (Figure 1). Transcription of either the *Bgl*II-*Cla*I fragment (~730 bp), or the *Mbo*II-*Taq*I fragment (164 bp) containing the *tufB* promoter was as sensitive to ppGpp as when the 6.4-kb *Eco*RI-*Sma*I fragment was used as a template. These results suggest that the DNA sequences involved in the stringent control reside within the 164-bp *Mbo*II-*Taq*I fragment.

Construction of deletion mutants using exonuclease Bal31

The 164-bp *Mbo*II-*Taq*I fragment was subcloned into pBR327 at the *Ava*I/*Eco*RI sites to yield plasmid pTUB2-mt8, which was cleaved with *Eco*RI, and various deletions were constructed using exonuclease *Bal*31. *Eco*RI linkers were ligated to the digested ends and the purified *Eco*RI-*Ava*I fragments were recloned into pBR327 at the *Eco*RI/*Ava*I site. The resultant deletion mutants were analyzed by restriction mapping and the regions of deletions were determined by DNA sequencing. As shown in Figure 2, deletion of plasmid pTUB2-mt8 from its *Eco*RI site (position +64) up to the position +2 did not destroy the sensitivity to ppGpp (mutants △39, △53 and △56). Deletion mutants △41 and △44, which lack the Pribnow box of the *tufB* promoter, gave no band of the *tufB* mRNA on the polyacrylamide gel. The conclusion drawn is that the sequence required for the stringent control resides within the DNA sequences upstream of position +2.

© IRL Press Limited, Oxford, England.

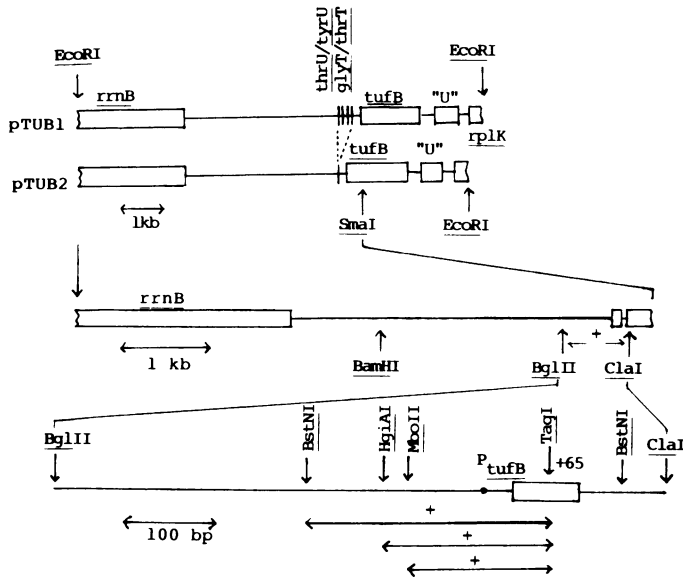
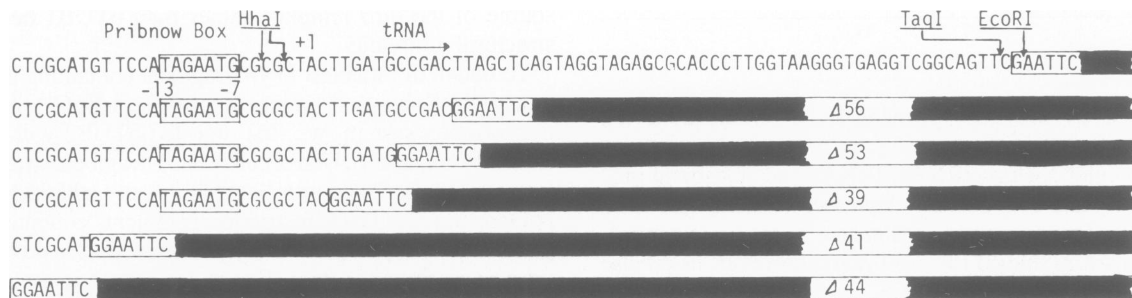


Fig. 1. Organization of the tRNA-*tufB* operon. The structure of the *Eco*RI fragment containing the tRNA-*tufB* region (derived from $\lambda rif^{d}18$) is shown in the upper two lanes (pTUB1 and pTUB2). In pTUB2, three out of four tRNA genes are deleted due to the fusion between homologous regions of *thrU* and *thrT* (Miyajima *et al.*, 1983). Restriction maps of the 6.4-kb *Eco*RI-*Sma*I fragment and the 0.7-kb *Bgl*II-*Cla*I fragment are shown in the lower two lanes. Various restriction fragments including the *Bgl*II-*Cla*I, *Bst*NI-*Taq*I, *HgiA*I-*Taq*I and *Mbo*II-*Taq*I fragments were prepared and tested for their response to ppGpp in the *in vitro* transcription system. The symbol (+) denotes the restriction fragments whose transcription showed sensitivity to ppGpp. The *Cla*I cleavage site is at the 5′ end of the structural gene of *tufB*.

