RpoS-dependent Promoters Require Guanosine Tetraphosphate for Induction Even in the Presence of High Levels of σ^S

Received for publication, February 28, 2000, and in revised form, March 22, 2000
Published, JBC Papers in Press, March 24, 2000, DOI 10.1074/jbc.C000122200

Kristian Kvint, Anne Farewell, and Thomas Nyström†
Department of Cell and Molecular Biology-Microbiology, Göteborg University, Box 462, 405 30 Göteborg, Sweden

RpoS-dependent promoters require ppGpp for induction in the stationary phase. This has been thought to be a simple consequence of σ^S itself requiring ppGpp for its production. By using four model promoters requiring σ^S for normal induction in the stationary phase, we demonstrate that σ^S-dependent promoters require ppGpp even in the presence of high levels of σ^S produced ectopically. Similar to σ^{30}-dependent promoters under positive control by ppGpp, the requirement of σ^S-dependent promoters for this alarmone is bypassed by specific “stringent” mutations in the β-subunit of RNA polymerase. The results suggest that stationary phase induction of both σ^S- and σ^{30}-dependent genes requires the stringent control modulon and that stringency confers dual control on the RpoS regulon by affecting promoter activity and the levels of the required σ-factor.

Cells of Escherichia coli elicit stringent control of ribosome production during amino acid starvation (1, 2). This control encompasses a rapid reduction in superfluous rRNA biosynthesis during cellular starvation (3). The effectors of the stringent control module are guanosine tetraphosphate, ppGpp, which, by binding to RNA polymerase (RNAS)^1 (4), causes a rapid reduction in rRNA transcription, probably by reducing the stability of the open promoter/RNAP complexes at rRNA promoters (5, 6). The alarmone ppGpp can also act as a positive effector of gene expression, and a large number of σ^{30}-dependent genes require this nucleotide for their induction during stationary phase and starvation (7, 8). For example, many operons encoding amino acid biosynthetic pathways require ppGpp for their transcription, and cells lacking ppGpp (relA spoT double mutants) are polyauxotrophic (7).

In addition, mutants lacking functional relA spoT or the stationary phase σ-factor, σ^S, have been found to express similar phenotypes; this has been explained by the fact that ppGpp-deficient mutants fail to induce the RpoS regulon, which, in turn, was demonstrated to be because σ^S itself requires ppGpp for its production (9–11). Thus, the failure of relA spoT mutants to induce a number of different σ^S-dependent genes, such as bolA and cfp, has been argued to be the result of the low levels of σ^S in these mutants (12, 13). If this notion is correct, it would be possible to restore stationary phase induction of σ^S-dependent genes in relA spoT mutants by ectopic overproduction of σ^S. We demonstrate here that this is not the case and that the σ^S-dependent model promoters analyzed, PuspB, bolAP1, Pefα, and PpadL, all require ppGpp for their induction even in the presence of wild-type levels of σ^S. Moreover, the requirement of these promoters for ppGpp is suppressed by the same σ^{30}-dependent promoters independent of ppGpp for their induction during stasis.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Growth Conditions—The E. coli strains used in this work are listed in Table I. Cultures were grown in liquid M9 defined medium (14) anaerobically in Erlenmeyer flasks in a rotary shaker at 37 °C. The media were supplemented with glucose (0.08%), thiamine (10 μM), and all 20 amino acids in excess (15). When appropriate, kanamycin (50 μg/ml), chloramphenicol (30 μg/ml), rifampicin (150 μg/ml), tetracycline (20 μg/ml), carbenicillin (100 μg/ml), and/or isopropyl-β-D-thiogalactopyranoside (200 μM) were added.

General Methods—P1 transductions and plasmid transformations were done as described previously by Miller (16) and Sambrook et al. (14). Plasmid purification was performed according to the manufacturer’s instructions (Qiagen, Inc.).

