Response of RNA polymerase to ppGpp: requirement for the ω subunit and relief of this requirement by DksA

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Previous studies have come to conflicting conclusions about the requirement for the ω subunit of RNA polymerase in bacterial transcription regulation. We demonstrate here that purified RNAP lacking ω does not respond in vitro to the effector of the stringent response, ppGpp. DksA, a transcription factor that works in concert with ppGpp to regulate rRNA expression in vivo and in vitro, fully rescues the ppGpp-unresponsiveness of RNAP lacking ω, likely explaining why strains lacking ω display a stringent response in vivo. These results demonstrate that ω plays a role in RNAP function (in addition to its previously reported role in RNAP assembly) and highlight the importance of inclusion of ω in RNAP purification protocols. Furthermore, these results suggest that either one or both of two short segments in the β′ subunit that physically link ω to the ppGpp-binding region of the enzyme may play crucial roles in ppGpp and DksA function.

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In Escherichia coli, transcription is carried out by a multi-subunit RNA polymerase (RNAP) composed of six subunits, including two copies of α and one copy each of β, β′, σ, and ω (for a recent review, see Geszvain and Landick 2004). α2, β, β′, and ω comprise core RNAP, which is catalytically active but unable to recognize promoters. The α2 dimer serves as the scaffold on which β and β′ assemble. β and β′ make up the vast majority of RNAP by mass and create the enzyme’s active center. To initiate transcription, one of several types of σ subunits, most commonly σ70, binds to core to form RNAP holoenzyme. σ and α are site-specific DNA-binding proteins that account for specific promoter recognition. Although a high-resolution structure of E. coli RNAP has not yet been determined, X-ray structures of the Thermus aquaticus and Thermus thermophilus holoenzymes (Murakami et al. 2002b; Vassylyev et al. 2002), as well as of a T. aquaticus RNAP holoenzyme–DNA complex (Murakami et al. 2002a), elucidate how the RNAP subunits interact with each other and with template DNA.

ω, encoded by the E. coli rpoZ gene, is the smallest RNAP subunit at only 10 kDa. ω has homologs in all three kingdoms of life. It is present in all sequenced free-living bacteria, such as Chlamydia sp., appear to lack an ω homolog, in archaea (RpoK), and in eukaryotes (RPB6) (Minakhin et al. 2001). The RNAP structures indicate that there is one copy of ω per RNAP, and that it interacts with β′ conserved regions D and G and wraps over and around the β′ C-terminal tail, latching β′ to the α3β subassembly [Minakhin et al. 2001]. The RNAP structures therefore are consistent with the model that ω functions as a chaperone in enzyme assembly by facilitating the binding of β′ to α3β (Gentry and Burgess 1993; Mukherjee et al. 1999; Ghosh et al. 2001, 2003). In support of this view, reconstitution of RNAP from its individual subunits is less efficient in the absence of ω [Mukherjee and Chatterji 1997].

In contrast to the insights that the structures of RNAP provide about a role for ω in enzyme assembly, the structures do not suggest an obvious role for ω in enzyme function. Furthermore, no defects have been reported in the specific activity of RNAP lacking ω or in the interactions of ω-deficient RNAP with transcription factors. Indeed, functional RNAP is produced by standard in vitro reconstitution procedures that do not include ω (Tang et al. 1995), and overproduction of core RNAP in vivo without co-overproduction of ω has been used extensively as a method for producing homogeneous mutant RNAP for structure–function analysis of RNAP in vitro [Artsimovitch et al. 2003, 2004].

In spite of the extraordinary evolutionary conservation of ω and its role in RNAP assembly, E. coli mutants
lacking rpoZ are viable. Some rpoZ mutants grow more slowly than wild type (Mukherjee and Chatterji 1997), but this phenotype has been proposed to result from polar effects on the downstream gene spoT (Gentry and Burgess 1989). SpoT is a bifunctional enzyme that can both synthesize and degrade the global transcriptional regulator guanosine 5’-diphosphate 3’-diphosphate, ppGpp (Xiao et al. 1991). ppGpp (used here to refer to both the tetraphosphate and its pentaphosphate precursor) is synthesized by the RelA and SpoT proteins in response to nutrient starvation (Cashel et al. 1996).

ppGpp strongly and directly inhibits promoters for ribosomal RNA (rRNA) and transfer RNA (tRNA) in vivo (Barker et al. 2001b; Paul et al. 2004a,b; Gralla 2005). In addition, ppGpp both directly and indirectly stimulates a set of promoters that make transcripts coding for enzymes for amino acid biosynthesis and transport (Barker et al. 2001a; Paul et al. 2005). The 151-amino-acid DksA protein binds directly to RNAP and is required for both negative control of rRNA promoters and positive control of amino acid promoters in response to changing concentrations of ppGpp in vivo (Paul et al. 2004a,b; Perederina et al. 2004). Thus, ppGpp and DksA together (ppGpp/DksA) result in a global response to amino acid starvation referred to as the stringent response.

