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Influence of the GCGC discriminator motif introduced into the ribosomal RNA P2- and *tac* promoter on growth-rate control and stringent sensitivity

M.Zacharias, H.U.Göringer and R.Wagner[1]

Max-Planck-Institut für Molekulare Genetik, Abteilung Wittmann, Ihnestrasse 73, D-1000 Berlin 33, and [1]Institut für Physikalische Biologie, Heinrich-Heine-Universität Düsseldorf, Universitätsstrasse 1, D-4000 Düsseldorf 1, FRG

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The synthesis of stable RNA in bacteria is known to be regulated by a stringent control mechanism. Characteristic of stringent-regulated promoters, all ribosomal RNA promoters P1, but not P2, contain a GC-rich discriminator sequence assumed to be important for such a control. Using site-directed mutagenesis we have altered both the *rrnB* P2 and the synthetic *tac* promoter to the consensus GCGC discriminator motif. The modified promoters were placed upstream of the structural gene encoding the chloramphenicol acetyltransferase. The response of the modified promoters to amino acid starvation, changes in the growth rate or differences in the basal level of guanosine tetraphosphate (ppGpp) were determined *in vivo*. The results clearly show, that the discriminator motif is sufficient to convert the ribosomal RNA promoter P2 to a stringent, as well as growth-rate regulated, promoter. By contrast, the same discriminator sequence linked to the synthetic *tac* promoter does not convert this promoter to either stringency or growth-rate regulation. Finally, the results presented in this study reinforce the view that stringent and growth-rate regulation utilize the same mechanism, with ppGpp being the common mediator.

Key words: discriminator motif / growth-rate control/ guanosine tetraphosphate/stringent control/transcriptional regulation

Introduction

The synthesis of ribosomal RNAs and tRNAs (stable RNAs) in bacteria is regulated over a wide range of growth conditions. To vary the concentration of these important molecules two regulatory phenomena exist, that can be distinguished physiologically, but which may have a common mechanism. In rapidly growing cells the mRNA synthesis rates increase linearly with the cell growth, while the rate of stable RNA synthesis is proportional to the square of the growth rate (Gausing, 1977; Nierlich, 1978). This kind of regulation is termed growth-rate control. Cells starved of amino acids exhibit coordinated depression of stable RNA synthesis (stringent control), which coincides with a rapid accumulation of the small effector molecule guanosine tetraphosphate (ppGpp) (Gallant, 1979; Lamond and Travers, 1985a). There is evidence to support the notion that the same mechanism underlies both growth-rate and stringent control (Baracchini and Bremer, 1988; Travers *et al.*, 1986). However, the details of such a mechanism remain elusive.

The seven *Escherichia coli* rRNA operons are transcribed from tandem promoters P1 and P2 (Lindahl and Zengel, 1986). Both promoters are regulated in a different manner. It has been shown that stringent as well as growth-rate control are linked to sequences close to the promoter P1 but not P2 (Glaser *et al.*, 1983; Sarmientos and Cashel, 1983). Sequence comparison of all known stringent regulated promoters revealed the presence of a highly conserved GC-rich sequence motif between the −10 region and the transcription start site (Travers, 1980). Indeed, mutagenesis studies of the GC-rich discriminator sequence of the *tufB* gene have demonstrated an altered response to ppGpp *in vitro* (Mizushima-Sugano and Kaziro, 1985). In addition mutations of the discriminator region of the *tyrT* promoter alter both stringent as well as growth-rate control, as has been shown by *in vivo* studies (Travers *et al.*, 1986; Lamond and Travers, 1985b).

