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Regulated communication between the upstream face of RNA polymerase and the β′ subunit jaw domain

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We used bacteriophage T7-encoded transcription inhibitor gene protein 2 (gp2) as a probe to study the contribution of the *Escherichia coli* RNA polymerase (RNAP) β′ subunit jaw domain—the site of gp2 binding—to activator and ATP hydrolysis-dependent open complex formation by the σ^{54}-RNAP. We show that, unlike σ^{70}-dependent transcription, activated transcription by σ^{54}-RNAP is resistant to gp2. In contrast, activator and ATP hydrolysis-independent transcription by σ^{54}-RNAP is highly sensitive to gp2. We provide evidence that an activator- and ATP hydrolysis-dependent conformational change involving the β′ jaw domain and promoter DNA is the basis for gp2-resistant transcription by σ^{54}-RNAP. Our results establish that accessory factors bound to the upstream face of the RNAP, communicate with the β′ jaw domain, and that such communication is subjected to regulation.

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Introduction

The central enzyme of gene expression, the DNA-dependent RNA polymerase (RNAP), is a complex and highly regulated molecular machine that is conserved between eukaryotes, archaea and bacteria (Ebright, 2000). In *Escherichia coli*, the catalytically competent core RNAP (E, subunit composition $\alpha_2\beta\beta'\omega$) binds one of the seven sigma (σ) subunits, at the so-called 'upstream face' of the core RNAP, to form an RNAP holoenzyme (Eσ) that is capable of promoter-specific transcription (Murakami *et al*, 2002; Chung *et al*, 2003; Bushnell *et al*, 2004).

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There are two distinct types of σ factors. RNAP holoenzymes containing the major σ^{70}-type factors (named after the prototypical housekeeping σ of *E. coli*, σ^{70}) recognize promoters characterized by conserved sequence elements centered around 35 and 10 base pairs upstream of the transcription start site at +1. Closed promoter complexes formed by RNAP holoenzymes containing σ^{70}-type factors readily isomerize into transcription-competent open promoter complexes. RNAP holoenzymes containing evolutionarily unrelated σ^{54}-type factors recognize promoters characterized by conserved sequence elements located 24 and 12 base pairs upstream of the transcription start site and form closed complexes that cannot spontaneously isomerize into open complexes (Buck *et al*, 2000). Conversion of Eσ^{54} closed complexes to open complexes requires ATP hydrolysis by a specialized activator protein belonging to the AAA (ATPases Associated with various cellular Activities) family (Zhang *et al*, 2002). The activator binds DNA upstream of the σ^{54} promoter, interacts with the Eσ^{54} closed complex and induces conformational changes that lead to the formation of a transcription-competent open complex (Buck *et al*, 2000; Zhang *et al*, 2002).

Core RNAP surfaces that are responsible for formation and maintenance of open promoter complexes are not yet fully characterized. For σ^{70}-dependent transcription, the interaction between the β′ jaw domain which is located at the so-called 'downstream face' of the RNAP (residues 1149–1190 of *E. coli* β′ subunit; Figure 1A) and the double-stranded DNA downstream of the transcription initiation start point has been shown to be important for stable open complex formation (Ederth *et al*, 2002). Binding of the bacteriophage T7-encoded gene 2 protein (gp2) to the β′ jaw domain strongly inhibits promoter complex formation by Eσ^{70}, further underscoring the importance of the β′ jaw domain in transcription (Nechaev and Severinov, 1999). In eukaryotic RNAPII, the jaw lobe of RPB1, a homologue of β′, forms part of the DNA-binding cleft and contacts bases in the downstream DNA (Bushnell *et al*, 2004). The role of the β′ jaw during σ^{54}-dependent transcription is not known. In this work, we have used gp2 as a probe to study the contribution of the *E. coli* RNAP β′ jaw domain to transcription by Eσ^{54}. We show that, unlike Eσ^{70} transcription, activator-dependent Eσ^{54} transcription is not inhibited by gp2. We provide evidence that activation induces a promoter DNA-dependent conformational change involving the β′ jaw domain, which allows Eσ^{54} to escape inhibition by gp2 and to engage promoter DNA.

Mutations in σ^{54} regulatory regions I and III (Figure 1B) result in Eσ^{54} that is capable of transcription in the absence of the activator (Casaz *et al*, 1999; Chaney and Buck, 1999; Wang and Gralla, 2001; Wigneshweraraj *et al*, 2002). We show that the integrity of Region I is important for the binding of gp2 to the β′ jaw domain, and thereby present evidence for a functional and/or structural link between σ^{54}

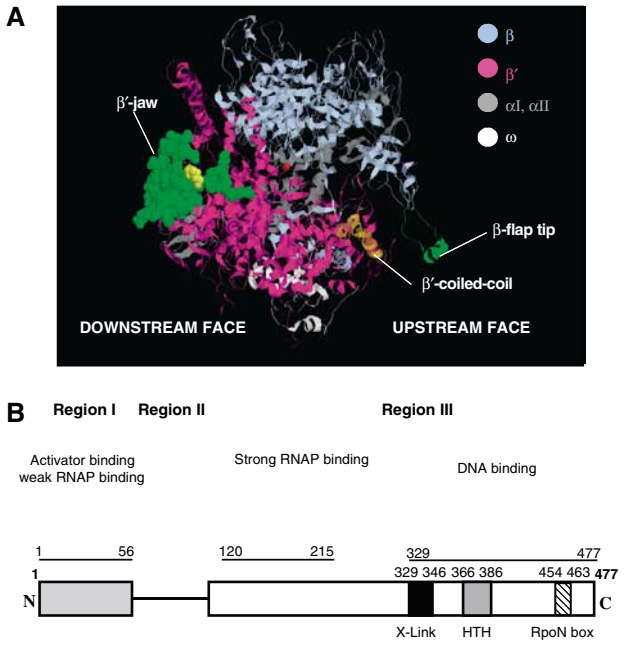


Figure 1 Proteins used in this study. (**A**) The crystal structure of the *T. aquaticus* core RNAP with the β′ jaw domain highlighted in green space fill and the E1188K mutation indicated in yellow. The β, β′, ω and αI and αII subunits are color coded as shown. The red sphere indicates the catalytic center. The downstream and upstream faces of the RNAP are labeled. (**B**) Domain organization of *K. pneumoniae* σ^{54}. The major activator, core RNAP and promoter DNA-binding determinants are shown. In Region III, residues 329–346 have been shown to UV-crosslink to DNA (X-link) (Cannon *et al*, 1994), residues 366–386 constitute the putative helix–turn–helix DNA-binding motif (HTH) (Merrick and Chambers, 1992) and residues 454–463 constitute a highly conserved patch (RpoN box) that interacts with the −24 consensus promoter element of σ^{54}-dependent promoters (Burrows *et al*, 2003).

Region I and the β′ jaw domain. Like $E\sigma^{70}$ transcription, activator-independent transcription by mutant $E\sigma^{54}$ is inhibited by gp2. We demonstrate that gp2 inhibits activator-independent transcription by preventing the step(s) leading to DNA melting. Overall, we present evidence for an internal conformational signaling pathway in $E\sigma^{54}$ that involves σ^{54} Region I and the β′ jaw domain, and provide clues as to how σ^{54} bound at the upstream face of the RNAP could regulate the activity of RNAP by controlling the β′ jaw function.

Results

Activator-dependent $E\sigma^{54}$ transcription is not inhibited by T7 gp2

We compared the effect of gp2 on $E\sigma^{70}$ and $E\sigma^{54}$ transcription in a single-round transcription assay using the *lac*UV5 (for $E\sigma^{70}$ transcription) and the *Sinorhizobium meliloti nifH* (for $E\sigma^{54}$ transcription) promoters. To activate $E\sigma^{54}$ transcription, we used a fragment of the *E. coli* σ^{54} transcription activator phage shock protein F ($PspF_{1-275}$), which lacks the DNA-binding domain and efficiently activates transcription from solution (Cannon *et al*, 2003). RNAP holoenzymes were reconstituted with purified σ proteins and combined with gp2 prior to the addition of promoter DNA. As a control, we used holoenzymes reconstituted with core RNAP harboring the E1188K substitution in the β′ jaw domain. This mutation prevents gp2 binding and makes $E\sigma^{70}$ transcription resistant to gp2 (Nechaev and Severinov, 1999; Figure 1A). As expected, transcription from *lac*UV5 was strongly inhibited by gp2 in reactions containing wild-type $E\sigma^{70}$, but not in reactions containing $^{E1188K}E\sigma^{70}$ (Figure 2A). In contrast, gp2 had no obvious effect on activated transcription from the *S. meliloti nifH* promoter by $E\sigma^{54}$ (Figure 2B). $E\sigma^{54}$ transcription from two other σ^{54}-dependent promoters (*E. coli glnHp2* and *pspA*) was also resistant to gp2 (data not shown). The order of addition experiments did not reveal any detectable inhibition of $E\sigma^{54}$-dependent transcription regarding whether gp2 was added before RNAP holoenzyme formation, after closed complex formation, or after open complex formation (data not shown). It therefore appears that activated $E\sigma^{54}$ transcription is fully resistant to gp2.