Ishihama and colleagues (Igarashi et al. 1989) found that separation of α from core enzyme by partial urea denaturation appeared to result in RNAP that was insensitive to ppGpp when assayed by transcription in vitro. Addition of refolded α resulted in partial inhibition by ppGpp. These results, in conjunction with the colocalization of rpoZ in the same operon as spoT, led to the conclusion that there was a functional link between α and ppGpp. This conclusion, however, was contradicted by the observation that cells lacking rpoZ still displayed stringent regulation of rRNA transcription in response to amino acid starvation (Gentry et al. 1991). Furthermore, the link between ppGpp and α was further clouded by the limited availability at that time of assays for examining effects of ppGpp in vitro, by subsequent reports that other polypeptides were present in some α preparations (Mukherjee and Chatterji 1997), and by the observation that RNAP lacking α was generally less active than wild-type RNAP (Mukherjee and Chatterji 1997). Finally, the positions of ppGpp and DksA in the RNAP holoenzyme indicate that neither ppGpp nor DksA is located in the positions of ppGpp and DksA in the RNAP holoenzyme (Mukherjee and Chatterji 1997). Finally, the immediate vicinity of the RNAP holoenzyme (Mukherjee and Chatterji 1997). Finally, the observation that RNAP lacking DksA functions, at least in part, by lowering the energy of a transition state, ppGpp decreases multiple-round transcription from rrrB P1 by native E. coli ErT10 RNAP holoenzyme by two- to threefold (Fig. 1A, “native RNAP”; Table 1, rows 1 and 2; Barker et al. 2001b). ppGpp also decreases the half-life (increases the dissociation constant, kD) of competitor-resistant complexes formed on rrrB P1 by two- to threefold (Fig. 1B, Table 1, rows 1 and 2; Barker et al. 2001b). However, when holoenzyme was purified by co-overproduction of α, β, and β’ in vivo (Artsimovitch et al. 2004), and this core enzyme was reconstituted with core enzyme, restores RNAP to its native state with respect to regulation by ppGpp. We further demonstrate that DksA eliminates the α requirement for the response of RNAP to ppGpp, resolving the discrepancy between previous conclusions about the requirement for α for ppGpp function in vitro versus in vivo. In addition to their importance for methodological purposes, these results provide important insights about the mechanism of action of ppGpp/DksA.

**Results**

**RNAP produced by overproduction of core subunits in vivo is insensitive to ppGpp**

ppGpp decreases multiple-round transcription from rrrB P1 by native E. coli ErT10 RNAP holoenzyme by two- to threefold (Fig. 1A, “native RNAP”; Table 1, rows 1 and 2; Barker et al. 2001b). ppGpp also decreases the half-life (increases the dissociation constant, kD) of competitor-resistant complexes formed on rrrB P1 by two- to threefold (Fig. 1B, Table 1, rows 1 and 2; Barker et al. 2001b). However, when holoenzyme was purified by co-overproduction of α, β, and β’ in vivo (Artsimovitch et al. 2004), and this core enzyme was reconstituted with core enzyme (hereafter referred to as “overproduced αββ’ RNAP”), this RNAP did not respond normally to ppGpp (Fig. 1C,D, Table 1, row 5). Specifically, transcription by overproduced αββ’ RNAP was inhibited slightly if at all by ppGpp, and the lifetime of overproduced αββ’ RNAP complexes was the same in the presence and absence of

**DksA compensates for α for ppGpp function**
ppGpp. We found that other RNAP preparations made by overproduction of αβ′ and purified using a chitin affinity tag also were insensitive to ppGpp (data not shown).

Native RNAP lacking the ω subunit is insensitive to ppGpp

Two explanations potentially could account for the inability of overproduced αβ′ RNAP to respond to ppGpp: Overproduction might result in a ppGpp-unresponsive conformation of the enzyme, or overproduced RNAP might be missing some necessary factor.