For the *E.coli* ribosomal RNA promoters the consensus discriminator sequence GCGC is found only in the P1 promoters and not in the P2 promoters. We have changed the response of the *rrnB* promoter P2 to that of stringent control, by creating a promoter-downstream discriminator sequence. This was achived by a single base substitution (A to G at position −6). We were interested to see whether such a sequence would be sufficient or necessary to confer stringent control *in vivo*. Furthermore, we set out to determine if and how stringent and growth-rate control are affected by the same DNA determinant. In addition we created an identical GCGC discriminator sequence downstream from the synthetic *tac* promoter (de Boer *et al.*, 1983). The response by the two modified promoters to alterations in the amino acid availability as well as different growth conditions was determined *in vivo* by measuring the activity of a fused chloramphenicol acetyltransferase gene (CAT). The results indicate that the GCGC discriminator sequence is a necessary but not sufficient prerequisite for stringent and growth-rate control. These findings are discussed with respect to a common mechanism for both types of regulation.

Results

Construction of plasmids with mutant promoters

Site-directed mutagenesis was used to create a consensus discriminator motif GCGC downstream of the −10 region in both the *rrnB* P2 and the *tac* promoter. The mutagenesis was performed according to Kunkel (1985), and the base changes verified by dideoxy sequencing (Sanger *et al.*, 1977). Identical DNA fragments with the wild-type or the mutated promoters were fused to the CAT gene present in the vector pKK232-8 (Brosius, 1984). They were designated pP1, pP2, pP2F, pPtacW and pPtacM. Figure 1 shows the sequences of the wild-type and mutated promoters with the base changes underlined.

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                    -35                 -10
P1    : AAATTTCCTCTTGTCAGGCCGGAATAACTCCCTATAATGCGCCACCAC
P2    : AAATAAATGCTTGACTCTGTAGCGGGAAGGCGTATTATGCACACCCCG
P2F   : AAATAAATGCTTGACTCTGTAGCGGGAAGGCGTATTATGCGCACCCCG
PtacW : AAATGAGCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATT
PtacM : AAATGAGCTGTTGACAATTAATCATCGGCTCGTATAATGCGCGGAATT
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Fig. 1. Primary sequences of the different promoter constructs. The −35, −10 and the discriminator motifs are marked accordingly. Mutated bases are underlined.

Efficiency of modified promoters under conditions of amino acid starvation

The stringent response of CP78 cells transformed with the plasmids pP1, pP2, pP2F, pPtacW and pPtacM was induced by addition of valine to cultures grown in a medium lacking valine and isoleucine (Gourse *et al.*, 1983). Transcription from the different promoters was determined by measuring CAT mRNA levels before and after amino acid starvation. To compensate for any differences in the plasmid copy numbers and extraction efficiencies of the individual clones, the activity of the β-lactamase (BLA) gene, also encoded on the vector pKK232-8, was used as an internal control (Klotsky and Schwartz, 1987) [the BLA promoter is known not to be under stringent control (Travers, 1980)]. The CAT and BLA mRNA levels were determined by Northern analysis 1 min before and 15 min after the addition of valine (see Figure 2A). Figure 2B shows a diagram of the CAT to BLA mRNA ratio for the various clones. It can be seen that promoter P1 shows the expected stringent repression (pP1), while the promoter P2 does not change its activity upon amino acid starvation. However, the mutated promoter P2 with the stringent discriminator sequence (pP2F) shows the same stringent repression as promoter P1. By contrast, the same discriminator sequence linked to the synthetic *tac* promoter (pPtacM) did not behave significantly differently from the wild-type *tac* promoter (pPtacW). The results show that a single base change did effectively alter the response of the ribosomal RNA promoter P2 to amino acid starvation, while an identical sequence linked to the synthetic *tac* promoter had no such effect.

Growth rate dependence of the CAT expression from different clones

Although the P2F mutant with the GCGC discriminator sequence shows stringent control regulation it still remained to be determined if the same sequence motif GCGC downstream from the −10 region would also confer growth-rate regulation. To answer this question we have studied the transcription efficiencies of the mutant promoters at various growth rates. The CAT and BLA activities were determined after transformants had been grown in different media to an optical density (OD_{600}) of 0.3−0.4 (see Materials and methods). The activity of the BLA gene was again used as internal standard. Figure 3 gives the CAT to BLA activity ratio as a function of growth rates for the different clones determined in the strain CP78. The transformant containing the promoter P1 shows the expected increase in promoter activity with increasing growth rates, while no such increase was detected for the promoter P2. Note, however, that the relative activity of the promoter P1 at all growth rates was

