Binding of transcriptional activators to sigma 54 in the presence of the transition state analog ADP–aluminum fluoride: insights into activator mechanochemical action

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Conformational changes in sigma 54 (σ^{54}) and σ^{54}-holoenzyme depend on nucleotide hydrolysis by an activator. We now show that σ^{54} and its holoenzyme bind to the central ATP-hydrolyzing domains of the transcriptional activators PspF and NifA in the presence of ADP–aluminum fluoride, an analog of ATP in the transition state for hydrolysis. Direct binding of σ^{54} Region I to activator in the presence of ADP–aluminum fluoride was shown and inferred from in vivo suppression genetics. Energy transduction appears to occur through activator contacts to σ^{54} Region I. ADP–aluminum fluoride-dependent interactions and consideration of other AAA+ proteins provide insight into activator mechanochemical action.

[*Key Words*: Sigma 54; activators; transcription; ADP · AlF_x; AAA+ proteins]

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Transcription by RNA polymerase (RNAP) is often regulated by interactions with control proteins to link specific gene expression to environmental signals and temporal cues. Often activators help recruit RNAP to promoters to increase initiation rates (Busby and Ebright 1999). In contrast, activity of the bacterial σ^{54} containing RNAP holoenzyme is regulated at the DNA melting step (for review, see Buck et al. 2000). Hydrolysis of an NTP by an activator drives a change in configuration of the σ^{54}-holoenzyme, converting the initial closed complex to an open complex to allow interaction with the template DNA for mRNA synthesis (Wedel and Kustu 1995). Preopening of DNA templates does not overcome the requirement for NTP hydrolysis by an activator to promote engagement of the holoenzyme with the melted DNA (Wedel and Kustu 1995; Cannon et al. 1999).

The activators of σ^{54}-holoenzyme are members of the large AAA+ protein family, which use ATP binding and hydrolysis to remodel their substrates (Neuwald et al. 1999; Cannon et al. 2000, 2001). The greater part of the central domain of σ^{54} activators corresponds to the AAA core structure, and includes ATP-binding and hydrolyzing determinants. The σ^{54} protein is known to be the primary target for the NTPase of activators, but how activators use NTP binding and hydrolysis is not well understood (Cannon et al. 2000). Similarly, the nature of the interaction between σ^{54} and the activator is not well described, but an interaction with σ^{54} can be detected in the case of the DctD activator by protein cross-linking (Lee and Hoover 1995). Here we show that the use of ADP–aluminum fluoride, an analog of ATP that mimics ATP in the transition state for hydrolysis, allows formation of a stable complex among the activator PspF, the PspF and NifA central activating domains, and σ^{54}. The binding assay was used to help define determinants in σ^{54} and the activator needed for their interaction, and to show that binding can lead to an altered σ^{54}–DNA footprint. The need for a transition-state analog of ATP for protein–protein binding is discussed in relation to the required ATPase activity of activators of σ^{54}-dependent transcription. In particular, it seems that altered functional states of activators exist as ATP is hydrolyzed. This suggests a parallel to some switch and motor proteins that use nucleotide binding and hydrolysis to establish alternate functional states (Hirose and Amos 1999).

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Results

Assay system

Enhancer-binding activators of the σ^{54}-holoenzyme are typically composed of three domains (Drummond et al. 1986; Morett and Segovia 1993). These include a C-terminal enhancer DNA-binding domain and an N-terminal domain. The latter functions in regulation, often by acting on the central domain (Lee et al. 2000). Interactions with the σ^{54}-holoenzyme and ATP-binding and hydrolyzing activities directly involve the activator central domain. The PspFΔHTH protein we have employed here represents mainly the central domain of the σ^{54} activators (Fig. 1a). The PspF activator of *Escherichia coli* lacks a regulatory N-terminal domain, being subject instead to control by PspA (Jovanovic et al. 1999; Dworkin et al. 2000). Interactions of σ^{54} and the activator PspFΔHTH were explored in the presence of MgADP and compounds that are known to mimic the transfer of the γ-phosphate at hydrolysis of ATP (transition state analogs) with several proteins, as defined by X-ray crystallography of the nucleotide-containing complexes (Fersht 1998). In particular, we were interested in exploring the possibility of isolating σ^{54}-activator complexes that depend on nucleotide interactions with the activator. The basic assay consisted of incubating the activator with the transition state analog ADP–aluminum fluoride ($ADP \cdot AlF_x$) together with σ^{54} (or holoenzyme), or a DNA complex thereof, and resolving the mixture on a native polyacrylamide gel. Typically either one of the protein components or DNA was ^{32}P-end labeled, and in some experiments complexes were visualized by Coomassie staining.

ADP–aluminum fluoride induces formation of a stable complex between the activator and σ^{54}

Initially either σ^{54} or PspFΔHTH was ^{32}P-end-labeled through an engineered heart muscle kinase (HMK) tag (Casaz and Buck 1997). Under conditions where

