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Structure-function comparisons of (p)ppApp vs (p)ppGpp for *Escherichia coli* RNA polymerase binding sites and for *rrnB* P1 promoter regulatory responses *in vitro*.

Bo ena Bruhn-Olszewska^a, Vadim Molodtsov^b, Michał Sobala^a, Maciej Dylewski^a, Katsuhiko S. Murakami^{b,*}, Michael Cashel^c, and Katarzyna Potrykus^{a,*}

^aDepartment of Bacterial Molecular Genetics, Faculty of Biology, University of Gdansk, Wita Stwosza 59, 80-308 Gdansk, Poland ^bDepartment of Biochemistry and Molecular Biology, Center of RNA Molecular Biology, Pennsylvania State University, University Park, PA 16802, USA ^cIntramural Research Program, Eunice Kennedy Shriver NICHD, National Institutes of Health, Bethesda, MD 20892, USA

Abstract

Precise regulation of gene expression is crucial for bacteria to respond to changing environmental conditions. In addition to protein factors affecting RNA polymerase (RNAP) activity, second messengers play an important role in transcription regulation, such as well-known effectors of the stringent response: guanosine 5'triphosphate-3'diphosphate and guanosine 3', 5'-bis(diphosphate) [(p)ppGpp]. Although much is known about importance of the 5' and 3' moieties of (p)ppGpp, the role of the guanine base remains somewhat cryptic. Here, we use (p)ppGpp's adenine analogs [(p)ppApp] to investigate how the nucleobase contributes to determine its binding site and transcriptional regulation. We determined X-ray crystal structure of *Escherichia coli* RNAP-(p)ppApp complex, which shows the analogs bind near the active site and switch regions of RNAP. We have also explored the regulatory effects of (p)ppApp on transcription initiating from the well-

*To whom correspondence should be addressed. Tel: +48 585236027; katarzyna.potrykus@biol.ug.edu.pl (K.P.); Tel: +1 8148652758; kum14@psu.edu (K.S.M.).
BBO: b.bruhn-olszewska@ug.edu.pl
VM: vum5@psu.edu
MS: michal.sobala@phdstud.edu.pl
MD: maciej.dylewski@phdstud.edu.pl
KSM: kum14@psu.edu
MC: cashelm@mail.nih.gov
KP: katarzyna.potrykus@biol.ug.edu.pl

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Availability of data and materials

Atomic coordinates and structure factors for the reported crystal structures have been deposited with the Protein Data bank under accession number 6BYU. All other data generated or analyzed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests.

studied *E. coli* *rnb* P1 promoter to assess and compare properties of (p)ppApp with (p)ppGpp. We demonstrate that contrary to (p)ppGpp, (p)ppApp activates transcription at this promoter and DksA hinders this effect. Moreover, pppApp exerts a stronger effect than ppApp. We also show that when ppGpp and pppApp are present together, the outcome depends on which one of them was pre-incubated with RNAP first. This behavior suggests a surprising Yin-Yang like reciprocal plasticity of RNAP responses at a single promoter, occasioned simply by pre-exposure to one or the other nucleotide. Our observations underscore the importance of the (p)ppNpp's purine nucleobase for interactions with RNAP, which may lead to a better fundamental understanding of (p)ppGpp regulation of RNAP activity.

Keywords

RNAP; transcription; *rnb* P1; ppGpp; (p)ppGpp; (p)ppApp

1. INTRODUCTION

In order to cope with changing environmental conditions, bacteria have evolved several different responses, often relying on a variety of noncanonical ribonucleotides as second messengers. These nucleotides are distinct by means of structural features as well as by the fact that they are not present as pathway intermediates and their sole function appears to be regulation.

One class of these analogs arises by 3',5'-cyclization of a single phosphate for each ribonucleotide; so far, examples include mononucleotides (cAMP and cGMP) as well as homomeric and heteromeric dinucleotides (c-di-AMP, c-di-GMP, c-GAMP). The mechanisms of action of these global regulators in bacteria range from affecting transcriptional regulators that bind to DNA (e.g. CRP and CRP-like proteins affected by cAMP, cGMP and c-di-GMP [1–4], to binding to specific proteins other than transcriptional factors or riboswitches (e.g. c-di-GMP [5] and c-di-AMP [6]).

Another class of second messengers consists of noncyclic guanosine derivatives with 3' and 5' hydroxyls blocked by linear phosphate chains; these analogs are guanosine 5'-triphosphate-3'diphosphate [pppGpp] and guanosine 3',5'-bis(diphosphate) [ppGpp], collectively termed (p)ppGpp [7; 8]. This class of nucleotides is ubiquitous in bacteria and plant plastids [8]. These molecules are effectors of the bacterial stringent response, characterized by inhibition of the rRNA and tRNA synthesis under different starvation and stress conditions [8]. The way (p)ppGpp causes the stringent response differs for Gram positive and negative bacteria. For example, in *Bacillus subtilis* (p)ppGpp indirectly regulates rRNA by inhibiting GTP biosynthesis, which limits rRNA transcripts that start with GTP. In *Escherichia coli*, a similar outcome is brought on by direct interactions of (p)ppGpp with the RNA polymerase (RNAP) leading to ribosomal RNA promoters' (*rnb*) transcriptional inhibition. Still, not all promoters are inhibited by this action, for example promoters for amino acid biosynthesis genes are activated [9; 10; 8]. Although (p)ppGpp is capable of exerting these effects on its own (particularly in the case of transcriptional inhibition), it has been shown that an RNAP secondary channel binding protein, DksA, may

amplify its action (especially true for instances of transcriptional activation) [10–12]. Still, there are instances where DksA and (p)ppGpp do not act in synergy but instead exert antagonistic effects [13–16].

Despite the vast amount of information available so far, until very recently it was largely unknown how each moiety of the (p)ppGpp molecule contributes to transcriptional regulation. It is almost certain that the 3' diphosphate plays a crucial role here since GTP and GDP do not display such regulatory effects. The nucleotide's 5' moiety also plays a role, as pppGpp and ppGpp were shown to affect the *E. coli* transcription to a different extent, with ppGpp being more potent than pppGpp [17; 18]. Indeed, guanosine pentaphosphate phosphatase (GppA) exists in *E. coli*, whose presence can be viewed as a means of fine tuning regulation. The importance of the 5' γ - β and 5' β phosphates was also demonstrated with yet another analog, pGpp, found to be synthesized in *Enterococcus faecalis* [19]. It was shown to inhibit *E. coli* *rrnB* P1 model promoter to a much lesser extent than either pppGpp or ppGpp, regardless of the presence of DksA in vitro [19].

Recent reports had pointed to two ppGpp binding sites on *E. coli* RNAP. Site 1 is DksA-independent, which is localized at the junction of the ω and β' subunits [17; 20; 21], while site 2 is DksA-dependent and locates at the binding interface between β' rim-helix of RNAP and DksA [22; 23]. Both pppGpp and ppGpp were shown to bind to sites 1 and 2 in a similar manner [17; 23], but it is uncertain whether potential differences in affinities might explain differences in their regulatory potency.