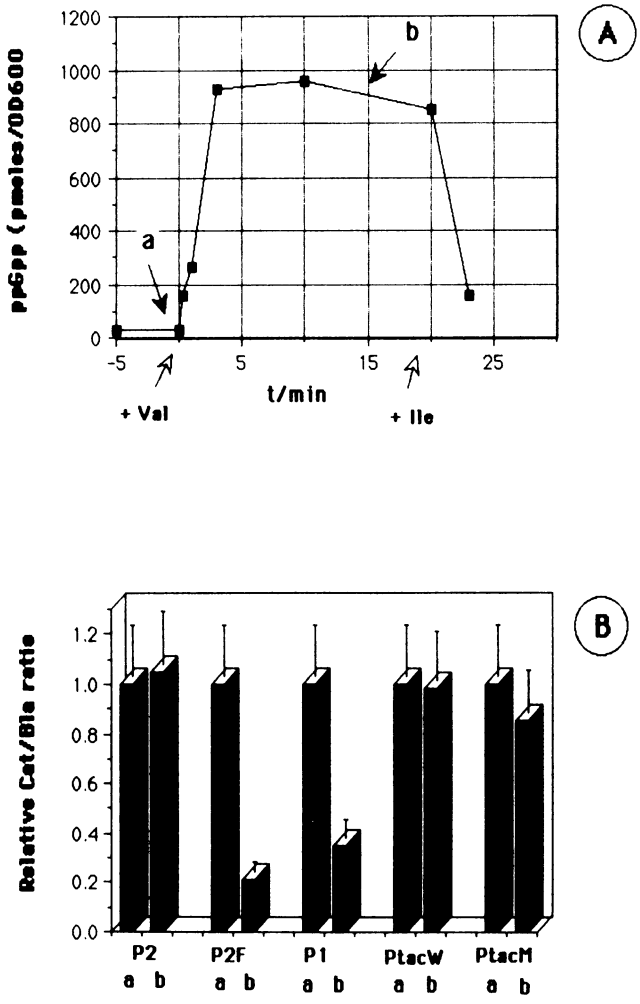

Fig. 2. (**A**) Time course of the cellular ppGpp level after amino acid starvation in CP78 cells transformed with pPtacW. a and b mark the exact time points when aliquots were withdrawn either before (a) or after (b) starvation. (**B**) CAT/BLA mRNA ratios of the different promoter constructs before (a) and 15 min after (b) amino acid starvation. The values before starvation are in every case set to one and the ratios after starvation are calculated accordingly. The BLA mRNA levels did not vary with the physiological conditions as has been shown before by Lamond and Travers (1985b). mRNA determinations were reproducible within an error rate of ≤20% as indicated by the error bars.

much lower than that of P2. This might be due to the fact that P1 was cloned without the complete upstream activating sequence (nucleotides −50 to −80), which is known to increase the activity of P1 by a factor of 10−20 (Gourse *et al.*, 1986). Nevertheless, the construct with promoter P1