Measurement of β-Galactosidase Activities—Relative β-galactosidase levels were determined as described by Miller (16) with modifications (17). The β-galactosidase activity is expressed as: 1000 × A_{600}/A_{600} culture × reaction time × sample volume. All experiments were repeated several times to ensure reproducibility, and the variation was less than 10%.

Measurement of σ^S Levels—SDS-polyacrylamide gel electrophoresis was performed as described by Sambrook et al. (14), and proteins were transferred to a polyvinylidene difluoride filter (Immobilon-P, Millipore) by semi-dry blotting (Schleicher and Schuell). σ^S was quantitated from Western blots of cell extracts using anti-σ^S antibodies provided by Dr. Hengge-Aronis and a peroxidase chemiluminescence kit (Roche Molecular Biochemicals).

RESULTS

PuspB Is Directly Dependent on Stringent Conditions for Induction—The uspB gene has a single, σ^S-dependent promoter that is induced when cells enter stationary phase (18, 19), and we report here that this induction requires ppGpp (Fig. 1A). We have confirmed that the stringent response (ppGpp accumulation) requires SpoT in this medium rather than RelA (i.e. relA+), relA1, and ΔrelA mutants all give identical results). The dependence of this σ^S-dependent gene on ppGpp confirms previous data (9–11) demonstrating that induction of σ^S-dependent genes and accumulation of σ^S are partly dependent on ppGpp. However, by introducing into our reporter strains the high copy number plasmid pMMKatF2 (20) that carries the structural gene for rpoS, we demonstrate that the uspB promoter cannot be rescued by producing σ^S at close to, or somewhat higher than, wild-type levels in the relA spoT mutant (Fig. 1, B and C). It should be noted that pMMKatF2 fully rescues the uspB promoter in an rpoS null mutant (19). Thus, to be induced, PuspB requires ppGpp in concert with the Ω^R holoenzyme.

This paper is available online at http://www.jbc.org
The Dual Requirement for ppGpp and Eσ70 Is Not Specific for PusB—To investigate whether the dual requirement for ppGpp and σ70 of PusB was promoter-specific, we tested the following σ70-dependent genes: bolA, cfa, and fadL. The bolA P1 promoter (used in this work) is strictly dependent on σ70 and ppGpp for stationary phase induction in vivo (Fig. 2A), whereas the cfa gene has two promoters, one of which, P1, is σ70-dependent and the other, P2, σ70-dependent (21). We used a construct of the region containing both promoters fused to lacZ and moved this construct into the λ att site of E. coli. When using this single-copy chromosomal fusion, we noted that about 75% of the stationary phase induction of cfa requires σ70 and that a residual induction occurs in the absence of this sigma factor, presumably because of transcription from P1 (Fig. 2B). Similar to cfa, stationary phase induction of a chromosomal fadL-lacZ fusion depends on both σ70 and σ70 in LB medium (18). However, in the defined medium used in this work, the stationary phase induction of fadL is primarily dependent on σ70 (Fig. 2C). As seen in Fig. 2, all of the promoters analyzed require ppGpp for induction, and none are rescued by σ70 overproduction in a relA spoT mutant. It is noteworthy that the residual induction of cfa observed in the absence of σ70 requires ppGpp (Fig. 2B, see below).

The Requirement for ppGpp Is Suppressed by the “Stringent” rpoB3449 Allele—The ppGpp dependence of Eσ70-dependent promoters can be totally or partially suppressed by the rpoB3449 allele (22, 23), which renders the RNA polymerase partly constitutively stringent in the absence of ppGpp. We tested whether this allele could alleviate the ppGpp requirement also of the σ70-dependent promoters analyzed. We found that this was the case, although the degree of suppression was promoter-dependent (Fig. 3). For instance, we noticed that the rpoB3449 allele up-regulated promoter activity somewhat also during exponential phase, at least for three of the promoters tested, which may be expected because the rpoB3449 mutant is constitutively “stringent.” To determine whether this activity was the result of the positive stringent control of σ70-associated Rnap, we introduced a rpoS null allele into the otherwise wild-type strain and into the rpoB3449 strain. As seen in Fig. 4, the levels of expression of cfa-lacZ in the rpoS rpoB3449 strain is suppressed by the rpoB3449 allele, whereas the levels of expression of fadL-lacZ are barely changed. This suggests that the σ70-independent promoters are not affected by the rpoB3449 allele and that the σ70-dependent promoters are.”
double mutant were identical to the levels of expression in the rpoB3449 mutant in exponential phase. Thus, the high level of expression of this fusion in the rpoB3449 strain during growth is due to Eσ^70 transcription, whereas the induction in stationary phase is dependent primarily on σ^70 (Fig. 4). Finally, it should be noted that the residual expression of cfa-lacZ in an rpoS mutant is also stationary phase inducible because of ppGpp-dependent transcription using Eσ^70.