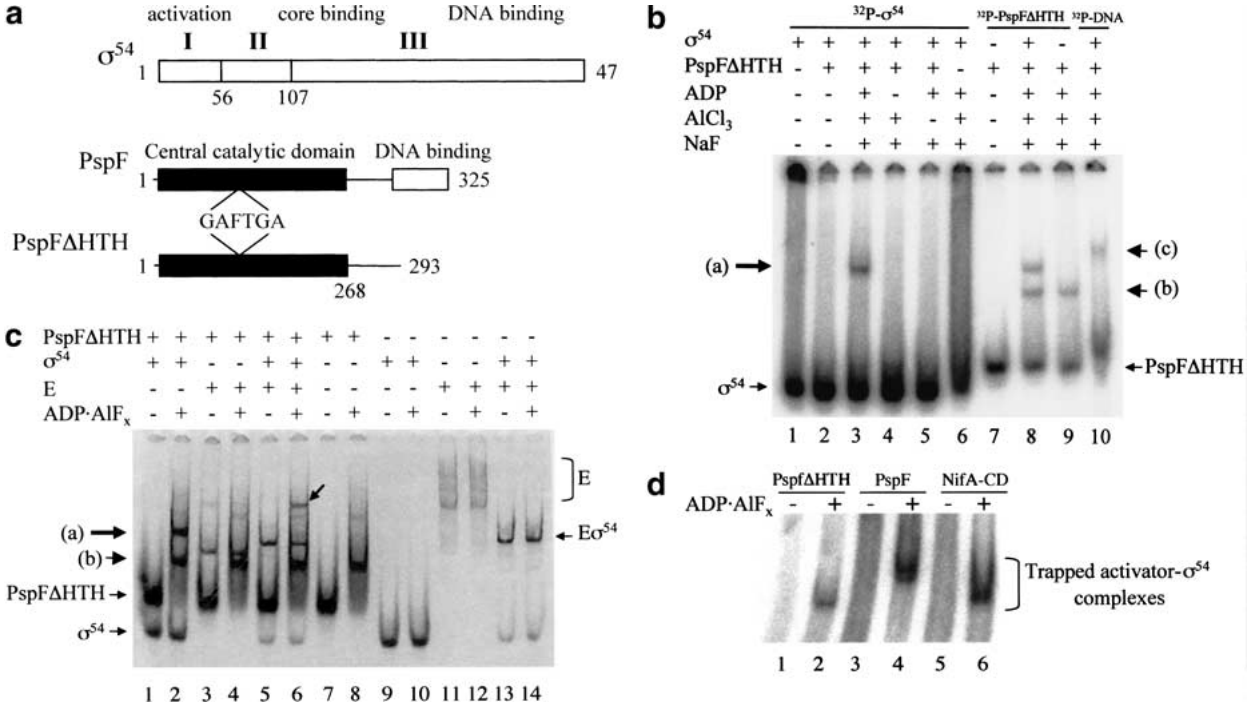

Figure 1. (*a*) Schematics of σ^{54} and the activator PspF. The three regions of *Klebsiella pneumoniae* σ^{54} and their associated functions are indicated (Buck et al. 2000). The functional domains of the *Escherichia coli* σ^{54} activator PspF and its derivative PspFΔHTH deleted for its DNA-binding domain are shown (Jovanovic et al. 1999). The approximate position of the highly conserved GAFTGA motif implicated as part of the Switch 1 region in σ^{54} activators (Rombel et al. 1998; Yan and Kustu 1999) is indicated. (*b*) Gel mobility-shift assay for ADP–aluminum fluoride-dependent complex formation between PspFΔHTH and σ^{54} using ^{32}P-HMK-tagged protein. Reactions were with ^{32}P-HMK-tagged σ^{54} or ^{32}P-HMK-tagged PspFΔHTH (100 nM), unlabeled PspFΔHTH (10 μM), and unlabeled σ^{54} (1 μM). Lane *10* contains σ^{54} (1 μM), PspFΔHTH (10 μM) with ^{32}P-end-labeled *Sinorhizobium meliloti nifH* promoter 88-nt DNA (16 nM). Arrow (a) indicates position of complexes formed between σ^{54} and PspFΔHTH in the presence of $ADP \cdot AlF_x$; arrow (b) indicates the position of PspFΔHTH complex formed in the presence of $ADP \cdot AlF_x$, and arrow (c) indicates the position of DNA–σ^{54}–PspFΔHTH complex in the presence of $ADP \cdot AlF_x$. (*c*) Gel mobility-shift assay for $ADP \cdot AlF_x$-dependent complex formation between PspFΔHTH and σ^{54} and σ^{54}-holoenzyme detected by Coomassie staining. Reaction conditions were as in *a* except for PspFΔHTH (20 μM), σ^{54} (4 μM or 600 nM when present with core RNAP [E]), and core RNAP (300nM). The arrow on the gel indicates the holoenzyme trapped activator complex in lane *6*. Arrows (a) and (b) point to complexes as indicated in *a*. (*d*) Wild-type PspF and the NifA central domain form an $ADP \cdot AlF_x$-dependent complex with σ^{54}. Reaction conditions were as in *b* with ^{32}P-HMK σ^{54} (50 nM), PspFΔHTH (10 μM), wild-type PspF (3 μM), and NifA central domain (3 μM). PspFΔHTH (~36 kD), wild-type PspF (~37.5 kD), and NifA-CD (~32 kD).

ADP · AlF_x (where x must be 3 or 4) can form, but not otherwise, the end-labeled protein (either σ^{54} or PspFΔHTH) was found in a new, slow-running complex when the nonlabeled protein (activator or σ^{54}, respectively) was added (Fig. 1b, lanes 3,8). Similar results were also achieved using proteins lacking the heart muscle kinase tag and in the absence of α-lactoalbumin (a nonspecific carrier protein, see Materials and Methods), with complexes being detected by Coomassie staining (Fig. 1c). PspFΔHTH in the presence of ADP · AlF_x also bound σ^{54}-holoenzyme ($E\sigma^{54}$; Fig. 1c, lane 6). Results show that σ^{54} and its holoenzyme can detectably associate with PspFΔHTH to form a stable complex in the presence of ADP · AlF_x. Hereafter we use the term "trapped" to refer to the form of activator bound to σ^{54} or σ^{54}-holoenzyme in the presence of ADP · AlF_x.

Controls using core RNAP (E) alone did not result in the increased formation of a complex between core RNAP and PspFΔHTH–ADP · AlF_x, suggesting that σ^{54} is the main target of the activator within the holoenzyme (Fig. 1c, cf. lanes 3 and 8 with lane 4). Interestingly, PspFΔHTH can interact with core RNAP in the absence of ADP · AlF_x (Fig. 1c, cf. lane 3 with lanes 1, 7, and 11).

We also used ADP · AlF_x with the full-length PspF activator (i.e., with its DNA-binding domain) and the central domain of the nitrogen fixation A protein, NifA-CD (Money et al. 2001), another activator of the σ^{54}-holoenzyme, so as to trap stable complexes with σ^{54} (Fig. 1d). As predicted from the presence of the activator PspFΔHTH within the trapped complex that formed with σ^{54}, and the different molecular weights of the PspFΔHTH, PspF, and NifA-CD, these three σ^{54} trapped complexes each had a different native gel mobility (Fig. 1d).

Order of addition experiments in which either ^{32}P-σ^{54} (50 nM) and PspFΔHTH (10 μM) were preincubated prior to formation of ADP · AlF_x (as in the standard reaction; see Materials and Methods) or PspFΔHTH was exposed to ADP · AlF_x before addition of σ^{54}, resulted in 24% and 1% of the ^{32}P-σ^{54} bound in the trapped complex, respectively. Formation of the σ^{54}-holoenzyme trapped complex was subject to the same order of addition effects (data not shown). This strongly suggests that the transition-state analog ADP · AlF_x acts to stabilize a preexisting unstable complex between σ^{54} and PspFΔHTH.

Addition of 20 mM phosphate or 10 mM ATP after trapped complexes had been allowed to form did not diminish the amount of trapped σ^{54}–PspFΔHTH complex, indicating that the ADP · AlF_x is stably bound in the complex (data not shown). ADP without aluminum fluoride, use of the alternative transition-state analog ADP · Vi (ADP in the presence of vanadate ion), or nonhydrolyzable analogs AMPPNP or ATPγS did not result in formation of a stable σ^{54}–PspFΔHTH complex (data not shown; Cannon et al. 2000, 2001). Other sigma factors (*E. coli* σ^{70} or σ^{38}) did not associate with PspFΔHTH–ADP · AlF_x to give the slow-migrating trapped complex (data not shown). Therefore, We conclude that ADP · AlF_x acts specifically to increase the binding of activator to σ^{54} and its holoenzyme.

Using Coomassie staining we estimated the amount of σ^{54} and PspFΔHTH in trapped complexes isolated from a native gel (data not shown). Repeated experiments indicated that not less than five PspFΔHTH monomers are present per σ^{54} monomer. This implies that an oligomeric form of activator binds to σ^{54}. Simple steric effects may also limit the number of σ^{54} molecules bound per activator oligomer.

ADP · AlF_x changes self-association and ATPase activity of PspFΔHTH

The native gel mobility of the PspFΔHTH activator is changed when ADP · AlF_x is allowed to form (Fig. 1b, cf. lanes 7 and 9; Fig. 1c, cf. lanes 7 and 8). This could be caused by differences in oligomerization state and/or conformation. Activators, in particular NtrC, of the σ^{54}-holoenzyme are known to form higher-order oligomers (Wyman et al. 1997), and this is also true for PspF and PspFΔHTH (see below). Activators of σ^{54} belong to the AAA+ protein family (Neuwald et al. 1999; Vale 2000), crystal structures of which show nucleotide interactions in one protomer and contact to the γ-phosphate from an adjacent protomer within a hexameric assembly (Neuwald et al. 1999). Preliminary gel filtration experiments and analytical ultracentrifugation analyses have shown that ADP · AlF_x does increase the association state of the PspFΔHTH protein (data not shown). Because the PspF protein is known to interact with ATP (Jovanovic et al. 1999), we infer that the self-associated activator is in an ADP · AlF_x-bound form. The ATPase activity of PspFΔHTH and PspF were inhibited by ADP · AlF_x. With PspFΔHTH (3.0 μM) or PspF (1.0 μM) and ATP (0.4 mM), the presence of ADP · AlF_x reduced ATPase activities by 40% and 95%, respectively. σ^{54} is not known to interact directly with nucleotides, suggesting that binding of σ^{54} to PspFΔHTH is stabilized through interactions made between ADP · AlF_x and activator.

Role of activator self-association in binding σ^{54}

Trapping experiments were performed using wild-type PspF at concentrations above that at which it fully self-associates and forms a higher-order oligomer (data not shown). Addition of ADP · AlF_x did not alter the native gel mobility of the wild-type PspF but did allow it to bind σ^{54} (data not shown; Fig. 1d, lane 4). Addition of ATP, ADP, or ATPγS did not allow wild-type PspF to bind stably to σ^{54} (data not shown). Formation of a higher-order oligomer per se does not therefore allow PspF to stably bind σ^{54}. Rather, a distinct form of PspF associated with the presence of the transition-state analog ADP · AlF_x is required for a stable interaction between activator and σ^{54}. The ADP · AlF_x-dependent self-association of PspFΔHTH may reflect loss of a contribution by the HTH to self-association that allows the effects of binding the ATP analog to be visualized in terms of oligomerization changes. Binding of ADP · AlF_x between protomers can help account for the self-association of PspFΔHTH.