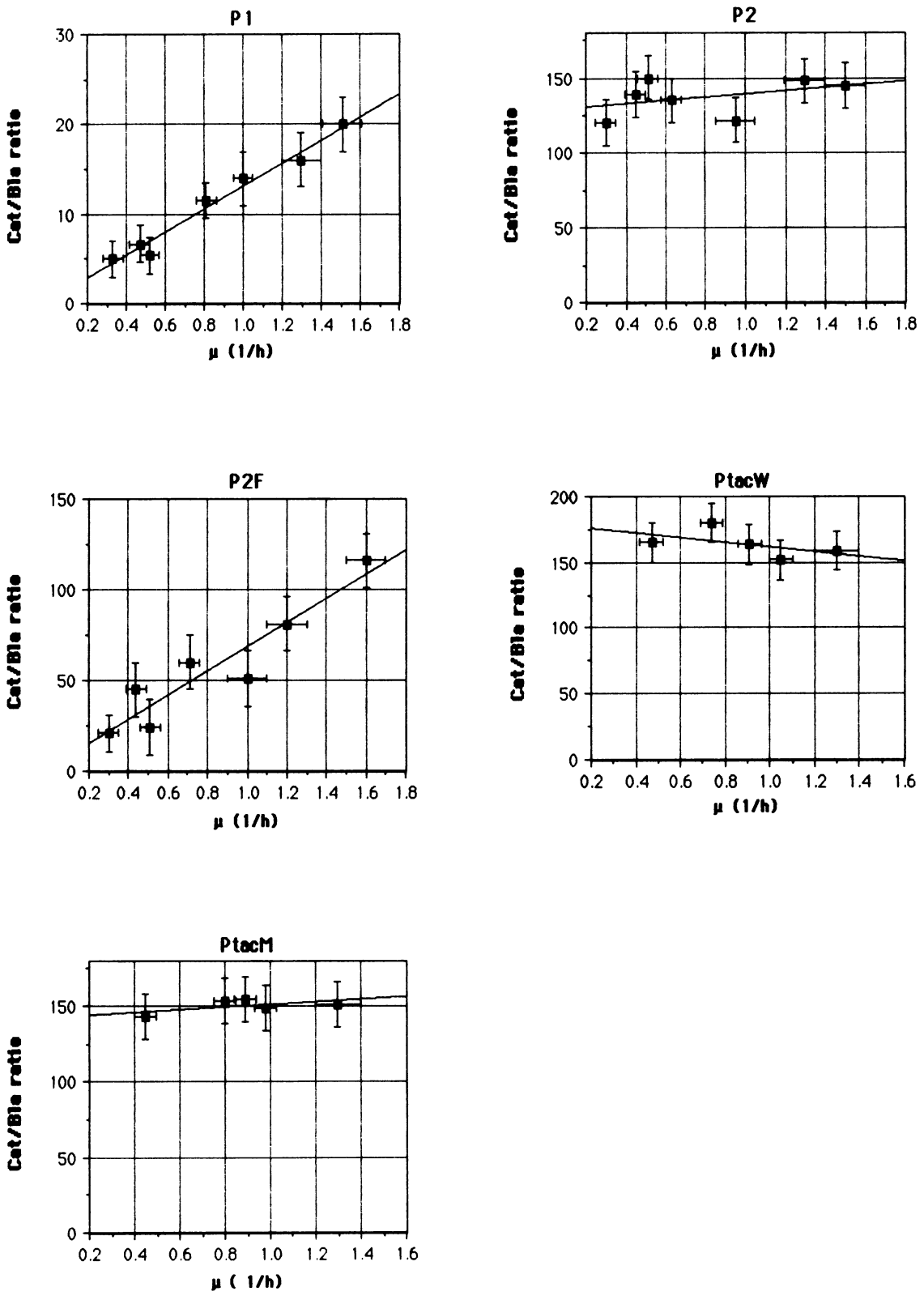

Fig. 3. Diagrams representing the variation of the CAT/BLA ratio of CP78 cells transformed with the different plasmids upon growth rate. The error bars indicate the standard deviations of a minimum of three independent measurements from different extracts. The CAT/BLA ratio is given as described in Materials and methods.

showed a clear growth-rate dependence. In contrast to the promoter P2, cells containing the mutated promoter P2F showed a growth-rate dependent increase in activity, similar to P1 (Figure 3). However, introducing the same discriminator sequence GCGC, into the synthetic *tac* promoter did not result in a growth-rate dependent response (Figure 3). Growth-rate control is known to occur in *relA*$^-$ strains as well (Ryals *et al.*, 1982). In support of this, P2F was also growth-rate regulated in the isogenic *relA*$^-$ strain CP79 (data not shown).