DISCUSSION

Many Eσ^70-dependent genes of E. coli are increasingly expressed as cells enter stationary phase and a large fraction of the corresponding promoters are positively regulated by ppGpp. We demonstrate here that this requirement for ppGpp also holds for Eσ^70-dependent promoters even in the presence of high levels of σ^70.

It is not completely understood how ppGpp activates transcription from some promoters while inhibiting transcription from other (stringent promoters), notably rRNA, promoters. Negative control by ppGpp is suggested to result from the dissociation of ppGpp-programmed RNAP from stringent promoters (22) because ppGpp drastically reduces the stability of the open complex with RNAP (5, 6). The rrrF1 promoter forms an intrinsically unstable open complex with RNAP; such promoters may be argued to be especially sensitive to the destabilizing effects of ppGpp (5, 6). In addition, the rpoB alleles that render the cell partly constitutively stringent form an RNAP that destabilizes promoter open complexes in vitro (5, 6, 22). Thus the β-mutant RNAPs mimic the effects of elevated ppGpp levels, which explains why rRNA promoters are down-regulated in cells expressing this mutant RNAP.

However, the reduction in the half-life of promoter open complexes by ppGpp or β-mutant RNAPs occurs also for promoters that are positively regulated by ppGpp (6). Possibly, as suggested by Gourse and co-workers (5, 6), a reduction in the stability of promoter open complexes can give rise to both negative and positive effects depending on the intrinsic kinetic properties of the target promoter. The half-lives of complexes of some promoters that are positively regulated by ppGpp and RNAP have been reported to be extremely long, and one could argue that this may be a kinetic problem that is mitigated by ppGpp. If this were the entire role of ppGpp in the positive regulation of these promoters, one might expect that ppGpp would also work as a positive factor of transcription from these promoters in an in vitro assay. However, as far as we know, this

**FIG. 2.** Expression patterns of lacZ fusions of bolA1, Pefu, and PfadL during growth and stationary phases. β-Galactosidase activities (closed symbols) and cell density (open symbols) are shown. A, bolA1-lacZ in relA1 (AD99, circles), relA1 ΔspoT (KK159, triangles), relA1 ΔspoT/pMMK27 (KK160, diamonds), and rpoS::Tn10 (KK243, squares). B, Pefu-lacZ in relA1 (AD218, circles), ΔrelA ΔspoT (KK229, triangles), ΔrelA ΔspoT/pMMK27 (KK229, diamonds), and rpoS (KK226, squares). C, PfadL-lacZ in relA1 (LS1349, circles), ΔrelA ΔspoT (KK235, triangles), ΔrelA ΔspoT/pMMK27 (KK229, diamonds), and rpoS (KK226, squares).

