A Role for the Conserved GAFTGA Motif of AAA+ Transcription Activators in Sensing Promoter DNA Conformation

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Transcription from $\sigma^{54}$-dependent bacterial promoters can be regarded as a second paradigm for bacterial gene transcription. The initial $\sigma^{54}$-RNA polymerase (RNAP) promoter complex, the closed complex, is transcriptionally silent. The transcriptionally proficient $\sigma^{54}$-RNAP promoter complex, the open complex, is formed upon remodeling of the closed complex by actions of a specialized activator protein that belongs to the AAA (ATPases associated with various cellular activities) protein family in an ATP hydrolysis-dependent reaction. The integrity of a highly conserved signature motif in the AAA activator (known as the GAFTGA motif) is important for the remodeling activity of the AAA activator and for open complex formation. We now provide evidence that the invariant threonine residue of the GAFTGA motif plays a role in the DNA downstream of the $\sigma^{54}$-RNAP-binding site and in coupling this information to $\sigma^{54}$-RNAP via the conserved regulatory Region I domain of $\sigma^{54}$ during open complex formation.

Gene transcription in bacteria is catalyzed by the multisubunit DNA-dependent RNA polymerase (RNAP). The catalytically competent core form of bacterial RNAP is a five-subunit enzyme ($\alpha_2\beta\beta'\omega$; E), which has to associate with a sixth subunit, the $\sigma$ factor, for promoter-specific and regulated initiation of gene transcription. On the basis of differences in mechanism of action and amino acid sequence, bacterial $\sigma$ factors are classified into two families. Most bacterial $\sigma$ factors belong to the $\sigma^{70}$ family, named after the prototypical housekeeping $\sigma$ factor, $\sigma^{70}$, of Escherichia coli. The major variant bacterial $\sigma$ factor belongs to the $\sigma^{54}$ class.

Transcription initiation by RNAP containing $\sigma^{70}$ (E$\sigma^{70}$) and $\sigma^{54}$ (E$\sigma^{54}$) is mechanistically distinct. E$\sigma^{70}$ recognizes promoters that contain consensus sequences centered at DNA positions $-35$ and $-10$, respectively, from the transcription start site (at +1). The initial transcriptionally inactive E$\sigma^{70}$-DNA complex, called the closed complex, can spontaneously isomerize to form the transcriptionally active open complex, in which the DNA strands are separated and the RNAP is poised for RNA synthesis. In contrast, E$\sigma^{54}$ forms closed complexes on promoters that contain consensus sequences centered at DNA positions $-24$ and $-12$ (1). Closed complexes formed by E$\sigma^{54}$ remain inactive for transcription unless activated by a specialized type of transcription activator protein that belongs to the AAA (ATPases associated with various cellular activities) protein family (2). E$\sigma^{54}$-dependent transcription activators (from now on referred to as AAA activators) bind to DNA sites located ($\sim150$–$200$ bases) upstream of the promoter (known as upstream activating sequences) and use the energy derived from ATP binding and hydrolysis to remodel the E$\sigma^{54}$ closed complex (2). The ATP hydrolysis-dependent binding interactions between the AAA activator and E$\sigma^{54}$ closed complex trigger a series of protein and DNA isomerization events in the E$\sigma^{54}$ closed complex, which result in the formation of the open complex. The major energetically favorable binding site for the AAA activator within the E$\sigma^{54}$ closed complex is the N-terminal Region I domain of $\sigma^{54}$ (see Fig. 1A) (3), which, in the closed complex, is located at the $-12$ consensus promoter region, where DNA opening for open complex formation nucleates (4). At the $-12$ promoter region, $\sigma^{54}$ Region I mediates tight binding to a repressive fork junction structure and so prevents open complex formation in the absence of activation. Region I of $\sigma^{54}$ is associated with a range of properties of E$\sigma^{54}$ (1). These include maintaining the closed complex transcriptionally silent prior to activation (5), stabilizing the open complex once it is formed (6), and conformational signaling to a structurally conserved DNA-interacting domain(s) of the catalytic $\beta'$ subunit of RNAP (7) required for stable open complex formation. Region I of $\sigma^{54}$ has been shown to make extensive interactions with the catalytic $\beta$ and $\beta'$ subunits of RNAP (8).

