Binding of the Transcription Effector ppGpp to Escherichia coli RNA Polymerase Is Allosteric, Modular, and Occurs Near the N Terminus of the β′-Subunit*

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Among the prokaryotes, the nucleotide ppGpp is a second messenger of physiological stress and starvation. The target of ppGpp is RNA polymerase, where it putatively binds and alters the enzyme’s activity. Previous data had implicated the β′-subunit of Escherichia coli RNA polymerase as containing a single ppGpp binding site. In this study, a photocross-linkable derivative of ppGpp, 6-thioguanosine-3′,5′-(bis)pyrophosphate (6-thio-ppGpp), was used to localize the ppGpp binding site. In vitro transcription assays, 6-thio-ppGpp inhibited transcription from the argT promoter identically to bona fide ppGpp. The thio group of 6-thio-ppGpp is directly photoactivatable and is thus a zero-length cross-linker. Cross-linking of RNA polymerase was directed primarily to the β′-subunit and could be competed efficiently by native ppGpp but not by GTP or GDP. Cyanogen bromide digestion analysis of the cross-linked β′-subunit was consistent with an extreme N-terminal cross-link. To assess allosteric consequences of ppGpp binding to RNA polymerase, high level trypsin resistance in the presence and absence of ppGpp was monitored. Trypsin digestion of RNA polymerase bound to ppGpp leads to protection of an N-terminal fragment of the β′-subunit and a C-terminal fragment of the β-subunit. We propose that the N terminus of β′ together with the C terminus of β constitute a modular ppGpp binding site.

Within the bacterial domain of the kingdom prokaryotaee, there is a general and ubiquitous response to nutritional and environmental stress, the stringent response (1). This general stress response is mediated by high level accumulation of the transcription effector guanosine-3′,5′-(bis)pyrophosphate (ppGpp). A major effect of elevated ppGpp levels is an immediate and severe reduction of stable rRNA and tRNA gene transcription (2). The cessation of stable RNA syntheses halts the major energy consuming activities of the cell, transcription and translation. This period of metabolic inactivity allows the cell to utilize its remaining energy reserves to adapt to stressful growth conditions through induction of specific “stress genes” (3). Once adaptation is near completion, ppGpp levels decrease and growth resumes. Failure to reduce ppGpp levels results in a severe reduction of cell viability (4).

Several lines of evidence suggest that ppGpp exerts its effects by directly binding to RNA polymerase (RNAP). Certain rifampicin-resistant mutants of the β′-subunit of RNAP display increased intracellular sensitivity to ppGpp (5, 6). Spontaneously occurring mutants that confer survival under artificial and prolonged exposure to toxic levels of ppGpp were mapped to the rpoB gene, encoding the β-subunit of RNAP (7). Mutant strains devoid of ppGpp, ppGpp² strains, are incapable of surviving nutritional deprivation; however, specific mutants of the σ⁷₀-, β′-, or β′-subunits of RNAP restores normal survivability to ppGpp² strains (8). Fluorescence quenching studies of RNAP in the presence of increasing concentrations of a fluorescently labeled ppGpp analogue (1-aminonaphthalene-5-sulfonate-ppGpp) is consistent with binding of ppGpp to a single binding site on RNAP (9). Cross-linking analyses by Chatterji et al. (10) using a radioactive photocross-linkable derivative of ppGpp, 8-azidoguanine-3′,5′-(bis)pyrophosphate (8-azido-ppGpp), demonstrated predominant cross-linking of ppGpp to the β′-subunit. In the same study (10), it was also observed that both N- and C-terminal partial trypsin digestion fragments of the β′-subunit were cross-linked by 8-azido-ppGpp, suggesting a modular ppGpp binding site analogous to that of the nucleotide binding site at the catalytic center of RNAP (11). Despite extensive studies on RNAP-ppGpp interactions, a precise localization of the ppGpp binding site on RNAP is lacking.