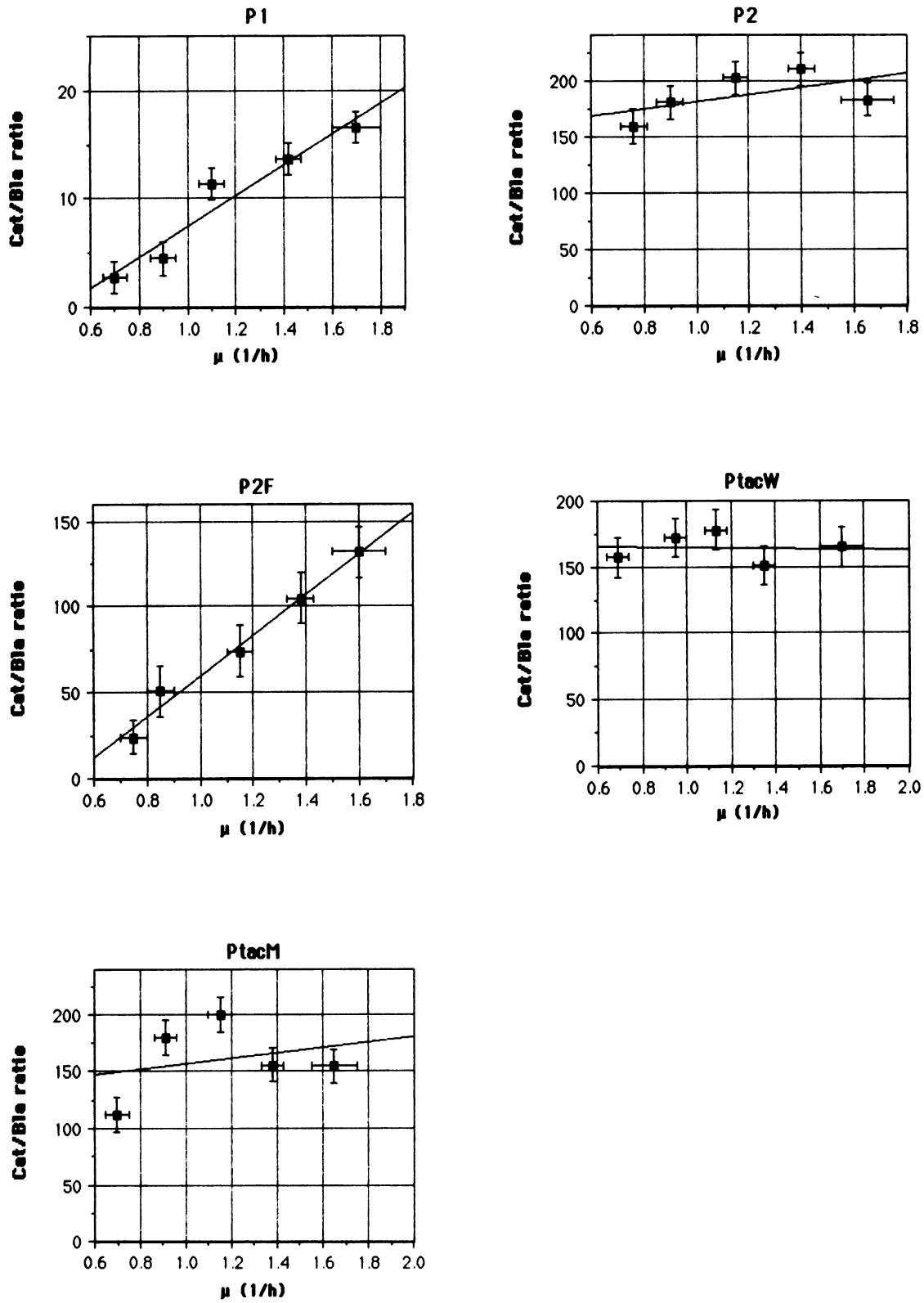

Fig. 4. Diagrams showing the CAT/BLA ratios of different *spoT* mutant strains transformed with the various plasmids. All strains were grown in the same medium and have cell-doubling times in the order of CF946 > CF945 > CF944 > CF943 > CF898, representing the order of the individual data points. Standard deviations were calculated from three or four independent measurements and are indicated as error bars.