We considered the possibility that gp2 might not be able to interact with the β′ jaw domain in the presence of σ^{54}. However, a native gel assay using ^{32}P-labeled gp2 confirmed that gp2 interacted stably and specifically with $E\sigma^{54}$ (Figure 2C). A radioactive band observed in reactions containing the wild-type core RNAP and gp2 corresponded to the RNAP core–gp2 complex (Figure 2C, lane 2) that was converted into a slightly slower migrating complex in reactions containing core RNAP, gp2 and σ^{54} (Figure 2C, lane 4). As expected, no such complexes were observed in reactions that contained core RNAP or $E\sigma^{54}$ harboring the E1188K substitution in β′ (Figure 2C, lanes 3 and 5). SDS–PAGE analysis of complexes shown in Figure 2C (lanes 2 and 4) confirmed the presence of σ^{54} in the complex seen in lane 4, but not lane 2 (data not shown).

Additional experiments using gp2 modified with lysine-specific protein-cleaving reagent 2IT-FeBABE (Datwyler and Meares, 2000) confirmed that gp2 was proximal to the β′ jaw domain in the context of RNAP core, $E\sigma^{70}$, and $E\sigma^{54}$ (data not shown). We therefore conclude that, even though gp2 specifically interacts with the β′ jaw domain in the presence of either σ^{70} or σ^{54} (Nechaev and Severinov, 1999; Figure 2C), it only inhibits $E\sigma^{70}$ transcription. The result thus suggests that the β′ jaw domain may make different interactions in transcription initiation by $E\sigma^{54}$ compared to $E\sigma^{70}$. Possibly, within $E\sigma^{54}$ promoter complexes, the interaction between the downstream DNA and the β′ jaw causes gp2 to dissociate from RNAP.

T7 gp2 binds to $E\sigma^{54}$ closed promoter complexes

To determine whether gp2 is able to bind to closed $E\sigma^{54}$ promoter complexes, we combined ^{32}P-labeled gp2 with $E\sigma^{54}$ complexes formed on an early-melted promoter probe that contains a heteroduplex segment near the −12 consensus promoter element and thus mimics the conformation of DNA in the closed complex (Cannon *et al*, 2003) (Figure 2D). An excess of early-melted promoter probe over $E\sigma^{54}$ was used to ensure that most $E\sigma^{54}$ was in the DNA-bound form. As can be seen, the mobility of $E\sigma^{54}$ bound to ^{32}P-labeled gp2 changed in the presence of DNA (Figure 2D, compare lanes 3 and 4), indicating that ^{32}P-labeled gp2 binds to the preformed $E\sigma^{54}$ closed promoter complex. Similar results were obtained when ^{32}P-labeled gp2 was incubated with $E\sigma^{54}$ prior to the addition of the early-melted probe (data not shown). The binding results thus indicate that, unlike $E\sigma^{70}$ closed promoter complexes (Nechaev and Severinov, 1999), $E\sigma^{54}$ closed promoter complexes are not disrupted by gp2.

T7 gp2 binds Eσ^{54} activator complex

We investigated whether gp2 remains bound to Eσ^{54} upon interaction with the activator. Stable, nucleotide-dependent binding of activator to Eσ^{54} can be measured by native PAGE in the presence of ADP-aluminum fluoride (ADP-AlF$_x$, where x is either 3 or 4), an analog of ATP in the transition state of hydrolysis (Chaney *et al*, 2001). We incubated the activator with ADP-AlF$_x$ and Eσ^{54} in the presence of ^{32}P-labeled gp2, and resolved the reaction products by native PAGE. As shown in Figure 3A, ^{32}P-labeled gp2 interacted with Eσ^{54} when added either before or after Eσ^{54}-PspF$_{1-275}$:ADP-AlF$_x$ complex formation (compare lanes 5 and 7). Similarly, ^{32}P-labeled gp2 interacted with Eσ^{54} when ATP was substituted for ADP-AlF$_x$ (data not shown). Therefore, gp2 has no effect on Eσ^{54} activator interaction. Further, this result also excludes the site of gp2 binding on the β′ jaw domain as a major site or determinant of activator interaction.

T7 gp2 does not bind intermediate Eσ^{54} promoter complex

To determine whether activator-induced conformational changes in the Eσ^{54} closed complex allow Eσ^{54} transcription to escape inhibition by gp2, we tested whether gp2 is able to interact with intermediate Eσ^{54} promoter complexes. The Eσ^{54}:DNA:PspF$_{1-275}$:ADP-AlF$_x$ complex represents an early intermediate *en route* to open complex formation by Eσ^{54} (Chaney *et al*, 2001; Cannon *et al*, 2003). We formed the Eσ^{54}:PspF$_{1-275}$:ADP-AlF$_x$ complex in the presence of the early-melted promoter probe (present at a four-fold molar excess over Eσ^{54} to ensure that most Eσ^{54} was in the DNA-bound form), added ^{32}P-labeled gp2 and analyzed the reaction by native PAGE. Silver staining of the native gel revealed a band (Figure 3B, lane 5, marked with the arrow) whose formation was dependent on the presence of DNA (Figure 3B, lane 6) and PspF$_{1-275}$:ADP-AlF$_x$ (Figure 3B, lane 4). Additional control reactions in the absence of core RNAP and σ^{54} did not result in the formation of the band marked with the arrow in Figure 3B, lane 5 (data not shown). Therefore, we conclude that this band (marked with the arrow in Figure 3B, lane 5) must correspond to Eσ^{54}:DNA:PspF$_{1-275}$:ADP-AlF$_x$ complex. However, the auto-

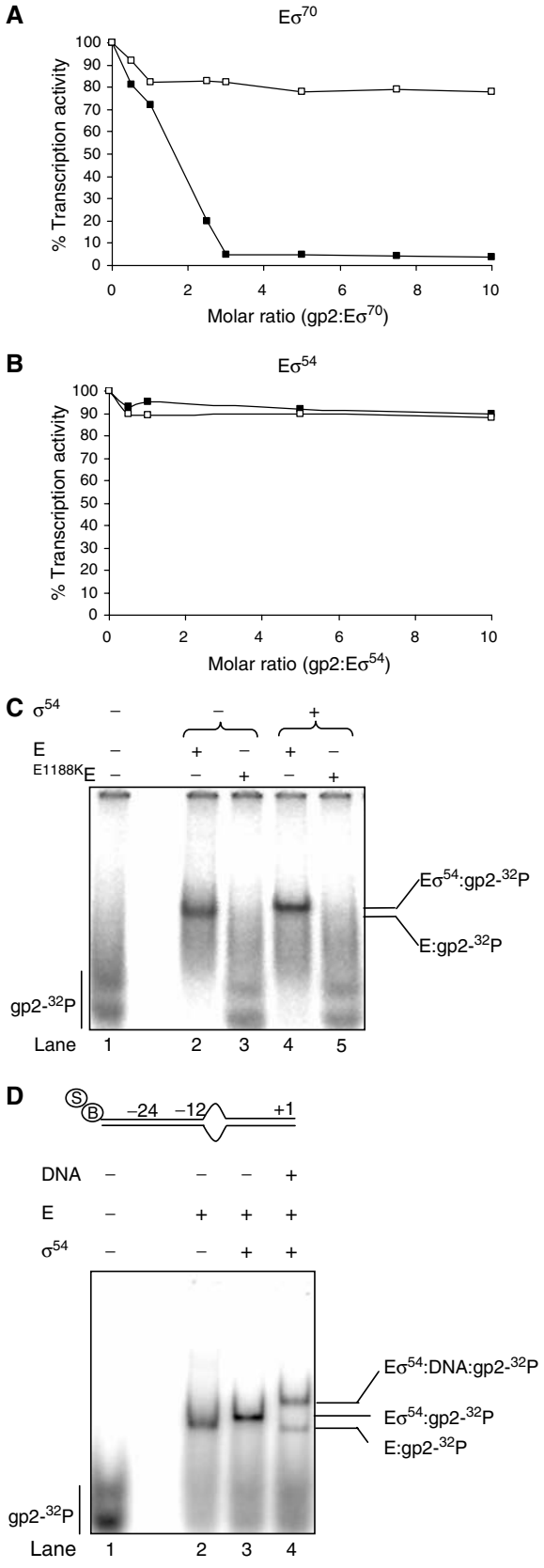
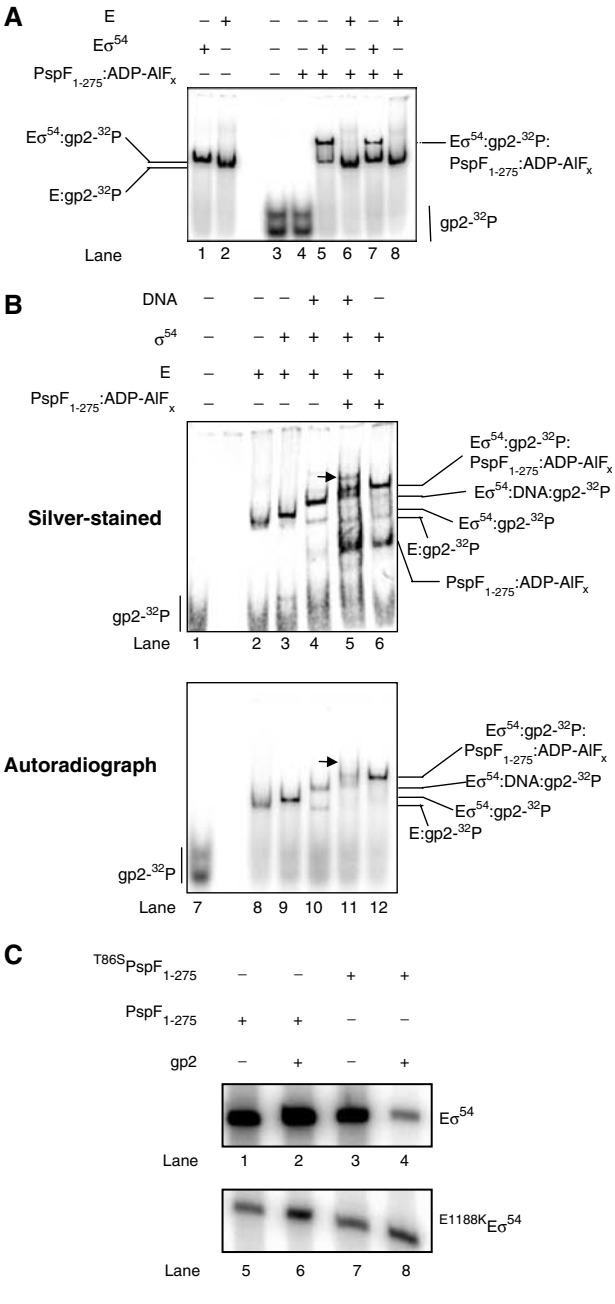