A recent crystal structure of the *E. coli* RNAP and DksA/ppGpp complex revealed a crucial role of the guanine base for accommodating ppGpp at both sites [23]. For site 1, the guanine base is recognized by the β' subunit amino acid residues (sandwiched by side chains of R362 and I619, and involved in polar interactions with H364 and D622), while both diphosphate groups establish salt bridges with basic residues (R3 and R52) of the ω subunit. For site 2, the guanine base interactions involve both the β' rim helix residues (Y679, I 683, D684 and N680) and DksA (L95).

So far, all noncanonical regulatory nucleotides seem to have purines as nucleobases. Evidently the cyclic class of analogs exists as adenine or guanine derivatives. Therefore, we ask if the same would be true for (p)ppGpp-like alarmones, i.e. what are the functional consequences of swapping the guanine of (p)ppGpp for adenine, and how nucleobase determines the (p)ppNpp-RNAP interactions.

2. MATERIALS AND METHODS

2.1. X-ray crystal structure determinations of the *E. coli* RNAP σ^{70} - holoenzyme in complex with (p)ppApp

Crystallizations of *E. coli* RNAP containing RpoB-H526Y or RpoB-S531L substitutions were performed as previously described [40]. To form co-crystals of RNAP with (p)ppApp, RNAP crystals were transferred to the cryoprotection solution (0.1 M HEPES (pH 7.0), 0.2 M MgCl₂, 30 % PEG400, 10 mM DTT) supplemented with 1 mM (p)ppApp and incubated overnight at 22 °C followed by flash-freezing in liquid N₂.

The X-ray dataset were collected at the Macromolecular Diffraction at the Cornell High Energy Synchrotron Source (MacCHESS) (Cornell University, Ithaca, NY) and the data were processed by HKL2000 [41]. The resolution limit for crystallographic dataset were determined based on $CC_{1/2}$ (>30%) rather than R_{merge} and $\langle I \rangle / \sigma I > 2$ criteria, since this approach prevents loss of useful crystallographic data for structure refinement as found in a recent study [24]. The structures were solved by molecular replacement using the suite of programs PHENIX [25]. Strong Fo-Fc maps corresponding to (p)ppApp were observed after the rigid body refinements. Structures of (p)ppApp were fitted into the extra density maps to continue the refinement. The program Coot [26] was used for manual adjustment of the models during refinements. The structures were refined by using the Phenix suite of programs for the rigid body and positional refinements with non-crystallographic symmetry and reference structure restraints to avoid over-fitting the data. Final coordinates and structure factors of RNAP-ppApp complex were submitted to the PDB depository with ID code listed in Table 1.

2.2. *In vitro* transcription

For the *rnnB* P1 promoter, *in vitro* transcription was performed basically as described in [12]. Briefly, the reactions were carried out in 20 μ l (final volume) at 30°C, using 10 nM linear template spanning - 180 to +109 bp (DNA template total length 289bp, full length transcript 109 nt) and 30 nM *E. coli* RNAP holoenzyme (Epicentre Technologies or New England Biolabs), in a buffer containing 50 mM Tris-acetate, pH 8.0, 10 mM Mg-acetate, 10 mM β -mercaptoethanol (β -ME), 10 μ g/ml BSA, 90 mM potassium glutamate (KGlu), 100 μ M ATP, GTP and CTP, and 10 μ M UTP (10 μ Ci/reaction [α -³²P] UTP), and either 250 μ M (p)ppGpp or 250 μ M (p)ppApp, unless stated otherwise. RNAP was preincubated (25°C) with ppGpp or (p)ppApp for 8-min prior to the addition of KGlu, and this was followed by 8-min incubation at 30°C with the DNA template and DksA, if present, unless stated otherwise. The reactions were initiated by adding NTP substrates and terminated after 10 min by the addition of an equal volume of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol). For single round reactions, heparin was added to 100 μ g/ml together with the NTPs. Samples were analyzed on 7M urea, 6% polyacrylamide sequencing gels and quantified by phosphorimaging with a Typhoon imaging system. The rationale for choosing 90mM KGlu salt concentration for *in vitro* transcription is described in Figure S1 and its accompanying legend. The ppApp and ppGpp order of addition experiments were carried out as depicted in Figure S2.

2.3. Template mutagenesis

The three promoter mutants were constructed by site-specific mutagenesis of the *rnnB* P1 promoter region. Mutations were introduced by PCR, at the +1 transcription start site: mut +1[A/G] (with primers: 5' – ATGCGCCACCGCTGACACGGAACAACGGC and 5' – TCCGTGTCAGCGGTGGCGCATTATAGGG), and in the discriminator region: C/G at position –7, relative to transcription start site (with primers: 5' – TATAATGGGCCACCACTGACACGG and 5' – TCAGTGGTGGCCCATTATAGGGAG), and CGC/ATA at position –7 to –5 (with primers: 5' – TATAATGATACCACTGACACGGAAC and 5' – GTGTCAGTGGTGTATCATTATAGGGAG).

2.4. Promoter escape and open complex stability assays

These assays were performed basically as described in [12], except that when present, either 250 μ M ppGpp or pppApp were added. Detailed information is provided in the Supplementary Information.

2.5. EMSA

The reactions were carried out in 20 μ L (final volume) at 30°C using 5 nM template (the same template as for *in vitro* transcription assays, but labeled with Cy-5 at both ends) and indicated RNAP concentrations (0–160 nM). Buffer contained 50 mM Tris-acetate, pH 8.0, 10 mM Mg-acetate, 10 mM β -ME, 10 μ g/ml BSA, 90 mM KGlu. RNAP was preincubated (25°C) in the presence of up to 500 μ M (p)ppApp and ppGpp for 8-min prior to the addition of KGlu and this was followed by 8-min incubation at 30°C with the DNA template. The reactions were supplemented with 25 μ g/ml poly(dI-dC) (Sigma Aldrich) and separated on 4% TBE-polyacrylamide gels (acrylamide to bis ratio 19:1) with 0.5x TBE as the running buffer. The pppApp and ppGpp order of addition experiments were carried out as depicted in Figure S3.

2.6. Synthesis and purification of ppGpp and (p)ppApp

Synthesis and purification of ppGpp has been carried out basically as described in [17]. Briefly, purified RelSeq1–385 was employed in a reaction with 8 mM ATP and 6 mM GTP in the presence of 100 mM NaCl, 25 mM bis-Tris propane (pH 9.0), 15 mM MgCl₂ and the reactions were incubated for 1 hr at 37°. GppA was used to obtain ppGpp. For (p)ppApp synthesis, a protocol for (p)ppGpp synthesis described in [28] was used, except that ATP and ADP (for ppApp) or only ATP (for pppApp) were used as substrates. Briefly, *Streptomyces morookaensis* extract was used with 9 mM ATP and 9 mM ADP for ppApp (or 18 mM ATP alone for pppApp), in the presence of 50 mM Tris-Cl (pH 8.9) and 20 mM MgCl₂. The samples were incubated at 32° for 30 min. The purity of the isolated (p)ppNpp's (i.e. ppGpp and (p)ppApp) assessed by thin layer chromatography revealed the absence of detectable UV absorbing nucleotides other than the product of interest. Note: within the manuscript, we have often shortened the abbreviation of pppApp and ppApp as a5p and a4p, as well as g5p and g4p for (p)ppGpp.