Since a previous report suggested that ω might play a role in the response to ppGpp (Igarashi et al. 1989), and since overproduced RNAP lacked ω, we tested the effect of ppGpp on native RNAP purified from a strain lacking rpoZ. RNAP purified from an rpoZ::kan strain (Gentry et al. 1991) was unresponsive to ppGpp in both the transcription and RNAP–promoter lifetime assays [Fig. 1E,F, Table 1, row 3], strongly suggesting that the unresponsiveness of overproduced αβ′ RNAP to ppGpp resulted from the absence of ω. E. coli RNAP reconstituted in vitro from individually purified α, β, and σ subunits was also insensitive to ppGpp (data not shown). Furthermore, the native RNAP preparations from the wild-type and rpoZ::kan strains were purified in the form of holoenzyme, in contrast to the overproduced RNAP preparations for which σ was added to core in vitro. Thus, reconstitution of core RNAP with σ in vitro was not responsible for the inability of RNAP lacking ω to respond to ppGpp (cf. Berghofer-Hochheimer et al. 2005).

Co-overproduction of ω with the other core subunits in vivo or reconstitution of ω with overproduced αβ′ RNAP in vitro restores sensitivity to ppGpp

We tested whether co-overproduction of ω with overproduced αβ′ core RNAP in vivo (to make overproduced αβ′ω RNAP) would restore sensitivity to ppGpp by overproduced RNAP. Transcription from rrnB P1 by overproduced αβ′ω RNAP was inhibited 3.6-fold by ppGpp [Fig. 2A; Table 1, row 4], and competitor-resistant complexes were 2.9-fold shorter-lived in the presence of ppGpp [Fig. 2B; Table 1, row 4]. Thus, in contrast to overproduced αβ′ RNAP, overproduced αβ′ω RNAP responded to ppGpp. In the absence of ppGpp, the dissociation constants of RNAP complexes containing overproduced αβ′ (Fig. 1D; Table 1, row 5) or overproduced αβ′ω RNAP (Fig. 2B; Table 1, row 4), measured in parallel on rrnB P1, were virtually identical, indicating that ω did not affect the intrinsic lifetime of the RNAP–promoter complex.

In the experiments reported above [Fig. 2A,B], ω coassembled with core RNAP in vivo before purification. In Figure 2C and D, we tested whether purified ω added in vitro to preassembled core RNAP, followed by addition of σ (overproduced αβ′ω + RNAP), would restore ppGpp sensitivity to RNAP. ppGpp inhibited transcription by the resulting holoenzyme 3.0-fold [Fig. 2C; Table 1, row 6], and it reduced the lifetime of the promoter–RNAP complex 2.7-fold [Fig. 2D; Table 1, row 6]. Thus, inclusion of ω, either by co-overproduction with other core subunits in vivo [Fig. 2A,B], or by addition of ω to
DksA compensates for ω for ppGpp function

Table 1. Effects of ω and ppGpp on transcription inhibition and promoter complex dissociation

| RNAP                                      | Presence of ω | −ppGpp/| +ppGpp| \(k_d \times 10^4\) [sec\(^{-1}\)] (−ppGpp) | \(k_d \times 10^4\) [sec\(^{-1}\)] (+ppGpp) | \(\frac{k_d + \text{ppGpp}}{k_d - \text{ppGpp}}\) |
|-------------------------------------------|---------------|--------|-------|------------------------------------------|------------------------------------------|------------------------------------------|
| Native holoenzyme\(^a\)                  | +             | 2.5 ± 0.5 | 0.24  | 0.53                                    | 2.2 ± 0.36                                |
| Native holoenzyme\(^b\)                  | +             | 2.8 ± 0.3 | 0.15  | 0.43                                    | 2.9                                     |
| Native \(rpoZ::kan\)                     | −             | 1.1 ± 0.03| 0.31  | 0.28                                    | 0.9 ± 0.05                               |
| Overproduced \(α_2β'β\) \(\omega\)\(^d\) | +             | 3.6 ± 0.5 | 0.32  | 0.94                                    | 2.9 ± 0.09                               |
| Overproduced \(α_2β'β\) \(\omega\)\(^e\) | −             | 1.3 ± 0.2 | 0.31  | 0.31                                    | 1.0 ± 0.05                               |
| Overproduced \(α_2β'β\) + \(\omega\)\(^f\) | +             | 3.0 ± 0.4 | 0.15  | 0.41                                    | 2.7 ± 0.19                               |

\(^a\)Native holoenzyme purified by method of Burgess and Jendrisak (1975).
\(^b\)Native holoenzyme purified by immunoaffinity chromatography.
\(^c\)Native holoenzyme purified from \(rpoZ::kan\) strain by immunoaffinity chromatography.
\(^d\)Core RNAP purified by overproduction of \(α_2β'β\) and \(\omega\), reconstituted in vitro with purified \(σ\).
\(^e\)Core RNAP purified by overproduction of \(α_2β'β\), reconstituted in vitro with purified \(σ\) and \(ω\).
\(^f\)Transcription was performed as described in Figure 1 and Materials and Methods. The ratio is the amount of transcription from the \(rrnB\) P1 promoter ± ppGpp. The ratio provided in row 2 is the mean and range from two experiments. All other means and standard deviations are from at least three experiments.
\(^g\)Values were calculated as described in Materials and Methods; representative assays are presented in Figures 1–4. Absolute \(k_d\) values varied slightly from day to day, most likely from slight changes in solution conditions. Effects of ppGpp were always compared directly in the same experiment. Enzymes produced with and without \(ω\) by overproduction (rows 4 and 5) were also compared directly in the same experiment. Associated errors represent standard deviations or ranges as appropriate from two or more experiments.