Construction of the linker insertion mutants

To locate more precisely the DNA sequence involved in the stringent control, the *tufB* promoter was excised at positions closer to the Pribnow box and ligated with the *Eco*RI or *Hind*III linker. One of the two *Hha*I sites, present downstream of the *tufB* Pribnow box, was cleaved and the staggered end was polished using DNA polymerase I Klenow fragment. Then, either *Hind*III (CAAGCTTG) or *Eco*RI (GGAATTCC) linker was ligated to this end and religated to the *Eco*RI site of pBR327 which had been converted to a blunt end. Two linker insertion mutants were obtained, pLIH9 and pLIE7 containing the *Hind*III and *Eco*RI linker, respectively.

Figure 3 shows the structure of pLIH9 and pLIE7, as well as effects of ppGpp on the transcription of these plasmids. pTUB2-mt8, pLIE7 and pLIH9 were digested with *Hind*II and used as templates. Transcription of these truncated templates should yield a fragment of *tufB* mRNA of 519 nucleotides for pTUB2-mt8, and 460 nucleotides for pLIE7 and pLIH9. Distinct bands corresponding to the expected size of *tufB* mRNA fragments were obtained on the polyacrylamide gel (Figure 3). Formation of *tufB* mRNA *in vitro* was almost abolished by 0.5 mM ppGpp when pTUB2-mt8 and pLIH9 were used as templates. Since all the sequences downstream of position −3 were replaced by other sequences in pLIH9, it is suggested that sequences downstream of position −3 are not required for the stringent response. In the case of pLIE7, in addition to the alteration of the sequences downstream of position −3, residue C at position −4 was displaced by residue G. As can be seen in Figure 3, transcription of pLIE7 was less inhibited by 0.5 mM ppGpp than was that of pLIH9. These results indicate that residue C at position −4 may be involved in the stringent response.



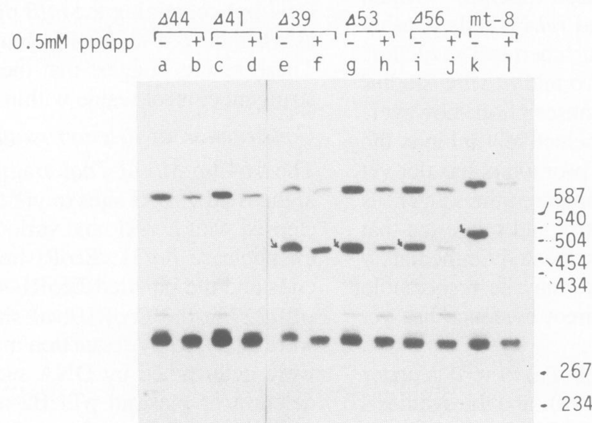


Fig. 2. Construction of deletion mutants of the *tufB* promoter region and the transcription of the mutant DNAs. Plasmid pTUB2-mt8 (shown in the uppermost lane) was cleaved at the *Eco*RI site and digested with exonuclease *Bal*31 for various times. *Eco*RI linkers were ligated to the digested ends and purified *Eco*RI-*Ava*I fragments were recloned into pBR327. Lengths of the individual deletions were determined by DNA sequencing, and the structure of the pTUB2-mt8 and its deletion mutants are shown in the top of the figure. Black bars indicate the sequence of pBR327 and the framed sequences are the Pribnow box and the sequences of *Eco*RI linkers. At the bottom, autoradiograms of transcripts of pTUB1-mt8 and its deletion mutants in the presence and absence of 0.5 mM ppGpp are shown.

