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Coupling σ Factor Conformation to RNA Polymerase Reorganisation for DNA Melting

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Abstract

ATP-driven remodelling of initial RNA polymerase (RNAP) promoter complexes occurs as a major post recruitment strategy used to control gene expression. Using a model-enhancer-dependent bacterial system (σ^{54} -RNAP, E σ^{54}) and a slowly hydrolysed ATP analogue (ATP γ S), we provide evidence for a nucleotide-dependent temporal pathway leading to DNA melting involving a small set of σ^{54} -DNA conformational states. We demonstrate that the ATP hydrolysis-dependent remodelling of E σ^{54} occurs in at least two distinct temporal steps. The first detected remodelling phase results in changes in the interactions between the promoter specificity σ^{54} factor and the promoter DNA. The second detected remodelling phase causes changes in the relationship between the promoter DNA and the core RNAP catalytic β/β' subunits, correlating with the loading of template DNA into the catalytic cleft of RNAP. It would appear that, for E σ^{54} promoters, loading of template DNA within the catalytic cleft of RNAP is dependent on fast ATP hydrolysis steps that trigger changes in the β' jaw domain, thereby allowing acquisition of the open complex status.

Keywords

ATPase; DNA melting; protein-DNA cross-linking; RNA polymerase; transcription regulation

Introduction

Gene expression is a fundamental process that is frequently controlled at the level of transcription initiation. The similarity in sequence and structure between bacterial and eukaryotic RNA polymerases (RNAPs) suggests that they share many functional states required to progress from initial promoter complexes to complexes competent for making RNA.¹ Understanding the nature of the conformational changes that occur during transcription

initiation and how they are controlled is important in elaborating how RNAPs work as intricate and regulated molecular machines.

A major adaptive response in bacteria relies upon regulation of gene expression at the DNA melting step.^{2,3} It requires RNAP containing the major variant σ^{54} promoter specificity factor and a specialised activator ATPase that belongs to the extended AAA+ (ATPases associated with various cellular activities) protein family. Transcription initiation from σ^{54} -dependent promoters in bacteria is functionally analogous to enhancer-dependent initiation of eukaryotic RNAP II, which requires an input of energy from ATP hydrolysis provided by transcription factor IIH.^{4,5} The bacterial activator ATPase causes the active remodelling of the initial transcriptionally silent σ^{54} -RNAP ($E\sigma^{54}$) promoter complex, termed the closed complex (CC), that otherwise persists. The activator ATPase remodels the $E\sigma^{54}$ CC to the transcriptionally proficient open complex (OC), in which the template DNA is found loaded within the catalytic cleft of RNAP.⁶⁻⁸ How ATP binding and hydrolysis by the activator ATPase are coupled to OC formation is only partially understood. Studying the interactions between the activator ATPase and $E\sigma^{54}$ has proven difficult due to the transient and dynamic nature of these interactions. Ground-state and transition-state analogues of ATP stabilise a highly conserved surface-exposed flexible σ^{54} -interacting loop, termed the L1 loop (Supplementary Fig. 1; the “GAFTGA loop”), located within the AAA⁺ domain of the activator ATPase, apparently causing it to engage $E\sigma^{54}$.⁹⁻¹³ However, the information obtained on the changes brought about in $E\sigma^{54}$ by the activator ATPase is restricted since complexes formed in the presence of these ATP analogues are “trapped,” unable to progress to the OC status and therefore remain transcriptionally inactive.

Here we demonstrate that the ATP analogue ATP γ S reacts slowly with the *Escherichia coli* σ^{54} -dependent activator ATPase phage shock protein F (PspF) to yield ADP and can activate $E\sigma^{54}$ transcription. Using a site-specific DNA-protein cross-linking assay,¹⁴⁻¹⁶ we follow, in a time-resolved manner, complete conversion of one binary σ^{54} promoter complex and the initial $E\sigma^{54}$ CC into their remodelled states. The work provides evidence for a nucleotide-dependent temporal pathway leading to DNA melting, which involves a small set of σ^{54} -DNA conformational states. We show that stimulation of OC formation by PspF₁₋₂₇₅-ATP γ S is dependent on the integrity of the L1 loop, and that nucleotide binding and subsequent hydrolysis cause changes in PspF₁₋₂₇₅-DNA and σ^{54} -DNA interactions that precede changes in RNAP β/β' -DNA interactions, with the latter being greatly limited by the slow hydrolysis rate. We also observe that compromising the functionality of the β' jaw domain [by using the bacteriophage T7 transcription inhibitor gene protein 2 (gp2)] results in significantly reduced ATP γ S-dependent, but not ATP-dependent, stimulation of OC formation. We thus provide evidence that at least two activator-dependent steps are required for productive OC formation and identify the β' jaw domain as one structurally conserved flexible feature of RNAP associated with a kinetic barrier to OC formation.¹⁷ We propose that when ATP is used, relatively fast hydrolysis steps in the activator ATPase overcome this barrier by linking changes in the relationship between activator ATPase, σ^{54} , and/or promoter DNA to major conformational reorganisations of the RNAP that accompany OC formation.

Results

ATP γ S is hydrolysed by PspF₁₋₂₇₅

OC formation by RNAPs is a multistep isomerisation process involving a number of intermediates, the characterisation of which has proved difficult due to their transient natures. For $E\sigma^{54}$, ground-state and transition-state ATP analogues (when bound to the activator ATPase) have allowed access to stable putative intermediates, but since these complexes are “trapped” and are therefore transcriptionally inactive, their relevance is uncertain.^{9-11,13} We reasoned that the poorly hydrolysable ATP analogue ATP γ S may allow the initial ATP-bound

form of the activator ATPase and early intermediates formed en route to the $E\sigma^{54}$ OC to be characterised (by slowing down one or more steps in the isomerisation process). Earlier studies suggested that ATP γ S would function far less efficiently, if at all, in $E\sigma^{54}$ -dependent OC formation.^{6,7,18} However, recently, it has been reported that ATP γ S is slowly hydrolysed by certain AAA⁺ proteins, including another $E\sigma^{54}$ activator ATPase, NtrC1.^{10,19,20} For experimental simplicity, we used a structurally characterised form of PspF (AAA⁺ domain amino acids 1-275, PspF₁₋₂₇₅), deleted for its DNA-binding domain, that is still capable of efficiently activating transcription from σ^{54} -dependent promoters *in vivo* and *in vitro* from solution.^{12,21,22}

To determine whether PspF₁₋₂₇₅ can hydrolyse ATP γ S to yield ADP, we performed *in situ* ADP-AIF “trapping” assays to monitor complex formation between $E\sigma^{54}$ and PspF₁₋₂₇₅-ADP-AIF⁹. Previously, we have shown that a stable complex containing (E) σ^{54} and a hexameric form of PspF₁₋₂₇₅ can be detected in the presence of the transition-state analogue ADP-AIF (formed in the presence of ADP, NaF, and AlCl₃).^{9,12} Importantly, recently, it has also been shown that by substituting ADP with ATP (in the presence of NaF and AlCl₃), we can form ADP-AIF trapped complexes, as ATP is hydrolysed by PspF₁₋₂₇₅ to ADP²³. We reasoned that if ATP γ S is hydrolysed by PspF₁₋₂₇₅, it also would support PspF₁₋₂₇₅-ADP-AIF trapped complex formation. As shown in Fig. 1a, the level of trapped complex formation, and thus ADP production, from PspF₁₋₂₇₅ in the presence of 0.2 mM ATP γ S (lane 2) is significantly lower than that observed with 0.2 mM ATP (lane 1) after 5 min incubation time. However, when the reaction time is increased, allowing accumulation of ADP from ATP γ S hydrolysis, the amount of trapped complex detected significantly increases (Fig. 1a, lanes 3-5). Importantly, in the absence of the trapping reagents (NaF and AlCl₃), ATP γ S hydrolysis does not result in the formation of a stable trapped complex (lane 6). Control reactions with a PspF₁₋₂₇₅ mutant harbouring the Walker B motif substitution D107A, which has significantly reduced (<1% of wild-type (WT) activity) ATP hydrolysis activity,^{23,24} failed to form trapped complexes in the presence of ATP γ S (data not shown). Furthermore, we observed that in ATP γ S-soaked PspF₁₋₂₇₅ crystals, ATP γ S occupies the same position in PspF₁₋₂₇₅ as ATP and AMP-PNP (Supplementary Fig. 122). In line with its slow hydrolysis rate, soaking crystals of PspF₁₋₂₇₅ with 40 mM ATP γ S did not lead to the crystals cracking.²²