Figure 2 Eσ^{54} is insensitive to inactivation by gp2. (**A**) Single-round transcription by Eσ^{70} from the *lac*UV5 promoter in the presence of increasing amounts of gp2. (**B**) Single-round transcription by Eσ^{54} from the *S. meliloti nifH* promoter in the presence of PspF$_{1-275}$, ATP and increasing amounts of gp2. In (A) and (B), empty boxes indicate the use of Eσ reconstituted with the gp2-resistant E1188K mutant core RNAP and filled boxes Eσ reconstituted with the gp2-sensitive wild-type core RNAP. The error range for the values graphed in (A) and (B) is within ±7%. (**C**) An autoradiograph of a 4.5% (w/v) native gel showing binding of ^{32}P-labeled gp2 to E and Eσ^{54}. The migration positions of gp2 (lane 1), E:gp2 (lane 2) and Eσ^{54}:gp2 (lane 4) are indicated. Protein components in each lane are indicated on top. (**D**) T7 gp2 binds to Eσ^{54}-closed promoter complexes. An autoradiograph of a 4.5% (w/v) native gel showing the binding of ^{32}P-labeled gp2 to E and Eσ^{54} in the presence of streptavidin-labeled *S. meliloti nifH* early-melted promoter probe (lane 4). The positions of gp2 (lane 1), E:gp2 (lane 2), Eσ^{54}:gp2 (lane 3) and Eσ^{54}:DNA:gp2 (lane 4) complexes are shown and the protein and DNA components of each lane indicated (top of the figure). A cartoon of the biotin (B)- and streptavidin (S)-tagged early-melted promoter probe is shown. The positions of the transcription start site (+1) and the start-site distal (−24) and proximal (−12) consensus promoter elements of σ^{54}-dependent promoters are shown.

radiograph of the native gel did not reveal any radioactivity in this band (Figure 3B, compare lanes 5 and 11, marked with the arrow). We therefore conclude that ^{32}P-labeled gp2 is not able to stably interact with the Eσ^{54}:DNA:PspF$_{1-275}$:ADP-AlF$_x$ complex. Identical results were obtained when Eσ^{54} was incubated with ^{32}P-labeled gp2 prior to the addition of DNA and PspF$_{1-275}$:ADP-AlF$_x$ (data not shown). Overall, the result indicates that activator-induced and promoter-dependent conformational changes in Eσ^{54} prevent the interaction of gp2 with the β′ jaw domain and may thereby allow activated transcription by Eσ^{54} to escape inhibition by gp2. Clearly, promoter DNA plays a critical role in inhibiting binding of gp2, since Eσ^{54}:PspF$_{1-275}$:ADP-AlF$_x$ complex binds gp2 well (Figure 3A, lanes 5 and 7).



Activator triggers conformational change(s) involving the β′ jaw domain

Evidence that the interactions between the Eσ^{54} closed complex and the activator somehow change the interaction between gp2 and the β′ jaw domain and allows Eσ^{54} to escape inhibition by gp2 prompted us to investigate whether ATP hydrolysis-dependent 'remodeling' of the Eσ^{54} closed complex is the reason for the gp2 resistance of Eσ^{54} transcription. We conducted single-round transcription assays from the *S. meliloti nifH* promoter using PspF$_{1-275}$ carrying a point substitution at the conserved position Thr^{86} (T86S; ^{T86S}PspF$_{1-275}$). The mutant activator is defective for interaction with the Eσ^{54} closed complex and is less efficient in ATP hydrolysis-dependent remodeling of Eσ^{54} closed complexes (Chaney *et al*, 2001). As shown in Figure 3C, we found that gp2 inhibited Eσ^{54} transcription activated by ^{T86S}PspF$_{1-275}$ by 6–7-fold (compare lanes 3 and 4). Similar levels of inhibition were observed in reactions using different activator mutants impaired for nucleotide binding and/or hydrolysis or Eσ^{54} interaction (data not shown). The inhibition was not detected in control reactions containing gp2-resistant ^{E1188K}Eσ^{54} (Figure 3C, compare lanes 7 and 8). As expected, in reactions containing wild-type PspF$_{1-275}$, no inhibition of Eσ^{54} transcription was observed (Figure 3C, compare lanes 1 and 2). Since the β′ jaw domain is not a direct target for the activator (Figure 3A and Wigneshweraraj *et al*, in preparation), we infer that, during open complex formation, the activator drives an ATP hydrolysis-dependent conformational change in the Eσ^{54} closed complex that causes, directly or indirectly, dissociation of gp2. The mutant activator (^{T86S}PspF$_{1-275}$) is less efficient in coupling ATP hydrolysis to conformational changes in Eσ^{54} closed complexes, which leads to gp2 sensitivity. Overall, the results point to activator- and ATP hydrolysis-dependent use of the β′ jaw domain during open complex formation by Eσ^{54}, which likely involves a β′ jaw domain-DNA interaction.

Figure 3 T7 gp2 does not bind an intermediate Eσ^{54} promoter complex. (**A**) Activator–Eσ^{54} interaction is not disrupted by T7 gp2. Autoradiograph of a 4.5% (w/v) native gel showing the ability of ^{32}P-labeled gp2 to bind a ternary Eσ^{54}:PspF$_{1-275}$:ADP-AlF$_x$ complex (lanes 5 and 7). In lane 5, gp2 was added prior to Eσ^{54}:PspF$_{1-275}$:ADP-AlF$_x$ complex formation and, in lane 7, gp2 was added after Eσ^{54}:PspF$_{1-275}$:ADP-AlF$_x$ complex formation. The protein components in each lane and the migration positions of gp2, E:gp2, Eσ^{54}:gp2 and Eσ^{54}:PspF$_{1-275}$:ADP-AlF$_x$:gp2 are indicated. (**B**) T7 gp2 does not bind an early intermediate promoter complex. Silver-stained 4.5% (w/v) native gel (top panel) and the autoradiograph of the same gel (bottom panel) showing that gp2 does not interact with an early intermediate Eσ^{54} promoter complex on streptavidin-tagged early-melted promoter probe in the presence of ^{32}P-labeled gp2 (see Materials and methods). The arrow in lanes 5 and 11 points to the migration position of Eσ^{54}:DNA:PspF$_{1-275}$:ADP-AlF$_x$ complex. The migration positions of other protein–protein and protein–DNA complexes are as indicated. The protein and DNA components in each lane are shown on the top of the figure. (**C**) Single-round transcription assays from the supercoiled *S. meliloti nifH* promoter using ATP and either wild-type PspF$_{1-275}$ (lanes 1 and 2) or ^{T86S}PspF$_{1-275}$ (lanes 3 and 4) for activation. The reactions in lanes 1–4 were conducted using the gp2-sensitive wild-type core RNAP, whereas the reactions in lanes 4–8 were conducted using the gp2-resistant E1188K mutant core RNAP. The protein components in each lane are indicated on the top of the figure.