**FIG. 3.** A mutated RNAP can suppress the lack of induction of PusPB, bolA1, Pefu, and PfadL in a ppGpp^8 background. A, PusPB-lacZ expression in relA1 (AF633, circles), ΔrelA ΔspoT (KK189, squares), and ΔrelA ΔspoT rpoB3449 (KK206, triangles) strains. B, bolA1-lacZ expression in relA1 (AD99, circles), relA1 ΔspoT (KK159, squares), and relA1 ΔspoT rpoB3449 (KK210, triangles) strains. C, Pefu-lacZ expression in relA1 (AD218, circles), ΔrelA ΔspoT (KK229, squares), and ΔrelA ΔspoT rpoB3449 (KK292, triangles) strains. D, PfadL-lacZ expression in relA1 (LS1349, circles), ΔrelA ΔspoT (KK235, squares), and ΔrelA ΔspoT rpoB3449 (KK236, triangles) strains.
FIG. 4. Pgal-lacZ expression requires stringency and σ^70 for full induction when cells enter stationary phase. β-Galactosidase activities (closed symbols) and cell density (open symbols) are shown in strains carrying rpoS (KK226, circles), rpoB3449 (KK224, squares), and rpoS rpoB3449 (KK227, triangles).

is not always the case. There are some examples of a positive effect of ppGpp on gene expression in vitro, but these experiments were performed using a coupled transcription translation assay (24–27).

It is possible that the effects of ppGpp on promoters that apparently require this alarmone for induction are due to more indirect effects, including ppGpp-dependent changes in RNA polymerase availability. According to a model by Zhou and Jin (22), RNAP recruitment is the rate-limiting step of promoters that are positively regulated by ppGpp and these promoters, therefore, are argued to be very sensitive to the concentration of free RNA polymerase. As elaborated above, the accumulation of ppGpp is suggested to result in the dissociation of RNAP from stringent promoters (22), and as a consequence, more RNAP becomes available to initiate transcription at promoters that have a relatively poor ability to recruit RNAP. Following the assumptions of this model, we would argue that all promoters induced during conditions of stringency, regardless of their dependence on different σ-factors, would require ppGpp for their induction; the results presented in this paper could be explained within the framework of the Zhou and Jin (22) model. For example, up-regulation of the σ^70-dependent promoters (which may have a relatively poor ability to recruit RNAP) in cells expressing the β- mutant RNAP may result from a higher level of free RNAP in these cells. In addition, Hernandez and Cashel (28) have shown that the amounts of σ^70 bound to E (RNAP core) is elevated in ppGpp\(^\beta\) cells and that a mutation in rpoD, which is epistatic to ppGpp-deficient defects, lowers the amount of E\(^\sigma^70\). Thus, it is possible that stringent conditions allow alternative σ-factors to compete more successfully for E by lowering the programming of E with σ^70.

Finally, ppGpp may directly or indirectly aid in the ability of σ^70 to transcribe σ^70-dependent promoters. σ^70 works poorly in in vitro transcription systems (29), which may indicate that other factors are needed for σ^70-dependent transcription. For example, the presence of polyphosphate, elevated levels of potassium glutamate, and the degree of supercoiling of the DNA template have been suggested to influence σ^70-dependent transcription (29, 30, 31, 33). The stringent response may alter any of these variables or other unknown factors and thus affect the ability of σ^70 to transcribe its cognate genes (e.g. Ref. 34). Future work in our laboratory will use both in vitro and in vivo methods to distinguish between these models.

Acknowledgments—Ding Jun Jin, Concetta C. DiRusso, Regine Henge-Aronis, Mårten Hammar, Miki Jishage, Mike Cashel, and Alfredo Diez are gratefully acknowledged for their contributions of strains, plasmids, and antibodies essential for this work.