σ^{54} Region I is essential for binding activator

σ^{54} fragments 57–477 (σ^{54} deleted for Region I, $\Delta I\sigma^{54}$), 1–324, 70–324, and a series of σ^{54} Region I three alanine substitution mutants from residue 6 to 50 (Casaz et al. 1999), both alone or as part of the holoenzyme, were screened for trapped complex formation with end-labeled PspFΔHTH activator (Fig. 2a; data not shown). It is clear that the σ^{54} N-terminal Region I sequences (residues 1–56) are important for the binding reaction with PspFΔHTH–ADP · AlF_x. No single three alanine substitution mutant in σ^{54} Region I diminished formation of the trapped complex with σ^{54} or σ^{54}-holoenzyme as greatly as did removal of Region I (Fig. 2a; data not shown). However, clear patterns of reduced binding were apparent, suggesting that several sequences in Region I contribute to binding of the activator (Casaz et al. 1999). With ^{32}P-HMK-tagged PspFΔHTH at 100 nM and σ^{54}-holoenzyme at 300 nM, Region I residues 6–11, 33–38, and 45–47 stood out as important patches for binding PspFΔHTH–ADP · AlF_x to holoenzyme. Triple alanine substitutions across these positions bound 20%, 30%, and 50% of the PspFΔHTH–ADP · AlF_x compared to wild-type holoenzyme, respectively (data not shown). For residues 33–38 and 45–47, binding activity correlates with the critical role of these patches in activated transcription (Syed and Gralla 1998; Casaz et al. 1999; Gallegos and Buck 2000). Importantly, two mutants in σ^{54} (deletion 310–328 and R336A) that share with certain σ^{54} Region I mutants and the Region I deletion form of σ^{54} the property of activator-independent transcription in vitro (Chaney and Buck 1999; Chaney et al. 2000), efficiently formed trapped complexes with PspFΔHTH (data not shown). This further supports the argument that σ^{54} Region I may directly interact with PspFΔHTH. Experiments using σ^{54} fragments 70–324 and 1–324 together with $\Delta I\sigma^{54}$ (residues 57–477) and wild-type σ^{54} showed that the NifA-CD had the same specificity for Region I as did PspFΔHTH in the trapping reaction (Fig. 2a; data not shown).

Interactions of σ^{54} Region I with activator

To explore the possibility that Region I (residues 1–56 of σ^{54}) sequences might directly bind activator, we added purified Region I to PspFΔHTH (Fig. 2b). A distinct, buffer-independent, small reduction in gel mobility was seen when PspFΔHTH–ADP · AlF_x was formed in the presence of Region I, compared to controls without Region I (Fig. 2b, cf. lanes 2 and 3). No stable interaction was seen between PspFΔHTH and Region I in the absence of ADP · AlF_x (Fig. 2b, lane 1; data not shown). This result provides direct evidence for a PspFΔHTH–ADP · AlF_x–Region I interaction. The absence of other regions of the σ^{54} protein may allow Region I to interact with the PspFΔHTH monomer so as to inhibit activator self-association in the presence of ADP · AlF_x. This could explain why lane 3 does not also contain a band with the mobility corresponding to self-associated activator as seen in lane 2.

Experiments using heterobifunctional cross-linking reagents revealed that determinants for nucleotide-independent binding of σ^{54} to the activator DctD were outside of σ^{54} Region I (Lee and Hoover 1995; Kelly et al. 2000). Therefore, we performed a competition assay wherein an increasing concentration of Region I or $\Delta I\sigma^{54}$

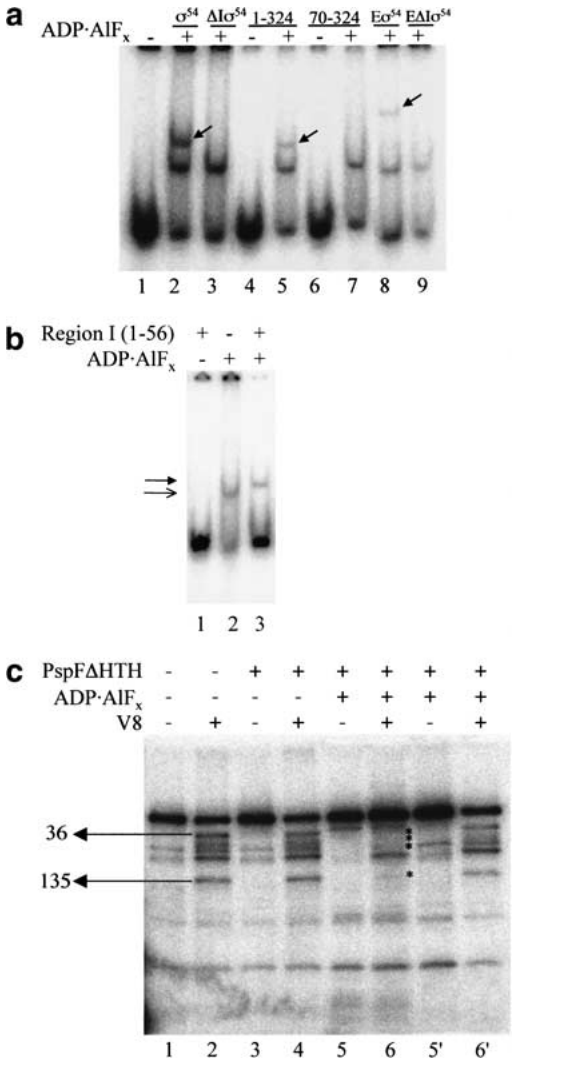

Figure 2. (*a*) Gel mobility-shift assay for ADP · AlF_x-dependent complex formation between PspFΔHTH and σ^{54}, σ^{54} peptides, and σ^{54}-holoenzyme with and without Region I. Reactions contained ^{32}P-HMK-tagged PspFΔHTH (100 nM), σ^{54}, and $\Delta I\sigma^{54}$ (1 μM). Peptides 1–324 and 70–324 (50 μM). Eσ^{54} and E$\Delta I\sigma^{54}$ were formed with σ^{54} (600 nM) and E (300 nM). Trapped activator–σ^{54} fragment complexes are marked with an arrow. (*b*) Gel mobility shift assay for ADP · AlF_x-dependent complex formation between PspFΔHTH and σ^{54} Region I. Reactions contained ^{32}P-HMK-tagged PspFΔHTH (100 nM) and σ^{54} Region I (50 μM). The lower, unfilled arrowhead indicates the PspFΔHTH–ADP · AlF_x complex and the upper, filled arrowhead the trapped PspFΔHTH–Region I complex. (*c*) V8 footprinting of the trapped PspFΔHTH–σ^{54} complex. Reactions contained ^{32}P-HMK-tagged σ^{54} (200nM) and PspFΔHTH (20 μM). V8-treated reactions are marked with + (lanes *2,4,6,6′*) and untreated reactions are marked with – (lanes *1,3,5,5′*). Lanes *5′* and *6′* contain the free σ^{54} isolated from reactions in lanes *5* and *6*, respectively. V8 cleavage sites are as marked.

was added to a fixed amount of σ^{54} and PspFΔHTH. At a ratio of 4:1 (Region I or $\Delta I\sigma^{54}$:^{32}P-σ^{54}) added prior to trapping, Region I reduced the amount of σ^{54} in the trapped complex by 68% and $\Delta I\sigma^{54}$ reduced the amount by 20% (data not shown). Together these results suggest that Region I is the region of primary contact between PspFΔHTH and σ^{54} before and after trapping but that additional determinants for an activator–σ^{54} interaction prior to trapping do exist outside of Region I. Consistent with the Region I–trapped activator interaction assays, protein footprints of the stable PspFΔHTH–σ^{54} complex formed with ADP · AlF_x showed that much of the Region I sequence was protected from protease attack (Fig. 2c, lane 6). In contrast, unbound σ^{54} from the same trapping and footprinting reaction was not protected across Region I (Fig. 2c, cf. lanes 6 and 6′). Protection in trapped complexes extended as far as amino acid 135, within the acidic Region II of σ^{54}. Overall we conclude that PspFΔHTH–ADP · AlF_x and σ^{54} form a complex that involves direct protein–protein contacts between Region I of σ^{54} and the activator.

σ^{54} mutants implicate interactions between the GAFTGA motif and Region I in vivo

A signature of activators of the σ^{54}-holoenzyme is the six-amino-acid GAFTGA motif within the C3 region, which is involved in transcriptional activation and implicated in energy coupling (Morett and Segovia 1993; Wang et al. 1997; Gonzalez et al. 1998; Rombel et al. 1998). In an attempt to identify the determinants of the σ^{54}-holoenzyme involved in the interaction with activator proteins, we searched for mutants of σ^{54} able to recover activator function of activation-defective mutants in the GAFTGA motif and in an adjacent residue in the C3 region of *Bradyrhizobium japonicum nifA* (Gonzalez et al. 1998). This strategy is based on the premise that in a macromolecular assembly the activity of a mutation that affects one of the members can be suppressed through a compensatory mutation in an interacting member.