2.7. Protein Purification

DksA and GreA were purified as described in [12].

2.8. Statistical analysis

In all cases, mean data is presented (in most cases experiments were done at least in triplicate), with standard deviation calculated with the Excel software. P-values were calculated by employing a 2-tailed t test assuming unequal variance.

3. RESULTS

3.1. Finding the (p)ppApp binding site in *E. coli* RNAP by X-ray crystal structure determination of RNAP - (p)ppApp complex

To directly identify the (p)ppApp binding site on RNAP, crystals of *E. coli* RNAP σ^{70} holoenzyme complexed with (p)ppApp were prepared by soaking 1 mM (p)ppApp into the preformed RNAP crystals and the structures were determined by X-ray crystallography.

In the RNAP-ppApp complex, only fragmented electron densities were found at the ppGpp binding site 1 (Figure S4), covering phosphate group binding areas of ppGpp, suggesting that ppApp binds only weakly at this site due to lack of base specific interactions observed in the RNAP-ppGpp complex. In contrast, clear, strong and triangular shape electron density was found near the active site of RNAP (distance between the Mg and a center of triangular shape density is ~ 13 Å) (Figure 1A). A clear map from the RNAP and pppApp complex was also observed at the same site as for the RNAP and ppApp complex. This is taken to indicate that both ppApp and pppApp associate with the RNAP at the same location.

The electron density map of ppApp showed three separated regions that correspond to 5'-diphosphates, adenosine (adenine base plus ribose ring) and 3'-diphosphates. We assigned larger and globular electron densities as electron rich phosphate groups whereas a smaller density as adenine base of ppApp. It is difficult to identify the 5' and 3' positions of phosphate groups based only on the density map, therefore we tested two alternative orientations of ppApp for refinement with either facing the 5' or 3' phosphate group to the active site Mg. We found that the orientation of ppApp with its 3' phosphate toward the active site Mg gives better stereochemistry, an anti-conformation of adenine base relative to sugar and good sugar puckering, compared with another orientation. We therefore used this orientation to refine the structures of RNAP in complex with ppApp and also pppApp. The (p)ppApp binding site is separated from the (p)ppGpp binding site 1 and site 2 by 33 Å and 47 Å, respectively (Figure 1C) indicating that they do not function via direct interactions with each other. The (p)ppApp binding site of *E. coli* RNAP is also distinct from the ppGpp binding site found in the *Thermus thermophilus* RNAP [29], which is also located at the active site of RNAP but positioned at a narrow pore region of the secondary channel of RNAP that is 21.5 Å away from the *E. coli* RNAP (p)ppApp binding site.

The (p)ppApp position is at a shallow cavity of the β' subunit formed by one of the double-psi β -barrel (DPBB) domains, which coordinates the catalytic Mg ions for RNA synthesis and degradation (Figures 1B and C). Basic residues, including R346 and R352 from the β' subunit and K1242 residue of the β subunit, interact with the phosphate groups of (p)ppApp, and these residues belong to the switch regions of RNAP (switch 2, β' subunit residues 331–348; switch 3, β subunit residues 1248–1270). The Q465 residue of the β' subunit may form a hydrogen bond with the 3'-phosphate group of (p)ppApp.

Within the adenine base binding pocket, no amino acid residue forms a hydrophilic interaction with the adenine base. Instead, the adenine binding site mainly uses hydrophobic and van der Waals interactions to recognize the shape of the adenine base. A side chain of A426 and a main chain carboxamide group of Q465 residues of β' subunit contact the C2

atom of adenine and this contact would not allow a guanine base in the same binding pocket due to steric clash with an amino group of guanine at its C2 position. Therefore, ppGpp is not likely to bind at this location.

3.2. (p)ppApp activates transcription initiating from the *rrnB* P1 promoter, but pppApp is a stronger activator than ppApp

To date, there has been only a single report of ppApp effects on transcription [30]. This report had suggested ppApp activates transcription of rRNA in a salt dependent manner, however this was done with phage lambda genomic DNA templates containing an *rrn* operon rather than an isolated promoter and cellular extracts containing RNAP and other cellular factors; in addition, pppApp has not been studied. Therefore, we first wanted to ask if we could reproduce the ppApp mediated activation of rRNA transcription, but using our well defined *in vitro* system where transcription is solely dependent on the *rrnB* P1 promoter. The *in vitro* system we employed has been successfully used in our previous studies with (p)ppGpp [12].

As depicted in Figure 2A and Figure S1, we observe activation by (p)ppApp of transcription initiating from the *rrnB* P1 promoter. Since it is known that the 5' moiety of (p)ppGpp (5' γ -phosphate) plays an important role with respect to the extent this molecule regulates the RNAP activity, where *rrnB* P1 transcription is more inhibited by ppGpp than pppGpp [17], we compared activation by ppApp with pppApp. In this set of experiments, ADP was used as control since one might argue that activation by ppApp is mainly due to the 5' diphosphate and not the 3' diphosphate of this nucleotide.

Contrary to the guanosine derivatives [17], pppApp displays a stronger regulatory effect than ppApp (Figure 2A). This observation is valid for all (p)ppApp concentrations tested (ranging from 25 to 500 μ M). In case of ppApp, the highest transcript level was obtained at a concentration of 250 μ M (1.36-fold higher than control; p value < 0.05 when compared to samples lacking or containing 25 μ M ppApp, as well as when compared to 250 μ M ADP), with reduced activation (1.13-fold) at 500 μ M. Surprisingly, for pppApp, activation reached 1.67- and 1.74-fold for 250 and 500 μ M, respectively (p value < 0.05 when compared to samples at no and 25 μ M pppApp, as well as when 250 μ M and 500 μ M pppApp is compared to 250 μ M and 500 μ M ppApp or ADP). Still, since the greatest effect was observed for pppApp and this effect differed only slightly between 250 and 500 μ M concentration, we used 250 μ M pppApp in all subsequent experiments. ADP in control experiments moderately inhibited transcription at high concentrations but did not activate at any concentrations tested, and accordingly was eliminated in most later experiments.