Previous studies showed that DksA and ppGpp work synergistically to inhibit transcription from rRNA promoters [Paul et al. 2004a]. Since RNAP nevertheless responds to ppGpp in strains lacking \(rpoZ\) [Gentry et al. 1991], we tested whether DksA might be able to restore ppGpp responsiveness to an RNAP preparation lacking \(ω\). \(rrnB\) P1 activity was measured using solution conditions in which ppGpp or DksA by themselves have little or no effects on transcription, but the two together severely inhibit transcription from \(rrnB\) P1 by native RNAP [Paul et al. 2004a]. Figure 5A shows that when both DksA and ppGpp were present, transcription by both overproduced \(α_2β'β\) and \(α_2β'β\) \(ω\) RNAP was strongly inhibited. The DksA concentration dependence of this inhibition [at 400 μM ppGpp] was similar for both RNAP preparations. DksA also rescued the ppGpp insensitivity of native \(α_2β'β\) RNAP made from the \(rpoZ::kan\) strain [data not shown].

To obtain a quantitative estimate of the ability of DksA to rescue the responsiveness of \(α_2β'β\) RNAP to ppGpp, we measured the half-lives of RNAP−lacUV5 promoter complexes at a range of ppGpp concentrations (and 500 nM DksA). The ppGpp concentration dependence of the effect of DksA and ppGpp together on half-life was indistinguishable for the overproduced \(α_2β'β\) and \(α_2β'β\) \(ω\) RNAP preparations [Fig. 5B].

We conclude that DksA restores ppGpp responsiveness to RNAP lacking \(ω\) and that this most likely accounts for the ability of the \(rpoZ\) mutant strain to maintain a stringent response [Gentry et al. 1991]. Strains lacking both \(rpoZ\) and \(dksA\) therefore should not exhibit a stringent response. We constructed the \(ΔrpoZ\ ΔdksA\) mutant, but we could not easily assess further loss of

preassembled purified core RNAP lacking \(ω\), restores the ability of RNAP to respond to ppGpp.

Insensitivity of overproduced RNAP lacking \(ω\) to ppGpp also occurs on non-rRNA promoters

ppGpp reduces the lifetimes of RNAP complexes on all promoters, although it inhibits transcription only from promoters that make intrinsically short-lived complexes [Barker et al. 2001b]. We tested whether \(ω\) affected RNAP’s insensitivity to ppGpp on a different promoter by comparing the behavior of overproduced \(α_2β'β\) RNAP and overproduced \(α_2β'β\) \(ω\) RNAP on the lacUV5 promoter. ppGpp reduced the lifetime of the lacUV5 promoter complex containing \(ω\) 3.8-fold [Fig. 3A], but had little or no effect on the complex formed without \(ω\) [1.2-fold decrease] [Fig. 3B]. Thus, the \(ω\) requirement for a response to ppGpp is not promoter-specific.

\(ω\) is not required for DksA function

DksA directly reduces the lifetimes of promoter complexes formed with RNAP, directly inhibits transcription from rRNA promoters, and greatly increases the effects of ppGpp in vivo and in vitro [Paul et al. 2004a]. Therefore, we next determined whether DksA function, like ppGpp function, requires \(ω\) [Fig. 4]. Transcription from \(rrnB\) P1 using enzymes purified by overproduction of core with and without co-overproduction of \(ω\) \(α_2β'β\) \(ω\) and \(α_2β'β\) RNAP, respectively) was inhibited similarly by DksA at each of several DksA concentrations tested [Fig. 4A, cf. black and gray bars]. Likewise, \(ω\) did not affect the ability of DksA to reduce the lifetimes of \(rrnB\) P1−RNAP complexes [Fig. 4B] or lacUV5−RNAP complexes [Fig. 4C].
rRNA regulation in this double mutant, since rRNA transcription is already derepressed in strains lacking dksA alone (Paul et al. 2004a).

ppGpp not only inhibits rRNA promoters, but it also activates amino acid promoters (Paul et al. 2005). Strains lacking dksA alone are defective in transcription from a subset of amino acid promoters and display complex amino acid requirements, because these promoters are unable to respond normally to ppGpp (Paul et al. 2005). As expected, strains lacking both rpoZ and dksA also displayed complex amino acid requirements [data not shown], strongly suggesting that they are unable to respond normally to ppGpp. Interestingly, the double mutant also displayed additional nutritional requirements compared with the strain lacking dksA alone. Further studies will be needed to address the basis for these phenotypes.