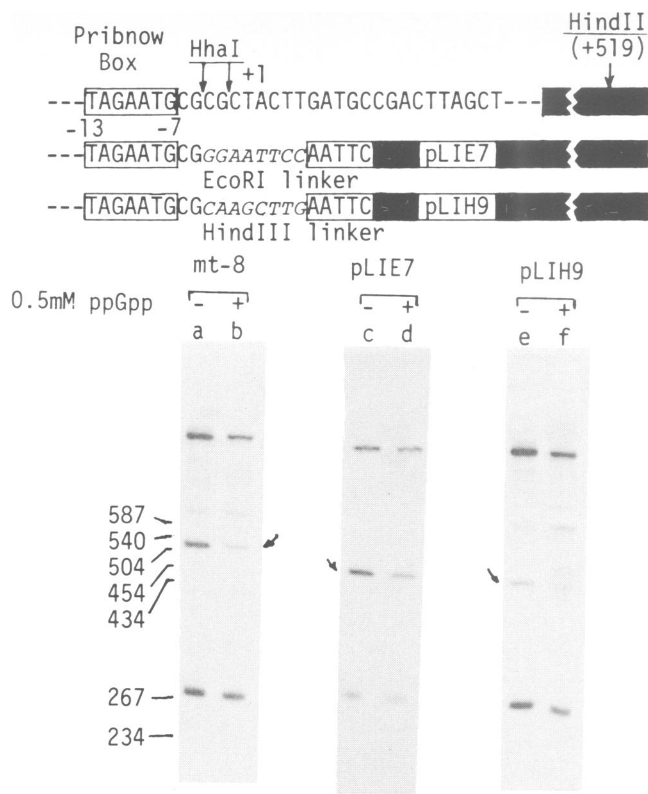


Fig. 3. Construction of the linker insertion mutants and transcription of the mutant DNAs. Plasmid pTUB2-mt8 (shown in the uppermost lane) was cleaved at the *Hha*I site (immediately 5′ to position −2) and treated with DNA polymerase I Klenow fragment to generate a blunt end. *Hind*III linker (CAAGCTTG) or *Eco*RI linker (GGAATTCC) was ligated to this end and religated to the *Eco*RI site of pBR327 which had been converted to the blunt end. The sequences of resultant linker insertion mutants were determined and the structure of pTUB2-mt8 and its linker insertion mutants are shown in the top of the figure. Black bars indicate the sequence of pBR327 and the framed sequences are the Pribnow box and the polished *Eco*RI sites of pBR327. At the bottom, radioautograms of transcripts of pTUB2-mt8 and its deletion mutants in the presence of 0.5 mM ppGpp are shown.

Deletion mutants which are resistant to ppGpp

Plasmid pLIH9 has only one cleavage site for *Hha*I at the adjacent position to the Pribnow box (see Figure 3). pLIH9 was digested with *Hha*I and the staggered end was polished using DNA polymerase I Klenow fragment. Two kinds of DNA fragments derived from pBR327 were ligated to this end to yield pDde1 and pDde4 plasmids (Figure 4). In pDde1, the Pribnow sequence was ligated to the *Dde*I site at position 4290 of pBR327, while in pDde4 it was ligated to the *Hind*II site at position 3905. Then, pDde1 and pDde4 were digested with *Hind*II and *Bgl*I, respectively, and the effects of ppGpp on the transcription of these truncated templates, in which all the sequences downstream of the Pribnow box were altered, were examined.

Transcription of the templates yielded fragments of *tufB* mRNA of 720 nucleotides for pDde1 and 420 nucleotides for pDde4. As shown in Figure 4, transcription of neither pDde1 nor pDde4 was inhibited by 0.5 mM ppGpp, in contrast to pTUB2-mt8. GC-rich sequences immediately adjacent to the Pribnow box were replaced by AT-rich sequences in pDde1, while in pDde4 residues C and G at positions −6 and −5 were replaced by G and A, respectively. These results, in combination with those obtained with linker insertion mutants, suggest that the sequence at positions −6 to −4 (CpGpC) or −6 and −5 (CpG) may be required

