Mutations in RNA Polymerase Bridge Helix and Switch Regions Affect Active-Site Networks and Transcript-Assisted Hydrolysis

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Abstract

In bacterial RNA polymerase (RNAP), the bridge helix and switch regions form an intricate network with the catalytic active centre and the main channel. These interactions are important for catalysis, hydrolysis and clamp domain movement. By targeting conserved residues in Escherichia coli RNAP, we are able to show that functions of these regions are differentially required during σ⁷⁰-dependent and the contrasting σ⁵⁴-dependent transcription activations and thus potentially underlie the key mechanistic differences between the two transcription paradigms. We further demonstrate that the transcription factor DksA directly regulates σ⁵⁴-dependent activation both positively and negatively. This finding is consistent with the observed impacts of DksA on σ⁷⁰-dependent promoters. DksA does not seem to significantly affect RNAP binding to a pre-melted promoter DNA but affects extensively activity at the stage of initial RNA synthesis on σ⁵⁴-regulated promoters. Strikingly, removal of the σ⁵⁴ Region I is sufficient to invert the action of DksA (from stimulation to inhibition or vice versa) at two test promoters. The RNAP mutants we generated also show a strong propensity to backtrack. These mutants increase the rate of transcript-hydrolysis cleavage to a level comparable to that seen in the Thermus aquaticus RNAP even in the absence of a non-complementary nucleotide. These novel phenotypes imply an important function of the bridge helix and switch regions as an anti-backtracking ratchet and an RNA hydrolysis regulator.

Introduction

Multisubunit RNA polymerases (RNAPs) carry out the essential function of DNA transcription and are highly conserved in structure and function across bacteria, archaea and eukaryotes [1–3]. The bacterial RNAP holoenzyme consists of a catalytic core (α₂ββ′ω or E) and a sigma factor (σ) required for promoter specific recognition [4,5]. The two large β and β′ subunits form a crab claw-like structure, with the mobile β lobe, β′ clamp and β′ jaw forming the two pincers [6]. Part of the β′ clamp hinges onto the RNAP body via the switch regions SW and has been shown to swing open for DNA entry and remain closed during initiation and elongation, respectively [7]. Five segments of the switch regions control the β′ clamp movement, termed switches 1–5 (SW 1–5), and they are also a new class of antibiotics target sites [8–10]. Myxopyronin is one antibiotic that targets the switch regions [9,11]. Structural analyses indicate that myxopyronin makes direct contacts with SW 1, 2, 4 and 5 [9]. Residues of SW 1–3 make direct contacts with the DNA template [8,12,13]. However, conformational changes of SW 1, 2, 4 and 5 are thought to promote the refolding of SW 3 [14]. These switches interact with one another and their conformational changes can be coupled to that of the bridge helix [8].

The bridge helix BH bifurcates the RNAP central cleft into a main DNA loading channel and a secondary channel [15]. As free nucleotide NTP is proposed to enter the secondary channel and binds to the “i+1” site within the active centre for base pairing,
the trigger loop TL locks in the correct NTP using its refolded helical conformation [16]. It has been proposed that the trigger helices kink the BH that subsequently shifts the nascent 3′-NMP from the ‘i + 1’ site to the ‘i’ site, driving the forward movement of the RNAP from a pre-translocated state to a post-translocated state [17]. The polymerisation can be reversed in cases of processive pyrophosphorolysis or intrinsic and assisted cleavages of the backtracked RNA [17–20].

The activity and conformation of the RNAP active centre are subject to modulation from transcription factors bound to the secondary channel [17,21–24]. For instance, DksA presents two Asp residues at the tip of its coiled-coil domain to the active centre during stringent response [25]. It has been proposed that DksA amplifies the inhibitory effect of ppGpp and reduces the half-life of certain open promoter complexes, possibly by allosterically affecting SW and/or BH/TL [26]. The allosteric regulation of the distant SW regions is thought to be achieved via the DksA's coiled-coil tip and the BH/TL three-helical bundle, which in turn could affect clamp opening and DNA contacts [22]. Consistent with this proposal, DksA-suppressor mutations were found in the BH/TL and four of the five SW regions (SW 1–3 and 5) [26]. The reason we chose BH/SW residues outside of the established DksA-suppressor mutations is twofold: (i) we would like to examine the impact of DksA on σ54-regulated promoters, and (ii) we would like to assess the potential interactions between DksA and the σ54 Region I.