**Discussion**

ω plays a role in RNAP function

We demonstrate here that RNAP holoenzymes lacking ω have a defect in function: They are completely unable to respond to ppGpp. Thus, the absence of ω might also compromise other properties of RNAP. In fact, preliminary data suggest that RNAP lacking ω associates more slowly than wild-type RNAP with the λPc promoter (R. Saeker and M.T. Record Jr., pers. comm.; see also Mukherjee and Chatterji 1997). Caution therefore should be exercised in interpreting results obtained with RNAP lacking ω.

We also show here that DksA, a protein recently discovered to work synergistically with ppGpp in vitro and in vivo (Paul et al. 2004a, 2005), suppresses the deficiency of RNAP lacking ω to respond to ppGpp in vitro. Since strains lacking rpoZ nevertheless still respond to ppGpp (Gentry et al. 1991), the presence of DksA in rpoZ mutant cells in vivo likely explains the previous discrepancy between the apparent requirement for ω for RNAP

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**Figure 2.** ω restores ppGpp sensitivity to overproduced core RNAP. Templates and methods as described in Figure 1. 

(A, B) RNAP made by overproduction of αββ' and ω followed by addition of purified σ in vitro. (C, D) RNAP made by overproduction of αββ' followed by addition of purified ω and σ in vitro. 

(A, C) Multiple-round transcription, 400 μM ppGpp and/or ω included as indicated. Quantitation (means and standard deviations from multiple independent experiments, −ppGpp/+ppGpp) is provided in Table 1. 

(B, D) RNAP–promoter lifetimes. Means and standard deviations of the resulting dissociation constants (k_d) for each RNAP from multiple independent experiments are provided in Table 1. The experiments reported in B and D were not performed in the exact same buffer, and slight differences in solution conditions, rather than the methods by which the holoenzymes were made, account for the differences in the intrinsic dissociation constants [0.32 × 10^{-4} vs. 0.15 × 10^{-4} sec^{-1}; see Materials and Methods].

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**Figure 3.** Insensitivity to ppGpp of RNAP lacking ω is not promoter-specific. The template was a DNA fragment containing the lacUV5 promoter. Half-lives were determined by filter-binding in transcription buffer containing 100 mM KCl in the absence (filled circles) and presence (open circles) of 400 μM ppGpp. Representative experiments are shown. 

(A) RNAP made by overproduction of core [ααββ'] followed by reconstitution with σ. The ratio of the complex lifetime with ppGpp/without ppGpp was 3.8 ± 0.7 (four independent assays).

(B) RNAP made by overproduction of core [ααββ'] without ω followed by reconstitution with σ. The ratio of the complex half-life with ppGpp/without ppGpp was 1.2 ± 0.1 (four independent assays).
Figure 4. ω is not required for DksA function. (A) Single-round transcription from rrnB P1 was performed on a supercoiled plasmid template (using transcription buffer containing 30 mM NaCl; see Materials and Methods) in the presence of different concentrations of purified hexa-histidine-tagged DksA (Paul et al. 2004a), added at the indicated concentrations. Transcription by RNAP holoenzyme made from overproduction of αββ′ (black bars) or αββ′ and ω (gray bars) at each DksA concentration is expressed relative to transcription without DksA. Pairwise comparisons of transcription in the presence of different concentrations of DksA yielded a ratio of 1.08 ± 0.19 for RNAP ± ω [nine independent experiments]. (B) Half-lives of rrnB P1 promoter–RNAP complexes ± DksA were determined by a transcription-based assay (see Materials and Methods and legend for Fig. 1). (Open symbols) 0.5 mM DksA; (filled symbols) no DksA; (triangles) RNAP made from overproduction of αββ′ and ω (gray bars) at each DksA concentration is expressed relative to transcription without DksA. Pairwise comparisons of transcription in the presence of different concentrations of DksA yielded a ratio of 1.08 ± 0.19 for RNAP ± ω [nine independent experiments]. (C) Half-lives of RNAP–lacUV5 promoter–RNAP complexes ± DksA were determined by a transcription-based assay (see Materials and Methods and legend for Fig. 3). (Open symbols) 0.5 mM DksA; (filled symbols) no DksA; (triangles) RNAP made from overproduction of αββ′; (circles) RNAP made from overproduction of αββ′ and ω. Two experiments were performed, and a representative one is shown. DksA decreased the lifetime of the complex an average of 3.0 ± 0.02-fold in the absence of ω and 3.7 ± 1.0-fold in the presence of ω. (C) Half-lives of RNAP–lacUV5 promoter–RNAP complexes ± DksA were determined by a transcription-based assay (see Materials and Methods and legend for Fig. 3). (Open symbols) 0.5 mM DksA; (filled symbols) no DksA; (triangles) RNAP made from overproduction of αββ′; (circles) RNAP made from overproduction of αββ′ and ω. Two experiments were performed, and a representative one is shown. DksA decreased the lifetime of the complex 8.5 ± 1.4-fold in the absence of ω and 6.1 ± 0.6-fold in the presence of ω.