An allosteric mechanism of ppGpp action on RNAP is generally invoked as mediating its transcriptional effects, although this has not been extensively studied. In this context, allostery refers to the inducement of functionally relevant conformational changes of RNAP as a result of ppGpp binding at a location other than the catalytic site. Consistent with this notion, ribonucleoside triphosphates do not compete with ppGpp for RNAP binding (12). In addition, circular dichroism studies have revealed a small but significant change in total α-helical content of RNAP following addition of ppGpp (13). To date, this single previous study (13) represents the only physical evidence of induced conformational change of RNAP by ppGpp. Clearly, further studies characterizing the nature of ppGpp allostery are warranted.

The aim of the present investigation is 2-fold: 1) to substantiate and refine the location of the ppGpp binding site on E. coli RNAP; and 2), to begin to elucidate the nature of ppGpp-induced conformational changes of RNAP. Toward the first of these goals, we synthesized a new photocross-linkable derivative...
ppGpp Binding to the β′-Subunit of E. coli RNA Polymerase

EXPERIMENTAL PROCEDURES

Enzymes and Chemicals—Escherichia coli RNA polymerase was prepared according to Cashel (18). Highly purified 6-thio-ppGpp was separated and characterized by Sepharose chromatography (17). Crude RelA-ribosome preparations were prepared according to Cashel (18). Chemicals and solvents were reagent grade, used without further purification, and purchased from Sigma-Aldrich. All radioactive nucleotides were obtained from ICN Pharmaceuticals Inc.

Synthesis of ppGpp and Derivatives—Synthesis conditions for ppGpp were as described (18). For synthesis of radioactive ppGpp, 0.5 mCi of [γ-32P]ATP (4500 Ci/mmol) were diluted with 1 mM cold ATP and 0.5 mM GDP or 6-thioguanosine-5′-triphosphate (6-thio-GTP) in a 0.1 ml reaction volume mixed with 60 A260 units of crude RelA-ribosomes at room temperature for 12 h. Final purification of ppGpp was on a QAE-Sephadex A-25 column as described (19). Nucleotides were typically eluted with a 0–0.5 M linear gradient of triethylammonium bicarbonate, pH 7.5. Elution products were visualized by autoradiography after separation by polyethyleneimine-cellulose thin layer chromatography (20). It was noted that 6-thioguanosine derivatives chromatographed with lower mobility on polyethyleneimine-cellulose plates than the corresponding guanosine containing compounds. The ppGpp-containing fractions were pooled and concentrated through removal of the reaction volume mixed with 60 mM GDP or 6-thioguanosine-5′-triphosphate as described previously (23). Final ppGpp was purified by gel filtration on a 2.5×107 Molar mass column in a Tris-glycine buffer system.

Trypsin Digestion Studies—RNA polymerase (0.25 μM) was incubated for 15 min at room temperature with 200 μM of either GDP or ppGpp in 20 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM magnesium acetate, 5% glycerol, 0.1 mM EDTA. After a 2-min warming at 37 °C, samples were mixed with increasing amounts of tosyl-l-phenylalanine chloromethylketone-treated trypsin (0, 0.1, 0.3, 0.6, 1.2, and 2.5 μM) in a 20-μl reaction volume and incubated at 37 °C for an additional 5 min. Trypsin digestions were stopped by the addition of phenylmethylsulfonyl fluoride to a final concentration of 10 mM, immediately followed by the addition of 5× SDS-sample buffer to a 1× concentration and incubation at 100 °C for 3 min. Total trypsin fragment were resolved in a 6× SDS-12% polyacrylamide gel and visualized by colloidal Coomassie G-250 staining (24). Specific trypsin fragments were identified by Western blot analyses using monoclonal antibodies against N-terminal, middle, and C-terminal epitopes of β′ (7RC78, 7RC74, and NT73, respectively) and β′ (7RB145, 7RB135, and NT63, respectively) (Ref. 25 and courtesy of Richard Burgess, University of Wisconsin-Madison). Reactive bands were visualized by chemiluminescence using the Renaissance Western blot Chemiluminescence Reagent Plus detection kit (PerkinElmer Life Science Products).