Randomly generated mutants across Regions I and II of σ^{54} were screened for suppression of the NifA E298D (outside of GAFTGA) and NifA T308S (within GAFTGA) mutants, which give a low (<1% compared to wild-type NifA) transcription activity in vivo (Gonzalez et al. 1998). From an initial pool of ~50,000 mutants, two rounds of screening resulted in selection of a mutant that consistently maintained the suppression phenotype. The nucleotide sequence of this clone revealed six nucleotide changes, resulting in four amino acid replacements and two silent changes (Q20L, CAG to CTG; H53N, CAC to AAC; D89D, GAT to GAC; D159G, GAA to GGA; R196R, CGT to CGC; and D231V, GAC to GTC). To determine which amino acid replacements were responsible for the phenotype, the four amino acid changes were segregated. The suppression phenotype was only maintained when Q20L and H53N mutations were present simultaneously (clone Q20L/H53N in Table 1a). The data presented in Table 1a suggest that mutant σ^{54} Q20L/H53N suppressed mutant NifA T308S in an allele-specific manner, because its activity was increased 23-fold but the activity of NifA E298D was increased only fivefold. Expression in combination with the wild-type NifA does not seem to be affected.

The suppression potential of σ^{54} Q20L/H53N was also examined with region C3 GAFTGA mutants in PspF. To do so, the corresponding amino acid substitutions T308S and T308V of *B. japonicum* NifA were made in PspFΔHTH by site-directed mutagenesis, resulting in PspFΔHTH T86S and PspFΔHTH T86V. A *Sinorhizobium meliloti nifH–lacZ* fusion was used to determine activity because this promoter can be activated in *trans* by PspFΔHTH. The data in Table 1b show that the changes generated in the GAFTGA motif of PspFΔHTH also strongly affected the activation function, as in the case of NifA, and that the σ^{54} Q20L/H53N suppressed the PspFΔHTH T86S mutant. As previously observed, in

Table 1. β-*galactosidase activity from* K. pneumoniae nifH *and* S. meliloti nifH *promoters in an* rpoN-*background*

(a) The mean β-galactosidase activity of σ^{54} wild type and the σ^{54} Q20L/H53N double mutant at the *K. pneumoniae nifH* promoter in the presence of NifA wild type and NifA T308S and E298D mutants.

NifA	σ^{54}	σ^{54} Q20L/H53N
wild type	35055 ± 2212	39640 ± 640
T308S	102 ± 10	2385 ± 408
E298D	50 ± 0.57	279 ± 112
–	55 ± 10	nd

(b) The mean β-galactosidase activity of σ^{54} wild type and the σ^{54} Q20L/H53N double mutant at the *S. meliloti nifH* promoter in the presence of PspFΔHTH and PspFΔHTH carrying mutations T86S or T86V.

PspFΔHTH	σ^{54}	σ^{54} Q20L/H53N
PspFΔHTH	9131 ± 701	17155 ± 774
T86S	2183 ± 107	10106 ± 845
T86V	2019 ± 99	3540 ± 475
–	2057 ± 211	4085 ± 89

(c) The β-galactosidase activity from the *K. pneumoniae nifH* promoter of a series of double mutations at position Q20 and H53 in the presence of NifA wild type and NifA T308S.

σ^{54}20/53	NifA	NifA T308S[a]
leu/leu	32044	1426 ± 3
leu/phe	34314	3998 ± 885
leu/asn	32860	1634 ± 333
leu/asn	28627	1699 ± 235
val/ala	29499	1076 ± 200
leu/ala	35444	1374 ± 243
leu/thr	46478	1734 ± 410
leu/glu	31568	1327 ± 109
leu/ser	32401	1537 ± 108
leu/asp	29293	1248 ± 144

[a]The β-galactosidase activity of σ^{54} wild type with NifA T308S was as in *a*.

vivo expression of the *S. meliloti nifH* promoter in the absence of a plasmid-borne activator is greater than the *Klebsiella pneumoniae nifH* promoter. This is likely caused by cross-activation at this strong promoter by other σ^{54} activators present in the cell. Interestingly, expression from σ^{54} Q20L/H53N in the absence of plasmids carrying PspFΔHTH was about twice that seen with wild-type σ^{54}, suggesting that cross-activation is more efficient with σ^{54} Q20L/H53N.

To identify other possible combinations of amino acids that could result in the same or a clearer suppression phenotype, codons 20 and 53 of σ^{54} were mutagenized to saturation using a pair of oligonucleotides with NNG/C at these positions. The resulting mutants were screened for suppression of the NifA T308S mutation. Ten colonies were detected that displayed a suppressor phenotype with the NifA T308S mutant. The plasmids of selected colonies were segregated and retransformed and their β-galactosidase activity was determined (Table 1c).

Nine out of the 10 σ^{54} suppressor mutants of NifA T308S had leucine at position 20, as did the original suppressor σ^{54} Q20L/H53N; the remaining mutant had Q20V. This indicates a clear requirement for a hydrophobic amino acid, leucine, at this position to suppress the NifA T308S phenotype. Moreover, the combination leucine/asparagine was selected several times with different codons for leucine, which discounts duplication of siblings. A range of functional substitutions at position 53, in addition to asparagine, suppressed the low transcription activity phenotype of NifA T308S (Table 1c). Position 53 seems to be more accessible to substitutions that lead to suppression than position 20. It is noteworthy that Q20L plus H53F had activity double that of the parental mutant (Table 1c). Although the double mutant had considerable activity with NifA T308A, no mutants were isolated that could recover the transcription activity of NifA E298D (data not shown).

Overall, the in vivo data provide strong indirect evidence for a functional interaction between the GAFTGA motif and Region I of σ^{54}. Q20 and H53 lie within σ^{54} sequences that are protected from protease attack by PspFΔHTH–ADP · AlF_x (Fig. 2c) and that directly bind to PspFΔHTH–ADP · AlF_x (Fig. 2b). Taken together, these data suggest that the Q20L and H53F suppression phenotype may be caused by an enhanced affinity for activator.

The PspF GAFTGA motif is a determinant of σ^{54} activator binding

Using PspFΔHTH GAFTGA mutants with T → S, T → A, or T → V mutations (amino acid 86), we examined whether the integrity of this motif was required for binding to the σ^{54} protein and its holoenzyme under trapping conditions (see below). In an in vitro transcription assay that allowed only one round of transcription from the *S. meliloti nifH* promoter (10 nM) with σ^{54}-holoenzyme (100 nM) and PspFΔHTH (4.0 μM), the T86S mutant gave 52% whereas the T86A and T86V mutants each gave less than 1% of the activity of the wild-type PspFΔHTH. The higher than expected activity of the T86S mutant compared to the in vivo data (Table 1b) may be explained in part by the effect of transcription reinitiation kinetics that are not measured in the single-round transcription assay. ATPase assays showed that at 2.0 μM protein monomer, the T86S, T86A, and T86V mutants had ATPase activities equivalent to wild type (data not shown). These data discount a simple defect in nucleotide binding. Trapping assays suggest that sequences within the GAFTGA motif function in binding σ^{54} Region I. The T86A and T86V mutants failed to give the characteristic self-associated complex seen with PspFΔHTH–ADP · AlF_x in the absence of σ^{54} and did not detectably form ADP · AlF_x-dependent complexes with σ^{54} or the holoenzyme (data not shown). The T → S mutant was defective for forming the ADP · AlF_x-dependent self-associated complex seen with PspFΔHTH in the absence of σ^{54} (cf. Fig. 3, lane 3 with Fig. 1c, lane 8) but did form trapped complexes with σ^{54} and holoenzyme (cf. Fig. 3, lane 4 with Fig. 1c, lane 2; data not shown). Binding of σ^{54} to the T86S mutant may stabilize its oligomeric state in the presence of ADP · AlF_x. Results with the T86 mutants correlate to the known defects in the GAFTGA motif for transcription activation and σ^{54} isomerization (Gonzalez et al. 1998; Cannon et al. 2000), and suggest they are closely linked to changes in binding one functional state of the activator that is established upon interaction with ADP · AlF_x. Clearly, although binding of σ^{54} need not be directly or exclusively to the GAFTGA motif, it critically involves it.