3.3. DksA hinders activation by (p)ppApp

It is well known that DksA, an RNAP secondary channel-binding factor, enhances the inhibitory effect of ppGpp on transcription initiating from the *rrnB* P1 promoter [10; 12]. We therefore asked if DksA would have the same effect on (p)ppApp mediated activity. Here, *in vitro* transcription reactions were carried out with increasing concentrations of DksA (0–300 nM) in the presence of constant concentrations of ADP, ppApp or pppApp (250 μ M). Interestingly, not only did we observe that DksA failed to activate (Figure 2B) but DksA

seems to abolish activation due to ppApp; at 300 nM DksA and 250 μ M ppApp, the transcript level was reduced from 137% at 0 DksA to 88 %, which corresponds to 35 % inhibition (p value < 0.05). In case of pppApp, DksA's effect does not seem to be as drastic since at 300 nM DksA we observe only a slight inhibition (transcript level was reduced from 167% with no DksA to 145% with DksA, corresponding to 13% inhibition, with p value > 0.05 (Figure 2B). However, it is notable that this experimental protocol (Figure 2B) required DksA to be added to RNAP that had been pre-incubated with (p)ppApp or ADP and with DNA. In all other experiments DksA was added to RNAP together with the DNA template. For these other experiments, the inhibitory effect of DksA was much stronger than when RNAP was pre-incubated with DNA before the DksA addition (see Figure 5A panel I, and Discussion – 186% and 86% transcript level was observed for 250 μ M pppApp in the absence and presence of 300 nM DksA). In the control reaction with ADP, 300nM DksA caused a 23% drop in the transcript level when compared to the reaction with template lacking DksA (Figure 2B). Evidently DksA is able to hinder activation due to (p)ppApp.

Similar experiments were performed with GreA, another RNAP secondary channel binding protein, as an additional control. We had previously shown that GreA can activate transcription initiating at the *rrnB* P1 promoter [12], however, we observed no significant effect of GreA (up to 300 nM) on (p)ppApp transcriptional activation (Figure S5).

3.4. The *rrnB* P1 activation by pppApp does not depend on the +1 initiating nucleotide being adenosine and is discriminator region specific

Next, we decided to explore the possibility that the observed pppApp transcriptional activation at the *rrnB* P1 promoter comes from the fact that ATP is the +1 nucleotide in the nascent RNA transcript. We therefore constructed a DNA template where +1 of *rrnB* P1 has been mutated from A to G (*rrnB* P1 mut+1 [A/G]). As can be seen in Figure 2C, pppApp is still able to activate transcription initiating from the mutated promoter, albeit to a lesser degree (1.45-fold vs 1.7-fold of the wild type template, at 500 μ M pppApp; p value < 0.05 when compared to samples with no pppApp present; p value > 0.05 when directly comparing the wild type with *rrnB* P1 mut+1 [A/G] at 500 μ M pppApp).

It is also known that (p)ppGpp mediated transcriptional inhibition or activation largely depends on the so-called discriminator region of the promoter, spanning from –10 to +1 bases relatively to the transcription start site. Promoters with GC rich discriminators are generally inhibited by (p)ppGpp, while those AT rich are activated [31]. We thus performed similar experiments with pppApp as above but with the known *rrnB* P1 discriminator mutants, previously shown to be insensitive to ppGpp [32]. We observed no significant (p)ppApp activation, indicating that similar to (p)ppGpp inhibition, (p)ppApp activation is discriminator region specific (Figure S6).

3.5. pppApp does not affect the rate of promoter escape, however pppApp stabilizes the *rrnB* P1-RNAP open complexes

Transcription regulation by (p)ppGpp predominately occurs at the initiation phase, and (p)ppGpp is known to destabilize open complexes at most promoters [33]. This step is a rate limiting step for some promoters, like *rrnB* P1, however (p)ppGpp might also affect other

steps, as it has been shown that a stable λ pR promoter is inhibited by ppGpp because of its effects on the first phosphodiester bond formation [34]. We thus asked if pppApp alters *rnb* P1 promoter escape and/or the DNA-RNAP open complex stability.

In the promoter escape experiments, the DNA – RNAP open complexes are first allowed to be formed and the reaction is then initiated by addition of NTP substrates together with heparin, which is a non-specific competitor that prevents RNAP from rebinding to the DNA template once one round of transcription reaction is completed. The samples are removed at specific time intervals which allows monitoring how many RNAP complexes have completed one full round of transcription. As can be seen in Figure 3A, the rate of *rnb* P1 promoter escape of RNAP in the presence of pppApp is very similar to the rate of the control reaction. It should be noted though, that the overall transcript level is decreased by 40%, which is somewhat surprising since transcriptional activation by pppApp was observed under multiround conditions (Figure 2A–C). Similar results were obtained for reactions carried out in the presence of 300 nM DksA. As an additional control, similar experiments were carried out with 250 μ M ppGpp and as expected, under our experimental conditions, ppGpp slows down the rate of promoter escape and decreases the overall transcript level, both in the presence and absence of DksA (Figure 3A).

The 40% decrease in overall transcript level suggests that pppApp might either destabilize the *rnb* P1-RNAP open complexes or convert the complexes formed in the presence of pppApp into “dead-end” complexes, i.e. complexes stuck at the promoter region and unable to produce full-length RNA. One of the symptoms of the dead end complexes could be an increased production of the short, abortive RNAs. In order to test this hypothesis, samples from the promoter-escape experiment were run on 23% gels, however, increased amounts of abortive RNA products were not detected (BBO and KP, personal communication).

We thus moved onto testing the stability of open complexes formed by RNAP at the *rnb* P1 promoter. In these experiments, DNA-RNAP open complexes are allowed to form and heparin is added at time 0 in order to prevent any RNAP from rebinding to the DNA template. Samples are taken at the indicated times and the transcription reaction is started by the addition of the NTP substrates with each sample assayed individually. This allows to monitor how many active (i.e. open) complexes are present at any given time point. As depicted in Figure 3B, evidently pppApp stabilizes the *rnb* P1-RNAP open complexes, with the open complex half-life (the time when 50% of open complexes are present) estimated to be 53 seconds in the presence of pppApp, and only 20 seconds in its absence. Interestingly, we note that open complexes formed in the presence of ppGpp also seem to be more stable than in the control reaction with no added (p)ppNpp, and the open complex half-life for ppGpp is estimated to be 45 seconds. In previous studies, done with the use of supercoiled templates instead of linear ones as used here, the open complex half-lives were estimated to be decreased by ppGpp [33].

On the other hand, the initial stabilizing effect due to pppApp is gone in the presence of 300 nM DksA (Figure 3B). This may explain the suppression by DksA observed in Figure 2C. Although DksA seems to drastically decrease the open complex half-lives in all cases, we can only estimate these half-lives to be under 15 seconds for the pppApp, ppGpp and control

reactions (we are unable to take the first sample time points earlier than 15 seconds, followed by 30s, 45s, 1 min, etc.). Still, there are complexes that are left bound past the half-life time point and those formed in the presence of pppApp are still more stable than those formed in the presence of ppGpp or without any (p)ppNpp added. When examining time points when only 25% of complexes are present (a “quarter-life”), for the control reaction, pppApp and ppGpp the values are 3 min, 5 min and 1.3 min, respectively. When DksA was absent, these values were 2 min, 4.6 min and 3.4 min, respectively (Figure 3).