Activator-bypass transcription by Eσ^{54} is inhibited by T7 gp2

The observation that activator and ATP hydrolysis-driven conformational changes involving the β′ jaw domain enable Eσ^{54} to escape inhibition by gp2 prompted us to investigate whether activator-independent (termed activator-bypass) transcription by Eσ^{54} is inhibited by gp2. We expected that, since no activator and ATP hydrolysis-driven conformational changes would occur during activator-bypass transcription, gp2 will be able to inhibit activator-bypass transcription by Eσ^{54}.

Certain substitutions in σ^{54} regulatory regions I and III allow Eσ^{54} *in vitro* transcription to occur without activation. In contrast to open promoter complexes formed by wild-type Eσ^{54}, which become heparin-resistant prior to initiation of RNA synthesis, activator-bypass mutant Eσ^{54} promoter complexes become heparin-resistant only after the initiation of RNA synthesis (Wang and Gralla, 1996). To check the effect of gp2 on activator-bypass transcription, we conducted single-round transcription assays using *S. meliloti nifH* promoter and Eσ^{54} reconstituted using an activator-bypass σ^{54} carrying the R336A substitution in Region III (Figure 1A, Chaney and Buck, 1999; Wang and Gralla, 2001; Wigneshweraraj *et al*, 2001). The first step of activator-bypass transcription assay from the *S. meliloti nifH* promoter involves the incubation of Eσ^{54} with the DNA template and GTP. Since the first three bases of the *nifH* RNA are G (Sundaresan *et al*, 1983), activator-bypass transcription initiation can occur under these conditions. In the second step of the assay, heparin and the remaining nucleotides are added to allow elongation of any initiated transcripts.

Activator-bypass transcription by Eσ$^{54}_{R336A}$ was strongly inhibited by gp2 (Figure 4A) and the inhibition was as effective as that with Eσ^{70} (compare Figures 2A and 4A). The inhibition of Eσ$^{54}_{R336A}$ transcription is specific, since a double mutant Eσ^{54} reconstituted from ^{E1188K}E and σ$^{54}_{R336A}$ was resistant to gp2 (Figure 4A). The order of addition experiments revealed that gp2 inhibited Eσ$^{54}_{R336A}$ transcription when added either before or after closed complex formation (Supplementary data Figure 1). Inhibition of transcription was not due to direct inhibition of closed complex formation by gp2, since ^{32}P-labeled gp2 was able to stably bind Eσ$^{54}_{R336A}$ closed promoter complexes formed on the early-melted promoter probe (Figure 4B, lane 1). Once activator-bypass transcript initiation had occurred, gp2 had no effect on elongation by Eσ$^{54}_{R336A}$ (Figure 4C). gp2 had a similar effect on activator-bypass transcription by RNAPs reconstituted with two additional Region III mutants, σ$^{54}_{F318A}$

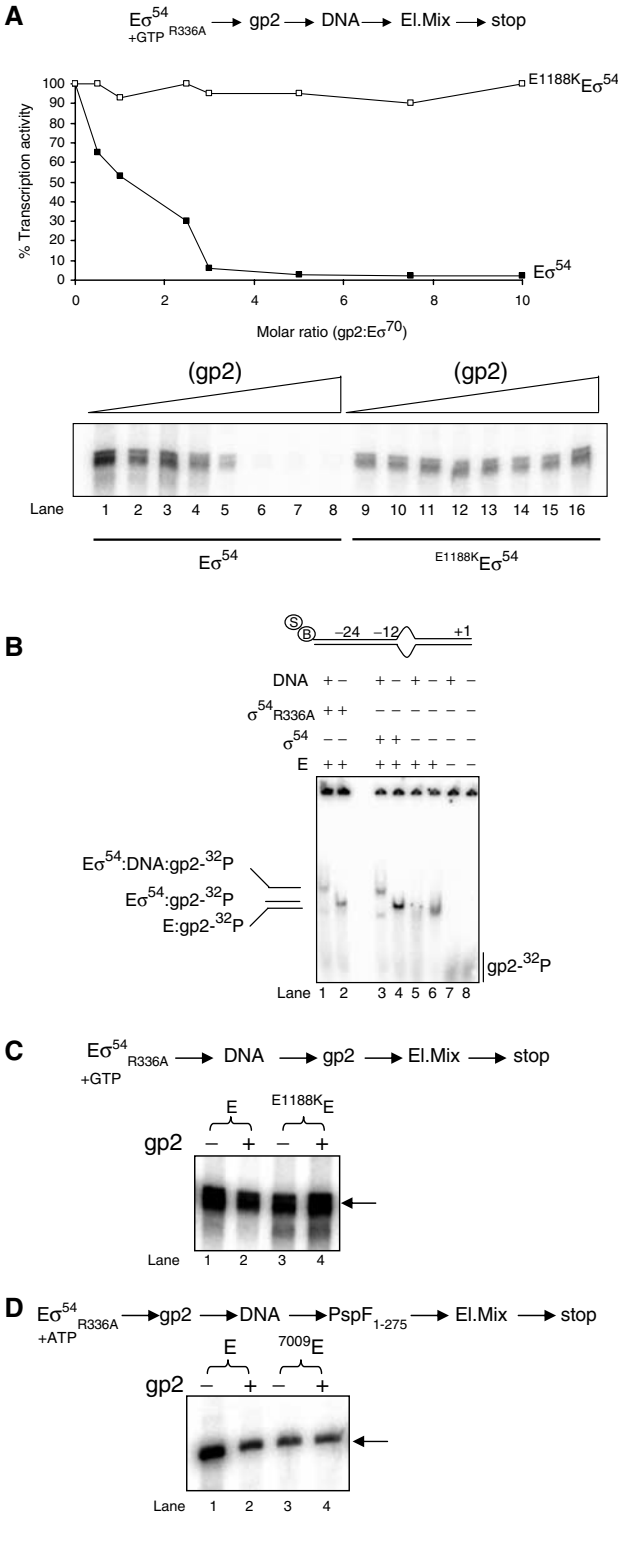


Figure 4 Activator-bypass transcription from supercoiled *S. meliloti nifH* promoter by Eσ$^{54}_{R336A}$ is inhibited by T7 gp2. (**A**) Titration of gp2 against Eσ$^{54}_{R336A}$ reconstituted with gp2-resistant E1188K mutant core RNAP (empty boxes) and gp2-sensitive wild-type core RNAP (filled boxes). The error range for the values shown in graph is ±5%. An autoradiograph of a 4% (w/v) denaturing gel showing the inhibition of activator-bypass transcription by Eσ$^{54}_{R336A}$ by gp2 is shown. (**B**) An autoradiograph of a 4.5% (w/v) native gel showing the binding of ^{32}P-labeled gp2 to Eσ^{54} (lane 3) and Eσ$^{54}_{R336A}$ (lane 1) promoter complexes formed on the *S. meliloti nifH* early-melted DNA probe. The migration positions of E:gp2, Eσ^{54}:gp2 and Eσ^{54}:DNA:gp2 are indicated. The protein and DNA components in each lane are shown on the top. (**C**) An autoradiograph of a 4% (w/v) denaturing gel showing that activator-bypass transcription by Eσ$^{54}_{R336A}$ is not inhibited when gp2 is added after transcription initiation. Reactions in lanes 1 and 2 were conducted using the gp2-sensitive wild-type core RNAP, whereas the gp2-resistant E1188K mutant RNAP was used in reactions in lanes 3 and 4. The arrow indicates the 470 nucleotide transcript from the *S. meliloti nifH* promoter on pMKC28. The reaction schematic is shown on the top of each figure (see Materials and methods). (**D**) An autoradiograph of a 4% (w/v) denaturing gel showing that activated transcription by Eσ$^{54}_{R336A}$ is not inhibited by gp2. The gel is labeled as in (C).

(Wigneshweraraj *et al*, 2002) and σ^{54}_{K388A} (Wang and Gralla, 2001) (data not shown). We therefore conclude that, during activator-bypass transcription by $E\sigma^{54}$ with lesions in σ^{54} Region III, gp2 binding interferes with the β′ jaw domain function during one or more steps that lead to activator-bypass open complex formation.