σ^{54}–DNA interactions in trapped complexes

To determine the DNA-binding properties of the trapped activator–σ^{54} complexes and compare these to σ^{54}, we conducted band shift and footprint assays. We wished to learn what the functional consequences of binding

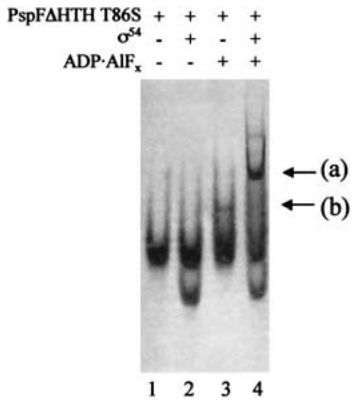

Figure 3. Gel mobility-shift assay for ADP · AlF_x-dependent complex formation between the PspFΔHTH T86S GAFTGA mutant and σ^{54} detected by Coomassie staining. Reactions contained PspFΔHTH T86S (20 μM) and σ^{54} (2 μM). Arrow (a), trapped σ^{54}–PspFΔHTH T86S complex; (b), PspFΔHTH T86S–ADP · AlF_x complex.

trapped activator were with respect to the DNA interacting properties of σ^{54} that are central to maintaining the closed promoter complex and to establishing the open promoter complex.

The trapped activator–σ^{54} complex binds promoter DNA PspFΔHTH lacks a DNA-binding domain and its use simplifies band-shift assays that employ DNA probes. We showed that σ^{54} bound to DNA probes derived from the *S. meliloti nifH* promoter also formed trapped complexes with PspFΔHTH (Fig. 4a). The new activator and ADP · AlF_x-dependent slow-running DNA complex has a slightly lower mobility than the DNA-free trapped PspFΔHTH–σ^{54} complex (Fig. 1b, cf. lane 10 with lanes 3 and 8). Controls showed that PspFΔHTH activator alone could not band shift the DNA irrespective of the presence of the trapping conditions (data not shown) and that the mobility of the σ^{54}–DNA complexes remained unchanged under trapping conditions when activator protein was omitted (data not shown). When ΔIσ^{54} (σ^{54} lacking Region I) was bound to DNA it did not form a new complex in the presence of PspFΔHTH–ADP · AlF_x, as was the case for trapping reactions without DNA (Fig. 2a). Trapped complexes containing promoter DNA were able to form when σ^{54} was bound to DNA before trapping and when the σ^{54} activator-trapped complex was allowed to form prior to addition of DNA. Compared to homoduplex DNA, a –12 to –11 heteroduplex (early melted DNA), representing DNA melted early in transcription initiation, is bound six- to eight-fold more strongly by σ^{54} (Cannon et al. 2000). The trapped complex did not show a loss of preference for binding the early melted DNA compared to the homoduplex probe, indicating that trapping does not greatly change σ^{54}–early melted DNA interactions (Fig. 4a, cf. lanes 3 and 6). Because Region I sequences of σ^{54} direct its tight binding to early melted DNA (Cannon et al. 1999, 2000; Gallegos and Buck 1999; Guo et al. 1999), this activity appears unaltered when σ^{54} is bound by PspFΔHTH–ADP · AlF_x, even though the activator is interacting with Region I.

A third DNA template used in the trapping experiments was an *S. meliloti nifH* heteroduplex from –10 to –1 (late melted DNA), representing DNA melted later in the transcription initiation process (Cannon et al. 1999, 2000). The late melted heteroduplex gave a close set of slow-running bands, suggesting that an interaction between σ^{54} and the region of melted DNA could be changed by binding of PspFΔHTH–ADP · AlF_x to σ^{54} Region 1 (Fig. 4a, lane 9).

Although homoduplex and heteroduplex DNAs each gave slow-running complexes under trapping conditions, only the early melted DNA gives the higher mobility nucleotide hydrolysis-dependent isomerized complex in

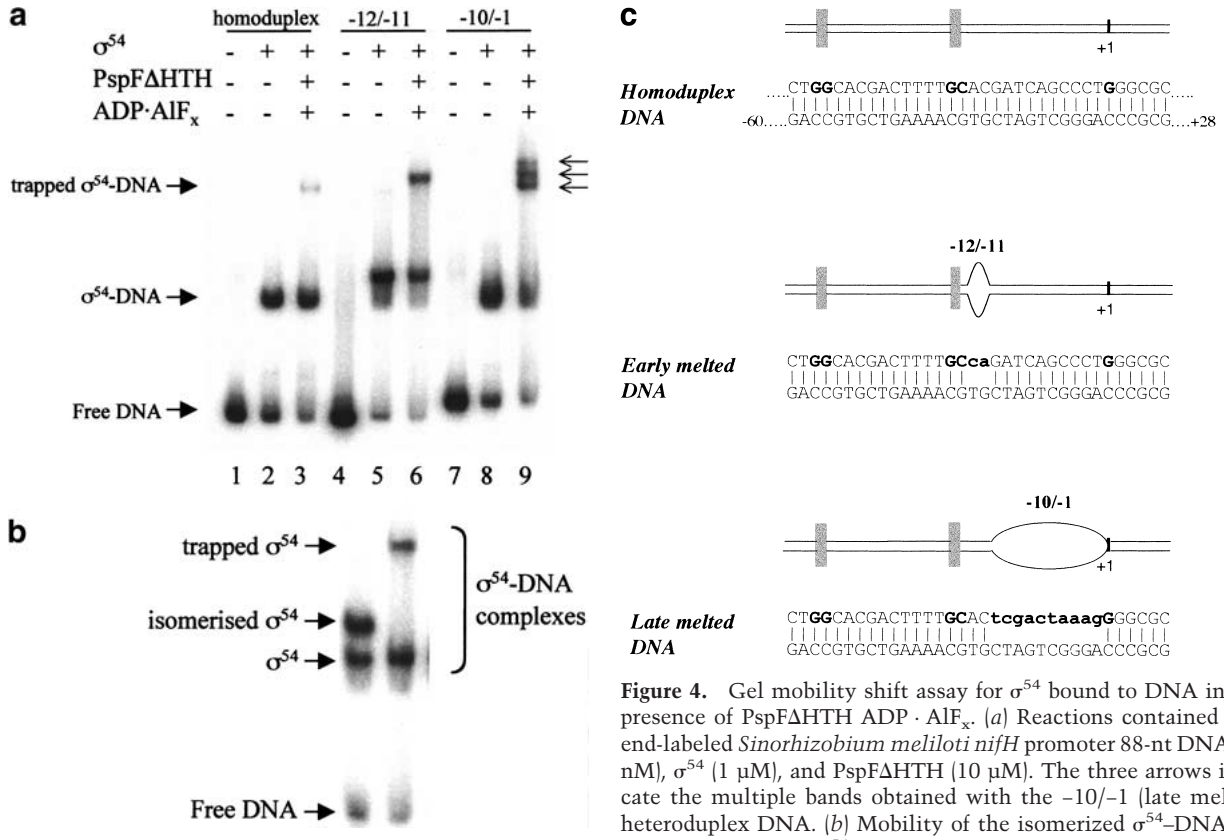

Figure 4. Gel mobility shift assay for σ^{54} bound to DNA in the presence of PspFΔHTH ADP · AlF_x. (*a*) Reactions contained ^{32}P-end-labeled *Sinorhizobium meliloti nifH* promoter 88-nt DNA (16 nM), σ^{54} (1 μM), and PspFΔHTH (10 μM). The three arrows indicate the multiple bands obtained with the –10/–1 (late melted) heteroduplex DNA. (*b*) Mobility of the isomerized σ^{54}–DNA supershifted complex (lane *1*; Cannon et al. 2000) compared with the trapped PspFΔHTH–σ^{54}–DNA complex (lane *2*). ATP was used for isomerization. (*c*) DNA molecules used in this experiment. The consensus GG and GC of the σ^{54} binding sites are indicated by the vertical bars. Mismatched regions that create the early and late melted DNA templates are indicated.

which activator is not stably bound (Fig. 4b, lane 1; Cannon et al. 2000).

The trapped complex produces an extended DNA footprint To explore further whether the trapped activator–σ^{54} complex interacts differently with promoter DNA, we conducted DNA footprinting of the trapped σ^{54}–promoter complexes. Using DNase I we found that the footprint of the trapped complex on homoduplex DNA was extended compared to σ^{54}, and was clearly increased toward the start of transcription (Fig. 5, lane 4a). Untrapped σ^{54}–DNA complexes from the same trapping and footprinting reaction did not show this footprint extension (Fig. 5, lane 4b). Unlike the ATP hydrolysis-dependent extended footprint of σ^{54}, which requires the use of the early melted heteroduplex (Cannon et al. 2000, 2001), the extended trapped complex footprint was not DNA template-dependent. The trapped complex gave a similar extended DNase I footprint on both the early melted and homoduplex DNA (data not shown). Similar extended footprints on both DNA templates were also observed with trapped σ^{54}-holoenzyme complexes (Fig. 5, lane 7a; data not shown). We conclude that the extended footprint occurs as a consequence of activator interacting with σ^{54} Region I at the point of ATP hydrolysis. The extended footprint could be caused by changed σ^{54}–DNA interactions and/or protection by the presence of PspFΔHTH at the downstream side of the promoter.