Imaging and Quantification—Radioactive samples were visualized by autoradiography and, when necessary, imaged on a Bio-Rad Molecular Imager and quantified using the Molecular Analyst software (Bio-Rad Laboratories Inc.).

Matrix-assisted Laser Desorption/Ionization Time-of-flight Mass Spectrometry (MALDI-TOF MS)—Protein fragments were excised from Coomassie-stained gels and analyzed at Borealics Biosciences Inc., Toronto Canada by MALDI-TOF MS.

RESULTS

Synthesis and Structure of 6-Thio-ppGpp—The nucleotide derivative 6-thioguanosine-5′-triphosphate (6-thio-GTP) was synthesized from 6-mercaptoguanosine and used as a substrate in the enzymatic reaction depicted in Reaction 1, to yield 6-thioguanosine-3′,5′-(bis)pyrophosphate (6-thio-ppGpp; Fig. 1). The reaction occurs in two steps, using crude RelA-ribosomes (see “Experimental Procedures”).

6-thio-GTP + ATP → 6-thio-pppGpp + AMP (Step 1)

6-thio-pppGpp → 6-thio-ppGpp + P (Step 2)

REACTION 1

The reactivity of 6-thio-GTP was comparable with GTP in the RelA-mediated reaction (data not shown). The conversion of ppGpp to ppGpp is mediated by the enzyme guanosine pentaphosphatase, which is present in crude RelA-ribosome preparations (18). Highly purified 6-thio-ppGpp was separated and

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obtained as described by Cashel (18).

The structure of purified 6-thio-ppGpp was verified by nuclear magnetic resonance (NMR). Both phosphorus (31P) and proton (1H) spectra of bona fide ppGpp and 6-thio-ppGpp were comparable and indicative of very similar structures (Fig. 1). Unlike the proton NMR spectra reported for 6-thio-GTP (14), 6-thio-ppGpp shows a strong chemical shift specific for a mercapto group proton (Fig. 1). Thus, in solution at pH 4.0, most of 6-thio-ppGpp bears a mercapto group at position 6 of the guanine moiety rather than a thio as depicted in Fig. 1. The predominance of the mercapto over the thio form of 6-thio-ppGpp may simply be because of the acidic pH under which NMR was performed. This is unclear, however, because the absolute pKₐ of the thio group of 6-thio-ppGpp is unknown, and sufficient material was not obtained to be able to determine this experimentally. Because the photoreactive conformation of the 6-thioguanosine moiety is the thio form, cross-linking to RNA polymerase is only possible if the bound form of 6-thio-ppGpp is in the thio form and not the mercapto form, as appears to be the case (Fig. 3).

Transcription Activity of 6-Thio-ppGpp—To test the activity of 6-thio-ppGpp compared with ppGpp, their ability to inhibit transcription of the tRNA promoter, P₁argT, was assayed. The activity of the argT promoter is negatively affected by increased ppGpp levels in vitro (26). Here, we demonstrate for the first time a specific inhibition of P₁argT in vitro by ppGpp under standard ionic and transcription conditions. At low ionic conditions, <50 mM KCl, the inhibitory effects of ppGpp were diminished (data not shown). A 4–6-fold inhibition of RNA synthesis from P₁argT was observed at the highest concentration of ppGpp tested (Fig. 2). As an internal control, transcription from a P₄lacUV₅ template was monitored in an equimolar mixed template reaction. The transcription from P₄lacUV₅ was also inhibited between 20–35%, as previously noted (27) but ppGpp preferentially inhibited transcription from P₁argT. The inhibition of transcription of P₁argT by 6-thio-ppGpp paralleled that of ppGpp.