Effect of the variation of the cellular ppGpp level by spoT mutations on the activity of the different promoters

The *E.coli* K12 strains CF943–946 (Sarubbi *et al.*, 1988) contain mutations in the coding region of the *spoT* gene necessary for the degradation of ppGpp. Due to these mutations they contain different levels of ppGpp (15–60 pmol/OD_{600}; see Figure 5) and grow at different rates in the same medium. Figure 4 shows that changes of growth rates can actually be obtained by using the different *spoT*

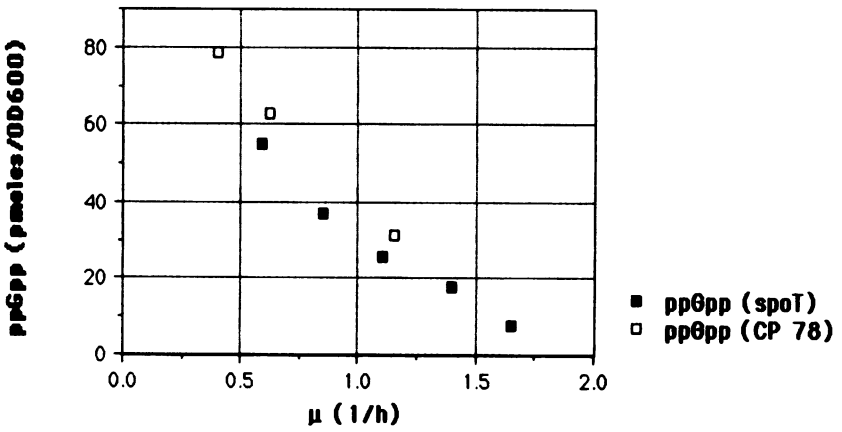

Fig. 5. The figure shows the growth-rate dependency of the cellular ppGpp concentration for both the *spoT* strains (filled squares) growing in one medium and strain CP78 (open squares) grown in different media (see Materials and methods). All bacteria were transformed with the pPtacW episome. The ppGpp level was determined following the method of Cashel (1969). For ppGpp concentrations >50 pmol/OD_{600} the reproducibility of the values was better than 10–15%, whereas for concentrations lower than 50 pmol/OD_{600} the relative error increased up to 20–25%.

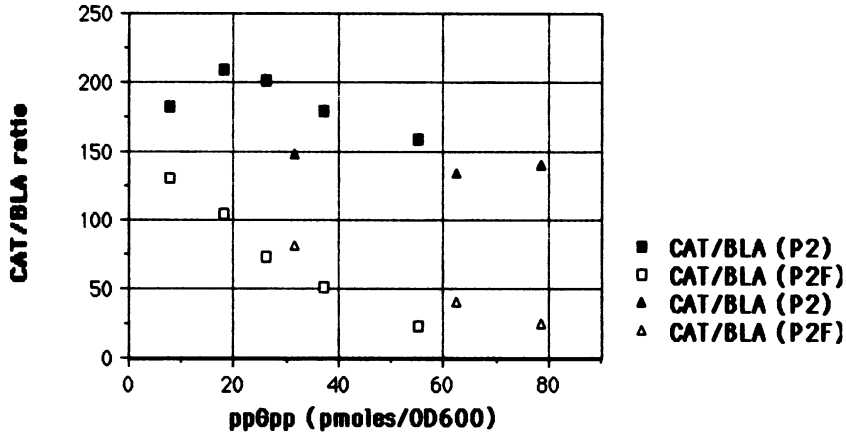

Fig. 6. The diagram shows the promoter strength of the promoters P2 and P2F as a function of the cellular ppGpp level. The CAT/BLA ratios as well as the ppGpp concentrations were taken from the data points presented in Figures 3–5.