Activation allows $E\sigma^{54}_{R336A}$ to escape inhibition by gp2

We next conducted the reciprocal experiment and investigated whether activation allows $E\sigma^{54}_{R336A}$ to overcome inhibition by gp2. In this experiment, escape from inhibition was interpreted to mean that activator and ATP hydrolysis drive conformational changes within the promoter complex that prevent gp2 action, most likely by changing gp2 binding to the β′ jaw domain (see above). As can be seen in Figure 4D, activator-dependent transcription by $E\sigma^{54}_{R336A}$ was slightly inhibited by gp2 when gp2 was added either before (compare lanes 1 and 2) or after closed complex formation (Supplementary data Figure 2). However, in titration assays using increasing amounts of gp2 in the presence of $E\sigma^{54}_{R336A}$, ATP and activator, the extent of inhibition of activator-dependent transcription by $E\sigma^{54}_{R336A}$ was significantly reduced (compare Figure 4A, lane 6 with Figure 4D, lane 2 and data not shown). Similarly, the addition of gp2 after activator-dependent open complex formation had no effect on $E\sigma^{54}_{R336A}$ transcription (Supplementary data Figure 2). Identical results were obtained with $E\sigma^{54}$ reconstituted with F318A and K388A activator-bypass σ^{54} proteins (data not shown). The results thus further confirm that, during open complex formation, activator-induced conformational changes lead to the loss of gp2 binding to the β′ jaw domain.

T7 gp2 prevents activator-bypass DNA melting by $E\sigma^{54}_{R336A}$

The results presented above point to an activator- and ATP hydrolysis-dependent use of the β′ jaw domain during DNA melting and open complex formation, and suggest that gp2 could inhibit activator-bypass transcription by preventing RNAP conformational changes and DNA interactions required for efficient DNA melting. To test this idea, we formed $E\sigma^{54}_{R336A}$ promoter complexes on supercoiled *S. meliloti nifH* promoter in the absence or presence of gp2 under conditions where activator-bypass DNA melting should occur, and detected DNA melting with potassium permanganate ($KMnO_4$) (see Materials and methods). In all experiments described below, gp2 was added prior to promoter complex formation. As shown in Figure 5A, no promoter DNA melting was detected in reactions that contained wild-type $E\sigma^{54}$, but lacked $PspF_{1-275}$ (compare lanes 4 and 5). In contrast, activator-bypass DNA melting (at position −8 on the nontemplate strand and position −12 on the template strand) was detected within promoter complexes formed with $E\sigma^{54}_{R336A}$ in the presence of GTP (recall that the first three bases transcribed from the *S. meliloti nifH* promoter are G) (Figure 5A, lane 7). $PspF_{1-275}$ moderately increased the efficiency of DNA melting by $E\sigma^{54}_{R336A}$ (Figure 5A, lane 8). In the presence of gp2, no DNA melting was detected in reactions containing $E\sigma^{54}_{R336A}$ and GTP (Figure 5A, lane 14). The extent of activator-dependent promoter melting by the wild-type $E\sigma^{54}$ was unaffected by gp2 (Figure 5A, lane 12). In agreement with the observation that activation allows $E\sigma^{54}_{R336A}$ to escape inhibition by gp2, we are able to detect

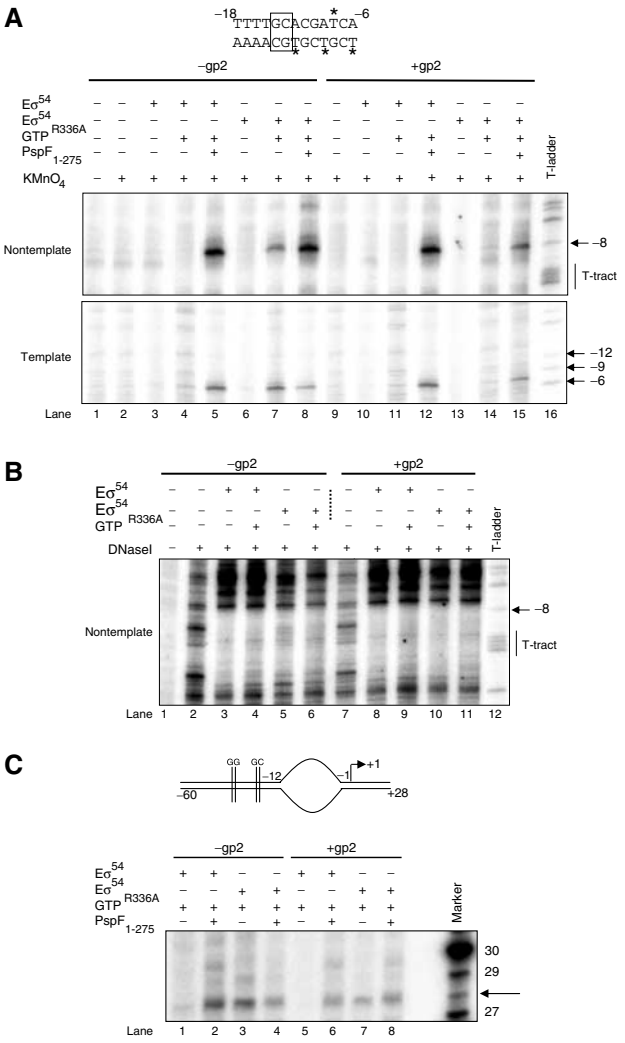


Figure 5 T7 gp2 prevents activator-bypass DNA melting within promoter complexes formed by $E\sigma^{54}_{R336A}$. (**A**) An autoradiograph of a 10% (w/v) denaturing gel showing $KMnO_4$ probing of *S. meliloti nifH* promoter complexes (on pMKC28) (reaction conditions as shown on the top of the figure) formed by $E\sigma^{54}$ and $E\sigma^{54}_{R336A}$ in the absence (lanes 1–8) and presence (lanes 9–16) of gp2. In (A), the DNA sequence of the *S. meliloti nifH* promoter is shown, with the asterisks indicating the thymine residues at position −8 on the nontemplate strand and −12, −9 and −6 on the template strand. (**B**) An autoradiograph of a 10% (w/v) denaturing gel showing DNase I probing of *S. meliloti nifH* promoter complexes (on pMKC28) (reaction conditions as shown on the top of the figure) formed by $E\sigma^{54}$ and $E\sigma^{54}_{R336A}$ in the absence (lanes 1–6) and presence (lanes 7–11) of gp2. In (A) and (B), lanes 16 and 12, respectively, contain chain termination DNA-sequencing reactions conducted with pMKC28 and ddTTP. In (B), lane 6, the dotted line indicates the extended protection within $E\sigma^{54}_{R336A}$ promoter complexes in the presence of GTP. (**C**) An autoradiograph of a 10% (w/v) denaturing gel showing run-off transcription from a linear *S. meliloti nifH* promoter probe containing a heteroduplex segment between positions −12 and −1. The arrow indicates the 28 nucleotide run-off product. The marker lane contains a mixture of end-labeled *S. meliloti nifH* promoter DNA fragments.

DNA melting by $E\sigma^{54}_{R336A}$ in the presence of gp2 and $PspF_{1-275}$ (Figure 5A, compare lanes 8 and 15). However, we note that the extent of DNA melting has been reduced in reactions containing gp2 and $PspF_{1-275}$ (lane 15) when compared to reactions containing no gp2 (lane 8).

DNase I footprinting of complexes formed by $E\sigma^{54}_{R336A}$ on supercoiled *S. meliloti nifH* showed clear protection of promoter DNA, both in the absence and presence of gp2 (Figure 5B, compare lanes 6 and 11). Thus, gp2 did not disrupt promoter complexes formed by $E\sigma^{54}_{R336A}$ under the assay conditions. In the footprint of the $E\sigma^{54}_{R336A}$ promoter complex, in the absence of gp2, a moderate extension of the footprint downstream of position −8 was observed (Figure 5B, lane 6; marked by the dotted line). This extended footprint is indicative of the activator-bypass isomerization of $E\sigma^{54}_{R336A}$ promoter complex in the presence of GTP (Figure 5B; compare lanes 5 and 6), and is also seen within wild-type $E\sigma^{54}$ promoter complexes in response to activation (data not shown; Wang and Gralla, 1996; Wigneshweraraj *et al*, 2002). No extended footprint was observed in the presence of gp2 in reactions containing $E\sigma^{54}_{R336A}$ and GTP (Figure 5B; compare lanes 6 and 11). Thus, gp2 binding to the β′ jaw domain interferes with activator-bypass isomerization and stable promoter DNA melting by $E\sigma^{54}_{R336A}$. In agreement with this, run-off activator-bypass transcription by $E\sigma^{54}_{R336A}$ from a heteroduplex *S. meliloti nifH* promoter (Cannon *et al*, 2000), where the DNA was stably pre-melted between positions −12 and −1, was unaffected by gp2 (Figure 5C, compare lanes 4 and 8).

Overall, the results strongly suggest that gp2 inhibits activator-bypass transcription by preventing step(s) leading to stable DNA melting and, by extension, suggest that the β′ jaw domain is required for DNA melting (possibly stabilizing the melted DNA) during $E\sigma^{54}$ transcription.