The catalytic dynamics of bacterial RNAP are defined by distinct multiple energetic states along the activation pathway. In Escherichia coli, the σ70 factor directs RNAP to the −10 and −35 elements and spontaneously isomerises the closed complex (RPc) to an open complex (RPo) via several intermediates (RPs). Under stress conditions, many genes that are involved in pathogenicity, nitrogen assimilation and biofilm formation use the alternative σ54 factor [27–31]. σ54 binds to the −12 and −24 elements to form the RPc and imposes a high-energy barrier to prevent DNA melting by inhibitory action of its Region I (σ54RI) [32,33]). Hexameric AAA+ σ54 activators (such as the E. coli phage shock protein PspF and the nitrogen regulation protein NtrC) help transcend the energy barrier at the expense of ATP hydrolysis, leading to full DNA melting in the RPo [34–36]. These properties mimic some eukaryotic Pol II characteristics, and distinct RPps of σ54-dependent transcription complexes can be efficiently captured by using non-hydrolysable ATP analogues bound to its activators [37–40].

Seven residues in (or in close proximity to) the BH and SW regions were selected for this study based on their sequence conservation (Fig. S1). These residues are located near the active centre and the secondary channel (Fig. 1), and these could have potential impacts on RNA extension, hydrolysis and functions of transcription factors. The majority of these selected residues were mutated to an Ala to assess their side-chain interactions. Position S1321 was further mutated to a Lys to assess the impact of

Fig. 1. Residues of the bridge helix and switch regions selected for this study. (A) The bridge helix (red), SW 1 (green), SW 2 (cyan), N-terminus of SW 5 (blue), C-terminus of SW 3 (magenta) and trigger loop (yellow) are highlighted in the E. coli crystal structure (PDB entry 4IGC). (B) The BH and SW regions were zoomed in to show residues of interest.
pair-swapping with K1348 on myxopyronin binding [11]. The wild-type (WT) and mutant RNAPs were normalised based on β and β′ concentrations (Fig. S2) and then reconstituted as holoenzymes with purified sigma factors (σ70 or σ54). The BH and SW mutations in general are detrimental to RPO formation, abortive synthesis and transcription elongation. We find that DksA imposes both positive and negative effects on σ54-dependent promoters. We speculate that, aside from the intrinsic properties of the promoter DNA, DksA works synergistically with σ54 Region I to regulate certain activator-dependent transcription complexes. The RNAP mutants we generated stay preferentially in a backtracked state and can efficiently perform an intrinsic penultimate phosphodiester bond cleavage without further requirement of mismatching NMP or inorganic pyrophosphate. Once locked in the backtracked state, many of the mutants do not elongate upon the addition of the next NTP.

Results

BH and SW residues impact on both RPO formation and initial RNA synthesis

The BH and SW residues form an intricate network around the RNAP active centre, in particular, with the promoter DNA and the trigger loop. Disruption of such network could potentially lead to defects in RPO formation and abortive synthesis. To assess the σ54-dependent RPO formation, we assembled the Eσ70 holoenzymes with activator PspF1-275 and activating nucleotide dATP on a test Sinorhizobium meliloti nifH linear promoter probe (harbouring a mismatch from −10 to −1 on the non-template strand to mimic the fully melted transcription bubble, −10 to −1/WT) and subsequently challenged them with heparin. Several RNAP mutants tested showed modest (50–90% WT activity) to severe defects (below 50% WT activity) in RPO formation (Fig. 2A). In contrast, the β′ K1348A mutant is more efficient in generating RPO (125% WT activity; Fig. 2A). To test if these mutants could carry the RPO into initial RNA synthesis, we added a dinucleotide primer and 32P-GTP to the transcription complex. The dinucleotide UpG and 32P-GTP form the tetranucleotide UpGpGpG (as the small primed RNA) whose level improves RPO formation on the other three promoters (compare Fig. 2B with 2A). The ability of each RNAP mutant to form RPO on different promoters is generally conserved. The exception lies with the β′ S1321A mutant that showed significantly improved RPO formation on the pspA promoter over the other three promoters (compare Fig. 3).