We next determined that the turnover rate of PspF₁₋₂₇₅ for ATP γ S under similar conditions used for the transcription assays (see below and Materials and Methods) is 0.03 min⁻¹ (± 0.0005), which is approximately 1/1000th the rate observed with ATP. The slow hydrolysis rate of ATP γ S is not the result of poor nucleotide binding since the affinities for ATP and ATP γ S are likely to be similar.²⁵ Importantly, since *in vitro* mutants of PspF₁₋₂₇₅ with greatly reduced ATPase activity support OC formation, we suggest that the very low rates of hydrolysis associated with ATP γ S would be sufficient for slowly making OCs.²³ Overall, these data establish that ATP γ S reacts slowly with PspF₁₋₂₇₅ to yield ADP, and we subsequently use the term “slow hydrolysis” without defining which enzymatic step(s) are slower when compared to ATP hydrolysis.

PspF₁₋₂₇₅-ATP γ S supports $E\sigma^{54}$ transcription

We next investigated whether ATP γ S can support *in vitro* transcription from the σ^{54} -dependent *Sinorhizobium meliloti* *nifH* promoter on a supercoiled plasmid. Figure 1b (lane 2) demonstrates that $E\sigma^{54}$ -dependent transcription can occur in the presence of PspF₁₋₂₇₅ and ATP γ S after 5 min activation time, the same time period when we observed a small amount of trapped complex formation (Fig. 1a, lane 2). Initially, we determined the optimal concentration of ATP γ S that resulted in the maximum number of transcripts from competitor (heparin)-resistant OCs. Unlike ATP or dATP, which functions optimally at 4 mM²⁵ (Fig. 1b, lane 7), the optimal concentration for ATP γ S under the experimental conditions used is 0.2 mM (Fig.

1b, lane 3). Previously, it has been demonstrated that PspF₁₋₂₇₅ functions in a mixed nucleotide state that is subject to inhibition by ATP at concentrations greater than 4 mM due to saturation of nucleoside triphosphate (NTP) binding sites by ATP.^{25,26} Since the rate of ATP γ S hydrolysis appears much slower compared to that of ATP (Fig. 1a) or dATP, we expect that this would lead to different optimal nucleotide requirements.

Previously, we have shown that in the presence of an ATPase defective mutant PspF₁₋₂₇₅ E108Q (1% of WT activity) and ATP, E σ ⁵⁴ transcription is greatly improved upon increased activation periods.²³ In line with this observation, E σ ⁵⁴ transcription from the supercoiled *nifH* promoter significantly increased at longer activation times with PspF₁₋₂₇₅-ATP γ S (Fig. 1c, lanes 2-4), more than 1000-fold above the level observed for control reactions in which ATP γ S (data not shown) or PspF₁₋₂₇₅ (lane 1) was omitted. These data are consistent with *in situ* trapping reactions, which demonstrate that more trapped complexes, and hence more ATP γ S hydrolysis, are observed when the incubation time of PspF₁₋₂₇₅ with ATP γ S is increased (Fig. 1a, lanes 2-5). However, we note that the ATP γ S-dependent transcript level is significantly lower than that observed in the presence of PspF₁₋₂₇₅ and either ATP or dATP (Fig. 1c, lanes 5-7 and 8-10). Separate experiments show that the decrease in transcripts observed after 60 min activation time with either ATP or dATP is the result of NTP depletion (and nonoptimal nucleotide ratios) and the decay of preformed OCs (data not shown).

We suggest that the effective use of ATP γ S in transcription activation (i.e., more transcripts formed at lower nucleotide concentrations; Fig. 1b, lane 3) is, in part, the result of a prolonged association between PspF₁₋₂₇₅ and ATP γ S, supporting a binding interaction with the E σ ⁵⁴ CC. In contrast with either ATP or dATP, where hydrolysis occurs much more rapidly, we envisage that a greater proportion of hydrolysis occurs when PspF₁₋₂₇₅ is not interacting with the E σ ⁵⁴ CC, thereby inefficiently coupling nucleotide hydrolysis to OC formation.

Preopening the DNA promotes OC formation by PspF₁₋₂₇₅-ATP γ S

Using the supercoiled DNA template, slow hydrolysis of ATP γ S clearly supports transcription activation by E σ ⁵⁴. We next considered whether preopening the DNA could increase the amount of OCs formed by PspF₁₋₂₇₅-ATP γ S, as might occur if, for example, rapid hydrolysis is linked to DNA melting and/or loading of the opened DNA into the catalytic cleft of RNAP. Using abortive transcription assays (to measure heparin-resistant OC formation)^{27,28} with two promoter probes, a native linear duplex (Fig. 1d; probe 1), and a linear pre-opened probe (Fig. 1e; probe 2; mismatched between positions -10 and -1, thereby mimicking the conformation of the promoter within the E σ ⁵⁴ OC),^{17,29} we determined whether the rate-limiting step in ATP γ S-dependent OC formation by PspF₁₋₂₇₅ occurs prior to, or after, DNA melting. Like the full-length transcription assays on the supercoiled template, the abortive transcription assays show that PspF₁₋₂₇₅ and 4 mM ATP γ S (lanes 1-3) are far less effective than PspF₁₋₂₇₅ and either 4 mM ATP (lanes 4-6) or 4 mM dATP (lanes 7-9) in stimulating OC formation when the promoter DNA template is fully double-stranded (Fig. 1d, probe 1). At the lower 0.2 mM concentration (optimal for ATP γ S), all the NTPs tested in the presence of PspF₁₋₂₇₅ form similar numbers of OCs on the duplex probe, implying that limiting amounts of NTP hydrolysis occur in each case. We note that the rates of decay of OCs (as judged by a heparin challenge experiment) are similar whether they are formed using ATP, dATP, or ATP γ S (data not shown), indicating that OCs formed in the presence of PspF₁₋₂₇₅-ATP γ S were indistinguishable from those formed in the presence of either PspF₁₋₂₇₅-ATP or PspF₁₋₂₇₅-dATP. This fact, and the rapid consumption of ATP or dATP but not ATP γ S, can explain the loss of abortive transcripts at longer activation times with the former.