σ^{54} Region I determines β′ jaw conformation

The observation that the β′ jaw domain is involved in activator and ATP hydrolysis-dependent conformational changes during $E\sigma^{54}$ transcription, but is not a direct binding target for the activator, prompted us to investigate the role of regulatory regions of σ^{54} in determining the β′ jaw domain function during activator-dependent transcription. The amino-terminal regulatory Region I of σ^{54} constitutes a binding site for the activator (Chaney *et al*, 2001) and undergoes major activator-dependent conformational changes during open complex formation (Casaz and Buck, 1999). Removal of, or mutations within, Region I confers an activator-bypass phenotype upon $E\sigma^{54}$ (Wang and Gralla, 1996; Casaz *et al*, 1999). We used σ^{54} lacking Region I ($\sigma^{54}_{\Delta RI}$) or containing a triple alanine substitution at Region I positions R24, L26 and L26 ($\sigma^{54}_{ala24-26}$) to reconstitute $E\sigma^{54}$ and investigate whether the removal of or mutations within Region I affected the β′ jaw domain–gp2 interaction. Native-PAGE analysis revealed that little or no stable complex between ^{32}P-labeled gp2 and $E\sigma^{54}_{\Delta RI}$ or $E\sigma^{54}_{ala24-26}$ was formed (Figure 6A). Since the free core RNAP, E:gp2, $E\sigma^{54}$ and $E\sigma^{54}$:gp2 complexes can be distinguished on native-PAGE gels, we silver-stained the native gel shown in Figure 6A to confirm that gp2 did not affect binding of $\sigma^{54}_{\Delta RI}$ or $\sigma^{54}_{ala24-26}$ to the RNAP (data not shown).

We considered the possibility that the addition of a peptide corresponding to σ^{54} Region I residues 1–56 *in trans* may restore the binding of gp2 to $E\sigma^{54}_{\Delta RI}$. This expectation was fulfilled as ^{32}P-labeled gp2 formed a stable complex with $E\sigma^{54}_{\Delta RI}$ when σ^{54} Region I peptide was combined with RNAP holoenzyme prior to the addition of ^{32}P-labeled gp2 (Figure 6B, lanes 5 and 6). By isolating the $E\sigma^{54}_{\Delta RI}$:gp2 complex (from Figure 6B, lane 6) and analyzing the protein components by SDS–PAGE, we confirmed that this complex

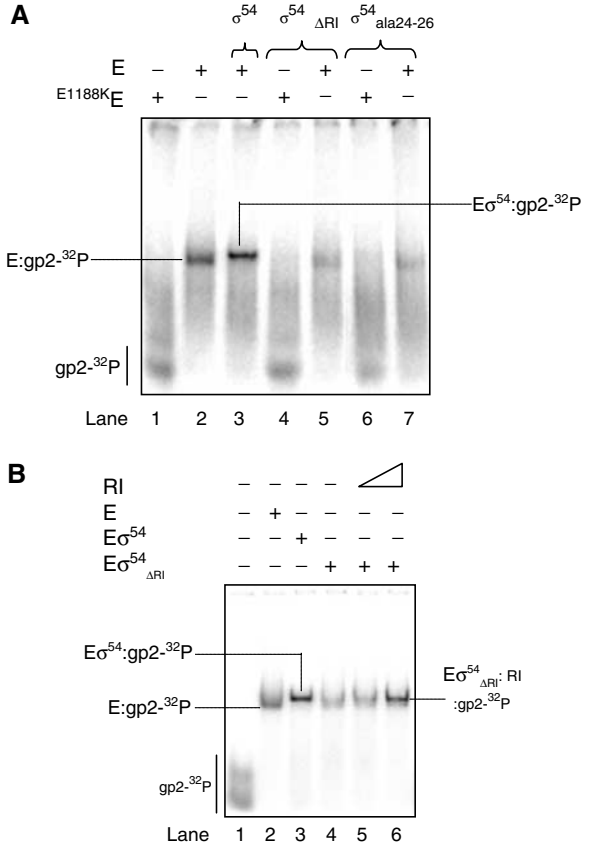


Figure 6 T7 gp2 does not bind $E\sigma^{54}$ reconstituted with σ^{54}-containing lesions in Region I. (**A**) An autoradiograph of a 4.5% (w/v) native gel showing the binding of ^{32}P-labeled gp2 to E (lane 2), $E\sigma^{54}$ (lane 3), $E\sigma^{54}_{\Delta RI}$ (lane 5) and $E\sigma^{54}_{ala24-26}$ (lane 7). The migration positions of gp2 (lane 1), E:gp2 (lane 2) and $E\sigma^{54}$:gp2 (lane 3) are indicated. Protein components in each lane are indicated on the top of the figure. (**B**) An autoradiograph of a 4.5% (w/v) native gel showing that ^{32}P-labeled gp2 can only detectably interact with $E\sigma^{54}_{\Delta RI}$ in the presence of σ^{54} Region I. Lanes 5 and 6 contain 2 and 4 μM σ^{54} Region I, respectively, that was added *in trans* to the reaction prior to the addition of ^{32}P-labeled gp2. The migration positions of gp2, E:gp2, $E\sigma^{54}$:gp2 and $E\sigma^{54}_{\Delta RI}$:RI:gp2 are as indicated. The protein components in each lane are indicated on top of the gel.

contained core RNAP, σ^{54} Region I, $\sigma^{54}_{\Delta RI}$ and ^{32}P-labeled gp2 (data not shown). *In trans* addition of σ^{54} Region I could somehow change the conformation of $\sigma^{54}_{\Delta RI}$ in such a manner that the gp2-binding site on the β′ jaw domain is revealed, thus allowing gp2 to stably bind. Control reactions demonstrated that gp2 did not bind Region I peptide or that the presence of σ^{54} Region I *in trans* did not alter the native gel migration properties of the E:gp2 complex (data not shown). Clearly, the results demonstrate that σ^{54} Region I, which is located at the 'upstream face' of the RNAP (Wigneshweraraj *et al*, 2000), affects the conformation of the gp2-binding site on the β′ subunit (located at the 'downstream face' of the RNAP; Figure 1a) and, by extension, determines the function of the β′ jaw domain during open complex formation.

σ^{54} Region I residues 24–26 and 42–47 have a role in determining β′ jaw domain conformation

To identify Region I determinants important for gp2 binding to $E\sigma^{54}$, we analyzed, by native PAGE, the binding of ^{32}P-labeled gp2 to $E\sigma^{54}$ reconstituted from σ^{54} Region I mutants