3.6. pppApp enhances RNAP binding to the *rnnB* P1 promoter region

In order to further explore the stabilizing effect of pppApp on the DNA-RNAP open complex stability and/or formation, we performed EMSA experiments. Here, RNAP (0 – 160 nM) was mixed with the DNA template (5 nM) in the presence or absence of 500 μ M pppApp (Figure 4). More EMSA-detectable DNA-RNAP complexes are formed even at low RNAP concentrations in the presence of pppApp than in its absence, although statistically significant differences were found only for 80 nM and 160 nM RNAP ($p < 0.05$). The corresponding values for RNAP bound in the complexes at 80 and 160 nM RNAP in the presence of pppApp were 65% and 75%, and in its absence they were 42% and 58%, respectively. Under our experimental conditions we observed two complexes, which we annotate as complex I and II in Figure 4. Adding pppApp seems to enhance formation of both of these complexes equally well. In a control experiment to Figure 4, 500 μ M ppGpp was employed. As expected from our previous studies [12], this time less stable complexes were formed with ppGpp than in control reactions without any additional (p)ppNpp (BBO and KP, personal communication). The discrepancy between Fig. 3B and those results might be due to the use of a different competitor (heparin vs poly(dIdC)). These observations support the notion that activation of transcription by pppApp involves enhanced promoter binding.

3.7. *In vitro* transcription order of addition experiments with pppApp and ppGpp reveal it is important which (p)ppNpp is added first

The X-ray structures of *E. coli* RNAP in complex with (p)ppApp (this study) and ppGpp [17; 23] revealed that (p)ppApp and ppGpp bind to different sites on RNAP and the biochemical assays so far suggest the two analogs influence *rnnB* P1 promoter transcription in various ways. This led to asking if pppApp and ppGpp might either compete when added together (with a net effect of cancelling their opposing effects) or if one or the other effect might dominate. Multiround *in vitro* transcription reactions were performed under conditions where either pppApp or ppGpp are titrated and: i) only one of the (p)ppNpp's was present (control), ii) the (p)ppNpp that was titrated was first preincubated with RNAP, and this was followed by another incubation period with a constant concentration of the other 3' diphosphate nucleotide, iii) both, the (p)ppNpp that was being titrated and the one present at a constant concentration were preincubated with RNAP at the same time. The preincubation periods with (p)ppNpp were followed by the addition of the DNA template together with DksA (when present), and then the reactions were started by the addition on the NTP substrates. A detailed scheme of the assays is presented in Figure S2. The results we obtained are presented in Figure 5A.

In the absence of DksA, increasing concentrations of pppApp cause a steady increase in transcript level (from 134% at 25 μ M pppApp to 203% at 500 μ M, with $p < 0.05$ when comparing these two conditions; p value was < 0.05 when comparing no pppApp control with pppApp at 150, 250 and 500 μ M) (Figure 5A, panel I). When RNAP is pre-incubated with pppApp and a constant concentration of ppGpp is added (125 μ M), the high level of transcripts is already saturated at lower pppApp concentrations (195% at 50 μ M pppApp; $p < 0.05$ for all pppApp concentrations equal or greater to 50 μ M when comparing to no pppApp control) (Figure 5A, panel II). However, when both (p)ppNpp's are added at the same time, the transcript level is the lowest – 113% at 25 μ M pppApp, rising to 139% at 150 μ M and reaching 126 % at 500 μ M pppApp (Figure 5A, panel III; $p < 0.05$ for 100 – 500 μ M pppApp when compared to no pppApp sample). The order of conditions yielding the most to the least transcripts is as follows: RNAP pre-incubated with pppApp and then ppGpp added; pppApp alone; pppApp and ppGpp added at the same time (conditions reflected in panels II>I>III).

In contrast, analogous experiments performed with increasing concentrations of ppGpp revealed a steady decrease in the transcript level (going down from 89% at 25 μ M ppGpp to 40% at 500 μ M ppGpp; $p < 0.05$ when comparing these two conditions; $p < 0.05$ for 100 – 500 μ M ppGpp when comparing to no ppGpp control) (Figure 5A, panel IV). Pre-incubation of RNAP with increasing concentrations of ppGpp and followed by addition of 125 μ M pppApp yields lesser inhibition than in the case just described (a steady decrease from 99% at 25 μ M ppGpp to 83% at 500 μ M ppGpp, although the p values were > 0.05 in all cases when compared to no ppGpp control) (Figure 5A, panel V). When both (p)ppNpp's are added at the same time, an intermediate effect is observed – the transcript level goes from 105% at 25 μ M ppGpp to 73% at 500 μ M ppGpp (Figure 5A, panel VI; $p < 0.05$ when comparing these two samples; $p < 0.05$ for 250 μ M and 500 μ M ppGpp when comparing to no ppGpp control). Under these conditions, the most transcripts are obtained when RNAP is preincubated with ppGpp and then pppApp is added; followed by ppGpp and pppApp added at the same time; and finally, ppGpp alone (panel V>VI>IV).

Adding DksA changes the observed dynamics, but only in the case when pppApp is titrated (Figure 5A, panels I-III). When pppApp is titrated alone, the transcript level stays at about 87%–80% (from 25 μ M to 500 μ M pppApp, $p > 0.05$; however p value was < 0.05 for all samples when compared to no pppApp control). When constant ppGpp concentration is added after RNAP pre-incubation with pppApp, the amount of transcripts decreases from 83% at 25 μ M pppApp to 60% at 500 μ M ($p < 0.05$ when compared to no pppApp control). Adding both (p)ppNpp's at the same time yields a steady decrease in transcript levels from 89% to 78% (at 25 μ M to 500 μ M pppApp, with $p < 0.05$ only for 500 μ M pppApp when compared to no pppApp control). Very similar levels of transcripts are seen when both, pppApp is present alone and when pppApp and ppGpp are added at the same time. The lowest level of transcript is when RNAP is pre-incubated with pppApp and then ppGpp is added (panel I=III>II) (Figure 5A, panel I-III).

On the other hand, addition of DksA when only ppGpp was present caused a decrease in transcript level from 59% to 19% (over a range of 25 μ M to 500 μ M ppGpp, with $p < 0.05$ for comparison of these two samples, as well as $p < 0.05$ when comparing all samples to no

ppGpp control). Pre-incubating RNAP with ppGpp and then adding constant concentration of pppApp somewhat alleviated this effect, causing a drop from 87% (25 μ M pppApp) to 50% (500 μ M pppApp), with $p < 0.05$; p value was also < 0.05 for all samples when compared to no ppGpp control. Adding both (p)ppNpp's at the same time leads to a decrease in transcript level from 56% to 31% (at 25 μ M to 500 μ M ppGpp, $p < 0.05$; p value was also < 0.05 when comparing all samples to no ppGpp control). Overall, these results reflect the same gradation as seen for data obtained without DksA, i.e. the most amount of transcripts was obtained when RNAP is pre-incubated with ppGpp and then pppApp is added; followed by ppGpp and pppApp added simultaneously; and the lowest amount when only ppGpp was present (panel V>VI>IV) (Figure 5A, panels IV-VI).