mutants transformed with the different plasmids. Again, as is evident from the determination of the CAT to BLA activity ratio, clear growth-rate regulation was observed for the promoters P1 and P2F. Thus, the same results were obtained whether growth-rate variations were achieved using different media or by the use of different *spoT* mutants (cf. Figures 3 and 4). As can be seen in Figure 5, there is a good inverse correlation between the different growth rates and the ppGpp level, independent of whether the different rates were achieved by *spoT* mutations or different media (see Sarubbi *et al.*, 1988). In Figure 6 the promoter activities of P2 and P2F are compared as a function of the cellular ppGpp concentration. The mutated promoter P2F showed a much greater repression in activity at higher ppGpp concentration by comparison to P2. The results strongly support the notion that ppGpp in combination with the discriminator sequence is not only the mediator for stringent control but also for growth-rate regulation. Apparently, the regulatory effect is independent of the manner in which the ppGpp level is altered.

Discussion

We were able to show that a selected point mutation in the *E.coli rrnB* P2 promoter motif was sufficient to make the promoter growth-rate regulated and sensitive to amino acid starvation. The mutation therefore, not only affected promoter activity but renders an unregulated promoter into a regulated one. The fact that the single point mutation conferred both stringent sensitivity and growth-rate control strongly supports the assumption that the two regulation modes are based on the same mechanism.

Although the sequence downstream from the −10 region is an important prerequisite for both growth-rate and stringent control the mutated *tac* promoter did not show a comparable regulative response. Sequence comparison of all known stable RNA promoters with that of the altered *tac* promoter, reveals that the regulated promoters in contrast to PtacM, always contain some G or C residues around the transcription start site. However, because there is no apparent additional primary structure homology the above data indicate that higher-order structures around the transcription start of stable RNA promoters are responsible for the regulation. Our results indicate that at least in the case of the *rrnB* P2 promoter neither the promoter upstream sequences nor alterations in the spacing between the −35 and −10 sequences, which had been suggested to affect growth-rate regulation (Gourse *et al.*, 1988), are of primary importance. We cannot exclude, however, that in addition to the GCGC discriminator sequence topological factors comprising the entire promoter region are crucial. In this context it should be noted that the *tac* promoter has a

consensus −35 region as opposed to the P1 or P2 promoters. This might explain the results obtained with the *tac* promoter mutant PtacM. To solve this question it is necessary to study the higher-order structure of the different promoters.

The correlation of the growth-rate regulation by ppGpp on P2F and P1 supports the RNA polymerase partition model (Ryals and Bremer, 1982; Little *et al.*, 1983). According to this hypothesis one form of RNA polymerase is modified by the binding of ppGpp and has a low affinity for stable RNA promoters. This form is highly abundant at low growth rates and during amino acid starvation. The mutated P2 promoter has a low affinity for the ppGpp-containing RNA polymerase and is therefore suppressed at low growth rates and under conditions of amino acid starvation. The fact that the correlation between the P2F activity and the ppGpp level is independent from the way the different ppGpp levels were achieved, in addition to the finding that both modes of regulation were altered, makes it highly evident that ppGpp is the ultimate effector molecule for stable RNA synthesis.

Materials and methods

Strains and media

All bacterial strains used in this study were *E.coli* K12 derivatives. RZ1032 (*ung*$^-$, *dut*$^-$; Kunkel, 1985), CP78 (*relA*$^+$), CP79 (isogenic to CP78 but *relA*$^-$; Fiil and Friesen, 1968). Strains CF943−946 contain different *spoT* mutations, CF898 (isogenic to CF946−943 but *spoT*$^+$) (Sarubbi *et al.*, 1988). The different media were derived from MOPS medium (Neidhardt *et al.*, 1974) and substituted with various carbon sources [succinate 0.2% (w/v), acetate 0.2% (w/v), glycerol 0.2% (v/v) or glucose 0.2% (w/v)] as well as casamino acid concentrations between 0.05 and 1% (w/v). The phosphate concentration was usually 5 mM. Medium SR for the ppGpp determination was MOPS containing 0.2 mM phosphate and 100−200 μCi [^{32}P]orthophosphate (Amersham), 0.2% (w/v) glucose and 40 μg/ml of all amino acids except valine and isoleucine. Medium S was MOPS supplemented with 0.2% (w/v) glucose and 0.2% (w/v) casamino acids.