Strikingly, when using the pre-opened (-10 to -1) template (Fig. 1e, probe 2), the number of OCs formed in the presence of PspF₁₋₂₇₅ and either 0.2 mM or 4 mM ATP γ S significantly increased (approximately 100-fold, or greater in the case of 4 mM ATP γ S) and approached the

number obtained with PspF₁₋₂₇₅-ATP after 60 min activation time (Fig. 1e). Notably, the number of OCs formed by PspF₁₋₂₇₅ and either 0.2 mM or 4 mM ATP or dATP is also increased by preopening the promoter DNA, yet by only 10-fold. Native gel shift experiments demonstrated that the number of CCs formed on the pre-opened probe in the presence of PspF₁₋₂₇₅ and either ATP γ S or dATP is similar (Supplementary Fig. 2a). The number of OCs formed (as judged by 5 min of heparin challenge) is significantly lower than the number of CCs observed, indicating that some CCs remain available for potentially making more OCs, and so CC formation is not limiting. We note that the number of OCs formed by PspF₁₋₂₇₅-ATP γ S increases linearly over the three time points assayed in this study (5 min, 30 min, and 60 min) (Supplementary Fig. 2a, lanes 2-4), yet the number of OCs formed by PspF₁₋₂₇₅-dATP is only linear at very early incubation time points (up to 2 min) (Supplementary Fig. 2a, lanes 5-13). Importantly, separate control reactions demonstrate that the reagents required to make OCs, including the promoter DNA concentration (Supplementary Fig. 2b), were not limiting, indicating that we would be able to detect an approximate 100-fold increase in OCs with either PspF₁₋₂₇₅-ATP or PspF₁₋₂₇₅-dATP if it were occurring. Taken together, these results suggest a role for rapid ATP hydrolysis-dependent steps in the loading of melted DNA within the catalytic cleft of the RNAP to form heparin (competitor)-resistant OCs.

Determinants in PspF₁₋₂₇₅ important for OC formation with ATP γ S

In principle, the ability of PspF₁₋₂₇₅-ATP γ S to support competitor-resistant OC formation could be due simply to the ATP γ S-bound form of PspF₁₋₂₇₅ mimicking the ATP-bound state, rather than it being slowly hydrolysed. Determinants in PspF required for both ATP binding (Walker A motif)²⁴ and ATP hydrolysis (Walker B motif)²³ were found to be essential for OC formation on the pre-opened promoter when using PspF₁₋₂₇₅ and either dATP or ATP γ S (data not shown). Determinants that are important for coupling the energy derived from ATP hydrolysis to isomerisation of the E σ ⁵⁴ CC, in particular residues F85 and T86^{6,21} (located within the GAFTGA motif of the σ ⁵⁴-interacting L1 loop; Supplementary Fig. 1), were also required for PspF₁₋₂₇₅-ATP γ S-dependent stimulation of OC formation (Fig. 2a). Previous studies have demonstrated that the PspF₁₋₂₇₅ mutants F85W and T86A were unable to interact with σ ⁵⁴, although their ability to hydrolyse ATP remained largely unaffected. Consistent with these findings, no abortive products on the pre-opened probe were detected when using PspF₁₋₂₇₅ harbouring either F85W or T86A substitutions in the presence of dATP (Fig. 2a, lanes 2 and 4) or ATP γ S (Fig. 2a, lanes 6 and 8). The T86S substitution, however, permits PspF₁₋₂₇₅ to interact with σ ⁵⁴ whilst having no significant effect on ATPase activity, which explains its ability to stimulate OC formation (and hence abortive products) in the presence of dATP at a level similar to that of WT PspF₁₋₂₇₅ (Fig. 2a, compare lanes 1 and 3). Significantly, T86S behaved differently in the presence of ATP γ S (Fig. 2a, compare lanes 3 and 7), displaying a severely reduced ability (<1% of wild-type levels) to form OCs and hence activate transcription from the pre-opened promoter, but, with respect to ATP γ S, had a modest effect on hydrolysis activity (80% of WT ATP γ S hydrolysis activity levels). These data show that post-DNA melting steps that require rapid ATP hydrolysis are linked to the functional integrity of the L1 loop for efficient OC formation.

OC formation by PspF₁₋₂₇₅-ATP γ S requires the functional integrity of the β' jaw domain

Stable OC formation by E σ ⁵⁴ relies upon the structural integrity of the β' jaw domain, which, together with the β' clamp domain, forms a trough where the DNA downstream of the active site is located within the OC. Thus, deletion of the β' jaw (*E. coli* β' residues 1149-1190) results in OCs with markedly reduced half-lives.^{27,31,32} We used the bacteriophage T7-encoded transcription inhibitor gp2, which specifically binds to the β' jaw domain, as a functional probe to study the contribution of the jaw domain to PspF₁₋₂₇₅-ATP γ S-dependent OC formation on the preopened (-10 to -1) promoter probe. As shown in Fig. 2b (lanes 1-9) and consistent with our previous findings,³⁰ transcription by E σ ⁵⁴ stimulated by PspF₁₋₂₇₅-dATP is resistant to

gp2, irrespective of when gp2 is added to the reaction (lanes 4-9). Significantly, in the presence of PspF₁₋₂₇₅-ATP γ S (Fig. 2b, lanes 12-20), gp2 severely reduced E σ^{54} transcription (and hence OC formation) but *only* when gp2 was added prior to PspF₁₋₂₇₅-ATP γ S (i.e., before OC formation has occurred) (Fig. 2b, compare lanes 15-17 and 18-20). As expected, control reactions with a gp2-resistant mutant core RNAP (harbouring the E1188K mutation in the β' jaw domain) showed no sensitivity to gp2 in PspF₁₋₂₇₅-ATP γ S-dependent stimulation of E σ^{54} transcription on the supercoiled template (Supplementary Fig. 3). Overall, the use of gp2 as a functional probe of E σ^{54} transcription suggests that the slower hydrolysis rates associated with ATP γ S are insufficient to trigger conformational changes in the β' jaw domain frequently enough to permit stable loading of the pre-opened DNA needed for OC formation. Notably, deletion of the β' jaw domain results in unstable OC formation, and pre-opening the DNA (specifically opening the DNA immediately upstream of the transcription start site) overcomes this phenotype,²⁷ resembling properties of PspF₁₋₂₇₅-ATP γ S-dependent OC formation (see Fig. 1e). We infer that the inefficiency of the PspF₁₋₂₇₅-ATP γ S-dependent stimulation of OC formation on the duplex probe (Fig. 1d) may be due to the inability of the β' jaw domain to frequently capture (and hence stabilise) melted DNA proximal to the transcription start site. Since regulatory sequences in σ^{54} Region I that are important for interacting with PspF are also important for cooperativity with the β' jaw domain for stable OC formation, it seems that conformational coupling between σ^{54} Region I and the β' jaw domain may be negatively affected by the slow hydrolysis rate associated with ATP γ S.

σ^{54} -DNA interactions are altered by PspF₁₋₂₇₅-ATP γ S

A site-specific protein-DNA cross-linking method was used to study protein-DNA relationship changes for σ^{54} -DNA, β/β' -DNA, and PspF₁₋₂₇₅-DNA interactions during PspF₁₋₂₇₅-ATP γ S-driven OC formation. The photoreactive DNA templates used in this assay were constructed by strategically placing the cross-linking reagent *p*-azidophenacyl bromide (Sigma) between positions -1 and +1 (-1) in the context of promoter probes 1, 2, and 3 (see below). The -1 site was chosen based on previous results, which revealed that the cross-linking pattern at this position allows a clear differentiation between the CC, an intermediate (ADP-AIF trapped) complex, and the OC, since: (i) only σ^{54} -DNA interactions are detected within the CC; (ii) in the trapped complex, both σ^{54} -DNA and PspF₁₋₂₇₅-DNA interactions are present; and (iii) only upon full ATP hydrolysis (i.e., within the OC) are interactions between the β/β' subunits and the promoter established.^{15,33}