3.8. EMSA experiments are mostly consistent with conclusions from *in vitro* transcription studies that it is important which (p)ppNpp is added first

Next, we wanted to test if EMSA assays would reflect our observations obtained with the *in vitro* transcription order of addition experiments. We have shown above (Figure 4) that 500 μ M pppApp increases the amount of stable RNAP-DNA complexes at 80 and 160 nM RNAP. Here, increasing concentrations of RNAP (20–160 nM) were pre-incubated with: i) either pppApp or ppGpp present at 500 μ M concentration (control), ii) first with either pppApp or ppGpp at 500 μ M, and then with the other (p)ppNpp at 250 μ M, iii) both nucleotides added together, one at 500 μ M and the other at 250 μ M concentration. Next, the DNA template was added together with the salt and/or 600 nM DksA, which was then followed by the addition of poly(dI-dC) competitor. The samples were then immediately loaded onto a running gel. The 2 fold molar excess of one (p)ppNpp over another would reflect conditions of 250 μ M pppApp (or ppGpp) challenged with 125 μ M ppGpp (or pppApp, respectively) in Figure 5A. A detailed scheme of the assays is presented in Figure S3.

To simplify, Figure 5B presents the combined data obtained for two DNA-RNAP complexes which were detected, with Table S1 summarizing the numerical data for each panel. Figure S7 and Figure S8 present scans of EMSA gels as well as data obtained for each complex separately. We did not observe notable differences between complex I and II, i.e. changes in the amount of one reflected the changes in the other one.

In the absence of DksA, the amount of EMSA-detected DNA-RNAP complexes (i.e. stable enough for detection) in the presence of 500 μ M pppApp had steadily increased with increasing RNAP concentrations under all conditions tested (Fig. 5B, panels I-III). However, the ultimate amount of stable complexes varied, with the highest level achieved under conditions where pppApp was present alone, followed by when the (p)ppNpp's were added at the same time, and finally the least stable when RNAP was pre-incubated with pppApp and then ppGpp was added (panels of Figure 5B organized according to the amount of stable complexes formed may be thus listed as I>III>II; p values calculated for 40–160 nM RNAP, when comparing panel I to panel II were < 0.05 ; when comparing panel I to panel III, or panel II to III, no significant differences were detected ($p > 0.05$)).

In case of 500 μ M ppGpp, the amount of EMSA-detected DNA-RNAP complexes also steadily increased with increasing RNAP concentrations (Figure 5B, panels IV-VI), although

in all instances it was lower than when 500 μ M pppApp was present (Figure 5B, panels I-III). Also, the hierarchy of conditions with the most stable complexes listed first is a little different, and could be summarized as: ppGpp and pppApp added at the same time; ppGpp present alone; RNAP pre-incubated with ppGpp and then pppApp added (i.e. panels VI>IV>V; p-values were <0.05 for 80 and 160 nM RNAP, when comparing panels VI and V; also, p<0.05 for 160 nM RNAP when comparing panel IV and V; no significant differences were found for all other comparisons). This signifies that it not only matters which (p)ppNpp is added first, but also which analog is present in excess over the other.

When DksA was present, in each case a rather similar gradation as without DksA is preserved. For 500 μ M pppApp, the hierarchy of conditions with the highest amount of detected DNARNAP complexes could be listed as panels I III>II. In case of 500 μ M, that order is panel VI>IV>V.

Overall, the EMSA studies seem to support the outcome of transcription measurements since they reveal notable differences in the amount of formed stable DNA-RNAP complexes depending on which (p)ppNpp has been added first, although these changes do not necessarily correspond with the effects observed in Figure 5A (see Discussion).

4. DISCUSSION

It has become evident that the diverse regulatory abilities of (p)ppGpp are due to its direct interactions with *E. coli* RNAP. Structure-function studies are but one approach to understanding this interaction. As mentioned earlier, so far focus has only been on varying the length of the ribose-5' phosphate chain but this has allowed assigning clear effects on regulatory potency of (p)(p)pGpp [17; 19]. The current work expands the focus to include the functional consequences of substituting guanine with adenine, as well as comparing the effects of the presence or absence of the 5' γ -phosphate in the adenine analog. For rigorous comparisons, it is crucial to use a well-defined experimental system. Studies of the functional effects of structural variants of (p)ppGpp are potentially of fundamental interest for understanding regulation of transcription. In addition, the ensuing understanding can add to a rational approach for the development of new antibiotics based on the stringent response.

It is intriguing that the binding site for (p)ppApp is discovered to be near the catalytic center of the enzyme, with adenine-specific contacts, where the chances for functional consequences are greatly enhanced. Despite specific (p)ppApp binding to RNAP, there is no evidence that (p)ppApp is of biological relevance for *E. coli* regulation. This puzzle becomes even more intriguing by finding that the functional consequences of (p)ppApp binding affects not just a few, but a whole set of regulatory properties. Even though the observed effects are not dramatically high, they are highly reproducible and notable. Moreover, these striking properties do not vary randomly but instead vary as a mirror image of those associated with (p)ppGpp binding.

From the X-ray crystal structures of *E. coli* RNAP and (p)ppApp complex, we found that (p)ppApp bind at the active site and switch regions of RNAP. Figure 1 D depicts basic

residues of RNAP interacting with the (p)ppApp 5' phosphate group (R346 and R352 from switch 2 region) and 3' phosphate group (K1242 from switch 3). The switch regions of RNAP form multiple conformations and serve as hinges for the RNAP clamp [35] and undergo conformational changes during the opening and closing of the clamp in the course of open complex formation [36; 37]. The bacterial RNAP inhibitors such as Myxopyronin (Myx) and Fidaxomicin (also known as lipiarmycin) bind at the switch regions [38; 39] and lock the clamp in distinct conformation. The (p)ppApp binding site is separated only by 13 Å from the Myx binding site (Figure 1D). Accordingly, the (p)ppApp binding at the switch regions may limit the clamp to a certain conformation favorable for stabilizing the open complex of RNAP at the *rrnB* P1 promoter. The extra 5' phosphate in pppApp facing the switch regions may well enhance the interactions with basic residues of these regions as compared to ppApp, making pppApp a stronger effector molecule than ppApp.

To understand functions of ppApp bound near the active site of RNAP during the open complex formation, we made a model of the open complex with ppApp by superposing the RNAP-ppApp complex structure with an X-ray structure of the *E. coli* RNAP transcription initiation complex [40] (Figures 1E and F). The ppApp occupies space at the template DNA binding site from -3 to -1 positions, indicating that the ppApp must dissociate from its binding site to permit formation of the open complex.

The 5'p of ppApp is located only 8 Å away from the acidic tip of $\sigma^{3.2}$ (also known as σ finger), which plays a role in facilitating transcription initiation [41]. Due to interactions with $\sigma^{3.2}$, the template DNA adopts an A-form helical conformation in a manner compatible with binding of initiating NTPs [42]. The $\sigma^{3.2}$ position shifts 7.3 Å from the apo-form to the open complex to prevent steric clash with template DNA (Figure 1F). If the likely electric repulsion between the negatively charged $\sigma^{3.2}$ and the phosphate groups of ppApp occurs, then the RNAP-ppApp complex shifts the σ finger 3.6 Å from its position in the apo-form RNAP. Interestingly, the $\sigma^{3.2}$ position in the RNAP-ppApp complex is halfway between its position in the apo-form and in the open complex of RNAP. This suggests ppApp might facilitate entrance of the template DNA to form an open complex. A role of ppApp may be similar to the role of the σ region 1.1 ($\sigma^{1.1}$) in that the highly negatively charged $\sigma^{1.1}$ in the main channel of RNAP was proposed to facilitate entrance of the downstream double-stranded; $\sigma^{1.1}$ must be displaced from the main channel to permit formation of the open complex [43; 44].