Cross-linking of 6-Thio-ppGpp to RNA Polymerase—Increasing amounts of radioactive 6-thio-ppGpp were mixed with 1 μM RNA polymerase and subjected to photoactivating ultraviolet light irradiation. Electrophoretic analysis of radioactively labeled RNAP on denaturing polyacrylamide gels revealed the presence of cross-linker on all RNAP subunits. At limiting concentrations of cross-linker, however, labeling appeared predominant for β- and/or β'-subunits (Fig. 3A). Further cross-linking experiments were all performed at an equimolar concentration of RNAP and 6-thio-ppGpp (1 μM ea). In competition experiments, cross-linking of β- and β'-subunits was dramatically reduced in the presence of a 200-fold excess of cold genuine ppGpp but not GTP (Fig. 3B). Electrophoresis of photoaffinity-labeled RNAP on low percentage denaturing 4% polyacrylamide gels to resolve β- and β'-subunits showed that the majority (~90%) of label was associated with the β' subunit (Fig. 3C). Competition with cold ppGpp had little effect on residual β'-subunit cross-linking, but it essentially eliminated cross-linking of the β'-subunits (Fig. 3D). No competition with 6-thio-ppGpp binding was observed in experiments with either GTP or GDP (Fig. 3D). In addition, we observed no effect on cross-linking in the presence of nonspecific or promoter containing DNA, RNA, or rifampicin (data not shown).

Mapping of 6-Thio-ppGpp Cross-linking on the β'-Subunit of RNAP—The labeled β'-subunit shown in Fig. 3C was excised and extracted from the 4% polyacrylamide gel and subjected to partial CNBr digestion. Following digestion, the fragments were resolved on a gradient polyacrylamide gel and visualized by phosphorimaging. The actual partial CNBr digest is shown in Fig. 4 in comparison with idealized computer-generated profiles of a single-hit partial CNBr digest of β'. Ideализed digestions were generated assuming either extreme C- or N-terminal labeling of β', respectively. The profile of the experimental CNBr digestion fragments aligns well with the idealized CNBr N-terminally labeled fragment ladder, particularly the three very specific bands of 102, 130, and 152 amino acid residues (Fig. 4). The pattern and intensity of bands is comparable with what has been obtained previously for N-terminally labeled β' (28). These results are consistent with 6-thio-ppGpp cross-linking between amino acid residues 29 and 102 of the β'-subunit of RNAP, because no label was found in association.
ppGpp Binding to the β'-Subunit of E. coli RNA Polymerase

Fig. 3. Cross-linking of 6-thio-ppGpp to RNAP. All cross-linking reactions were performed with 1 μM RNAP and radioactively labeled 6-thio-ppGpp. A, increasing concentrations of 6-thio-ppGpp were incubated with RNAP, as indicated, followed by photocross-linking and fractionation on a denaturing 10% polyacrylamide gel. B, 1 μM 6-thio-ppGpp was competed with 200 μM GTP or ppGpp prior to photocross-linking and fractionation as described in A. C, six duplicate cross-linking reactions with 1 μM 6-thio-ppGpp and RNAP were performed followed by fractionation on a denaturing 4% polyacrylamide gel to resolve β- and β'-subunits. D, 1 μM 6-thio-ppGpp was competed with 200 μM GTP, GDP, or ppGpp prior to photocross-linking and fractionation as described for panel C.

with the extreme N-terminal 29 amino acid residue fragment.

ppGpp-dependent Trypsin-resistant Fragments of RNAP—To begin to explore the allosteric consequences of ppGpp binding to RNAP, resistance to trypsin proteolysis was used to probe for ppGpp stabilized domains of RNAP. Limiting proteolysis has been used as a means of defining subtle alterations in the conformation of RNAP upon promoter binding (28) as well as for the definition of major RNAP structural domains (30). In contrast, we employed in this study an excess of trypsin to cleave RNAP cross-linked with radioactive 6-thio-ppGpp did not give a clear or consistent pattern of digestion; we conjecture that this failure was the result of technical problems, e.g. an interference with trypsin activity by the presence of nonspecific UV-induced cross-links. Perhaps in future experiments, the exact identification of the conformational change of RNAP induced following 6-thio-ppGpp cross-linking may be achieved using methods other than trypsin digestion to probe for changes in protein conformation.