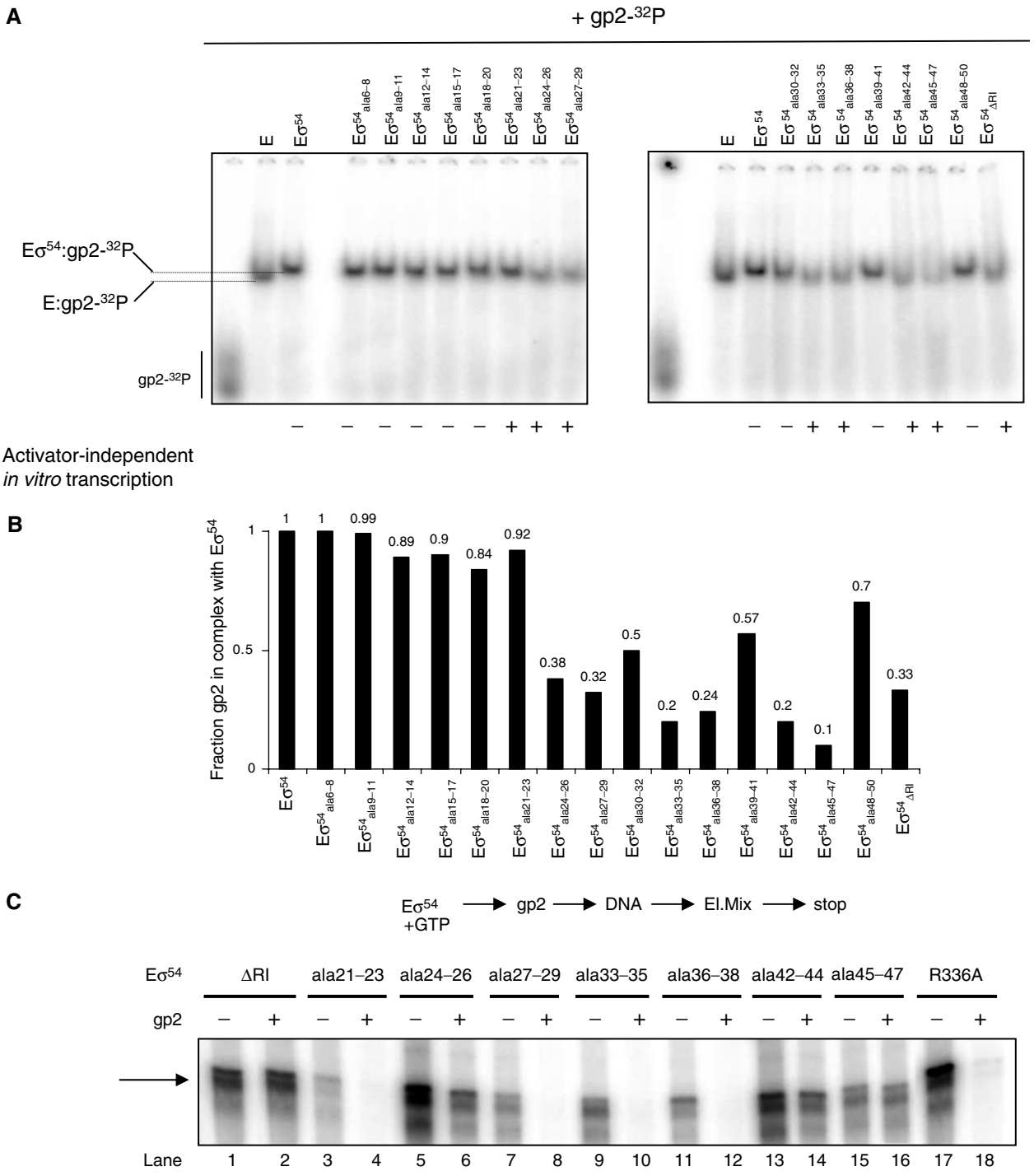


Figure 7 σ^{54} Region I residues 24–26 and 42–47 are important for T7 gp2 binding to Eσ^{54}. (**A**) An autoradiograph of a 4.5% (w/v) native gel showing the ability of ^{32}P-labeled gp2 to bind Eσ^{54} reconstituted with an array of σ^{54} Region I mutants. The migration positions of gp2, E:gp2 and Eσ^{54}:gp2 are indicated. At the bottom of the gel, '+' indicates that the corresponding mutant Eσ^{54} is active and '−' indicates that it is not active for activator-bypass transcription. The error range for the graphed values is within ±9%. (**B**) Graph showing the efficiency of ^{32}P-labeled gp2 binding to the Eσ^{54} mutants relative to its binding to the wild-type Eσ^{54} (see text). (**C**) An autoradiograph of a 4% (w/v) denaturing gel showing the effects of gp2 on activator-bypass transcription by Eσ^{54} reconstituted with selected σ^{54} Region I mutants (see text). The arrow indicates the 470 nucleotide transcript from the *S. meliloti nifH* promoter on pMKC28. The reaction schematic is shown on the top of the gel.

in which residues 6–50 have been systematically changed to alanines, three consecutive residues at a time (Casaz *et al*, 1999). Densitometry analysis of the gel presented in Figure 7A revealed that Eσ^{54} reconstituted with $\sigma^{54}_{ala24-26}$, $\sigma^{54}_{ala27-29}$, $\sigma^{54}_{ala33-35}$, $\sigma^{54}_{ala36-38}$, $\sigma^{54}_{ala42-44}$ and $\sigma^{54}_{ala45-47}$ bound gp2 60–90% less efficiently than wild-type Eσ^{54} (Figure 7B), suggesting that σ^{54} residues 24–29, 33–38 and 42–47 are important for gp2 binding to Eσ^{54}.

Since σ^{54} Region I residues important for gp2 binding to Eσ^{54} result in strong deregulation of Eσ^{54} when mutated (Casaz *et al*, 1999), we conducted activator-bypass transcription assays to check whether activator-bypass transcription

by $E\sigma^{54}$'s reconstituted with $\sigma^{54}_{ala24-26}$, $\sigma^{54}_{ala27-29}$, $\sigma^{54}_{ala33-35}$, $\sigma^{54}_{ala36-38}$, $\sigma^{54}_{ala42-44}$ and $\sigma^{54}_{ala45-47}$ was resistant to inhibition by gp2. As shown in Figure 7C and, as expected, activator-bypass transcription by $E\sigma^{54}_{\Delta RI}$ (lanes 1 and 2), $E\sigma^{54}_{ala24-26}$ (lanes 5 and 6), $E\sigma^{54}_{ala42-44}$ (lanes 13 and 14) and $E\sigma^{54}_{ala45-47}$ (lanes 15 and 16) was not inhibited by gp2, consistent with the fact that these mutant $E\sigma^{54}$'s bound gp2 with 10–30% efficiency compared to the wild-type $E\sigma^{54}$ (Figure 7A and B). Surprisingly, activator-bypass transcription by $E\sigma^{54}_{ala27-29}$, $E\sigma^{54}_{ala33-35}$ and $E\sigma^{54}_{ala36-38}$, which was deficient in gp2 binding in a native-PAGE assay (Figure 7A and B), was inhibited by gp2 (Figure 7C, lanes 8, 10 and 12). We explain this by suggesting that binding of gp2 to $E\sigma^{54}_{ala27-29}$, $E\sigma^{54}_{ala33-35}$ and $E\sigma^{54}_{ala36-38}$ may not be sufficiently stable to survive native PAGE, but is stable enough to prevent activator-bypass transcription in solution. Indeed, reducing the incubation time with gp2 (from 5 min to 30 s) before the addition of promoter DNA to the transcription reaction resulted in a detectable recovery of activator-bypass transcription by $E\sigma^{54}_{ala27-29}$, $E\sigma^{54}_{ala33-35}$ and $E\sigma^{54}_{ala36-38}$, while transcription in the control reaction containing $E\sigma^{54}_{R336A}$ was efficiently inhibited by gp2 under these conditions (data not shown).

Overall, the results support the view that σ^{54} Region I, particularly Region I residues 24–26 and 42–47, are important for gp2 binding to $E\sigma^{54}$ and may contribute to the β′ jaw domain function in the context of wild-type $E\sigma^{54}$.

Discussion

Multisubunit RNAPs must exist in several different functional states for the process of transcription initiation to progress. Bacterial RNAP containing σ^{54} relies on an activator- and ATP-hydrolysis-driven remodeling step for transcription initiation. We have used bacteriophage T7-encoded transcription inhibitor of σ^{70}-dependent transcription, the gp2 protein, as a tool to study the contribution of the β′ jaw domain—the gp2-binding site—to transcription initiation by $E\sigma^{54}$. Our analysis has revealed that even though gp2 binds to the same β′ site in σ^{54}- and σ^{70}-containing holoenzymes, activator- and ATP hydrolysis-dependent $E\sigma^{54}$ transcription is resistant to inhibition by gp2. We have shown that unlike $E\sigma^{70}$ closed complexes, $E\sigma^{54}$ closed complexes are not disrupted by gp2. Using an analogue of ATP in the transition state of hydrolysis, we demonstrated that gp2 is not able to stably interact with β′ jaw domain within an early intermediate promoter complex. Thus, we suggest that the basis for the resistance of activated $E\sigma^{54}$-dependent transcription to gp2 is due to activator- and ATP hydrolysis-driven conformational changes that disrupt inhibitory interaction(s) between gp2 and the β′ jaw domain during normal open complex formation. Supporting this view, the use of activator mutants defective in efficient coupling of energy derived from ATP hydrolysis to conformational changes in $E\sigma^{54}$ resulted in some inhibition of $E\sigma^{54}$ transcription by gp2. Further, in assays using the wild-type activator, reducing the incubation time with the activator (from 5 min to 30 s) resulted in significant inhibition of $E\sigma^{54}$ transcription in reactions containing the wild-type core RNAP, but not the gp2-resistant E1188K mutant core RNAP (our unpublished observations).

The requirement for σ^{54} Region I for the binding of gp2 to $E\sigma^{54}$ suggests a functional and/or structural link(s) between σ^{54} Region I and the β′ jaw domain. The precise nature of this link is currently unclear, but must have a role in regulating the functionality of the RNAP. Region I constitutes the major activator-binding site (Chaney *et al*, 2001) and it undergoes activator- and ATP hydrolysis-dependent conformational changes during open complex formation (Casaz and Buck, 1999; Wigneshweraraj *et al*, 2001; Burrows *et al*, 2003, 2004). Thus, an attractive possibility is that 'activating signals' from the 'upstream face' of the RNAP (where Region I is located; Wigneshweraraj *et al*, 2000) are somehow relayed to the β′ jaw domain during open complex formation. Since the β′ jaw domain is modeled to contact the downstream DNA within the crystal structure of the *Thermus aquaticus* $E\sigma^{A}$ promoter complex (Murakami *et al*, 2002) and is suggested to make stabilizing interactions with downstream DNA during open promoter complex formation by $E\sigma^{70}$ (Ederth *et al*, 2002), we suggest that the β′ jaw domain, upon activation, becomes engaged with downstream DNA during open complex formation by $E\sigma^{54}$. Similarly, the homologue of the β′ jaw domain in eukaryotic RNAP II, the RPB1 jaw lobe, undergoes a conformational change and moves towards DNA upon binding of transcription factor TFIIB (Bushnell *et al*, 2004).