Use of a hydrolyzable nucleotide and activator results in some σ^{54}-dependent DNA melting with early melted heteroduplex DNA, suggestive of a single strand DNA-binding activity being revealed within σ^{54} (Cannon et al. 2000, 2001). We attempted to measure DNA opening within DNA complexes formed between σ^{54} and PspFΔHTH in the presence of ADP · AlF_x. However, complexes were rapidly destroyed by $KMnO_4$ or diethylpyrocarbonate, and we were unable to obtain data showing any DNA melting.

Trapped activator does not induce efficient single-stranded DNA binding or open complex formation We investigated whether trapped activator can induce single strand DNA binding on a preopened DNA template by examining trapped holoenzyme interactions with the late melted heteroduplex DNA. Holoenzyme formed with wild-type σ^{54} is able to form a heparin stable complex on late melted *S. meliloti nifH* promoter DNA if activator and a hydrolyzable nucleoside triphosphate are present (Wedel and Kustu 1995; Cannon et al. 1999). Trapping of activator did not allow the σ^{54}-holoenzyme to form a heparin stable complex on the late melted DNA, even when initiating nucleotide was present (data not shown). This suggests that the interaction of trapped activator with the σ^{54}-holoenzyme does not induce all the conformational changes in the holoenzyme that allow stable interactions with melted DNA. Consistent with this, the σ^{54}-holoenzyme bound to promoter DNA with heteroduplex from –7 to –3, from –5 to –1, or from –3 to –1 did not acquire heparin stability in the presence of activator and ADP · AlF_x (data not shown). It seems that although trapping can alter σ^{54}–DNA interactions (Fig. 4a), these changes are not sufficient to lead to properties seen in activated complexes of the σ^{54}-holoenzyme.

To test further if the trapped activator can induce formation of an open complex, we attempted to make specific transcripts from the trapped σ^{54}-holoenzyme using supercoiled *S. meliloti nifH* promoter DNA templates.

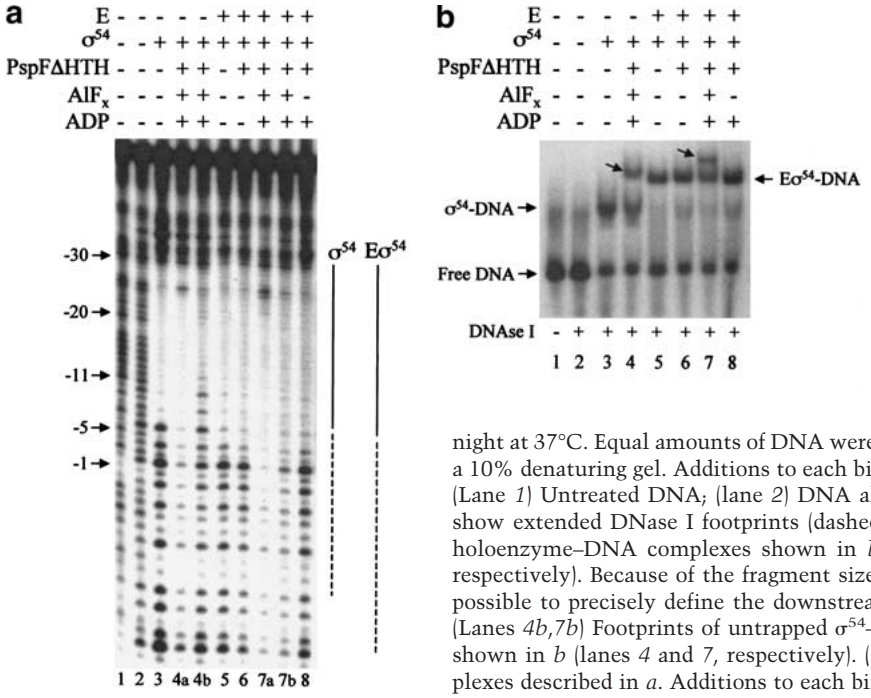

Figure 5. DNase I footprints of trapped complexes bound to promoter DNA. (*a*) Complexes were formed with *Escherichia coli glnH*p2 homoduplex 88-nt DNA (100 nM, bottom strand end-labeled), σ^{54} (1 μM) or its holoenzyme ($E\sigma^{54}$, 100 nM), and PspFΔHTH (20 μM) in the absence or presence of ADP · AlF_x. Samples were treated with DNase I (1.75×10^{-3} units; Amersham Life Sciences) for 1 min and the reaction was stopped by the addition of EDTA (10 mM). Bound and unbound complexes were separated and excised from a native gel (*b*) and the DNA was eluted into H_2O overnight at 37°C. Equal amounts of DNA were denatured and then electrophoresed through a 10% denaturing gel. Additions to each binding reaction are indicated above each lane. (Lane *1*) Untreated DNA; (lane *2*) DNA alone treated with DNase I. Lanes *4a* and *7a* show extended DNase I footprints (dashed lines) from the trapped σ^{54}–DNA and σ^{54}-holoenzyme–DNA complexes shown in *b* (marked with an arrow in lanes *4* and *7*, respectively). Because of the fragment sizes migrating close to the gel front it was not possible to precisely define the downstream end of the extended footprint in lane *7a*. (Lanes *4b*,*7b*) Footprints of untrapped σ^{54}–DNA and σ^{54}-holoenzyme–DNA complexes shown in *b* (lanes *4* and *7*, respectively). (*b*) Native gel showing DNase I-treated complexes described in *a*. Additions to each binding reaction are indicated above each lane.

This failed, but can be rationalized through the demonstrated failure of trapped holoenzyme to efficiently engage melted DNA (see above) and the known requirement of σ^{54} Region I sequences for stabilizing the open complex (Cannon et al. 1999; Gallegos et al. 1999). The latter requirement may not be efficiently met when Region I remains bound by the trapped activator, possibly because of restricting movements in Region I that seem to occur during stable open-complex formation (Casaz and Buck 1997; Wigneshweraraj et al. 2001). Results suggest that although trapped activator binds Region I of σ^{54}, the binding does not lead to engagement of the holoenzyme with melted DNA. Further conformational changes in σ^{54} likely to be associated with completion of the ATP hydrolysis cycle appear necessary.

Discussion

Activator–RNAP contacts are known to be critical for regulated transcription initiation, and activators of the σ^{54}-holoenzyme catalyze formation of open promoter complexes through hydrolysis of a nucleoside triphosphate. We have shown, using the central ATP-hydrolyzing domains of the activators PspF and NifA together with wild-type PspF, that in the presence of ADP–aluminum fluoride a stable complex between the activator and σ^{54} or its holoenzyme is formed. It seems that we may have isolated a new functional state of the activator. The combination of ADP and ions (Al^{+3} and F^{-}) is likely to form a planar complex that mimics the atomic arrangement of the ATP γ-phosphate in the transition state (Wittinghofer 1997). We propose that this transition-state analog of ATP (ADP · AlF_x) is interacting with activator to allow adoption of a functional state that has increased affinity for σ^{54}, compared to the nucleotide-free form of the activator, or when nonhydrolyzable analogs such as GTPγS and ATPγS are used. ADP did not substitute for its aluminum fluoride form, suggesting a critical role for the ATP γ-phosphate in establishing the conformation of activator that binds σ^{54}. We infer that one role of ATP hydrolysis in activation of the σ^{54}-holoenzyme is to promote formation of a functional state of the activator that tightly binds σ^{54}. Hydrolysis of ATP would indicate this functional state is not long lived, and that Pi and ADP release return the activator to an alternate state that has decreased binding for σ^{54} (Fig. 4b).