DksA significantly affects abortive synthesis but not RPO formation on four σ54-dependent promoters

DksA can independently and directly regulate promoters transcribed by Eσ70 [41,42]. However, in the case of σ54-dependent promoters, the effects of DksA on in vitro transcription are passive, as its addition does not significantly change the RPO lifetime or transcript level on a Pseudomonas putida promoter [43]. We suspect that the marked regulatory differences of DksA on the two transcription systems may involve intrinsic promoter properties. Thus, four linear σ54-dependent promoter probes were constructed: S. meliloti nifH (nitrogen fixation [32]), E. coli pspA and pspG (membrane stress response [44]) and Pseudomonas syringae hrpL (type III secretion system [45]), all harbouring a pre-melted transcription bubble from −10 to −1 (Fig. 3, diagrams). The RPO formation assays were performed as described above and analysed on native gels. In agreement with the passive model as previously proposed, the presence of DksA does not significantly change the stability of RPO on all four σ54-dependent promoters (Fig. 3). The ability of each RNAP mutant to form RPO on different promoters is generally conserved. The exception lies with the β′ S1321A mutant that showed significantly improved RPO formation on the pspA promoter over the other three promoters (compare Fig. 3).

As the rate of abortive synthesis was assessed, the first striking observation was that DksA had direct and differential impacts on all four σ54-dependent promoters (Fig. 4). The abortive synthesis with the WT RNAP was stimulated by DksA by more than 2-fold on the nifH promoter (Fig. 4A) and by nearly 50% on the pspG promoter (Fig. 4C) but inhibited by approximately 30% on the hrpL promoter (Fig. 4D). The BH and SW mutants reacted to σ54-dependent promoters to different extents, with the β′ I1309A and...
β′ K1348A mutants showing the most detrimental defects in abortive synthesis on nifH and pspG promoters (Fig. 4A and C). The majority of the RNAP mutants seem to follow the effects of DksA on WT RNAP on three out of four promoters tested (with the exception of the pspA promoter). Thus, we propose that, in general, the BH and SW mutations do not override the impacts of DksA on σ54-dependent promoters. The observed effects on nifH, pspG and hrpL likely rely on intrinsic properties of each promoter sequence. Interestingly, both the pspA and pspG promoters share identical –12 and –24 elements and the transcription start site nucleotide (Fig. 4B and C, diagrams), yet they were activated by DksA to different extents (Fig. 4B and C). We propose that the sequence from −10 region to −1 region that constitutes the transcription bubble might be a major factor involved in fine-tuning DksA regulation via the BH and SW regions (see below).

**DksA works in synergy with σ54RI to impact on abortive synthesis**

During DNA melting and loading, σ54 provides extensive interactions at the transcription bubble from −10 to −1 [46]. These interactions are probably mediated by σ54 Region I (residues 1–56). The σ54 Region I forms inhibitory interactions at the −12 site prior to transcription activation and later couples the ATP hydrolysis energy to DNA melting. The σ54 Region II (residues 57–107) is functionally dispensable as it is absent in many σ54 species [47]. The σ54
Region III (residues 108–477) contains a winged helix–turn–helix domain that predominantly binds to the $-24$ element [48]. We suspect that $\sigma_{54}$RI may interact with the transcription bubble and the BH and SW network in the active centre either directly or indirectly and that it may play a role in forming RPO and abortive synthesis. To test this hypothesis, we generated $\sigma_{54}$ with Region I deleted ($\sigma_{54} \Delta$RI) and performed abortive transcription assays on $pspG_{-10/-1}/WT$ and $hrpL_{-10/-1}/WT$ promoters (Fig. 4E and F).