Since the *Escherichia coli rrnB* P1 promoter is one of the best studied (p)ppGpp-regulated promoters, we have employed it in our system. Our data indicate that (p)ppApp activates transcription initiating from this promoter *in vitro*, with pppApp being more potent than ppApp (Figure 2A). This regulatory behavior is opposite to what is observed for ppGpp, which is a stronger inhibitor than pppGpp *in vivo* and *in vitro* [17]. We find that this activation does not depend on ATP as the +1 initiating nucleotide, since changing the promoter sequence at this position from A to G did not prevent pppApp mediated activation (Figure 2C). On the other hand, we show that the known promoter discriminator mutants, previously shown to be insensitive to ppGpp [32], now also become largely immune to pppApp (Figure S6). This can be taken to argue that the discriminator region is a key element for regulation by both analogs.

In order to further verify that the effects we observe are pppApp specific, we performed a control experiment where we titrated ATP under our experimental conditions. This is because one might argue that since the ribosomal promoters are thought by many to strongly depend on the initiating nucleotide concentration (ATP in case of *rrnB* P1) [45], the activating effect exerted by pppApp comes only from the presence of the 5' triphosphate moiety and is not promoter specific *per se*. On the other hand, if true, it would be expected that the 3' diphosphate of pppApp would block the formation of the first diester in transcription initiation and transcriptional inhibition would be observed instead of activation. Figure S9 demonstrates that indeed, over the range of ATP concentrations tested (0 – 500 μ M, in addition to the constant 100 μ M ATP already present in each reaction), the increase in *rrnB* P1 transcript level is negligible (in the range of 1.1 – 1.2 fold).

We have also asked if the pppApp activation would occur at promoters other than *rrnB* P1. As an example, we had used *pthr* which is activated by (p)ppGpp in the presence of DksA. We find that (p)ppApp does not have a pronounced effect on transcription initiating from the *pthr* promoter, either in the presence or absence of DksA (Figure S10). This and the control experiments mentioned above lead us to conclude that the observed transcriptional activation by (p)ppApp at *rrnB* P1 is promoter specific.

We found that pppApp does not affect the rate of promoter escape despite the facts that: 1) pppApp enhances RNAP binding to DNA, as judged by EMSA approaches; 2) open-complexes are rendered more stable (Figure 3 and Figure 4); and 3) structural considerations suggest pppApp might confer stability through its interactions with the switch region (see above). So far we are nevertheless unable to validate these results by DNase I footprints of RNAP on the *rrnB* P1 promoter region in the presence of pppApp (BBO and KP, personal communication). This inconclusive outcome might be due to inherent limitations of the DNase I footprinting method because it is very difficult to obtain an RNAP footprint at this very unstable promoter [12]. It is also puzzling why the overall transcript level in a single round transcription reaction, as evidenced by promoter escape studies, is lower in the presence of pppApp than in the control reaction. One hypothesis would be that dead-end complexes are being formed, however, as mentioned above, we did not observe increased abortive transcript production in that case. Still, formation of dead-end complexes would affect single and multiple-round transcription in a similar way.

Our data also indicate that DksA hinders activation by pppApp. It could be that while pppApp stabilizes the DNA-RNAP complexes, DksA does not affect the open complex stability but rather hinders the phosphodiester bond formation, as was already proposed for the ppGpp-RNAP-DksA complex [23], making the complexes transcriptionally inactive. The data presented in Figure 5A panel I, and Figure 5B panel I, seems to support this notion because the abundance of stable complexes detected by the EMSA experiments in the presence and absence of DksA is similar, yet the amount of transcripts varies greatly (Figure 5). This could also explain why in the open complex stability experiments (Figure 3B), as opposed to EMSA assays, we observe a seemingly destabilizing effect of DksA, since that type of experiments relies on detecting only transcriptionally active complexes.

It is also revealing that it matters at which point DksA has been added to the reaction mix, which was not the case of DksA and ppGpp [12]. Here, when DksA is added to the already pre-formed DNA-RNAP-pppApp complexes, its counter-activation effect is much weaker than when DksA is added together with the DNA template to the pppApp-RNAP complexes (13% vs 46% inhibition when compared to no DksA controls; compare Figure 2B and 5A, panel I). It could be that binding of DNA by pppApp-RNAP induces some conformational changes that now preclude DksA from its otherwise proper binding to RNAP. Alternatively, it has been shown that DksA has a higher affinity for free RNAP than for RNAP-DNA open complexes [46]. Although we did not see such a difference for ppGpp [12], it may matter for pppApp. Also, recent studies reveal three alterations of RNAP holoenzyme structure that accompany the presence of DksA alone, ppGpp alone and DksA and ppGpp together [23]. Thus, it is not unreasonable to suspect that unique RNAP structural differences might accompany the presence of various combinations of ppApp or pppApp in the presence of DksA, ppGpp and DksA together with ppGpp.

We also show that when two (p)ppNpp's, i.e. ppGpp and pppApp, are present together, the outcome as to whether activation or inhibition will take place depends on the order of addition of each nucleotide. For example, adding pppApp first and then ppGpp, leads to enhanced activation; whereas pre-incubation with ppGpp before adding pppApp results in less inhibition than if ppGpp was present alone (Figure 5A). Adding them both at the same time leads to very little activation (when titrating pppApp and keeping ppGpp at a constant level), or rather low inhibition (when titrating ppGpp and keeping pppApp at a constant level) (Figure 5A). It is particularly notable that the spectrum of RNAP regulatory effects in the combined presence of DksA and ppGpp appears to represent a mirror image of those mediated by pppApp. These features include: activation/inhibition of transcription initiation, stabilization/inhibition of open complexes, synergistic effects of DksA are absent/present and the 5'- γ phosphate potentiates/weakens regulation for (p)ppApp vs (p)ppGpp, respectively.

5. CONCLUSIONS

Our experiments reveal a unique RNAP binding site for (p)ppApp that is 13 Å away from the catalytic center Mg ion and contacting both switch 2 and 3 regions. Analog-specific interactions with this site and the (p)ppGpp-specific sites 1 and 2 hold substantial promise for understanding how a substitution of guanine with the adenine base in (p)ppGpp might account for many of the regulatory differences observed. Since ppApp and pppApp appear to bind to the same site, a structural explanation for differences in regulatory potency between the two analogs remains puzzling. Possible affinity differences between pppApp and ppGpp also remain unexplored. It seems that binding of pppApp or ppGpp could induce conformational changes in RNAP that affect the binding of the other. In this sense competition could occur at the level of which one binds to RNAP first. Although very tempting, it is too early to speculate whether binding of one might create or destroy a novel site for the other one.