Figure 5. DksA rescues the ppGpp-sensitivity of RNAP lacking ω. (A) Single-round transcription was performed on a supercoiled plasmid template containing the rrnB P1 promoter. The reactions contained transcription buffer containing 30 mM KCl, 400 µM ppGpp and/or different concentrations of DksA were included as indicated. (Left panel) RNAP made by overproduction of αββ′ but not ω. (Right panel) RNAP made by overproduction of αββ′ and ω. (B) Lifetimes of RNAP–lacUV5 promoter complexes were determined in transcription buffer containing 100 mM KCl at different ppGpp concentrations in the presence of 0.5 µM DksA using the filter-binding assay. (Filled circles) RNAP made by overproduction of αββ′ but not ω; (open circles) RNAP made by co-overproduction of αββ′ and ω. Half-lives at each ppGpp concentration are plotted relative to the half-lives in the absence of ppGpp. Each fraction was generated by averaging the half-lives from several independent experiments. Error bars represent standard deviations from the mean.

DksA compensates for ω for ppGpp function

function in vitro versus in vivo. We emphasize that although DksA can bypass the requirement for ω with respect to ppGpp function, the two proteins are not redundant: ω cannot substitute for DksA function in regulation of transcription initiation, and there is no evidence that DksA can substitute for ω function in RNAP assembly.

Mechanism by which ω affects the response of RNAP to ppGpp

The recent cocrystal of the T. thermophilus RNAP complex containing ppGpp defines a ppGpp-binding site adjacent to, but not overlapping, the active center (Artsimovitch et al. 2004). Assuming this is the functionally relevant binding site for ppGpp, the structure clearly indicates that ω and ppGpp are much too far apart to interact directly [Fig. 6A]. Thus, the observed effect of ω on ppGpp function demonstrates that the proposed ppGpp-binding region is affected by parts of the enzyme located relatively distant from the ppGpp-binding site.
Effects of $\omega$ on ppGpp function could therefore involve (1) an allosteric effect of $\omega$ on ppGpp binding or (2) an allosteric effect of $\omega$ on ppGpp action. We note that there are two segments in $\beta'$ [Fig. 6B] that directly interact with the ppGpp-binding site [see also Minakhin et al. 2001]. Specifically, conserved region I of $\omega$ interacts with $\beta'$ residues N910–K911 (amino acid numbering refers to E. coli RNAP). These residues are connected through a single $\alpha$-helix (red in Fig. 6A,B) to $\beta'$ residues E925 and Q929, which, in turn, likely interact with ppGpp [Artsimovitch et al. 2004]. $\omega$ also connects with the ppGpp-binding region through $\beta'$ N458–L483 [yellow in Fig. 6A,B], in which L483 directly interacts with $\omega$; N458 interacts with the guanine base of ppGpp [Artsimovitch et al. 2004], D460, D462, and D464 coordinate a catalytic Mg$^{2+}$; and D460 also helps coordinate a ppGpp-associated Mg$^{2+}$ [Artsimovitch et al. 2004].

We suggest that $\omega$ and the ppGpp-binding region might communicate through these segments in $\beta'$, providing a possible structural explanation for the effect of $\omega$ on ppGpp function. For example, interactions of $\omega$ with $\beta'$ might shift the orientation of the $\beta'$ N910–Q929 $\alpha$-helix, facilitating ppGpp binding by altering the position of Q929 relative to ppGpp [model 1]. Alternatively, $\omega$-dependent rearrangements in either (or both) of the segments of $\beta'$ connecting $\omega$ and the ppGpp-binding region might alter the stabilities of kinetic intermediates on the pathway to open complex formation/dissociation, amplifying effects of ppGpp [model 2]. In this context, we note that bacteriophage N4 single-stranded binding protein (N4SSB), an activator of N4 late gene transcription [Minakhin et al. 2001], has a conserved region that interacts with the ppGpp-binding site (see also Minakhin et al. 2001). Alternatively, DksA could facilitate ppGpp binding or function allosterically by working through a segment of $\beta'$ in contact with $\omega$, possibly through a similar allosteric communication mechanism.