The AAA activators of E$\sigma^{54}$ are mechanochemical P-loop ATPases of the AAA family (2, 9) and can use ATP hydrolysis to remodel the E$\sigma^{54}$ closed complex to trigger open complex formation. Structural analysis of one model AAA activator, E. coli
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PspF (phage shock protein F) (10), suggests that mobile loops, called loops 1 (L1) and 2 (L2), within a hexameric assembly contact Region I of σ54 (see Fig. 1, B and C) and that these contacts form during the energy-coupling process that transmits a conformational change around the γ-phosphate of the ATP-binding site on PspF to changes in the Eσ54 closed complex conformation, which subsequently triggers open complex formation. Specifically, an invariant threonine residue in L1 (Thr86 in PspF) (see Fig. 1C) is thought to engage with σ54 Region I during the energy-coupling process (10, 11). The ATP hydrolysis-dependent movements of L1 and L2 have also been observed in the AAA activator Aquifex aeolicus NtrC1 (nitrogen regulatory protein C1) (12).

Current models for energy coupling by PspF indicate that a range of functional states of the AAA domain of PspF will exist, depending upon its nucleotide-bound state, and that more than one conformation of σ54 Region I will exist for open complex formation to occur (10). To elucidate the role of σ54 Region I and the invariant Thr86 residue in the energy-coupling process, we have sought altered forms of σ54 that can compensate for defects displayed by a partially functional PspF variant containing a serine substitution at Thr86 in L1 (3, 11). In a previous study, we established that the T86S form of L1 of PspF is defective in interaction with the Eσ54 closed complex in the presence of the ATP hydrolysis transition state analog ADP-AlF3 (11).

The ADP-AlF3-dependent complex between PspF and the Eσ54 closed complex (referred to as the ternary complex) is regarded as an intermediate state that is established en route to full open complex formation (3, 13). Here, we describe the isolation of a new mutant form of σ54, σ54(G4L) (see Fig. 1A), capable of suppressing the defects of PspF(T86S) evident in ternary complex formation with the Eσ54 closed complex and during in vitro transcription initiation. We show that the “to-be-melted” DNA downstream of the −12 consensus promoter region somehow negatively affects the ability of PspF(T86S) to stably interact with the Eσ54 closed complex. The properties of PspF(T86S) and σ54(G4L) imply a role for the conserved GAFTGA motif in PspF and, by extension, in other AAA activators of Eσ54 in “sensing” the conformation adopted by the DNA downstream of the −12 promoter region and coupling this information to Eσ54 via Region I to allow open complex formation.

EXPERIMENTAL PROCEDURES

Design and Construction of a σ54 Region I Fragment 2−14 Mutant Library—Klebsiella pneumoniae σ54 Region I amino acids 2−14 were subjected to random mutagenesis using a novel combinatorial codon-based mutagenesis approach that enabled the mutagenic rate to be controlled to obtain σ54 variants with few amino acids substitutions (14). A detailed background of this mutagenesis method is described by Gaytan et al. (14). This method is based on the use of five dinucleotides designated B1B2, (AA, TT, AT, GC, and CG). Mutant codons are assembled by tandem addition of a diluted mixture of the five dinucleotides to the growing oligonucleotide and a mixture of four nucleotides to generate 20 trinucleotides that encode a set of 18 amino acids. Thus, in this method, wild-type codons are doped with a set of mutant codons during oligonucleotide synthesis to generate random combinatorial libraries of primers that contain few codon replacements per variant. The mutagenic rate is controlled by varying the percentage of B1B2 dimers used to “dope” the second and third positions of the wild-type codons targeted for mutagenesis. An oligonucleotide, called oligonucleotide A (72-mer), with sequence 5′-tattgcccgtgcatATG-AAG/B1B2B3-CAA/B1B2B3-GGT/B1B2B3-TTG/B1B2B3-CAA/B1B2B3-TTA/B1B2B3-AGG/B1B2B3-CTA/B1B2B3-AGC/B1B2B3-CAA/B1B2B3-AGG/B1B2B3-CTT/B1B2B3-GCC/B1B2B3-atgagccgcaactg-3′ was designed to complement the 3′-flanking arm of oligonucleotide A. The complementary sequences of both oligonucleotides are shown in boldface. The flanking arms included two restriction sites, NdeI and XbaI (underlined). The introduction of the restriction sites did not change the wild-type coding sequence of σ54. The duplex DNA library of the target region was generated by the extension of complementary oligonucleotide B over mutagenic oligonucleotide A using the Klenow fragment (3′ → 5′, exonuclease −) of DNA polymerase I. Briefly, an equimolar concentration of oligonucleotides A and B (500 pmol) was mixed in Klenow buffer (50 mM Tris-HCl (pH 7.2 at 25 °C), 10 mM MgSO4, and 1 mM DTT) containing all four dNTPs. For the annealing step, the mixture was heated at 70 °C for 15 min and allowed to cool to room temperature. 1 µl of Klenow fragment (5 units/µl) was added, and the reaction was incubated at 37 °C for 1 h. Following this, the Klenow fragment was heat-inactivated at 70 °C for 15 min. The duplex DNA library was separated by gel electrophoresis and purified from single primer species on a 2% (w/v) agarose gel using a Qiagen gel extraction kit following the manufacturer’s instructions. The duplex DNA library was digested with NdeI and XbaI and used to replace the wild-type σ54 residues corresponding to positions 2−14 in pVB009 (15). The in vivo screening for the rpoN clones capable of recovering the activation defect of NifA(T308S) (nitrogen fixation protein A) was performed as described previously (3). Briefly, the mutant library in pVB009 (ampicillin resistance) was then transformed into E. coli strain (TH1 (ΔrpoN)) and selected on X-gal (20 µg/µl) containing nitrogen-free medium.