In agreement with the activator- and ATP hydrolysis-dependent use of the β′ jaw domain during $E\sigma^{54}$ open complex formation, gp2 is able to inhibit activator-bypass transcription by some activator-bypass forms of $E\sigma^{54}$ as efficiently as $E\sigma^{70}$ transcription. Notably, activator-bypass mutant $E\sigma^{54}_{R336A}$ is gp2-resistant in activator-dependent assays, but not in activator-bypass assays. The striking difference in gp2 sensitivity between activator-dependent and activator-bypass transcription further underscores the activator-dependent engagement of the β′ jaw with DNA in a σ^{54} Region I-dependent manner during $E\sigma^{54}$ transcription. Since gp2 inhibited activator-bypass $E\sigma^{54}$ transcription by preventing stable DNA melting, we suggest that the β′ jaw domain has a role in stable DNA melting during open complex formation by $E\sigma^{54}$. Supporting this idea, $E\sigma^{54}$ reconstituted using a mutant RNAP core form lacking the β′ jaw domain (β′Δ1149–1190) is defective for DNA melting and transcription (Wigneshweraraj *et al*, in preparation).

In bacterial RNAP, access to the cleft containing the catalytic center is restricted by the β′ clamp domain (Murakami *et al*, 2002). This conformation is referred to as the 'closed clamp state'. Promoter DNA must enter the cleft, either as melted DNA or as DNA 'to be melted' during or after the 'clamp-opening' event. Several groups (Murakami *et al*, 2002; Armache *et al*, 2003; Bushnell and Kornberg, 2003) proposed that initiation of DNA melting 'outside' the cleft may allosterically induce conformational changes that result in 'clamp opening' and propagation of melting. The β′ clamp and jaw domains are integral parts of the β′ subunit and thus are likely to undergo interdependent conformational changes during 'clamp opening'. Supporting this view, recent structural information on yeast RNAPII revealed that the RPB1 clamp and jaw-lobe domains undergo inter-dependent conformational changes upon interaction with transcription factor TFIIB (Bushnell *et al*, 2004). Based on different gp2 sensitivities of $E\sigma^{70}$ and $E\sigma^{54}$ transcription, below, we discuss the role of the β′ jaw domain in 'clamp opening' and transcription by bacterial RNAP below.

σ^{54} can initiate DNA melting in response to activation in the absence of RNAP core (Cannon *et al*, 2001). Our results suggest that activation somehow results in conformational

changes in the β′ jaw domain and allows the β′ jaw domain to engage downstream DNA, which leads to stable melting. Based on the two-step model proposed by Wang and Gralla (1996) for open complex formation by $E\sigma^{54}$, we suggest that the first activation step results in a core RNAP-independent initiation of DNA melting, which occurs in the 'closed clamp' conformation outside the catalytic cleft (Cannon *et al*, 2001). During the second activation step, conformational changes in the β′ jaw domain trigger 'clamp opening' and so facilitate the entry of the partly melted DNA into the RNAP catalytic cleft to allow propagation of melting in an RNAP core-dependent manner. Within the RNAP catalytic cleft, the RNAP catalytic site proximal downstream DNA contacts are made by the β′ clamp domain, whereas the RNAP catalytic site distal downstream DNA contacts are made by the β′ jaw domain (Murakami *et al*, 2002). We envisage that the gp2 dissociates from the β′ jaw domain upon activator- and ATP hydrolysis-dependent loading of DNA onto the β′ jaw domain. Consistent with the view that activator 'drives' the DNA onto the β′ jaw domain, the interaction between core RNAP and gp2 is disrupted by heparin or nonspecific DNA (our unpublished observations). Similarly, the interaction between gp2 and $E\sigma^{54}$ is highly heparin-sensitive (see Supplementary data Figure 3). In the case of activator-bypass transcription, the DNA enters the RNAP catalytic cleft and contacts the β′ jaw domain independently of the activation event. Since activator-bypass open complexes are significantly more heparin sensitive than activator-dependent open complexes, we argue that during activator-bypass transcription the DNA-β′ jaw domain contacts are weak and cannot result in dissociation of gp2.

In contrast, σ^{70} strictly relies on the use of core RNAP subunits for DNA melting (Young *et al*, 2004). Hence, core RNAP-dependent DNA melting may allosterically induce 'clamp opening' and subsequently facilitate further DNA melting within the catalytic cleft. Therefore, as proposed by Ederth *et al* (2002), the presence of gp2 could prevent β′ jaw–DNA contacts during promoter DNA melting and thereby destabilize intermediate open promoter complexes. However, once complete promoter DNA melting and 'clamp opening' has occurred, gp2 is not able to bind open promoter complexes and therefore has no effect on $E\sigma^{70}$ open promoter complexes (Nechaev and Severinov, 1999)—presumably because the gp2-binding site on the β′ jaw domain is bound by DNA. Clearly, accessory factors such as σ factors which bind the upstream face of the RNAP are able to control the activity of other parts of the RNAP (Figure 1A), allowing control of RNAP functionality (Chung *et al*, 2003; Bushnell *et al*, 2004 and this work).

Materials and methods

Protein purification

All wild-type and mutant *Klebsiella pneumoniae* σ^{54} and *E. coli* $PspF_{1-275}$ were purified as described in Wigneshweraraj *et al* (2003). T7 gp2 was purified from *E. coli* 7009(DE3) cells essentially as detailed in Nechaev and Severinov (1999). The protocol for the overexpression and purification of wild-type *E. coli* σ^{70} is described in Nechaev and Severinov (1999). Wild-type *E. coli* core RNAP was purchased from Epicentre Technology (Madison, WI). T7 gp2-resistant *E. coli* core RNAP containing the E1188K mutation was purified from *E. coli* 7009 cells as detailed in Nechaev and Severinov (1999).

In vitro transcription assays

Single-round activator-dependent and activator-bypass transcription assays from supercoiled (pMKC28) and linear *S. meliloti nifH* templates were conducted as detailed in Wigneshweraraj *et al* (2003). The protocol for $E\sigma^{70}$-dependent transcription from pOM*lac*UV5 is described in Colland *et al* (1999). Where indicated, gp2 was present in four-fold molar excess over Eσ (unless otherwise stated). The final concentrations of template DNA, Eσ (reconstituted using 1:4 molar ratio of core RNAP over σ), $PspF_{1-275}$ (where indicated) and ATP (where indicated) were at 20, 100 nM, 4 μM and 4 mM, respectively. The elongation mix (abbreviated as 'El.mix' in the figures) contained 1 mM ATP, CTP, 0.05 mM UTP, 3 μCi of [α-^{32}P]UTP and 100 μg/ml heparin. The point at which gp2 was added to the reactions is specified in the figure and legends thereof.

T7 gp2-Eσ^{54}-binding assays

All binding reactions (10 μl) were conducted in STA (25 mM Tris-acetate, pH 8.0, 8 mM Mg-acetate, 10 mM KCl, 3.5% w/v PEG 6000) buffer at 37°C. $E\sigma^{54}$ was present at 100 nM (reconstituted as above) and ^{32}P-gp2 at 800 nM. Where indicated, the streptavidin–biotin-labeled *S. meliloti nifH* promoter probe (positions −35 to +1) was present at 400 nM. Biotin-tagged (at the 5′ end of the nontemplate strand) *S. meliloti nifH* promoter probe was labeled with streptavidin (Sigma) by incubating with four-fold streptavidin for 5 min at 37°C in STA buffer. Where indicated, σ^{54} Region I residues 1–56 were present at the indicated concentrations (see figure legends). Assays were started by reconstituting the $E\sigma^{54}$ for 5 min, followed by adding of gp2 and incubating for a further 5 min. Where indicated, a streptavidin-labeled promoter probe was added for 5 min. The reactions were stopped and analyzed for native PAGE as in Wigneshweraraj *et al* (2003).

Activator interaction assays

The $E\sigma^{54}$:gp2 or $E\sigma^{54}$:DNA:gp2 complexes were formed as described above. The ADP-AlF_x-dependent activator-binding assays were conducted as described in Chaney *et al* (2001) using final concentrations of 5 μM $PspF_{1-275}$, 0.2 mM ADP, 0.2 mM $AlCl_2$ and 5 mM NaF to form the $PspF_{1-275}$:ADP-AlF_x complex.

DNA melting and footprinting assays

Promoter complexes were formed on pMKC28 in the absence and presence of gp2 at concentrations as outlined above for the transcription assays. $KMnO_4$ and/or DNase I footprinting reactions were conducted as detailed in Cannon *et al* (2001) and the reactions were processed as detailed in Burrows *et al* (2003).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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