It is also intriguing that (p)ppApp were shown to be synthesized along with (p)ppGpp by *Bacillus subtilis* under certain stress conditions *in vivo* [47, 48] and by a promiscuous

(purine nucleotide) pyrophosphotransferase from *Streptomyces morookaensis in vitro* [49]. Perhaps these adenosine derivatives may exert regulatory effects in species where they naturally occur (i.e. Firmicutes and Streptomyces).

Finally, it has been proposed recently that ppApp or its analogues could be used as alternatives to antibiotics since it has been shown that ppApp inhibits *E. coli* RelA and thus could block (p)ppGpp synthesis [50]. We now know that caution should be taken, as ppApp, if successfully transported into *E. coli* cells, might alter the RNAP activity and thus exert an effect opposite to the one expected.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights:

- RNAP/(p)ppApp crystal structure reveals a unique binding site
- (p)ppApp activate transcription at *E. coli* *rrnB* P1; pppApp is the stronger effector
- There is competition between pppApp and ppGpp, influencing the effects they exert

Figure 1. Structure of the ppApp-RNAP complex.

A. Electron density map of the ppApp and its binding site. Fo-Fc map (σ cut off = 4) is calculated by omitting ppApp for phase calculation and is shown as blue mesh with stick models of β (cyan) and β' subunits (pink) of RNAP, as well as ppApp. Positions of adenine base and phosphate groups of ppApp are indicated. The catalytic Mg^{2+} ion is shown as a magenta sphere. Amino acid residues involved in the interaction with phosphate groups of ppApp are indicated. **B.** ppApp binding site. The β (cyan) and β' (pink) subunits are shown as surface representation and ppApp is shown as a CPK/stick representation. Amino acid residues contact with adenine base via C2 position are highlighted and indicated (A426, red; Q465, yellow). **C.** The ppApp and ppGpp binding sites are shown in the *E. coli* RNAP σ^{70} holoenzyme (RNAP: cartoon model with transparent surface; α , white; β , cyan; β' , pink; ω , gray; σ , orange; β flap and insert 11 domains are removed to see the ppApp binding site). ppApp and ppGpp are depicted as CPK representations. The clamp domain of β' subunit is highlighted in green and the main channel of RNAP (MC) is indicated. Opening and closing

of the clamp is indicated by a black arrow. **D.** magnified view of the ppApp binding site in (C). The DPBB domain (brown) and switch regions of RNAP involved in the ppApp binding are indicated. The $\sigma_{3.2}$ is highlighted in black. Myx bound at the switch regions is depicted in CPK/stick representation, which was constructed by superposing the RNAP-ppApp complex structure (this study) and the RNAP-Myx complex (PDB: 4YFX). **E.** model of open complex with ppApp was constructed by superposing the structures of the RNAP-ppApp complex (this study) and the transcription initiation complex (PDB: 4YLN). Template (t-DNA) and non-template DNA (nt-DNA) strands are depicted in white and black CPK/stick representation and the transcription start site (+1, green) is highlighted. A position of downstream DNA (dsDNA) is indicated. ppApp and active site Mg are depicted as CPK representation and magenta sphere. **F.** A magnified view of the ppApp binding site in (E). The transcription start site (+1, green) and the template DNA from -1 to -3 positions (brown) that occupy the ppApp binding site are indicated. Three positions of $\sigma^{3.2}$ in the apo-form, in the RNAP-ppApp complex and in the transcription initiation complex are depicted and indicated. Movement of $\sigma^{3.2}$ from the apo-form to the transcription initiation complex is indicated by a black arrow.

Figure 2. Effect of (p)ppApp and ppGpp on the transcription initiating from the *rrnB* P1 promoter.

A. B. Transcription was performed at 90 mM KGlu and assessed in the presence of increasing concentrations of ADP, ppApp and pppApp (25–500 μ M). **C.** Comparison of constant amount of ADP, a4p and a5p (250 μ M) in the presence of increasing concentration of DksA (0–300 nM). **D.** Titration of a5p in an *in vitro* transcription initiating from the *rrnB* P1 promoter mutant (+1 transcription start site: A/G). The data are from at least 3 independent experiments, error bars represent S.D. Examples of autoradiograms displaying bands corresponding to *rrnB* P1 initiated transcripts resolved on sequencing gels are provided.

Figure 3. Influence of pppApp and ppGpp on the transcription initiation stages.

A. Effect of 250 μ M pppApp (a5p) and 250 μ M ppGpp (g4p) on promoter escape in the absence and presence of DksA (300 nM). **B.** Effect of 250 μ M pppApp (a5p) and 250 μ M ppGpp (g4p) on stability of open complexes at the *rnnB* P1 promoter. Half-lives of the open complexes were estimated by „run-off” transcription following heparin addition at time 0. The data are from at least 3 independent experiments, error bars represent S.D.

Figure 4. RNA polymerase binding to the DNA fragment containing *rrnB* P1 promoter in the presence of pppApp.

A. Representative electrophoretic mobility shift assay (EMSA) gel of Cy5-labeled DNA fragment (5 nM) and indicated amounts of RNAP (0–160 nM). **B.** Quantifications of the percentage of RNAP–DNA complexes (shifted DNA) formed with RNAP only or with addition of 500 μ M pppApp, normalized to the unbound DNA in each experiment. The data are from 3 independent experiments, error bars represent S.D.

A *In vitro* Transcription

B EMSA

Figure 5. The effect of the order of addition of pppApp and ppGpp in *in vitro* transcription and EMSA experiments.

A. *In vitro* transcription carried out at different order of (p)ppNpp addition, as indicated. Details are provided in the text and Figure S1. **B.** EMSA experiments measuring stable DNA-RNAP complexes, carried out at different order of (p)ppNpp addition, as indicated. The amount of bound DNA is normalized to 20 nM RNAP in the presence of pppApp in panel I. Details are provided in the text and Figure S2. The data are from at least 3 independent experiments, error bars represent S.D.

Table 1:

Data collection and refinement statistics

RNAP complex with PDB code	ppApp 6BYU
Data collection	
Space group	P2 ₁ 2 ₁ 2 ₁
Cell dimensions	
<i>a</i> (Å)	186.3
<i>b</i> (Å)	203.7
<i>c</i> (Å)	308.3
Resolution (Å)	30 – 3.6
Total reflections	792,327
Unique reflections	135,308
Redundancy	5.9 (6.0)
Completeness (%)	100 (100)
<i>I</i> / σ	10.1 (2.16)
<i>R</i> _{sym} (%)	21.7 (101.8)
CC ^{1/2}	(0.478)
Refinement	
Resolution (Å)	30 – 3.6
<i>R</i> _{work}	0.244
<i>R</i> _{free}	0.311
No. of atoms	54,996
<i>B</i> factors	153.5
R.m.s deviations	
Bond length (Å)	0.004
Bond angles (°)	0.775
Clashscore	14.2
Ramachandran favored, %	90.4
Ramachandran outliers, %	1.53

Data sets were collected at MacCHESS F1 line, Ithaca, NY

* Highest resolution shells are shown in parentheses