The identity of the tryptic fragments stabilized in the presence of ppGpp was determined by Western blot analyses. The trypsinized samples shown in Fig. 5A were processed for Western analyses and probed with a battery of monoclonal antibodies (mAb) against various epitopes of the β- and β'-subunits (see "Experimental Procedures"). The β'-subunit-specific mAb, 7RC78, recognizes an epitope located between amino acid residues 115 and 236. Western blots of trypsin fragments with 7RC78 reveals that the 100- and 28-kDa ppGpp-protected fragments contain the N terminus of the β'-subunit (Fig. 5B). The β-subunit-specific mAb, NT63, recognizes an epitope between amino acid residues 922 and 1099 and is the most C-terminal epitope recognized by available RNAP mAbs (Burgess and colleagues (15, 16, 25, 29, 37)). Western blot analysis with NT63 is consistent with the 40kDa fragment containing the C terminus of β (Fig. 5C). Given the fact, however, that the epitope of NT63 is a considerable distance from the actual C terminus of the β-subunit, it was difficult to make a definitive C-terminal assignment to this fragment. For this reason, MALDI-TOF MS analysis was performed on the 40-kDa trypsin stable fragment. MALDI-TOF MS analysis (see "Experimental Procedures") indicated that the 40-kDa β fragment spans amino acid residues 958–1328 ± 10. Thus, the C-terminal assignment of the 40-kDa β-subunit band was confirmed. Assignment of the approximate end points of the 28-kDa fragment by MALDI-TOF MS was not successful because of contamination by identical size fragments of the α-subunit. Fortunately, the close proximity of the β'-specific mAb, 7RC78, allows confident N-terminal assignment to the trypsin stable 28-kDa fragment.
ppGpp Binding to the β'-Subunit of E. coli RNA Polymerase

ppGpp at the sixth position of the guanine moiety at pH 4.0 (Fig. 1). At this same pH level, a mercapto group was not observed for the precursor, 6-thio-GTP (14). Thus, the presence of the 3'-pyrophosphate on ppGpp may change the local chemistry of the thio group on the guanine ring. No enol proton was detectable in *bona fide* ppGpp, indicating that this property is unique for the thiolated ppGpp. It is not clear which is the preferred form of 6-thio-ppGpp at pH 7.9, at which crosslinking and transcription analyses were performed. Equilibrium competition experiments and relative affinity measurements of 6-thio-ppGpp compared with ppGpp could not be performed because of the nonequilibrium conditions necessary for efficient 6-thio-ppGpp cross-linking (see “Experimental Procedures”). If 6-thio-ppGpp at pH 7.9 is predominantly in the mercapto conformation, however, it does not appear to affect dramatically its efficacy in inhibiting transcription (Fig. 2). Fundamental chemistry dictates that the thio and not the mercapto conformation of the 6-thioguanosine moiety is subject to photoactivation. With this in mind, the observed cross-linking of RNA by 6-thio-ppGpp (Fig. 3) is consistent with a preferred binding to RNA of the thio form of 6-thio-ppGpp. It is likely, therefore, that in natural ppGpp the keto group at position 6 of the guanine moiety is important for the binding of ppGpp to RNA.

Predominant cross-linking of radioactively labeled 6-thio-ppGpp occurs within the first 102 amino acid residues of the N terminus of the β'-subunit of RNA polymerase (Figs. 3 and 4). This location overlaps with the conserved region A of β' (β′ₐ). In the recently solved crystal structure model of *Thermus aquaticus* RNA polymerase, the extreme N terminus of the β'-subunit, including β′ₐ, is disordered and lacks electron density (32). The crystal data together with our finding that ppGpp binds to and induces trypsin resistance to the N-terminal portion of β' (Fig. 5B) leads us to propose that ppGpp binding induces a higher order structure of this region.