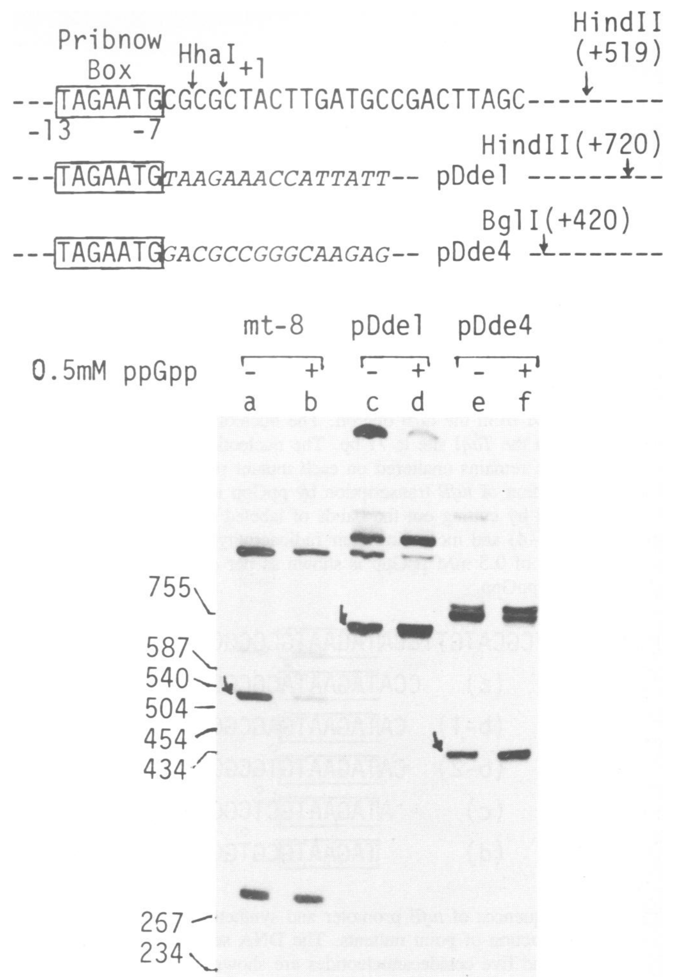


Fig. 4. Construction of deletion mutants which are resistant to ppGpp and the transcription of the mutant DNAs. Linker insertion mutant pLIH9 was cleaved at the *Hha*I site immediately 3′ to the Pribnow box of *tufB* promoter and polished with DNA polymerase I Klenow fragment. Two kinds of DNA fragment derived from pBR327 were ligated to this end to yield plasmids pDde1 and pDde4. One of the two fragments was obtained by digesting pBR327 at position 4290 with *Dde*I, and the other was obtained by digesting pBR327 at position 3905 with *Hind*II. The sequences of resultant mutants were determined and the structure of the pTUB2-mt8 (in the uppermost lane) and deletion mutants are shown. At the bottom, radioautograms of transcripts of pTUB2-mt8 and deletion mutants in the presence and absence of 0.5 mM ppGpp are shown. Arrows indicate the *tufB* transcripts.

for the stringent response.

The region of *tufB* DNA conserved in each mutant plasmid and the extent of inhibition by ppGpp are shown in Figure 5, after determination of the radioactivity of each band of the labeled *tufB* mRNA from the gels (Figures 2−4). Transcription of pTUB2-mt8 was markedly inhibited by 0.5 mM ppGpp, whereas that of pDde1 and pDde4 was not inhibited at all. In the case of pDde1, transcription was even stimulated by ppGpp. The sensitivity to ppGpp of transcription of pLIE7 and pLIH9 was examined several times; transcription of pLIE7 was less sensitive to ppGpp as compared with that of pLIH9.

Construction of the point mutants by oligonucleotide-directed mutagenesis and their expression

From the data obtained above, the sequence which is required for the stringent response appeared to be the CpGpC sequence at positions −6 to −4 (or CpG at −6 and −5). To test the func-

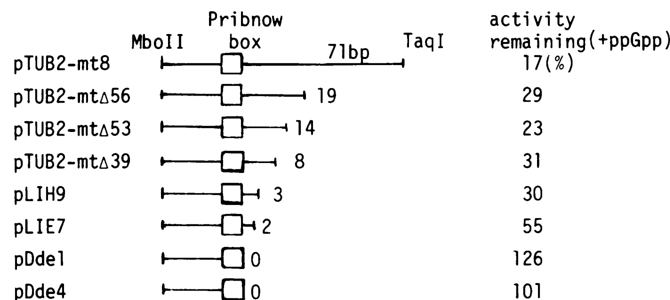


Fig. 5. The structure of deletion and linker insertion mutants and their sensitivity to ppGpp. The region which is derived from *tufB* operon in each plasmid is indicated. Plamid pTUB2-mt8 contains the *Mbo*II-*Taq*I fragment which is derived from the *tufB* operon. The nucleotide length from the Pribnow box to the *Taq*I site is 71 bp. The nucleotide length of the DNA sequence which remains unaltered on each mutant plasmid is shown. The extent of inhibition of *tufB* transcription by ppGpp in each mutant plasmid was determined by cutting out the bands of labeled *tufB* mRNA from the gel (Figures 2–4) and measuring their radioactivity. The remaining activity in the presence of 0.5 mM ppGpp is shown as per cent of the activity in the absence of ppGpp.

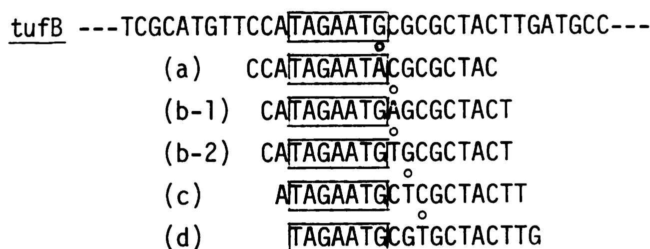


Fig. 6. The sequences of *tufB* promoter and synthetic octadecanucleotides used for construction of point mutants. The DNA sequences around the Pribnow box and five octadecanucleotides are shown. Each synthetic octadecanucleotide contains one base mismatch to the wild-type *tufB* promoter in its center.

tion of these sequences more precisely, we constructed several point mutants of this region and examined the sensitivity of their transcription to ppGpp. In addition to the CpGpC sequence at position −6 to −4, the residue G at position −7 was also altered, since this G residue is highly conserved among the stringently controlled promoters.