Initially, we analysed by native PAGE a binary σ^{54} -promoter complex formed in the presence of PspF₁₋₂₇₅ and either 0.2 mM ATP γ S, 4 mM ATP, or 4 mM dATP on promoter probe 3 (Fig. 3a, top), which contains an unpaired segment at positions -12 and -11 on the non-template strand, thereby resembling the DNA conformation found within the E σ^{54} CC. Previously, we demonstrated that the initial binary σ^{54} -probe 3 complex can be remodelled by PspF₁₋₂₇₅-ATP (or dATP) to a “supershift” complex (ss σ^{54} -DNA; Fig. 3a, lanes 5 and 8) that migrates differently in native PAGE analysis but does not contain the activator ATPase PspF₁₋₂₇₅; instead, the DNA in this complex is melted (as judged by KMnO₄ footprinting) to -5.^{6,34} Using this assay, we can establish whether early steps in OC formation that rely on σ^{54} isomerisation can occur in the presence of PspF₁₋₂₇₅-ATP γ S (and in the absence of the core RNAP). We observed that when PspF₁₋₂₇₅ and ATP γ S are present, supershift complexes are no longer detected, compared to PspF₁₋₂₇₅ and either ATP or dATP (Fig. 3a, compare lanes 2, 5, and 8). Extending the incubation time with PspF₁₋₂₇₅-ATP γ S failed to result in any detectable accumulation of the isomerised form of σ^{54} (lanes 3-4). In reactions containing ATP or dATP where supershift complexes were observed in native PAGE analysis (Fig. 3a, lanes 5 and 8, respectively), the cross-linking profile of σ^{54} -DNA in these reactions is altered (Fig. 3b, lanes 5 and 8; the extra band is highlighted with an arrow), compared to σ^{54} alone (Fig. 3b, lane 1), suggesting that, within the supershift complex, the organisation of σ^{54} with respect to promoter

DNA has changed. These cross-linking data are consistent with the prior observation that, within the supershift complex, no activator protein was present and that σ^{54} had been remodelled.⁶ When the supershift complexes were allowed to decay with time (Fig. 3a, compare lane 5 with lanes 6 and 7, and lane 8 with lanes 9 and 10), the cross-linking profile of σ^{54} -DNA in these reactions (Fig. 3b, lanes 6-7 and 9-10) then resembles that of σ^{54} in the initial binary complex (Fig. 3b, lane 1; a single band is observed). Strikingly, in reactions containing PspF₁₋₂₇₅ and ATP γ S, we observed an additional cross-linked species that was identified using different molecular weight versions of PspF¹⁴ to correspond to PspF₁₋₂₇₅-DNA (Fig. 3b, lanes 2-4). Control reactions (Supplementary Fig. 4) lacking σ^{54} (lane 3), PspF₁₋₂₇₅ (lane 1), or ATP γ S (lane 2) indicated that σ^{54} , PspF₁₋₂₇₅, and ATP γ S were all required in the reaction to obtain the cross-linked PspF₁₋₂₇₅-DNA species (lane 4). Furthermore, we also established that the cross-linked PspF₁₋₂₇₅-DNA species was also dependent on a form of PspF₁₋₂₇₅ that was capable of interacting with σ^{54} (Supplementary Fig. 4, lanes 6 and 8). Since we are unable to resolve a σ^{54} -DNA-PspF₁₋₂₇₅-ATP γ S complex by native PAGE, we suggest that the σ^{54} -dependent cross-linked PspF₁₋₂₇₅ in the ATP γ S reactions probably reflects a relatively unstable interaction between σ^{54} and PspF₁₋₂₇₅, made possible by the slow hydrolysis rate (compared to ATP or dATP) associated with ATP γ S. We also note that the cross-linking profile of σ^{54} -DNA has changed in the ATP γ S reactions (Fig. 3b, lanes 2-4, marked with an arrow), although the relative amount of promoter bound by σ^{54} remains similar to that observed for σ^{54} alone (Fig. 3a, lanes 1-4). The change in the cross-linking profile of σ^{54} -DNA (i.e., the disappearance of the more intense faster-migrating σ^{54} -DNA species present in the σ^{54} -alone reactions) seems to be due to the cross-linkable PspF₁₋₂₇₅-DNA species, suggesting that the organisation of σ^{54} within these reactions is altered. These data indicate that one action of PspF₁₋₂₇₅-ATP is to bind to and reorganise σ^{54} with respect to promoter DNA. Since we observed significantly reduced, if any, cross-linked PspF₁₋₂₇₅-DNA in the ATP or dATP reactions, we envisage that fast hydrolysis steps may be required for the 'release' of PspF from the σ^{54} -DNA complex.

β/β' -DNA interactions follow rearrangements in σ^{54}

Only upon stable OC formation are promoter DNA cross-links with the catalytic β and β' subunits established, reflecting the loading of DNA within the catalytic cleft of RNAP.¹⁵ Since the rate of accumulation of OCs in the presence of PspF₁₋₂₇₅-ATP γ S is much slower than in the presence of PspF₁₋₂₇₅-ATP (or dATP) (Fig. 1c and d), we were able to use the cross-linking assay to monitor β/β' interactions with the promoter DNA in a time-resolved manner. Importantly, the cross-linking reactions were not competitor-challenged, so that unstable transcription intermediates formed en route to the OC could be captured and analysed. The results shown in Fig. 4a indicate that no interactions with the β/β' subunits were detected within complexes exposed to PspF₁₋₂₇₅-ATP γ S even after 60 min incubation time (lanes 2-4), contrasting reactions with ATP (lanes 5-7) or dATP (lanes 8-10). This is consistent with the dramatically reduced transcription activity associated with PspF₁₋₂₇₅-ATP γ S on promoter probe 1 (Fig. 1d). We note that a very weak band (lane 4, indicated by an arrow) is observed; however, this band is also present in the E σ^{54} -alone reactions and therefore represents 'free' core interacting non-specially with the promoter DNA probe. Furthermore, in the presence of PspF₁₋₂₇₅-ATP γ S, we note that the profile of the σ^{54} -DNA interactions within E σ^{54} complexes has changed from a double band (lane 2, indicated by an arrow) to a single band (lane 3, indicated by an arrow), yet it is not identical to the σ^{54} -DNA species obtained in reactions containing ATP or dATP (e.g., lane 5). Control reactions in which ATP γ S is absent show no change in the σ^{54} -DNA cross-linking pattern even after 60 min incubation time (data not shown). This is consistent with our earlier observations (Fig. 3b), suggesting that one primary action of the activator ATPase is to reorganise σ^{54} (within E σ^{54}). It would appear that later steps associated with rapid ATP hydrolysis involve the loading of DNA within the catalytic cleft of the RNAP, more specifically engagement of the β' jaw domain with the promoter DNA

to help stabilise OCs (since there was no effect of gp2 on reactions stimulated by either ATP or dATP). We also note that, in line with our earlier observations, PspF₁₋₂₇₅-DNA cross-links are detected in E σ^{54} reactions containing PspF₁₋₂₇₅-ATP γ S (Fig. 4a, lanes 2-4), but not in similar reactions containing PspF₁₋₂₇₅ and either ATP or dATP (Fig. 4a, lanes 5-10) where OCs efficiently form and prior intermediate complexes containing PspF₁₋₂₇₅ have not accumulated.