The N terminus of β' is implicated as playing a crucial role in many aspects of the transcription process. Region β′ₐ contains a zinc finger, which is essential for stable DNA association of the elongating transcription complex (33) and has been crosslinked to the double-stranded DNA at the lagging end of the transcription bubble (34). Additionally, within the transcription complex the extreme N terminus of the β'-subunit cross-links along the entire length of the “extruded” nascent RNA (34, 35). This close proximity of the extruded RNA to the N terminus of β' is “replaced” in a paused transcription complex by an alternate proximity of the RNA to the so-called “flap” structure of the β-subunit (36). This repositioning or “switching” of the path of RNA within the complex is thought to be part of the mechanism of transcriptional pausing (36). Finally, a primary determinant of σ⁷₀ binding is found between amino acid residues 260 and 309 of the β'-subunit (37). These multifaceted functions associated with the N terminus of β' will be considered below in light of the putative binding of ppGpp near this region and the known effects of ppGpp.

The binding of ppGpp to RNA polymerase has been proposed to destabilize specifically the open complex of rRNA promoters (27, 38). Thus, ppGpp binding and restructuring of the region of RNA polymerase near the N terminus could disrupt the function of the β′ₐ zinc finger and/or σ⁷₀ interaction and could lead to collapse of the open complex. Another well documented effect of ppGpp is that it decreases transcription elongation rates, primarily by increasing the pause times at naturally occurring pause sites (12, 39–41). Because the path of nascent RNA, extruded across the N terminus of β' in elongating RNA polymerase, is altered to putatively bring about transcriptional pausing (36), ppGpp binding might directly influence this process. Thus, the binding of ppGpp to

**DISCUSSION**

The global stress regulator, ppGpp, a ligand and effector of RNA polymerase, has been studied since its discovery by Cashel and Gallant (31) over 30 years ago, yet little is known about how it binds to and modifies RNA polymerase. Here, we report the synthesis of a novel photocross-linkable derivative of ppGpp, 6-thioguanosine-3',5'-bispyrophosphate. This cross-linkable ppGpp derivative has allowed us to obtain the first indication that ppGpp binds to the N terminus of the large subunit of RNA polymerase, β'ₐ. This compound has the distinct advantage over preceding cross-linkable ppGpp derivatives of carrying a zero-length cross-linking group. The chemical structure of 6-thio-ppGpp in solution, confirmed by NMR spectroscopy, reveals a predominance of the mercapto group (analogous to an enol group in ppGpp) rather than the thio group (analogous to a keto group in ppGpp) at the sixth position of the guanine moiety at pH 4.0 (Fig. 1). At this same pH level, a mercapto group was not observed for the precursor, 6-thio-GTP (14). Thus, the presence of the 3'-pyrophosphate on ppGpp may change the local chemistry of the thio group on the guanine ring. No enol proton was detectable in *bona fide* ppGpp, indicating that this property is unique for the thiolated ppGpp. It is not clear which is the preferred form of 6-thio-ppGpp at pH 7.9, at which cross-linking and transcription analyses were performed. Equilibrium competition experiments and relative affinity measurements of 6-thio-ppGpp compared with ppGpp could not be performed because of the nonequilibrium conditions necessary for efficient 6-thio-ppGpp cross-linking (see “Experimental Procedures”). If 6-thio-ppGpp at pH 7.9 is predominantly in the mercapto conformation, however, it does not appear to affect dramatically its efficacy in inhibiting transcription (Fig. 2). Fundamental chemistry dictates that the thio and not the mercapto conformation of the 6-thioguanosine moiety is subject to photoactivation. With this in mind, the observed cross-linking of RNA by 6-thio-ppGpp (Fig. 3) is consistent with a preferred binding to RNA of the thio form of 6-thio-ppGpp. It is likely, therefore, that in natural ppGpp the keto group at position 6 of the guanine moiety is important for the binding of ppGpp to RNA.