Deletion of $\sigma_{54}$RI drastically reduced the ability of most BH and SW mutants to form abortive products on both promoters (compare Fig. 4E and F with Fig. 4C and D), suggesting that $\sigma_{54}$RI indeed contributes to the BH and SW network. The presence of DksA seemed to partially rescue the abortive synthesis with $\beta'$S1321A and $\beta'$S1321K mutants (Fig. 4E), which may suggest that DksA adds another layer of interactions (direct or indirect) with the $\sigma_{54}$RI-BH-SW network. Most intriguingly, deletion of $\sigma_{54}$RI enables DksA to switch roles from stimulation to inhibition on the $pspG$ promoter with the WT RNAP (compare Fig. 4C with 4E) and from inhibition to stimulation on the $hrpL$ promoter with the WT and $\beta'$D802A RNAPs (compare Fig. 4D with 4F). These observations argue that the influence of DksA relies not only on the intrinsic properties of the $\sigma_{54}$-dependent promoter itself but also on the integrity of $\sigma_{54}$RI.

BH and SW mutants are prone to backtracking and exhibit accelerated transcript-assisted hydrolysis

After assessing the impacts of BH and SW mutants on RPO formation and abortive synthesis, we next examined their abilities to elongate on a $\sigma$-independent DNA/RNA minimal scaffold [49]. The minimal scaffold is assembled with an 18-bp downstream DNA duplex and an 8-bp upstream RNA/DNA hybrid (MS8; Fig. 5). A gap was introduced after the “$i+1$” site to accommodate the kinked DNA path [13]. An alternative minimal scaffold that harbours a 3′-CMP mismatch on the RNA was constructed for studying transcript-assisted hydrolysis [MS8(C); Fig. 5].

We first tested the elongation complex formation on a native gel (in the absence of $\sigma$ factors). As seen in Fig. 5A, the majority of mutant RNAPs showed moderate (60–90% WT activity) to better-than-WT activities in the elongation complex formation. The $\beta'$
K1348A mutant showed the most detrimental defect (20% WT activity; Fig. 5A). Interestingly, many mutants showed different migration patterns to that of the WT RNAP, possibly due to differential clamp movements. To test whether the RNAP mutants could polymerise efficiently, we added a complementary nucleotide (cold UTP) to the 3′-end of the radiolabelled RNA. As seen in Fig. 5B, many of the tested RNAP mutants showed clear defects (below 50% WT activity) in transcript extension; such defects could not be entirely accounted for by weakened complex formation (compare Fig. 5B with 5A). In addition, many RNAP mutants generated RNA 10 through mis-incorporation (Fig. 5B).

To test whether these polymerisation-defective RNAP mutants favour backtracking and/or intrinsic hydrolysis, we performed transcript-assisted cleavage assays. Transcript-assisted cleavage occurs in a 1-bp backtracked state from the pre-translocated state. As the cleavage rate on fully matched 3′-NMP is extremely low, acceleration is usually achieved by mismatching the 3′-NMP [20,50]. In the 1-bp backtracked state, the mismatched 3′-NMP occupies the E site (a non-base-pairing binding site) and assists...
and P2 cleavage, respectively. The penultimate phosphodiester bonds are referred to as the P1 cleavage (a), possibly by enhancing Mg\(^{2+}\) coordination. Re-initiation of elongation was confirmed with WT and β' D802A RNAPs only on the MS8 scaffold (Fig. S5). This observation suggests that not all backtracked states of the RNAP mutants are functionally equivalent, as some can be reversed back to elongation whilst others were driven towards further cleavage.