The octadecanucleotides used for the *in vitro* mutagenesis are shown in Figure 6. Each synthetic octadecanucleotide contained one base mismatch to the sequence of the wild-type *tufB* promoter in its center. The fragment containing the *tufB* promoter was first cloned into the M13 vector M13mp8 together with the *rrnB* terminator T_1 and T_2 to obtain the recombinant phage ETT1. The octadecanucleotide was hybridized to the single-stranded DNA of ETT1, and used as a primer for synthesis of the complementary strand in the presence of DNA polymerase I Klenow fragment and T4 DNA ligase. After treatment with S1 nuclease, DNA was introduced to *E. coli* JM101 to obtain single-stranded phage DNA. The complementary strand was again synthesized *in vitro* using the same octadecanucleotide as a primer. After successive S1 nuclease digestion and transformation, single-stranded phage DNA from individual plaques were screened by plaque hybridization or by direct DNA sequencing. About 25–50% of the plaques were found to contain mutated DNA. The replicative forms of mutant phage DNA were then prepared and digested with *Taq*I at position +65 of the *tufB* operon. Using this DNA as template, the sensitivity to ppGpp was tested in an *in vitro* transcription system (Figure 7).

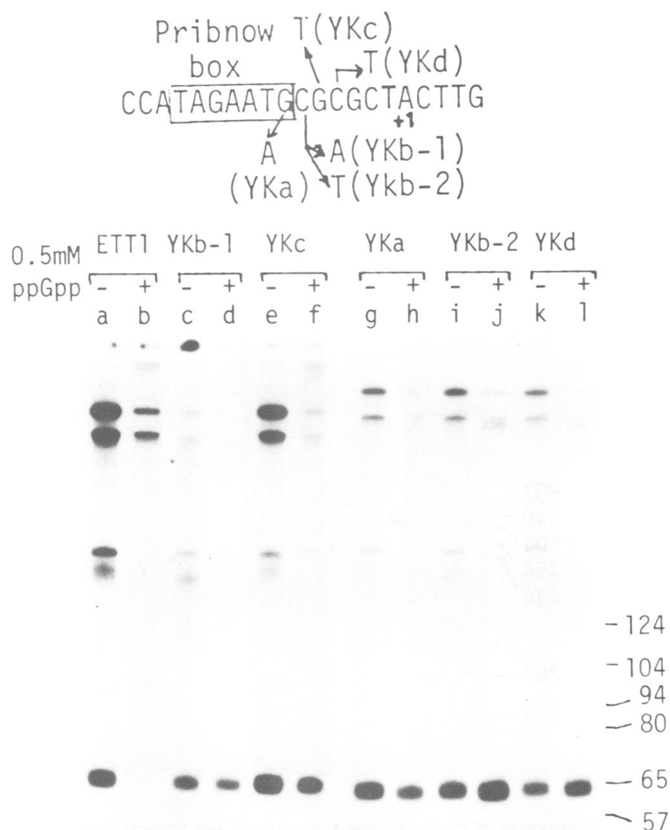


Fig. 7. The altered base of each point mutant and the transcription of the mutant DNAs. The sequence of wild-type *tufB* promoter and altered base of each point mutant is shown at the top. The fragment containing the *tufB* promoter sequence was cloned in M13 vector M13mp8 together with the terminator of *rrnB* to obtain recombinant phage ETT1. The octadecanucleotide, complementary to the *tufB* promoter region except for one base, was hybridized to ssDNA of ETT1, and the complementary strand was synthesized in the presence of DNA polymerase I Klenow fragment and T4 DNA ligase. After treatment by S1 nuclease, DNA was introduced into the *E. coli* JM101 and ssDNA was prepared. Again, using the same octadecanucleotide as a primer, a complementary strand of this ssDNA was synthesized *in vitro*. After S1 nuclease digestion and transformation, ssDNA was prepared to screen for point mutants. Replicative forms of the point mutants were prepared and used as templates for transcription. At the bottom, autoradiograms of transcripts of ETT1, which contain wild-type *tufB* promoter and its point mutants in the presence and absence of 0.5 mM ppGpp are shown.

Recombinant phage ETT1, in which the wild-type *tufB* promoter is cloned, showed a marked sensitivity to 0.5 mM ppGpp. In contrast to ETT1, each point mutant was less inhibited by ppGpp. Point mutants YKb-2 and YKd in which the C residue at positions −6 and −4, respectively, was altered to a T residue showed rather an enhancement of transcription by ppGpp. For quantitation, the bands corresponding to the truncated mRNA of *tufB* (Figure 7) were excised, and their radioactivity was counted. The extent of inhibition of transcription is shown in Figure 8. The results indicate that the alteration of any one of the nucleotides in the GpCpGpC sequence at positions −7 to −4 leads to the loss of the stringent response.

Discussion

We have shown that the transcription of the *tufB* operon in a cell-free system consisting of purified RNA polymerase holoenzyme was strongly inhibited by ppGpp whereas that of *recA* was little affected (Mizushima-Sugano *et al.*, 1983). This suggests the presence of a specific sequence in the *tufB* promoter responsible for the inhibition by ppGpp.