Plasmids

The plasmids constructed for the analysis of promoter efficiency were derivatives of the promoter test vector pKK232-8 (Brosius, 1984), which contains the CAT reporter gene. pP1 was constructed by cloning a *DraI*−*DdeI* fragment isolated from the plasmid pKK3535 (position 1177−1275; Brosius *et al.*, 1981) into the *SmaI* and *HindIII*-restricted pKK232-8, together with a *HindIII*−*DdeI* adaptor. In the case of pP2 and pP2F a *DdeI* fragment of pKK3535 (position 1275−1353) containing either P2 or P2F was made blunt with Klenow polymerase and cloned into the *SmaI* site of pKK232-8. pPtacW and pPtacM were cloned as *Sau*3A fragments (376 bp) containing the *tac* promoter (from pKK223; position 4334−4589; Brosius, 1984) and a small portion of the *lacZ* gene (121 bp) from M13mp9 into the *BamHI* site of pKK232-8. The presence of the mutations and the correct orientations of the promoter fragments were verified by restriction enzyme analysis. M13 single-stranded probes for either the CAT mRNA or the BLA mRNA were generated by cloning parts of the corresponding genes into M13mp8 or mp9.

Site-directed mutagenesis

The point mutation at position −6 (A to G) of the *rrnB* P2 promoter and the base changes at −7 and −5 relative to the transcription start of the *tac* promoter were introduced by oligonucleotide-directed mutagenesis (Kunkel, 1985). Mutagenic oligonucleotides were synthesized automatically, using β-cyanoethyl phosphoramidite chemistry.

Assay for CAT

Cells were lysed according to Zacharias and Wagner (1989) and the CAT-activity level was determined as the rate of chloramphenicol acetylation according to Gorman *et al.* (1982) with [^{14}C]chloramphenicol. Acetylated reaction products were separated from non-reacted material on silica gel thin-layer plates. The rate is given as nanomoles acetylated chloramphenicol synthesized per minute and normalized to a cell density equivalent 1 OD_{600}.

β-Lactamase activity measurements

The activity of β-lactamase was measured according to Lupski *et al.* (1984). One BLA unit is defined as the decrease in optical density at 255 nm per minute of a 0.1 M Cephalosporine solution and finally normalized to a cell density equivalent 1 OD_{600}.

Determination of ppGpp

The ppGpp level was determined according to Cashel (1969).

CAT and BLA messenger RNA determination

10 ml of cells grown to 0.3−0.5 OD_{600} were suspended in a mixture of 7.5 ml ethanol, 2 ml 3 M NaOAc solution and 0.5 ml phenol (cooled to −70°C). After centrifugation at 12 000 *g* for 10 min the cells were resuspended in 0.5 ml lysozyme solution (5 mg/ml) at 0°C. After 2 min 50 ml of 10% SDS and 0.5 ml phenol were added, mixed and heated for 2 min at 65°C. After centrifugation the aqueous phase was collected and the nucleic acids were precipitated with ethanol. Up to 20 μg of the isolated nucleic acids were either directly analysed by Northern blot analysis according to Maniatis *et al.* (1982), using CAT gene and BLA gene fragments as hybridization probes. These probes were labelled with ^{32}P by the random primer method (Feinberg and Vogelstein, 1984) prior to the hybridization. To improve the resolution of the Northern blot 20−30 μg of the extracted RNA were hybridized with single-stranded M13 CAT or BLA probes prior to the electrophoresis (2 h in 6 × SSC) followed by a RNase T1 (100 μg/ml, 20 min, 37°C) and S1 digestion (40 U/ml, 20 min, 37°C). The resulting RNA−DNA hybrids were then analysed in the same way as the Northern analysis described above. Amounts of CAT mRNA and BLA mRNA were determined by counting the radioactivity of the CAT or BLA corresponding bands. The BLA mRNA was determined for corrections of the hybridization efficiencies and differences in plasmid copy number.

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