**Discussion**

The differences in sequence between σ\(^{70}\) and σ\(^{54}\) are reflected in the different mechanisms of RPO formation at play for these two transcription paradigms [34,35]. Although it is difficult to define precisely the relative location of the three σ\(^{54}\) domains within holoenzymes based on existing structural knowledge of σ\(^{70}\), a cryo-electron microscopy contour of the Eσ\(^{54}-\)PspF complex suggests that σ\(^{54}_{RI}\) blocks access to the DNA loading channel, somewhat functionally reminiscent to the “place-holder” function of σ\(^{70}_{1.1}\) [52]. Here, we report an additional function of σ\(^{54}_{RI}\) through its interactions with the BH and SW regions (and possibly also DksA). σ\(^{54}_{RI}\) strongly stabilises the transcription bubble near the +1 site on both strands, and RPO formed without σ\(^{54}_{RI}\) is unstable [45,52]. A co-localisation of σ\(^{54}_{RI}\) with the BH/TL/SW at the +1 site could explain why deletion of σ\(^{54}_{RI}\) has a detrimental impact on RPO formation and abortive synthesis with the mutant RNAPs (Fig. 2A and B). Although both σ\(^{70}_{1.1}\) and σ\(^{54}_{RI}\) may interact with the DNA loading channel at some point along the pathway from RP\(_C\) to RP\(_O\), their precise local interactions with RNAP and their physical routes of displacement for or after DNA loading may well be different. This could explain why transcription activation of σ\(^{54}\) and of σ\(^{70}\) are differentially impacted by BH and SW mutations (this study) and why σ\(^{54}\) is not as sensitive to the T7 Gp2 protein that disrupts the normal displacement of σ\(^{70}_{1.1}\) [53,54].

Three mechanistic models for DksA regulation on alternative σ-dependent transcription have been proposed [55]: (i) DksA potentiates alternative σ factors for their competitiveness, (ii) intrinsic properties of promoter DNA lead to different impacts by DksA and/or (iii) the availability of RNAP increases when DksA regulates σ\(^{70}\)-dependent promoters (the passive model). Experiments carried out by Bernado et al. favour the passive model, where the authors did not observe any increased competitiveness for core RNAP of σ\(^{54}\) or transcription stimulation by DksA in vitro [43]. In this study, we provide evidence
that DksA has direct and kinetically diverse impacts on $\sigma^{54}$ promoters at the stage of abortive synthesis. We demonstrated that DksA stimulated abortive synthesis on nifH, pspA and pspG promoters and inhibited abortive synthesis on the hrpL promoter (Fig. 4). Notably, the TraR protein (a DksA homologue) has been shown to stimulate extracytoplasmic stress genes as regulated by the alternative $\sigma^E$ in the absence of the cofactor ppGpp [56]. It is possible that DksA alters the energetic state on $\sigma^{54}$-regulated promoters prior to the progression to the abortive cycle [42], or it works in synergy with the bridge helix and trigger loop to affect abortive synthesis. However, these possibilities do not explain why deletion of $\sigma^{54}_{RI}$ affects DksA's role on abortive synthesis on pspG and hrpL promoters (Fig. 4E and F). Hence, we propose that a tripartite network involving $\sigma^{54}_{RI}$, DksA and the BH and SW regions is at play for DksA's regulation on $\sigma^{54}$-dependent transcription.

Transcript-assisted RNA cleavage proceeds in a 1-bp backtracked conformation with the mismatched 3′-NMP slipping into the E site to facilitate the penultimate phosphodiester bond cleavage [20]. Without supplementing a mismatched 3′-NMP (either in cis or in trans), the rate of reaction is extremely slow [20,50]. In this study, we have identified BH and SW mutants that are extremely efficient at RNA hydrolysis even on full-matched RNA/DNA hybrids. The accelerated cleavage is comparable to that observed in Thermus aquaticus RNAP that is usually 1-2 orders of magnitude faster than the hydrolysis mechanism in E. coli [16,50]. The comparative studies with a mismatched scaffold showed that the hydrolysis activities were enhanced with our RNAP mutants. One possibility is that the 3′-RNA binds in the wrong register with respect to the transcription start site in the transcription complexes formed with the mutant enzymes. We believe that the observed phenotype points to a novel function of the BH and SW regions as an anti-backtracking ratchet and an RNA hydrolysis regulator. Disruption of such functions is particularly interesting in the β′ V803A mutant, as the RNAP is trapped in a sliding state (with both P1 and P2 cleavage equally likely; Fig. 5C).