From the comparison of the promoter sequences, Travers (1980a, 1984) proposed the existence of a consensus sequence rich in G and C residues immediately 3′ to −10 region. Travers (1980b) and Kingston (1983) altered the sequences in the $tRNA^{Tyr}$ and *rrnB* P2 promoters, respectively, and found that their response to ppGpp in the *in vitro* system was altered. However, the *in vitro* transcription system used in their experiments did not reflect the *in vivo* expression of these genes. For example, Travers (1980b) reported that 20−40 μM ppGpp stimulated the transcription of the wild-type $tRNA^{Tyr}$ gene in his *in vitro* system; however, it is not certain whether the transcription of the gene *in vivo* is also stimulated by ppGpp. Kingston (1983) used an *in vitro* transcription system in which transcription of the *rrnB* P2 promoter was inhibited by ppGpp; however, it has recently been reported that expression of the P2 promoter of the *rrnA* gene is not under stringent control either *in vivo* (Sarmientos *et al.*, 1983; Gourse *et al.*, 1983; Sarmientos and Cashel, 1983) or *in vitro* (Kajitani and Ishihama, 1984; Glaser *et al.*, 1983).

In contrast, the *in vitro* transcription system used in our previous and present studies seems to reflect more faithfully the *in vivo* regulation. Transcription of the *tufB* operon in our system is inhibited by ppGpp in the presence of 2 mM Mg^{2+}, and 80−120 mM KCl, and this inhibition is specific for the stringently controlled promoter since transcription of non-stringently controlled *recA* promoter is refractory to ppGpp. To locate the DNA sequence involved in the stringent control using this *in vitro* transcription system, we constructed various deletion, insertion and point mutants of the *tufB* operon and examined their response to ppGpp.

Since transcription of the *tufB* operon was inhibited at the step of initiation (Mizushima-Sugano *et al.*, 1983), we first looked for the smallest size of DNA restriction fragments which still showed sensitivity to ppGpp and we found that the 164-bp *Mbo*II-*Taq*I fragment contains the DNA sequences required for the stringent response. Various deletion mutants were then constructed to show that the deletion of plasmid pTUB2-mt8 from its *Eco*RI site (position +64) up to the position +2 did not destroy the sensitivity to ppGpp (plasmid pTUB2-mt△39). Therefore, the structural gene for tRNA is not required for the stringent response.

It may be noted that the sequence of the *tufB* operon determined here differs by one base from that determined by An and Friesen (1980). Their sequence contained a C residue between the two T residues at positions −17 and −18 (Figure 2). Both DNA strands of this region were sequenced, but we did not find the additional C residue.

Furthermore, the linker insertion mutants and the deletion mutants were constructed and it was concluded that the sequences downstream of position −3 are not required for the stringent response, but the C residue at position −4 is partially, and the G residue at position −5 is fully, required for the stringent response.

To test the function of this region in a more precise manner, we constructed point mutants in which each one of the nucleotides from positions −7 to −4 (GpCpGpC) was altered to A or T. The G residue at position −7 corresponds to the 3′ end of the Pribnow box. The nucleotide at this position of the stringently controlled promoters mostly contain G, while non-stringent type promoters contain any one of the four bases at an equal frequency (Hawley and McClure, 1983; Travers, 1984). To construct point mutants, we first cloned the fragment containing the *tufB* promoter region into phage M13mp8, and the resultant recombinant phage was used as a template to construct point mutants. However, each mutant obtained was found to possess an unexpected additional base substitution or deletion in the Pribnow box or in the −35 region. This is probably due to the read-through of the mRNA started from the *tufB* promoter into the genes which are required for growth of M13 phage. Therefore, we inserted the *rrnB* terminator downstream of the *tufB* promoter, and succeeded in constructing the desired point mutants. Only the point mutant YKb-2, in which the residue C at position −6 was altered to a T, had one additional base substitution at position −73 (A→G). However, since the sequence upstream of −70 is not required for the stringent response of the *tufB* operon *in vitro* (Wakabayashi, Mizushima-Sugano and Kaziro, unpublished), the substitution at position −73 does not seem to affect the effect of ppGpp. The results obtained from the transcription of these point mutants (Figures 7 and 8) suggest that the sequence GpCpGpC at position −7 to −4 is responsible for the stringent control of the *tufB* operon.