REFERENCES
1. Sands, M. K., and Roberts, R. B. (1952) J. Bacteriol. 65, 505–511
2. Stent, G. S., and Brenner, S. (1961) Proc. Natl. Acad. Sci. U. S. A. 47, 2065–2014
3. Cashel, M., Gentry, D. R., Hernandez, V. J., and Vinella, D. (1996) in Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology (Neidhardt, F. C., Ingraham, J. L., Low, K. B., Magasanik, B., Schaechter, M., and Umbarger, H. E., eds) pp. 1458–1496, American Society for Microbiology Press, Washington, D. C.
4. Chatterji, D., Fujita, N., and Ishihama, A. (1998) Genes Cells 5, 279–287
5. Bartlett, M. S., Gaal, T., Ross, W., and Gourse, R. L. (1998) J. Mol. Biol. 279, 331–345
6. Gourse, R. L., Gaal, T., Aiyar, S. E., Barker, M. M., Estrem, S. T., Hirveno, C. A., and Ross, W. (1998) Cold Spring Harbor Symp. Quant. Biol. 63, 131–139
7. Xiao, H., Kalman, M., Ikehara, K., Zemel, S., Glaser, G., and Cashel, M. (1991) J. Biol. Chem. 266, 5980–5990
8. Nyström, T. (1994) Mol. Gen. Genet. 245, 355–362
9. Gentry, D. R., Hernandez, V. J., Nguyen, L. H., Jensen, D. B., and Cashel, M. (1993) J. Bacteriol. 175, 7862–7869
10. Lange, R., Fischer, D., and Henge-Aronis, R. (1995) J. Bacteriol. 177, 4676–4680
11. Zgurskaya, H. I., Keyhan, M., and Matin, A. (1997) Mol. Microbiol. 24, 643–651
12. Eichel, J., Chang, Y., Riesenberg, D., and Cronan, J. E., Jr. (1999) J. Bacteriol. 181, 572–576
13. Lange, R., and Henge-Aronis, R. (1991) J. Bacteriol. 173, 4474–4481
14. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., pp. 1.82–1.84, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
15. Albertson, N. H., and Nystroem, T. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 180, 2172–2182
16. Miller, J. (1972) Experiments in Molecular Genetics, pp. 201–205, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
17. Albertson, N. H., and Nyström, T. (1994) FEMS Microbiol. Lett. 117, 181–188
18. Farewell, A., Kvint, K., and Nyström, T. (1998) Mol. Microbiol. 29, 1039–1051
19. Farewell, A., Kvint, K., and Nyström, T. (1998) J. Bacteriol. 180, 6140–6147
20. Mulvey, M. R., Sorby, P. A., Triggs-Raine, B. L., and Loewen, P. C. (1988) Mol. Microbiol. 2, 212–222
21. Miller, J. (1972) Experiments in Molecular Genetics, pp. 201–205, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
22. Zhou, Y. N., and Jin, D. J. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 2988–2993
23. Kvint, K., Hosbond, C., Farewell, A., Nybroe, O., and Nyström, T. (2000) Mol. Microbiol. 35, 435–443
24. Stephens, J. C., Arzt, S. W., and Ames, B. N. (1975) Proc. Natl. Acad. Sci. U. S. A. 72, 4389–4393
25. Primackoff, P., and Arzt, S. W. (1979) Proc. Natl. Acad. Sci. U. S. A. 76, 1726–1730
26. Riggs, D. L., Mueller, R. D., Kwan, H. S., and Arzt, S. W. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 9333–9337
27. Choy, H. E. (2000) J. Biol. Chem. 275, 6783–6789
28. Hernandez, V. J., and Cashel, M. (1995) J. Mol. Biol. 252, 536–549
29. Kusano, S., Ding, Q., Fujita, N., and Ishihama, A. (1996) J. Biol. Chem. 271, 1998–2004
30. Ding, Q., Kusano, S., Villarejo, M., and Ishihama, A. (1995) Mol. Microbiol. 4, 649–656
31. Ohiaka, K. L., and Gralla, J. D. (1992) Mol. Microbiol. 6, 2243–2251
32. Jin, D. J., and Gross, C. A. (1988) J. Mol. Biol. 202, 45–58
33. Shiba, T., Tsutsui, K., Yano, H., Ibara, Y., Kameda, A., Tanaka, K., Takahashi, H., Munekata, M., Raa, N. N., and Kornberg, A. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11210–11215
34. Rao, N. N., Liu, S., and Kornberg, A. (1998) J. Bacteriol. 180, 2186–2193
35. DiRusso, C. C., Metzger, A. K., and Heimert, T. L., (1993) Mol. Microbiol. 7, 311–322