To determine whether the ATP γ S-dependent interaction with PspF₁₋₂₇₅ could correspond to an 'on-pathway' intermediate, we next analysed these same E σ^{54} reactions on the pre-opened promoter since with this probe we were able to detect significantly more abortive products (Fig. 1e). As shown in Fig. 4b, pre-opening the DNA results in the formation of a β/β' cross-linked species (lanes 3-4) in reactions containing PspF₁₋₂₇₅-ATP γ S, identical to that observed for PspF₁₋₂₇₅ and either ATP or dATP (lanes 5-10). Similar to reactions on the duplex promoter probe (Fig. 4a), a small amount of non-specific 'free' core (lane 2, indicated by an arrow) was detected prior to formation of the cross-linked β/β' species. We also note for the PspF₁₋₂₇₅-ATP γ S reactions that (i) the σ^{54} -DNA cross-linking profile has changed from a double band (lane 2, indicated by an arrow) to a single band (lane 3, indicated by an arrow), and (ii) cross-linked PspF₁₋₂₇₅-DNA is also present (as observed with the linear duplex probe 1; Fig. 4a). Since we observed a similar PspF₁₋₂₇₅-ATP γ S-dependent change in the σ^{54} -DNA relationship on both promoter probes, we propose that one early action of the activator ATPase, potentially with ATP bound in some of its subunits, is to reorganise σ^{54} , an action that precedes changes in the β/β' -DNA relationship. The presence of a cross-linked PspF₁₋₂₇₅-DNA species only in reactions containing ATP γ S (and not ATP or dATP) indicates that the relationship between the activator ATPase and the promoter DNA changes before the change in β/β' -DNA relationship. We observed cross-linked PspF₁₋₂₇₅-DNA in reactions containing ATP γ S in which transcription levels approach those observed with ATP, consistent with a subset of the E σ^{54} population that has not yet formed the transcriptionally proficient OC. Presumably due to its slow hydrolysis rate, ATP γ S remains bound by PspF₁₋₂₇₅, in association with E σ^{54} . Pre-opening the DNA, in contrast to reactions using duplex (fully double-stranded) DNA, results in increased numbers of PspF₁₋₂₇₅-ATP γ S-dependent β/β' -DNA cross-linked species (and transcripts; Fig. 1e); one phase of activation in which stable OCs accumulate and melted DNA is loaded into the RNAP is usually linked to relatively rapid ATP hydrolysis used to trigger changes in the location or conformation of the β' jaw domain required for stable OC formation. With the pre-opened DNA, less frequent changes in the β' jaw domain, caused by the slow hydrolysis of ATP γ S, can be productive.

PspF₁₋₂₇₅-ATP γ S and its relationship to ATP ground-state and transition-state activator ATPase interactions

Results so far indicate that one action of the activator ATPase interacting with ATP γ S is to alter the initial relationship between σ^{54} and the promoter DNA, and that outcomes from this early event are more apparent after 30 min incubation time (Fig. 4a and b, lane 3, single band with arrow). Interaction with PspF₁₋₂₇₅-ATP γ S at 5 min of incubation time (Fig. 4a and b, lane 2, double band with arrow, which resembles the σ^{54} -DNA pattern observed in the E σ^{54} -alone reactions, lane 1) could reflect an initially important—potentially prior to ATP hydrolysis—association of the activator ATPase with the CC. To examine this further, we performed comparative cross-linking assays on the duplex probe (probe 1) with E σ^{54} , PspF₁₋₂₇₅, and either ATP γ S or the trapping reagents ADP-BeF, ADP-AlF, and AMP-AlF. It has been proposed that both ADP-BeF¹⁰ and AMP-AlF¹³ reflect ground-state analogues of ATP, whilst ADP-AlF resembles the ATP hydrolysis transition state.⁹ As shown in Fig. 4c, at 5 min incubation time with PspF₁₋₂₇₅-ATP γ S, the σ^{54} -DNA cross-linking profile (lane 2, indicated by an arrow) looks similar to the σ^{54} -DNA pattern of the E σ^{54} -alone reaction (lane 1), but changes to a single band at 30 min incubation time (lane 3, indicated by an arrow). In contrast, each of the trapping

reagents tested resulted in a fully altered σ^{54} -DNA profile (lane 8, a single σ^{54} -DNA species indicated by an arrow) after 5 min incubation time with PspF₁₋₂₇₅ (lanes 5, 8, and 11). However, the cross-linked PspF₁₋₂₇₅-DNA species can be detected at all time points, prior to any changes in the σ^{54} -DNA pattern being detected (in reactions with ATP γ S, lane 2), implying that interactions between ATP-bound PspF₁₋₂₇₅ and σ^{54} might well occur prior to any ATP γ S hydrolysis, although we cannot exclude the possibility that some ATP γ S hydrolysis has occurred in some of the subunits even at the 5-min incubation time point.

In order to delineate between ATP binding and ATP hydrolysis, we conducted control reactions using the ATPase defective mutants D107A^{23,24} (<1% of WT activity) and E108Q²³ (1% of WT activity) in the presence of both ATP γ S and dATP—our rationale being that when an ATP hydrolysis defective mutant is combined with ATP γ S, we would be able to significantly restrict ATP hydrolysis (at early time points), enabling access to states associated with ATP binding prior to hydrolysis. Results indicate that in reactions containing E108Q-dATP, a cross-linked PspF₁₋₂₇₅-DNA species is observed after 5 min incubation time (Supplementary Fig. 5a, lane 8). Interestingly, these conditions permit the formation of a new species with E108Q identified by native PAGE analysis (Supplementary Fig. 5b, lane 9), which migrates similarly to the ADP-AIF trapped complex (data not shown). If the incubation time is increased to 60 min to compensate for the reduced ATP turnover of the mutants, a cross-linked PspF₁₋₂₇₅-DNA species is now observed in the E108Q-ATP γ S reactions (Supplementary Fig. 5a, lane 9, arrow), when no change in the σ^{54} -DNA interactions is observed (double band, lane 9). Importantly, in the E108Q-dATP reactions, after 60 min incubation time, the σ^{54} -DNA relationship is fully altered (lane 10, single band with arrow). Significantly, and consistent with the findings of the *in situ* trapping reactions (data not shown), no PspF₁₋₂₇₅-DNA cross-links or changes in the σ^{54} -DNA profile were obtained with D107A (Supplementary Fig. 5a, lanes 1-5), even though the ATPase activity is similar to E108Q, implying that the integrity of residue D107 may be required for ATP-dependent binding interactions with σ^{54} -DNA, and hence for DNA cross-links with PspF₁₋₂₇₅, to be established. Importantly, taken together, these results establish that the cross-link between PspF₁₋₂₇₅ and DNA, and the subsequent rearrangement of σ^{54} with respect to the promoter DNA require conformational changes in some PspF₁₋₂₇₅ subunits that occur with ATP binding or after some ATP hydrolysis.

Interestingly, the intensity of the cross-linked σ^{54} -DNA species is different between the PspF₁₋₂₇₅-ATP γ S (Fig. 4c, lanes 2-4) reactions and the reactions with the trapping reagents (Fig. 4c, lanes 5-13), even though the relative intensity of the cross-linked PspF₁₋₂₇₅-DNA species remains constant. These data suggest that the organisation of the activator ATPase (PspF₁₋₂₇₅) with respect to DNA within the ATP γ S reactions and each of the trapped complexes (ADP-BeF, ADP-AIF, and AMP-AIF) is similar, but the conformation of σ^{54} in the trapped reactions (Fig. 4c, lanes 5-13) may not necessarily be identical with that found in the ATP γ S reactions, especially at early time points (Fig. 4c, lanes 2-4). Since PspF₁₋₂₇₅-ATP γ S is capable of stimulating OC formation and since ATP γ S can be hydrolysed, a more native initial set of interactions between E σ^{54} and PspF₁₋₂₇₅—distinct from the biochemically stable complexes of the metal-fluoride-dependent trapping reactions—may occur. Such differences may well be attributed to ATP γ S giving rise, through its reactivity in yielding ADP, to a mixed nucleotide-bound state, with several different conformations of PspF₁₋₂₇₅ subunits existing within one PspF₁₋₂₇₅ hexamer.