The above sequence is remarkably well conserved in many stringently controlled promoters (Travers, 1984). It is especially well conserved among the promoters for stable RNAs such as tRNA and rRNA. In this respect, it is noteworthy that the expression of the P2 promoters of rRNA genes are not under stringent control, although their sequences downstream of the

-13 -7 +1		activity remaining(+ppGpp)
TAGAATGCGCGCTAC	ETT1	27(%)
A	YKa	63
A	YKb-1	85
T	YKb-2	150
T	YKc	72
T	YKd	131

Fig. 8. The altered base of each point mutant and their sensitivity to ppGpp. The DNA sequence of wild-type *tufB* promoter (ETT1) and altered base of each point mutant is shown. The extent of inhibition of *tufB* transcription by 0.5 mM ppGpp in each point mutant was determined by cutting out the bands of labeled *tufB* mRNA from the gel (Figure 7) and measuring their radioactivity. The extent of inhibition was indicated as shown in Figure 5.

Sequences of rrn promotor

P1	rrnA, B, G	TATAATGCGCCAC
	rrnD, E, X	TATAATGCGCCTC
P2	rrnA, B, G	TATTATGCACACC
	rrnD, E, X	TAATATACGCCAC
		-13 -7

Fig. 9. The nucleotide sequence of the P1 and P2 promoters of rRNA genes.

Pribnow box are rich in G and C residues. As shown in Figure 9, the P2 promoter sequences contain residue A at position −7 for *rrnD*, *rrnE* and *rrnX*, and at position −5 for *rrnA*, *rrnB* and *rrnG*. This is in line with the present results which demonstrated that the G residues at positions −7 and −5 are essential for the ppGpp sensitivity. It will be interesting to see whether the alteration of the P2 promoter sequences to the (−7)GpCpGpC(−4) consensus sequence may confer the ppGpp sensitivity to the transcription of P2 promoters.

As these results were obtained in the system consisting of pure RNA polymerase holoenzyme, the question arises as to how RNA polymerase could distinguish between stringently and non-stringently controlled promoters. Our current hypothesis is that when RNA polymerase is bound to a stringent promoter its conformation may be altered to the form to which ppGpp is accessible. An alternate possibility is that, in the presence of ppGpp, RNA polymerase can bind only to the non-stringently controlled promoters. These points are now currently under investigation. Also, it remains to be seen whether the observed alteration of the stringently controlled promoter to the non-stringently controlled form by *in vitro* mutagenesis can also be reproduced in whole cell studies *in vivo*.

Materials and methods

Labeled compounds

[α-^{32}P]GTP (400 Ci/mol) and [α-^{32}P]dATP (3000 Ci/mmol) were purchased from the Radiochemical Center, Amersham. Inorganic ^{32}P was obtained from New England Nuclear and [γ-^{32}P]ATP ($\sim$8000 Ci/mmol) was prepared by the method of Walseth and Johnson (1979).

Enzymes

E. coli RNA polymerase was purified from PR13 as described by Mizushima-Sugano *et al.* (1983). The purified preparation consisted of $\sim$90% holoenzyme, as judged by the content of the sigma subunit. Restriction enzymes were purchased from Takara Shuzo (Kyoto), New England Biolabs, and Bethesda Research Laboratories. *E. coli* DNA polymerase I Klenow fragment and T4 polynucleotide kinase were from Takara Shuzo. Exonuclease *Bal*31 was from Bethesda Research Laboratories. S1 nuclease was from P.L.Biochemicals.

DNA preparations

Plasmid DNA and replicative forms of M13 phage DNA were prepared by method B of Wilkie *et al.* (1979) with some modifications. DNA fragments were isolated according to the modified procedure of Vogelstein and Gillespie (1979).

In vitro transcription

In vitro transcription reactions were carried out under the reaction conditions described by Mizushima-Sugano *et al.* (1983). After incubation for 20 min at 37°C, the reaction was terminated by adding 185 μl of 20 mM Tris-HCl (pH 7.5), 1 mM EDTA containing 20 μg carrier tRNA at 0°C. The mixture was precipitated with ethanol and analyzed by polyacrylamide gel electrophoresis. When necessary, the bands of labeled mRNA were cut out and the radioactivity was measured.

Construction of point mutants

Point mutants were constructed according to the method of Wasylyk *et al.* (1980) and Temple *et al.* (1982). Synthetic octadecanucleotides containing one base mismatch in the center of their sequences were kindly prepared by Dr H.Ozawa, Toray Research Laboratories. A mixture (7.5 μl) containing 40 pmol of a phosphorylated primer, 0.5 pmol of ssDNA M13 recombinant (containing the *tufB* promoter fragment), 40 mM Tris-HCl pH 7.5, 20 mM $MgCl_2$, 0.1 M NaCl, and 2 mM 2-mercaptoethanol was incubated for 3 min at 80°C and for 60 min at 0°C. A mixture (11.3 μl) containing 22 mM Tris-HCl (pH 7.5), 11 mM $MgCl_2$, 1 mM 2-mercaptoethanol, dATP, dGTP, dCTP and TTP each at 0.83 mM, 0.4 mM ATP, 2.5 units of DNA polymerase I Klenow fragment, and 0.5 unit T4 DNA ligase were added and incubation was continued for 30 min at 0°C and 5 h at 23°C. Samples (1 μl) were analyzed on 1% agarose gels to check for the complete conversion to double-stranded circles. After treatment with S1 nuclease, DNA was used to transform *E. coli* JM101 and ssDNA was prepared. Again, using the same octadecanucleotide as a primer, a complementary strand to this ssDNA was synthesized *in vitro*. The reaction mixture used was the same as described above except the ratio of primer to ssDNA was 25. Also, the initial incubation at 0°C was omitted. After successive S1 nuclease digestion and transformation, plaques were obtained and analyzed.