Characteristics of the σ^{54}-activator binding interaction

Because activators exist in different functional states depending on the progress of nucleotide hydrolysis, different sets of binding interactions between the activator and σ^{54} seem likely, and may relate to different steps in the process of formation of the open complex. σ^{54} Region I sequences function before open-complex formation to maintain the closed complex, and after to stabilize the open complex, suggesting that a series of linked interactions between the activator and σ^{54} Region I may occur and need not all be direct (Cannon et al. 1999; Gallegos et al. 1999; Gallegos and Buck 2000; Guo et al. 2000). The trapped σ^{54} and σ^{54}-holoenzyme–PspFΔHTH complexes were only able to form efficiently when the proteins were allowed to associate prior to formation of ADP · AlF_x. This suggests a pathway in which σ^{54} and activator associate weakly prior to interaction with nucleotide. We also showed that σ^{54} deleted for Region I ($\Delta I\sigma^{54}$) was able to compete weakly with full-length σ^{54} for PspFΔHTH prior to trapping, implying that at least some of the interactions between activator and σ^{54} prior to trapping lie outside of Region I. Additional evidence for a range of interactions is provided by results of cross-linking assays between σ^{54} and the activator DctD (Lee and Hoover 1995; Wang et al. 1997; Kelly et al. 2000). In these experiments the determinants for binding σ^{54} to DctD were outside of σ^{54} Region I, and in the N-terminal half of the activator C3 region.

In our experiments the addition of ADP · AlF_x results in a new stable complex between σ^{54} and activator that directly requires the regulatory Region I of σ^{54} for its formation. This suggests that ATP hydrolysis is used to promote a new binding interaction between σ^{54} Region I and activator. The dependence on ADP · AlF_x for detecting the complex indicates that the stable complex with σ^{54} is normally transient and is not seen when using ATP because of the short life of the transition state of ATP at hydrolysis. One inference is that coupling σ^{54} and activator interactions to ATP hydrolysis can lead to high rates of transcription through rapidly directing σ^{54} to a new functional state suitable for open-complex formation.

Activator–nucleotide interactions

The changed native gel mobility of PspFΔHTH resulting from incubation with ADP · AlF_x supports the idea that nucleotide binding can change the quaternary structure of this mutant activator. Tight binding of the transition state of ATP by PspFΔHTH may stabilize formation of an oligomer through one protomer binding the nucleotide base while an adjacent protomer contributes a contact to the γ-phosphate. Implicit in this view is the idea that the activator senses the γ-phosphate of the hydrolyzable nucleoside triphosphate, and that hydrolysis and changing interactions with the γ-phosphate allow the activator to interconvert between different functional states. The ATP-bound form of the activator NtrC is reported to interact with σ^{54} in a manner dependent on the presence of the γ-phosphate (Guo et al. 2000). These interconversions may also involve the amino acids located in regions of the ATP-binding fold, and may include residues equivalent to those that are known to comprise the Switch 1 and Switch 2 sequences of GTP-hydrolyzing signaling proteins (Gamblin and Smerdon 1998; Rombel et al. 1998). There appears to be a strong similarity between activators of the σ^{54}-holoenzyme and motor proteins and signaling switch proteins that use nucleotide binding and hydrolysis to interconvert between functional states with different affinities for their targets. In the case of activators of the σ^{54}-holoenzyme,

activator–σ^{54} interactions that depend on ATP hydrolysis lead to an increased DNA interaction by σ^{54} to enable open complex formation (Cannon et al. 2000, 2001).

A notable property of the mutants in the GAFTGA motif of PSPFΔHTH was their failure to efficiently self-associate in the presence of ADP · AlF_x (Fig. 3; data not shown). The normal levels of ATPase activity and its unchanged dependence on activator concentration for these mutants (J. Schumacher, unpubl.) support the argument that the self-association and interactions of the mutant proteins with ATP for hydrolytic cleavage are largely intact. Differences seen with ADP · AlF_x could be related to alterations in interactions with ADP or a subtle defect in self-association at some step after the hydrolytic cleavage of ATP. Although the PSPFΔHTH T86S mutant gave little self-associated product in the absence of σ^{54} (Fig. 3), the level of trapped σ^{54}–PSPFΔHTH T86S complex was normal, suggesting that σ^{54} stabilizes an interaction with ADP · AlF_x that occurs through the GAFTGA motif.

The tight binding of trapped activator with σ^{54} and holoenzyme seen in the absence of promoter DNA raises the possibility that the enhancer-bound activator might recruit the holoenzyme to promoters that are weak binding sites for the holoenzyme. However, careful kinetic analysis to include consideration of the short lifetime of the transition state of ATP hydrolysis and the dissociation rate of the closed complex would be required to show such an effect was meaningful when ATP was being hydrolyzed. Nevertheless, it is clear that one particular binding interaction between σ^{54} and activator only occurs efficiently when the activator is in a particular "on" state that is transiently created when ATP is being turned over.

The role of σ^{54} Region I

The regulatory Region I of σ^{54} was clearly a determinant in the formation of the stable trapped complex with activator. This is consistent with the central role Region I has in activated transcription and in controlling the DNA-binding properties of σ^{54} and its holoenzyme that partly distinguish the closed and open promoter complexes (Wang et al. 1995; Casaz and Buck 1999; Guo et al. 1999; Gallegos and Buck 2000; Pitt et al. 2000). Binding assays with σ^{54} Region I alone strongly suggest that activator makes a direct contact to it. Analysis of 15 triple alanine substitution mutants spanning Region I (amino acids 6–50) showed none had a defect as great as deletion of the entire Region I, implying multiple determinants. Region I sequences localize in the core RNAP near the active site for RNA synthesis (Wigneshweraraj et al. 2000) and over the promoter region that is near the start of DNA melting. We have termed this protein–promoter DNA focus the regulatory center of the holoenzyme closed complex (Wigneshweraraj et al. 2001). Mutations in Region I lead to changes in the holoenzyme and σ^{54}–DNA interactions that can lead to activator-independent transcription in vitro (Wang et al. 1995; Syed and Gralla 1998; Casaz et al. 1999). The trapped state of the activator may therefore make a contact to σ^{54} Region I within the regulatory center to start to change the conformation of the protein components of the closed complex.

DNA interactions of the trapped complex

In the light of the distinctive changes in σ^{54} binding to late melted DNA, it appears that binding of σ^{54} by the trapped activator may begin to change the DNA-binding properties of σ^{54}. Experiments using DNA heteroduplex from –10 to –1 showed multiple banding with the trapped σ^{54}–PspFΔHTH complex. This suggests that an altered interaction with start site proximal single strand DNA is possible when σ^{54} Region I is stably engaged with trapped activator. When ATP or GTP is used, the activator drives σ^{54} to melt DNA from –9 to –6 (Cannon et al. 2001). We attempted to see whether the σ^{54} trapped complex had resulted in any DNA strand denaturation, using $KMnO_4$ or diethylpyrocarbonate as a probe for DNA base unstacking. Unfortunately, the sensitivity of the protein complex to both $KMnO_4$ and diethylpyrocarbonate precluded any meaningful interpretation of the DNA footprints.

The extended promoter DNase I footprint we see with σ^{54} and holoenzyme trapped activator complexes could be directly caused by any single component in the complex being proximal to the DNA downstream of the –12 promoter DNA. Because Region I of σ^{54}, the binding site for activator, is located over the –12 promoter element (Wigneshweraraj et al. 2001), activator bound to Region I could be responsible, at least in part, for directly blocking DNase I access. Two observations are consistent with this idea. First, the isomerized complexes that form with the σ^{54}, activator, and hydrolyzable NTP and give extended DNase I footprints require the use of early melted DNA (Cannon et al. 2000), whereas the extended footprint in trapped complexes was evident with homoduplex and early melted DNA. Therefore, the extended footprint seen with the trapped complex may not be owing to σ^{54} isomerization. Rather, the footprint may reflect an intermediate state of σ^{54}, not the fully isomerized form, bound to activator. Second, the isomerized σ^{54}–DNA complexes bind core RNAP poorly, and holoenzyme does not efficiently form the isomerized complex (Cannon et al. 2001). This contrasts with the clear extended footprint seen with the trapped holoenzyme–activator complex on homoduplex and early melted DNA, again supporting the argument that the extended footprint may not be caused by σ^{54} isomerization but, rather, to the presence of activator downstream of the –12 promoter DNA.