Discussion

Transcription initiation is a multistep process. For the E σ^{54} system, the experimental accessibility of the initial CC has allowed us, with the use of ATP γ S, to probe the activation pathway in terms of protein component to DNA proximities in a time-resolved manner. In doing so, we have established that large-scale conformational rearrangements (associated with

OC formation) of the catalytic core RNAP subunits β/β' -DNA relationship follow changes in the σ -DNA organisation, and that both rely on nucleotide binding and hydrolysis activities of the activator ATPase (Fig. 4a and b).

Use of ATP γ S and ATP γ S hydrolysis

The PspF₁₋₂₇₅-dependent formation of ADP from ATP γ S was much slower than the formation of ADP from ATP, but ATP γ S hydrolysis could be coupled to the large-scale productive conformational changes in E σ^{54} required for OC formation. We infer that ATP γ S binding to PspF₁₋₂₇₅, catalysed cleavage to yield ADP and thiophosphate, and ADP and thiophosphate product release steps support the same mechanism of large-scale protein conformational changes that leads to NTP hydrolysis-dependent OC formation when ATP is supplied. In the absence of a detailed kinetic analysis of the steps involved in the enzymatic cleavage of γ -thiophosphate from ATP γ S, we can not say precisely which steps occurring in PspF₁₋₂₇₅ are rate-limiting for OC formation, although steps leading to the successful nucleophilic attack of the γ - β bond, rather than bond scission *per se*, may be slowed considerably. For example, the precise positioning of protein (PspF₁₋₂₇₅) structure around ATP γ S, to form the active site, may be slower than with ATP, and activation of the attacking water molecule or positioning of the R-finger needed to stabilise the transition state may be somewhat disfavoured by a non-native γ - β phosphate bond. Furthermore, the ease with which the modified γ - β phosphate bond in ATP γ S can be activated for scission may differ from the γ - β phosphate bond in ATP and thus change the probability of populating the transition state for hydrolysis.

Initial complexes and early phase of activation

In the presence of ATP γ S, we detected an unstable (compared to the trapped complexes) σ^{54} -DNA-PspF₁₋₂₇₅-ATP γ S complex. Given the slow rate of hydrolysis associated with ATP γ S, we assume that PspF₁₋₂₇₅ in such complexes is predominantly bound by ATP γ S. Utilisation of an ATP γ S-bound form of PspF₁₋₂₇₅ (Figs. 3b and 4a and b) and mutant forms of PspF₁₋₂₇₅ with very low ATPase activity (Supplementary Fig. 5a) suggests that a productive (in terms of OC formation) interaction between σ^{54} and PspF₁₋₂₇₅ occurs prior to, or after a small amount of, ATP hydrolysis. With PspF₁₋₂₇₅-ATP γ S, changes in the σ^{54} -DNA cross-linking profile were detected when no cross-links were observed with the β/β' subunits (Fig. 4a), suggesting that activator ATPase-dependent alterations occurring in the σ^{54} factor precede changes in RNAP organisation. Since close-proximity relationships between the β/β' subunits and DNA were not observed at early incubation times (Fig. 4b, lane 2), we propose that the conformational changes in RNAP organisation occur after any further ATP has been hydrolysed. Although the stabilities of σ^{54} -PspF₁₋₂₇₅ and E σ^{54} -PspF₁₋₂₇₅ complexes formed in the presence of ATP γ S are different compared to ADP-AlF, they clearly share an ability to interact with σ^{54} (Fig. 4c). Therefore, the organisation of the σ^{54} -interacting Loop 1 of PspF is, in structural terms, favourably equilibrated with the nucleotide binding pocket when ATP is bound, and not just at the transition state (Fig. 4c), since it would be difficult to envisage how the protein structure would equilibrate at the transition state, as the transition state is so shortlived. The cross-linking data with PspF₁₋₂₇₅ and ATP γ S (Figs. 3b and 4a-c) and the ground-state analogue ADP-BeF (Fig. 4c) suggest that, upon ATP binding, the engagement of Loop 1 with σ^{54} is retained up to and throughout the life of the ATP transition state (mimicked with ADP-AlF) (Fig. 4c).

Time-dependent σ^{54} -DNA cross-links within E σ^{54}

Changes in the σ^{54} -DNA cross-linking profile (from a double band to a single band) within E σ^{54} complexes were observed at position -1 in the DNA in response to an interaction with PspF₁₋₂₇₅-ATP γ S. However, these PspF₁₋₂₇₅-ATP γ S-dependent changes in the σ^{54} -DNA cross-linking profile are less apparent at early (5 min) time points (Fig. 4a and b, lane 2). It

appears that conversion of the CC to the OC is dominated by two distinct conformational states of σ^{54} -DNA (evident as two σ^{54} -DNA bands in the cross-linking assay), where the slower migrating species becomes more intense in a NTP-hydrolysis-dependent and time-dependent manner. It seems likely that some change in the σ^{54} -DNA organisation takes place after the initial interaction between σ^{54} -DNA and PspF₁₋₂₇₅-ATP γ S (Fig. 4a and b, lane 2), and that further changes occur either upon ATP γ S hydrolysis or as PspF₁₋₂₇₅ positions ATP γ S, ready for hydrolysis to occur, thereby adopting a new conformation (Fig. 4a, lane 3). Results with PspF₁₋₂₇₅ E108Q are in agreement with either proposition (Supplementary Fig. 5a). If the σ^{54} -DNA-PspF₁₋₂₇₅-ATP γ S state persists without hydrolysis, some reorganisation of the CC may represent the outcomes of an 'on' enzyme equilibrium between activator ATPase and σ^{54} -DNA rather than a hydrolysis-dependent set of changes. Similarities between the σ^{54} -DNA and PspF₁₋₂₇₅-DNA cross-linked species in the presence of ATP γ S and ADP-BeF (Fig. 4c) suggest that hydrolysis-independent σ^{54} -DNA reorganisations can occur with ATP in its ground state.

Accumulation of β/β' -DNA cross-links

Cross-links between the catalytic β/β' subunits and promoter DNA position -1 were both time-dependent and DNA structure-dependent (Fig. 4a and b). With the use of either ATP γ S (Fig. 4a and b) or the PspF₁₋₂₇₅ E108Q mutant (Supplementary Fig. 5a) to slow the transition from the CC to the OC, the cross-links between β/β' and the promoter DNA accumulated *after* changes in the σ^{54} -DNA cross-linking profile had been detected, suggesting that reorganisation of the catalytic β/β' subunits follows reorganisation of the σ^{54} -DNA relationship. This is consistent with the observation that the interaction between σ^{54} and core RNAP is altered following ATP hydrolysis-dependent isomerisation of σ^{54} .³⁴ Recovery of β/β' cross-links when using the preopened (-10 to -1) probe in reactions containing PspF₁₋₂₇₅-ATP γ S (Fig. 4b, lanes 3 and 4) and inhibition of transcription by gp2 (Fig. 2b, lanes 15-17) suggest that rapid ATP hydrolysis favours formation of the OC, and that slow hydrolysis of ATP γ S populates unstable competitor (heparin)-sensitive intermediates that rarely convert into stable OCs. Rapid conformational coupling within promoter complexes may be required for stable OC formation and may rely on concerted changes in σ^{54} and structurally flexible features of the catalytic β and β' subunits, including the β' jaw domain. The proximity of σ^{54} to the -1 DNA position in the cross-linking experiments may reflect the existence of a microdomain bounded somewhere between -12 and -1 within the CC that is potentially used for DNA melting, somewhat analogous to the situation in E σ^{70} , where a subdomain comprising less than one-fifth of E σ^{70} is sufficient for DNA melting.³⁵ Recent structural studies of PspF₁₋₂₇₅-bound E σ^{54} suggest that such a microdomain would come into play (for DNA melting) after ADP and P_i have been formed by the activator.³⁶