**Mechanism by which DksA affects the response of RNAP to ppGpp**

The overall structure of DksA resembles that of the bacterial transcription elongation factors, GreA and GreB [Perederina et al. 2004]. Like the Gre factors, DksA binds in the secondary channel of RNAP [Perederina et al. 2004; I. Toulokhonov, J. Mukhopadhyay, R.H. Ebright, and R.L. Gourse, unpubl.]. There is currently no structure-based model that fully explains the effects of DksA on transcription initiation and its synergy with ppGpp. However, DksA rescued the effect of ppGpp on RNAP lacking $\omega$ and thus must facilitate binding or function of ppGpp, either directly or allosterically. For example, DksA could potentially facilitate binding or function of ppGpp by interacting with ppGpp directly in the RNAP secondary channel, as proposed previously [Perederina et al. 2004]. Alternatively, DksA could facilitate ppGpp binding or function allosterically by working through the pathway of structural communication between $\omega$ and the ppGpp-binding site proposed above, potentially enhancing ppGpp binding or function by the same mechanism as $\omega$. Protein–protein footprinting experiments [I. Toulokhonov and R.L. Gourse, unpubl.] indi-
icate that DksA, positioned in the secondary channel, is in close proximity to residues in the segments of β’ linking ω and ppGpp.

We also note that higher concentrations of DksA on its own (i.e., in the absence of ppGpp and ω) can directly inhibit or stimulate transcription initiation, depending on the kinetic characteristics of the promoter (Fig. 4; Paul et al. 2004a, 2005). In this case, DksA bypasses requirements for both ppGpp and ω. One simple model is that DksA affects RNAP–promoter lifetime and transcription initiation through interactions with one or both of the segments of β’ connecting ω and the ppGpp-binding region.

Additional roles for ω in vivo

As indicated above, a strain lacking both rpoZ and dksA is viable, but its growth is impaired, and it has nutritional requirements in addition to those resulting from mutations inactivating either dksA or rpoZ alone [data not shown]. It is possible that the additional phenotypes of the double mutant reflect some further impairment in the response of RNAP to ppGpp (by virtue of the loss of both DksA and ω). Alternatively, additional effects of the double mutant may be unrelated to ppGpp function. As noted above, ω has effects on transcription in the absence of ppGpp [R. Saecker and M.T. Record Jr., pers. comm.].

Finally, antibiotic production and morphogenesis in Streptomyces sp. are positively controlled by ppGpp (Bibb 2005). Disruption of rpoZ drastically disrupts these processes in Streptomyces kasugaensis [Kojima et al. 2002], suggesting that the effects of ppGpp on RNAP in this organism are dependent on ω in vivo, perhaps by the same mechanism described here for E. coli.

Materials and methods

Purification of overproduced αββ’ RNAP

Holoenzyme purified by purification of core RNAP after overproduction of α, β, and β’ in vivo, followed by reconstitution with purified α70, is referred to as “overproduced αββ’ RNAP.” Overproduced αββ’ω RNAP was purified by RLG7651 (BL21ADE3 containing both pLA299, which overexpresses αββ’, and pCDFω, which overexpresses ω. pCDFω was constructed by insertion of the E. coli rpoZ gene between the NdeI and XhoI sites of pCDF-1 [Novagen] [Y. Kim and R.H. Ebright, unpubl.]. RLG7651 was grown in 2 L of LB with 100 µg/mL ampicillin and 50 µg/mL spectinomycin at 30°C to an OD600 of 0.35 and induced for 3 h with 1 mM IPTG. Overproduced αββ’ω RNAP was purified exactly as described above for overproduced αββ’RNAP, the two preparations were purified in parallel and were of similar purity as assessed by SDS-PAGE stained with Coomassie blue or silver. ω was a prominent band in SDS-PAGE of the overproduced αββ’ω RNAP.