The transition state–dependent interaction between activator and σ^{54} occurs prior to open complex formation

The full ATP-hydrolysis cycle by activator results in a remodeling of the σ^{54}-holoenzyme–DNA complex, with conformational changes being evident in σ^{54} and DNA

(Cannon et al. 2000, 2001). Characterization of the trapped PspFΔHTH σ^{54}-holoenzyme complex interaction with DNA (see above) and the failure to transcribe from supercoiled DNA suggest that these changes have not fully occurred when ADP · AlF_x replaces ATP. It seems that an enzymatic transition in the activator needed for the major conformational change in σ^{54} and its holoenzyme occurs after the transition state of ATP hydrolysis has been established. We envisage that on formation of the transition state between activator and ATP the switch in σ^{54}-holoenzyme, probably Region I of σ^{54}, tightly interacts with activator through direct protein–protein contact. It seems that ADP · AlF_x locks the σ^{54}–activator complex in a tense state. Subsequently, upon phosphate and/or ADP release, enzymatic transitions in the activator operate the switch (Region I) in σ^{54} to allow holoenzyme to form an open complex and so complete the mechanochemical cycle that couples ATP hydrolysis to a change in configuration of the σ^{54} protein and its holoenzyme. Region I of σ^{54} interacts with other parts of σ^{54} and core RNAP and is involved in establishing the closed complex and in maintaining open complexes (Cannon et al. 1999; Gallegos et al. 1999; Gallegos and Buck 2000). Together these activities point to a need for Region I to be repositioned in order for transcription initiation to occur.

For AAA+ family proteins, nucleotide is located close to the interface between protomers, with the active site being contributed to by residues from the adjacent protomer (Vale 2000). Our results with ADP · AlF_x strongly suggest that for PspF and NifA, and likely all other activators of the σ^{54}-holoenzyme, sensing the state of the γ-phosphate of ATP may serve to critically link ATP hydrolysis to changes in protomer structure to enable engagement with the σ^{54} subunit. Hence the basis of the mechanochemical functioning of activator may be in propagating interactions with the γ-phosphate to changes in activator conformation to allow cycles of binding and rebinding of activator and σ^{54} that rely on an ATP hydrolysis cycle. Intriguingly, structural alignment of PspF with the AAA+ protein p97 shows that the position of the GAFTGA motif of PspF, important for ADP · AlF_x-dependent binding to σ^{54}, is predicted to be dependent on the nucleotide-bound state of the activator (X. Zhang, pers. comm.). We suggest that other AAA+ proteins may also use ATP hydrolysis to manipulate the structure of their targets through the formation of a new functional state at the transition state of ATP hydrolysis.

Materials and methods

DNA manipulations

Plasmid pSRW–HMKPSPFΔHTH encodes *E. coli* PspFΔHTH protein with a 6-His tag followed by a heart muscle kinase (HMK) site at its N-terminal end. Plasmid pSRW–HMKPSPFΔHTH was constructed from pMJ15 (Jovanovic et al. 1999). The *Nla*III site, containing the *pspF* ATG codon, was replaced with an *Nde*I site by PCR mutagenesis. The PCR product was digested with *Nde*I and *Hin*dIII, and the resulting mutagenized fragment was cloned into plasmid HMK–pET28b+, in which the sequence SSGLV in pET28b+ (Novagen) was changed to RRASV to create an HMK site. Construction of mutant plasmids encoding PspFΔHTH T86S, T86A, or T86V was by oligonucleotide site-directed mutagenesis using pMJ15 (Jovanovic et al. 1999). For protein expression the mutated *PspFΔHTH* DNA was inserted into pQE32 (QIAGEN). The full-length *pspF* gene was amplified by PCR from the chromosome of *E. coli* (MRE600) and inserted into the expression vector pET28b+. Random PCR mutagenisis (6mM $MgCl_2$) of Regions I and II of σ^{54} was performed using plasmid pMM70 as template (Merrick and Gibbins 1985). The resulting mutagenic fragment was inserted into plasmid pVB009, a derivative of pTZ19R (Pharmacia) that contains sequences encoding Region III of σ^{54} from pMM70. Codons 20 and 53 of *rpoN* were mutagenized to saturation using a pair of oligonucleotides with NNG/C at these positions (Miyazaki and Arnold 1999).

Protein purification

The σ^{54} and PspFΔHTH proteins were overproduced and purified as described previously (Cannon et al. 1995, 1999; Gallegos and Buck 1999; Jovanovic et al. 1999). The Δ$I\sigma^{54}$ (residues 57–477) was without His tag. σ^{54} (residues 1–477) was used with and without an N-terminal His tag. PspFΔHTH and all other σ^{54} fragments had an N-terminal His tag. For ^{32}P end-labeling, a heart muscle kinase site was added to the C-terminal end of σ^{54} (Casaz and Buck 1997) or at the N-terminal end of PspFΔHTH (Cannon et al. 2000). Expression of the full-length PspF protein was carried out in *E. coli* strain C41(DE3) (Miroux and Walker 1996). Essentially, growth of cells was in LB to an A_{600} of 0.6 at 37°C. Induction was carried out with 0.5 mM IPTG, and cultures were transferred to 16°C (with shaking) for a further 12 h. Cells were disrupted, and subsequent purification was by Ni-chelate affinity chromatography using a Hi-Trap column (Pharmacia) and FPLC (Pharmacia). Purified protein was eluted in TGED (10 mM Tris at pH 8.0, 5% glycerol, 0.1 mM EDTA, 1 mM DTT) containing approximately 300 mM NaCl, and protein was dialyzed and stored in TGED (as before with 50% glycerol) containing 500 mM NaCl. Purified protein was stored at –80°C.

Promoter DNA

Purified synthetic DNA fragments based on the –60- to –28-bp sequence of the *S. meliloti nifH* promoter were used to construct the DNA molecules used in this study (Cannon et al. 2000). DNA strands were annealed to create a duplex, with one strand ^{32}P-kinased and the other unlabeled strand at a twofold molar excess.

Trapping

ADP–aluminum fluoride trapping was essentially performed as described previously for myosin subfragment 1 (Moshe et al. 1992). The desired amount of activator was incubated at 30°C for 5 min in STA buffer (25 mM Tris-actetate at pH 8.0, 8 mM Mg-actetate, 10 mM KCl, 1 mM DTT, 3.5% w/v PEG 6000) with ADP (0.2 mM), NaF (5.0 mM), and other proteins as required. After addition of $AlCl_3$ (0.2 mM) the reactions were incubated for a further 10 min and directly loaded onto a native gel. Reactions (10 µL) containing ^{32}P-end-labeled proteins also contained α-lactoalbumin (50 ng).

Gel-shift assays

Gel-shift assays were conducted using either end-labeled protein or linear end-labeled *S. meliloti nifH* promoter fragments as

described previously (Cannon et al. 2000). Either 88-nt homoduplex or heteroduplex molecules from –60 to +28 bp, the latter with non-template-strand sequences mismatched, were used. Binding reactions were in STA buffer at 30°C, and bound and unbound protein or DNA was resolved on 4.5% native polyacrylamide Bio Rad mini protean II gels run at room temperature at 120 V in 25 mM Tris, 200 mM glycine buffer (pH 8.6). Where added, ATP was at 4 mM.

V8 protease footprints

Reactions (20 µL) contained the trapped activator–^{32}P end-labeled σ^{54} complex (see above). After incubation for 10 min at 37°C, 150 ng of V8 protease (Sigma) was added for 2 min followed by the addition of 500 µM dichloroisocoumarin (Sigma) to stop digestion. Samples were run on a 4.5% native gel. Complexes were isolated from gel either as free protein or as part of a trapped activator complex, eluted in 1× Lamelli dye (Sigma), and run on a 12.5% SDS gel.

In vivo activation assays

Plasmids carrying the mutated *rpoN* gene were transformed into the *E. coli* Δ*rpoN* TH1 strain harboring a *K. pneumoniae nifH–lacZ* fusion in plasmid pRT22 (Cm^r) and plasmid pRJ7511 (Tc^r) carrying the wild-type or mutant derivatives of the *B. japonicum nifA* gene. Plasmid pWKS130 (Km^r) carried wild-type or mutant derivatives of the *E. coli pspF*Δ*HTH* gene. The screen for suppressor mutants was made on minimal medium plates containing X-gal to select those colonies that displayed an enhanced blue color (Grande et al. 1999). The β-galactosidase assays were performed as described previously (Grande et al. 1999).

Estimation of stoichiometry

Trapped complexes containing PspFΔHTH and σ^{54} were resolved on a native gel. The center of the trapped-complex band was isolated. The proteins contained in the gel slice were then separated on denaturing 12.5% SDS PAGE and stained with Coomassie blue, and the respective bands were analyzed by densitometry. The ratio of PspFΔHTH to σ^{54} in the trapped complex was calculated by comparing optical density (OD) readings with standard curves (mass/OD) prepared at the same time for PspFΔHTH and σ^{54}.

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