Time-dependent PspF₁₋₂₇₅-DNA cross-links

In the presence of σ^{54} and ATP γ S (Figs. 3b and 4a and b, lanes 2-4), but not ATP (Figs. 3b and 4a and b, lanes 5-7) or dATP (Figs. 3b and 4a and b, lanes 8-10), PspF₁₋₂₇₅ can be cross-linked to DNA, indicating that, prior to the occurrence of DNA proximity changes with the β/β' subunits, the proximity relationship between PspF₁₋₂₇₅ and DNA is distinct. The subsequent loss of cross-links to PspF₁₋₂₇₅ correlates with the reorganisation of RNAP being evident and suggests that PspF₁₋₂₇₅ is not engaged within the RNAP promoter complex once cross-links characteristic of the OC are detected. Hence we propose that activator ATPase release from E σ^{54} occurs upon, or just prior to, completion of stable OC formation. Failure to see PspF₁₋₂₇₅-DNA cross-links when using dATP or ATP can be attributed to their rapid (compared to ATP γ S) turnover and hence only transient support of an association between PspF₁₋₂₇₅ and the CC.

Pathways for making OCs

In several respects, activation by PspF₁₋₂₇₅-ATP γ S resembles certain activator bypass forms of σ^{54} , which fail to effectively form stable OCs and can not initiate transcription from duplex linear DNA templates, but can do so from supercoiled or pre-opened DNA.^{37,38} We suggest that the promoter and core RNAP interactions made by an activator bypass form of σ^{54} and the reorganisation of wild-type σ^{54} evident when using PspF₁₋₂₇₅-ATP γ S (Fig. 4a and b, lanes 2-4) or PspF₁₋₂₇₅ E108Q-dATP (Supplementary Fig. 5a) are functionally related to early events in transcription activation. Changes in the binding interactions between RNAP and σ^{54} , as altered by the activator ATPase, link the energy derived from ATP hydrolysis to reorganisation of the RNAP to accommodate the DNA within the catalytic cleft of RNAP. Importantly, inhibition of PspF₁₋₂₇₅-ATP γ S-dependent transcription activation in the presence of gp2 (Fig. 2b, lanes 15-17) and its relative resistance when PspF₁₋₂₇₅ and either ATP (data not shown) or dATP (Fig. 2b, lanes 4-9) are used strongly support this view. One possibility is that DNA contacts by core RNAP associated with the opening of double-stranded DNA might not efficiently, or frequently enough, form in PspF₁₋₂₇₅-ATP γ S-driven activation reactions to capture transiently opened DNA, but that sufficient change in the accessibility of the downstream channel of RNAP does occur to allow effective utilisation of pre-opened DNA. Although our experiments with ATP γ S do not reveal where (in terms of inside or outside of the RNAP catalytic cleft) DNA melting occurs, the results imply that, for E σ^{54} , DNA melting outside of the catalytic cleft is not disallowed.³⁹

Overall, our data provide clear evidence for a coupling of activator ATPase-driven changes in the organisation of σ^{54} -DNA to changes in RNAP conformation used for OC formation, and uncover a kinetic barrier to gene expression. Importantly, we also demonstrate how ATP γ S can be utilised as a tool for studying the activities of other ATPases, in particular the initial interactions that the ATP-bound form of the ATPase makes with its substrate, as well as the formation of the final products of the ATP hydrolysis event.

Materials and Methods

Proteins and DNA probes

E. coli core RNAP was purchased from Epicentre Technology (Cambio). *Klebsiella pneumoniae* σ^{54} and *E. coli* PspF₁₋₂₇₅ were purified as described previously.²⁸ The *S. meliloti* *nifH* phosphorothioated (Operon) DNA probes were derivatised with *p*-azidophenacyl bromide at the -1/+1 position, as described previously.^{14,15} The (modified) promoter strands were ³²P-labelled and annealed to the complementary strand, as described previously.^{15,28}

ADP-AIF trapping experiments

ADP-AIF trapping experiments were performed in STA buffer [25 mM Tris-acetate (pH 8.0), 8 mM Mg-acetate, 10 mM KCl, and 3.5% wt/vol polyethylene glycol 6000] in a 10 μ l volume containing 5 μ M PspF₁₋₂₇₅, 1 μ M σ^{54} , and 5 mM NaF. This mix was incubated at 37 °C for 10 min, and then 0.2 mM ATP γ S or 0.2 mM ATP was added, and the reaction was incubated for the specified times. The “trapping” reactions were initiated upon addition of 0.4 mM AlCl₃ and incubated for 10 min at 37 °C. The samples were analysed using a 4.5% nondenaturing gel run at 100 V for 55 min. Proteins were detected by Coomassie blue staining and quantified using a Fuji FLA-5000 densitometer.

In vitro full-length or abortive transcription assays

Full-length or abortive transcription assays were performed in STA buffer in a 10 μ l reaction volume containing 100 nM E σ^{54} (reconstituted using a 1:4 ratio of E: σ^{54}) and either dATP, ATP, or ATP γ S (at the specified concentration) and 20 nM promoter DNA probe. The mix

was incubated at 37 °C for 5 min, and the reaction was started by the addition of 5 μ M PspF₁₋₂₇₅ (or PspF₁₋₂₇₅ mutants, where indicated) and incubated for the indicated time at 37 °C. Full-length transcription was initiated by adding a mix containing 100 μ g/ml heparin; 1 mM ATP, cytidine triphosphate, and guanosine triphosphate; 0.05 mM UTP; and 3 μ Ci of [α -³²P]UTP for a further 20 min. The reaction was quenched by addition of loading buffer and analysed on a 6% sequencing gel. The synthesis of abortive transcript (UpGGG) was initiated by adding a mix containing 100 μ g/ml heparin, 0.5 mM UpG, and 4 μ Ci [α -³²P]guanosine triphosphate for a further 20 min. Where indicated, gp2 was present in a fivefold molar excess over E σ ⁵⁴. In +gp2 reactions, gp2 was added prior to PspF₁₋₂₇₅ (to holoenzyme assemblies); in +gp2* reactions, gp2 was added after PspF₁₋₂₇₅ (to the OC). The reaction was quenched by addition of loading buffer and analysed on a 20% denaturing gel. Full-length transcription gels were dried, and transcripts were visualised and quantified using a Fuji FLA-5000 PhosphorImager.

ATPase activity

ATPase activity assays were performed using the ATPase colourimetric assay kit Pi Colorlock Gold (Innova Biosciences, UK). The reaction mix containing 35 mM Tri-acetate (pH 8.0), 70 mM K-acetate, 15 mM Mg-acetate, 19 mM NH₄-acetate, 0.7 mM DTT, and varying concentrations of PspF₁₋₂₇₅ was incubated at 37 °C for 10 min, and the colour reaction was started by adding 1 mM ATP or 0.1 mM ATP γ S. Reactions were stopped by addition of kit reactant, and phosphate release was measured.