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**FIG. 5.** Trypsin-resistant fragments of RNA polymerase in the presence of ppGpp. Increasing concentrations of trypsin, as indicated, were incubated with 250 nM RNA polymerase for 5 min and fractionated on denaturing 10% polyacrylamide gels. A, Coomassie-stained gel of total protein. Known proteins and the position of migration of relative molecular weight markers are indicated. The major trypsin-resistant fragments are indicated by asterisks. B, Western blot of the same total protein samples as shown in A probed with monoclonal antibody 7RC78, which recognizes an epitope between amino acid residues 115 and 236 of the β'-subunit. C, Western blot of the same total protein samples as shown in A probed with monoclonal antibody NT63, which recognizes an epitope between amino acid residues 922 and 1090 of the β'-subunit.
ppGpp Binding to the β'-Subunit of E. coli RNA Polymerase

this particular region of RNAP may be linked mechanistically to many of the observed effects of ppGpp-RNAP interactions.

Previous results (see the Introduction) have indicated that ppGpp binding is localized to the β-subunit of RNAP. However, in this study we have determined that the N terminus of the β'-subunit is in close proximity to bound ppGpp. Although our results seemingly conflict with these previous observations, upon closer examination they actually corroborate earlier data concerning ppGpp-RNAP binding. We have synthesized and used for the first time a zero-length cross-linking ppGpp derivative, 6-thio-ppGpp. Preceding studies (10) used an azido group cross-linker, which replaces the proton at position 8 of the guanine moiety of ppGpp with an azido group (-N₃). The thio group at position 6 of the guanine moiety of 6-thio-ppGpp is 8–10 Å distant from the azido group of 8-azido-ppGpp. Given the considerable separation of these two cross-linking groups, the thio group of 6-thio-ppGpp predictably would be in a different location and thus provide unique cross-links compared to 8-azido-ppGpp. Our trypsin resistance studies (Fig. 5) are congruent with the formation of a highly structured N-terminal interface comprising the N- and C-terminal portions of the β'- and β-subunits, respectively.

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REFERENCES

1. Cashel, M., Gentry, D. R., Hernandez, V. J., and Vinella, D. (1996) *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology* (Niedhardt, F. C., Curtiss, R., Ingraham, J. L., Lin, E. C. C., Low, K. B., Magasanik, B., Reznikoff, W. S., Riley, M., Schaechter, M., and Umbarger, H. E., eds) 2nd Ed., pp. 1458–1496, American Society for Microbiology, Wash. D. C.

2. Ryals, J., Little, R., and Bremer, H. (1982) *J. Bacteriol.* **151**, 1261–1268.

3. Gentry, D. R., Hernandez, V. J., Nguyen, L. H., Jensen, D. B., and Cashel, M. (1993) *J. Bacteriol.* **175**, 7982–7989.

4. Aizenman, E., Engelberg-Kalpa, H., and Glaser, G. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 6059–6063.

5. Little, R., Ryals, J., and Bremer, H. (1983) *J. Bacteriol.* **154**, 787–792.

6. Little, R., Ryals, J., and Bremer, H. (1983) *J. Bacteriol.* **155**, 1162–1170.

7. Tedin, K., and Bremer, H. (1992) *J. Biol. Chem.* **267**, 2537–2544.

8. Hernandez, V. J., and Cashel, M. (1995) *J. Mol. Biol.* **252**, 536–549.

9. Reddy, P. S., Raghaven, A., and Chatterji, D. (1995) *Mol. Microbiol.* **15**, 255–265.

10. Chatterji, D., Fujita, N., and Ishihama, A. (1996) *Genes Cells* **3**, 279–287.

11. Mustaev, A., Kozlov, M., Markovtsov, V., Zaychikov, E., Denissova, L., and Goldfarb, A. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 6641–6645.

12. Kingston, R. E., Newman, W. C., and Chamberlin, M. J. (1981) *J. Biol. Chem.* **256**, 2787–2797.

13. Woody, A. Y., Woody, R. W., and Malcolm A. D. (1987) *Biochem. Biophys. Acta* **909**, 115–125.

14. Sergiev, P. V., Lavrik, I. N., Wlassoff, V. A., Dokudovskaya, S. S., Dentsouva, O. A., Boganov, A. A., and Brimacombe, R. (1997) *RNA* **3**, 464–475.