Materials and Methods

Protein construction and purification

E. coli rpoB and rpoC genes harbouring single substitutions were mutagenised in plasmids pLA458, pLA545 and pLA661 and were sub-cloned into pVS10 vectors [57]. The reconstituted RNAP mutants were over-expressed in Novablaue (Novagen) and purified as previously described [14]. E. coli PspF₁-275 (the AAA+ domain, residues 1–275) and Klebsiella pneumoniae $\sigma^{54}$ were over-expressed in BL21(DE3) and purified as previously described [36]. E. coli $\sigma^{55}$ was over-expressed in BL21(DE3) and purified via a nickel affinity column followed by a heparin chelating step. E. coli DksA was over-expressed in BL21(DE3) and purified via a nickel affinity column. Proteins were stored in TGED buffer [20 mM Tris–HCl (pH 8.0), 50 mM NaCl, 1 mM DTT, 0.1 mM ethylenediaminetetraacetic acid and 5% glycerol] at −80 °C.

DNA probes

Linear DNA probes (88 nt long) representing E. coli lacUV5, S. mellioti nifH, E. coli pspA, E. coli pspG and P. syringae hrpL promoters were synthesised by Sigma-Aldrich with the highest purity (>98%). The template strands were 5′-32P labelled and annealed to the mismatched non-template strands for binding assays [36].

RP₉ formation and minimal scaffold binding assays

The heparin-resistant RP₉ formation was assessed by mixing 50 nM radiolabelled DNA, 100 nM holoenzyme (1:4 ratio of E to σ), 4 μM PspF₁-275 and 4 mM dATP (for $\sigma^{54}$-dependent RP₉ only) at 37 °C for 15 min. The complexes were challenged with heparin at 37 °C for 5 min before loaded on a 4% native gel and quantified by Aida.

Minimal scaffold binding assays were conducted by mixing an equimolar amount of RNAP and radiolabelled minimal scaffold (100 nM) at 37 °C for 15 min. Stable elongation complexes were resolved on a 4% native gel and quantified by Aida.

In vitro abortive transcription assays

In vitro abortive assays were performed as previously described [36]. Holoenzymes (100 nM, 1:4 ratio of E to σ) were allowed to assemble on specific promoter DNA probes, followed by addition of initiating dinucleotide primers and radiolabelled NTP for 15 min at 37 °C. In $\sigma^{54}$-dependent transcription, 4 μM PspF₁-275 and 4 mM dATP were added to initiate transcription. To examine the impact of transcription factor on RP₉ formation, we incubated 2 μM DksA with the holoenzyme and DNA for 5 min at 37 °C before extension starts. To ensure that RP₉ formation was compared in a single-round transcription initiation, we added 0.2 mg/ml heparin during elongation (10 min at 37 °C). Promoter- and $\sigma$-independent minimal scaffold transcription assays were conducted using 100 nM core RNAP on 50 nM MS8 scaffolds at 37 °C for 15 min [58]. The forward polymerisation was performed by assessing the incorporation of 32P-UTP into RNA on MS8.

Cleavage assays

To assess backtracking and intrinsic cleavage of RNAP mutations, we radiolabelled the MS8 and MS8(C) minimal scaffolds on RNA 5′-ends. Intrinsic cleavage of RNA was allowed to proceed for 15 min at 37 °C before quenched. To test the pyrophosphorolytic activities of the RNAP mutants, we added 0.5 mM PPI to the reaction mixture as previously described [14]. To re-initiate elongation on backtracked complexes, we added 1 mM NTP mixture (ATP, GTP, CTP, UTP) to each cleavage
reaction and incubated it 60 min to allow for transcript extension at 37 °C.

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Conflict of Interest: All authors declared no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jmb.2015.09.005.

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