Photo cross-linking assays

Photo cross-linking reactions were conducted at 37 °C in STA buffer in a total reaction volume of 10 μ l, as previously described.¹⁵ Briefly, where indicated, either 1 μ M σ ⁵⁴ or 200 nM E σ ⁵⁴ holoenzyme (reconstituted using a 1:2 ratio of E: σ ⁵⁴), 0.2 mM ATP γ S, 4 mM ATP or dATP, and 20 nM modified ³²P-labelled promoter DNA probe were incubated for 5 min at 37 °C. PspF₁₋₂₇₅ (5 μ M) was then added to the reactions and incubated for either 5 min, 30 min, or 60 min at 37 °C. Trapped complexes were formed *in situ* by adding 5 μ M PspF₁₋₂₇₅, 5 mM NaF, 1 mM ADP, and 0.2 mM BeCl₃ for the ADP-BeF reactions; 1 mM ADP and 0.2 mM AlCl₃ for the ADP-AlF reactions; and 1 mM AMP and 0.2 mM AlCl₃ for the AMP-AlF reactions, and incubated for either 5 min, 30 min, or 60 min at 37 °C. To eliminate free core RNAP binding the promoter probe, all reactions contained 100 ng/ μ l salmon sperm DNA. Reactions were UV-irradiated at 365 nm for 30 s using a UV-Stratalinker 1800 (Stratagene). A 2 μ l sample of the cross-linking reaction was analysed by native PAGE (4.5%) gel run at 100 V for 55 min. The rest of the cross-linking reaction was diluted by addition of 5 μ l of 10 M urea and 5 μ l of 2 $\times$ SDS loading buffer (Sigma). The samples were heated at 95 °C for 3 min, and 10 μ l was loaded onto a 7.5% SDS-PAGE gel run at 200 V for 50 min. The gels were dried, and cross-linked protein-DNA complexes were visualised and quantified using a FLA-5000 PhosphorImager. The cross-linked proteins were identified using antibodies, as described previously.¹⁵

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

RNAP, RNA polymerase; CC, closed complex; OC, open complex; PspF, phage shock protein F; gp2, gene protein 2; NTP, nucleoside triphosphate; BBSRC, Biotechnology and Biological Sciences Research Council; WT, wild-type.

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Fig. 1.

ATPyS is hydrolysed and supports full-length (FL) $E\sigma^{54}$ transcription. (a) *In situ* trapping reactions demonstrate that, given sufficient incubation times, PspF₁₋₂₇₅-ATPyS (hydrolysed by PspF₁₋₂₇₅ to ADP) supports the formation of an ADP metal/fluoride trapped complex. (b) Denaturing gels showing that PspF₁₋₂₇₅-ATPyS can support $E\sigma^{54}$ transcription from the supercoiled *S. meliloti nifH* promoter, and the results of different ATPyS (lanes 2-7), ATP (lanes 8-13), and dATP (lanes 14-19) concentrations in the transcription assays. The activation time for reactions was 5 min. The FL transcript is as indicated. The relative amount of transcription product formed in the presence of ATPyS (light gray), ATP (dark gray), and dATP (black) is depicted in the bar graph below. (c) Denaturing gel showing transcription from the

supercoiled *nifH* promoter by PspF₁₋₂₇₅ and either 0.2 mM ATP γ S (lanes 2-4), 4 mM ATP (lanes 5-7), or 4 mM dATP (lanes 8-10) after 5 min, 30 min, and 60 min activation time. The FL transcript is as indicated. The bar graph is as indicated in (b). (d) Nucleotide sequence of the *S. meliloti nifH* duplex promoter probe (probe 1) used in this study. Underlined are the two consensus dinucleotide promoter elements GG (positions -26 and -25, with respect to the transcription start site at +1) and GC (positions -14 and -13), which characterise σ^{54} -dependent promoters. The symbol used to represent this promoter probe is shown on the right. Abortive transcription gels showing the effect of increased activation time on E σ^{54} OC formation with PspF₁₋₂₇₅ and either 0.2 mM or 4 mM ATP γ S (lanes 1-3), 0.2 mM or 4 mM ATP (lanes 4-6), and 0.2 mM or 4 mM dATP (lanes 7-9) on probe 1. The relative number of productive OCs formed on probe 1 in the presence of ATP γ S (dark gray), ATP (light gray), and dATP (black) following 5 min, 30 min, and 60 min activation time is indicated in the bar graph. The abortive transcript 5' UpGGG is indicated. (e) As in (d), but using the pre-opened promoter probe (probe 2). Lower-case letters indicate non-complementary residues in probe 2.

Fig. 2.

OC formation by PspF₁₋₂₇₅-ATPγS is dependent on the β' jaw domain. (a) Abortive transcription assays (see Materials and Methods) on pre-opened probe 2 demonstrating that separate determinants in PspF₁₋₂₇₅ used for coupling the energy derived from ATP hydrolysis to OC formation (T86A or S and F85W located within the σ⁵⁴-interacting L1 loop) are required for PspF₁₋₂₇₅-ATPγS-dependent stimulation of OC formation. The percentage of WT PspF₁₋₂₇₅ ATPase activity in the presence of ATP (dATP) or ATPγS is as indicated. (b) Abortive transcription assays on probe 2 demonstrating the sensitivity of the PspF₁₋₂₇₅-ATPγS-dependent stimulation of OC formation to the bacteriophage T7 transcription inhibitor gp2. Only when gp2 is added prior to activation (+gp2; lanes 15-17) is PspF₁₋₂₇₅-ATPγS-dependent OC formation affected. When gp2 is added after activation (+gp2*; lanes 18-20), no effect on OC formation is observed. Consistent with previous findings³⁰ in the presence of PspF₁₋₂₇₅-dATP, no effect of gp2 (lanes 4-9) on OC formation is observed. In (a) and (b), the abortive transcript 5' UpGGG is indicated.

Fig. 3.

PspF₁₋₂₇₅-ATPyS alters the DNA proximities of σ^{54} and PspF₁₋₂₇₅. (a) Nucleotide sequence of the *S. meliloti nifH* mismatch promoter probe (probe 3) used in this study. Underlined are the two consensus dinucleotide promoter elements GG and GC, which characterise σ^{54} -dependent promoters. Lower-case letters indicate non-complementary residues in probe 3. The symbol used to represent this promoter probe is shown on the right. Native PAGE gel demonstrating that supershift complexes (ss σ^{54} -DNA) are only observed in the presence of PspF₁₋₂₇₅ and either ATP or dATP following 5 min incubation time (lanes 5 and 8). The supershift complexes decay (back to the initial σ^{54} -DNA complex) at longer incubation time points (lanes 6-7 and 9-10). The migration positions of the supershift (ss σ^{54} -DNA), initial σ^{54} -DNA (σ^{54} -DNA), and free DNA are as indicated. (b) Denaturing gel showing the cross-linking profiles of σ^{54} -probe 3 complexes in the presence of PspF₁₋₂₇₅ and either 0.2 mM ATP γ S (lanes 2-4), 4 mM ATP (lanes 5-7), or 4 mM dATP (lanes 8-10) following 5 min, 30 min, and 60 min incubation time. The migration positions of the cross-linked σ^{54} -DNA and PspF₁₋₂₇₅-DNA species are as indicated.

Fig. 4. PspF₁₋₂₇₅-ATP γ S alters the DNA proximities of σ^{54} , PspF₁₋₂₇₅, and core (β/β') RNAP. Denaturing gels showing the cross-linking profiles of E σ^{54} promoter complexes formed on (a) probe 1 and (b) probe 2 in the presence of PspF₁₋₂₇₅ and either 0.2 mM ATP γ S (lanes 2-4), 4 mM ATP (lanes 5-7), or 4 mM dATP (lanes 8-10) following 5 min, 30 min, and 60 min incubation time. (c) Denaturing gel showing the cross-linking profile of E σ^{54} probe 1 complexes formed in the presence of PspF₁₋₂₇₅ and either 0.2 mM ATP γ S (lanes 2-4) or the trapping reagents ADP-BeF (lanes 5-7), ADP-AIF (lanes 8-10), or AMP-AIF (lanes 11-13) following 5 min, 30 min, and 60 min of incubation time. In (a)-(c), the migration positions of the cross-linked σ^{54} -DNA, PspF₁₋₂₇₅-DNA, and β/β' -DNA species are as indicated.