15. Burgess, R. R., and Jendrisak, J. J. (1975) *Biochemistry* **14**, 4634–4638.

16. Lowe, P. A., Hager, D. A., and Burgess, R. R. (1979) *Biochemistry* **18**, 1344–1352.

17. Wellington, S. R., and Spiegelman, G. B. (1991) *Biochem. Biophys. Res. Commun.* **179**, 1107–1114.

18. Cashel, M. (1974) *Annu. Rev. Biochem.* **43**, 100–107.

19. Cashel, M., and Kalbacher, B. (1970) *J. Biol. Chem.* **245**, 2309–2318.

20. Cashel, M. (1990) *Methods Mol. Genet.* **3**, 341–355.

21. Hsu, L. M. (1990) *Methods Enzymol.* **179**, 59–71.

22. Markovtsov, V., Mustaev, A., and Goldfarb, A. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 3221–3226.

23. Grachev, M. A., Lukhtanov, E. A., Mustaev, A. A., Zaychikov, E. F., Abdakayumov, M. N., Rabinov, I. V., Richter, V. I., Shklov, Y. S., and Chistyakov, P. G. (1989) *Eur. J. Biochem.* **180**, 577–585.

24. Neuloff, V., Arold, N., Taube, D., and Elharadit, W. (1988) *Electrophoresis* **9**, 255–262.

25. Thompson, N. E., Hager, D. A., and Burgess R. R. (1992) *Biochemistry* **31**, 7003–7008.

26. Rowley, K. E., Elford, R. M., Roberts, I., and Holmes, W. M. (1993) *J. Bacteriol.* **175**, 1309–1315.

27. Kajitani, M., and Ishihama, A. (1984) *J. Biol. Chem.* **259**, 1951–1957.

28. Nudler, E., Avetissova, E., Markovtsov, V., and Goldfarb, A. (1996) *Science* **273**, 211–217.

29. McMahan, S. A., and Burgess, R. R. (1999) *Biochemistry* **38**, 12424–12431.

30. Borukhov, S., Severinov, K., Kashlev, M., Lebedev, A., Bass, I., Rowland, G. C., Lim P. P., Glass, R. E., Nikiforov, V., and Goldfarb, A. (1991) *J. Biol. Chem.* **266**, 239621–239626.

31. Cashel, M., and Gallant, J. (1969) *Nature* **221**, 838–841.

32. Zhang, G., Campbell, E. A., Minakinni, L., Richter, C., Severinov, K., and Darst, S. A. (1999) *Cell* **98**, 811–824.

33. Nudler, E., Avetissova, E., Markovtsov, V., and Goldfarb, A. (1996) *Science* **273**, 211–217.

34. Korzhova, N., Mustaev, A., Kozlov, M., Malhotra, A., Nikiforov, V., Goldfarb, A., and Darst, S. A. (2000) *Science* **290**, 619–625.

35. Nudler, E., Gusarov, I., Avetissova, E., Kozlov, M., and Goldfarb, A. (1998) *Science* **281**, 424–428.

36. Wang, D, Severinov, K., and Landick, R. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 8433–8438.

37. Arthur, T. M., and Burgess, R. R. (1999) *J. Biol. Chem.* **273**, 31381–31387.

38. Gourse, R. L., Gaal, T., Aiyar, S. E., Barker, M. M., Estrem, S. T., Hirvenen, C. A., and Ross, W. (1998) *Cold Spring Harbor Symp. Quant. Biol.* **63**, 131–139.

39. Vogel, U., and Jensen, K. F. (1994) *J. Biol. Chem.* **269**, 16236–16241.

40. Vogel, U., and Jensen, K. F. (1999) *J. Biol. Chem.* **274**, 18335–18340.

41. Vogel, U., and Jensen, K. F. (1997) *J. Biol. Chem.* **272**, 12265–12271.