Purification of ω and reconstitution with core RNAP in vitro

Holoenzyme prepared by purification of core RNAP after overproduction of α, β, β’ in vivo, followed by reconstitution with purified ω and α70 in vitro, is referred to as “overproduced αββ’ + ω RNAP.” Native (untagged) ω was purified from 2 L of BL21ADE3 carrying pCDFω grown at 30°C in LB with 50 µg/mL spectinomycin to an OD600 of 0.4–0.6 and induced for 2 h with 1 mM IPTG. ω was purified as described [Gentry and Burgess 1990]. Since the majority of ω remained in the soluble fraction, only the protocol relevant to the soluble fraction was used. Furthermore, since ω was >99% pure after the Q-Sepharose Fast Flow and Red-Sepharose column steps, the final Mono-Q chromatographic step was omitted. Overproduced αββ’RNAP core was incubated at 37°C with fivefold to 10-fold molar excess of ω in 1× transcription buffer and either 45 or 250 mM NaCl for 20 min as described [Gentry and Burgess 1990] before addition of two- to fivefold molar excess of ω for 40 min. Although we did not determine the stoichiometry of ωRNAP, the response to ppGpp of RNAP reconstituted with ω in vitro was within error of that of overexpressed RNAP with overproduced ω (Table 1), suggesting that these preparations were saturated with ω to the same extent. We note that incubation of ω with overproduced αββ’RNAP in a buffer containing 50% glycerol resulted in only partial restoration of ppGpp sensitivity to RNAP, suggesting that glycerol might inhibit the rate of association of ω with core.

Purification of native RNAP

Holoenzyme purified without overproduction from cells wild-type for rpoZ is referred to as “native,” and holoenzyme purified...
from rpoZ::kan cells without overproduction is referred to as “native rpoZ::kan.” Native holoenzymes were purified by standard methods (Burgess and Jendrisak 1975) or by immuno-affinity chromatography using the poly-l-lysine-reactive antibody NTT3 [Neoclone] as described [Thompson et al. 1992]. The purities of the resulting holoenzymes were confirmed by SDS-PAGE. No ω protein was detected in the preparation made from the rpoZ mutant strain.

Transcription assays

Multiple-round assays were carried out with 10–30 nM RNAP in transcription buffer (40 mM Tris-HCl at pH 7.9, 10 mM MgCl₂, 1 mM DTT, 0.1 mg/ml BSA, and KCl or NaCl at the concentrations indicated in the figure legends) at 30°C [Ross et al. 1990]. Reactions containing transcription buffer, NTPs (200 µM ATP, GTP, and CTP; 10 µM UTP; and 1 µCi of [α-32P]UTP), and rrnB P1 template (with 400 µM ppGpp or 0.5 µM DksA or both as indicated) were initiated by addition of RNAP and stopped after 10 min. For single-round reactions, the template was incubated with RNAP for 10 min, 10 µg/ml heparin was added, and after 10 sec transcription was initiated by addition of NTPs. In addition, 0–400 µM ppGpp and 0.2–1.0 µM DksA were included when indicated. Reactions were terminated by addition of an equal volume of urea stop buffer [Ross et al. 1990] after 10 min, followed by analysis by gel electrophoresis and phosphorimaging.

Dissociation kinetics

Half-lives of open complexes were determined from semilog plots of fraction remaining versus time. Dissociation rate constants, k_d, were determined from the first-order decay equation, c_{pm ret} = c_{pm max}e^{-k_d t}. Half-lives of RNAP on rrnB P1 were measured by a transcription-based assay [Barker et al. 2001b]. Superoxoled plasmid pRLG4264 (0.5 nM), containing the rrnB P1 promoter, was incubated with 10–30 nM RNAP in transcription buffer [see above; 30 mM KCl] for 10 min at 30°C to form complexes, and 400 µM ppGpp (Tri-link, Inc.) and 0.2–1.0 µM DksA were included when indicated. After addition of 10 µg/ml heparin, samples were removed at intervals, and transcription was initiated by addition of NTPs and allowed to proceed for 10 min. Half-lives of RNAP on the lacUV5 promoter were measured by filter-binding [Ross and Gourse 2000]. lacUV5 promoter fragments (end points −140 to +88) from pRLG4264 [Ross and Gourse 2005] were incubated with 10–30 nM RNAP in transcription buffer containing 100 mM KCl, and 0.5 µM DksA and/or 2–400 µM ppGpp were added as indicated before RNA polymerase addition. After addition of 10 µg/ml heparin, samples were removed at intervals and filtered through nitrocellulose disks. The filter-bound complexes were washed with 100 mM NaCl, 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA, dried, and counted with a Packard scintillation counter.

We emphasize that intrinsic lifetimes of promoter–RNAP complexes are very sensitive to slight variations in solution conditions, topology of DNA, or heparin concentrations. Therefore, RNA polymerase preparations under comparison were always tested with the same solutions in the same experiment.

DksA preparations

N-Terminal hexa-histidine-tagged DksA was prepared as described previously [Paul et al. 2004a]. No band migrating at the position of ω was detected under conditions where 0.1% potential contamination with ω would have been detectable. Since 500 nM DksA was used in transcription reactions, ω could therefore have been present at no more than 0.5 nM, which would have been greatly substoichiometric with the 5–30 nM RNA polymerase used.

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Response of RNA polymerase to ppGpp: requirement for the ω subunit and relief of this requirement by DksA
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