Nucleotide sequence determination

The DNA sequence of deletion or insertion mutants was determined by the method of Maxam and Gilbert (1977), and that of point mutants was determined according to the method of Sanger *et al.* (1977).

Plaque hybridization

Plaque hybridization was carried out as described by Benton and Davis (1977) and Zoller and Smith (1982). Phage DNA on the filters was hybridized to oligonucleotide, which contains one mismatch, at 23°C for 24 h and the filters were washed at three different temperatures (37°C, 45°C, 50°C) with 4 x SET (1 x SET: 0.15 M NaCl, 30 mM Tris-HCl pH 8.0, 1 mM EDTA) three times and then 1 x SET.

Acknowledgements

We are very grateful to Dr H.Ozawa for kindly supplying us with synthetic oligonucleotides. We thank Dr M.Cashel for supplying plasmid pPS1 and Dr S.Nagata for valuable advice.

References

An,G. and Friesen,J.D. (1980) *Gene*, **12**, 33-39.
Benton,W.D. and Davis,R.W. (1977) *Science (Wash.)*, **196**, 180-182.
Cashel,M. and Gallant,J. (1969) *Nature*, **221**, 838-841.
Gallant,J.A. (1979) *Annu. Rev. Genet.*, **13**, 393-415.
Glaser,G., Sarmientos,P. and Cashel,M. (1983) *Nature*, **302**, 74-76.
Gourse,R.L., Stark,M.J.R. and Dahlberg,A.E. (1983) *Cell*, **32**, 1347-1354.
Hawley,D.K. and McClure,W.R. (1983) *Nucleic Acids Res.*, **8**, 2237-2255.
Hudson,L., Rossi,J. and Landy,A. (1981) *Cell*, **25**, 251-258.
Kajitani,M. and Ishihama,A. (1984) *J. Biol. Chem.*, **259**, 1951-1957.
Kingston,R.E. (1983) *Biochemistry (Wash.)*, **22**, 5249-5254.
Lee,J., An,G., Friesen,J. and Fill,N. (1981) *Cell*, **25**, 251-258.
Maxam,A.M. and Gilbert,W. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 560-564.
Miyajima,A. and Kaziro,Y. (1978) *J. Biochem.*, **83**, 453-462.
Miyajima,A., Shibuya,M. and Kaziro,Y. (1979) *FEBS Lett.*, **102**, 207-210.
Miyajima,A., Shibuya,M., Kuchino,Y. and Kaziro,Y. (1981) *Mol. Gen. Genet.*, **183**, 13-19.
Miyajima,A., Yokota,T., Takebe,Y, Nakamura,M. and Kaziro,Y. (1983). *J. Biochem.*, **93**, 1101-1108.
Mizushima-Sugano,J., Miyajima,A. and Kaziro,Y. (1983) *Mol. Gen. Genet.*, **189**, 185-192.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463-5467.
Sarmientos,P. and Cashel,M. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 7010-7013.
Sarmientos,P., Sylvester,J.E., Contente,S. and Cashel,M. (1983) *Cell*, **32**, 1337-1346.
Shibuya,M. and Kaziro,Y. (1979) *J. Biochem.*, **86**, 403-411.
Temple,G.F., Dozy,A.M., Roy,K.L. and Kan,Y.W. (1982) *Nature*, **296**, 537-540.
Travers,A.A. (1980a) *J. Bacteriol.*, **141**, 973-976
Travers,A.A. (1980b) *J. Mol. Biol.*, **141**, 91-97.
Travers,A.A. (1984) *Nucleic Acids Res.*, **12**, 2605-2618.
Vogelstein,B. and Gillespie,D. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 615-619.
Walseth,T.F. and Johnson,R.A. (1979) *Biochim. Biophys. Acta*, **562**, 11-31.
Wasylyk,B., Derbyshire,R., Guy,A., Molko,D., Roget,A., Teoule,R. and Chambon,P. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 7024-7028.
Wilkie,N.M., Clements,J.B., Boll,W., Mantei,N., Lonsdale,D. and Weismann,C. (1979) *Nucleic Acids Res.*, **7**, 859-877.
Zoller,M.J. and Smith,M. (1982) *Nucleic Acids Res.*, **10**, 6487-6500.

Received on 25 January 1985