In Vivo Activity Assays—The in vivo β-galactosidase assays were conducted as described previously (3, 15) in E. coli strain TH1 (ΔrpoN) containing plasmids pRT22 (chloramphenicol resistance) and pWKS130 (kanamycin resistance). pRT22 is a reporter plasmid and contains the σ54-dependent K. pneumoniae nifH promoter fused to the lacZ gene. pWKS130 carries the three different Bradyrhizobium japonicum nifA alleles (wild-type nifA, nifA(T308S), and nifA(T308V)) used in this study. The clones that displayed the desired phenotype were sequenced to establish that mutation(s) were located only within amino acids 2−14 of σ54.

Site-directed Mutagenesis—σ54 and PspF(1−275) containing single amino acid substitutions were constructed using the Stratagene QuikChange mutagenesis kit following the manufacturer’s instructions. The templates used for the σ54 and PspF(1−275) mutagenesis reaction were pVB009 and pPB1 (16), respectively.
Proteins—For protein overproduction, NdeI-HindIII fragments containing wild-type and mutant (G4L) *rpoN* genes were cloned into pET28b (Novagen) and overproduced and purified as described previously (17). Wild-type and mutant variants of *E. coli* PspF-(1–275) were constructed, overproduced, and purified exactly as described (16, 17). Wild-type *E. coli* core RNAP was purchased from Epicentre Technologies (Madison, WI). The concentrations of all protein preparations were determined using a Bio-Rad protein assay kit accordingly to the manufacturer's instruction. To further ascertain that equal quantities of proteins were used in the assays, each preparation was checked by SDS-PAGE analysis.

Promoter DNA Probes and Proteins—The *Sinorhizobium meliloti nifH* homoduplex and heteroduplex promoter probes used in this study were constructed and 32P-5'-end-labeled exactly as described previously (17).

Native Gel Mobility Shift Assays—These were performed exactly as described previously (17). The gels were analyzed, and the complexes were quantified using a Fuji FLA-5000 fluorescent image analyzer. All native gel mobility assays were repeated at least twice, and the values shown in the figures represent an average of these replicates with an error range of ±6%.

In Vitro Transcription Assays—These were performed exactly as described previously (7), but with a range of incubation times (specified in the figure legends). The transcription reactions were repeated at least twice, and the values shown in the figures represent an average of these replicates with an error range of ±5%.

RESULTS

In Vivo Isolation and Characterization of α54 Mutants That Recover the Activity of the Activation-defective T308S Mutation in the AAA Activator NifA—Initially, we screened *in vivo* for altered forms of *K. pneumoniae* α54 that were able to recover the activity of the AAA activator *B. japonicum* NifA containing the T308S substitution in L1 (18). The *B. japonicum* NifA(T308S) protein is drastically impaired only in transcription activation. Neither ATP hydrolysis nor oligomerization functions are affected by the T308S mutation, indicating a defect in coupling ATP hydrolysis to open complex formation (18). We used *B. japonicum* NifA instead of *E. coli* PspF for the *in vivo* experiments because *in vivo* assay systems for measuring activation of transcription are well established and documented for the NifA protein (3, 18). Previous results indicated that α54 Region I amino acids 6–14 are important for binding to the AAA activator (11). Thus, we subjected α54 amino acids 2–14 to a codon-based mutagenesis approach (14) (see "Experimental Procedures"). The mutant α54 library was transformed into *E. coli* strain TH1, which has a deletion of the *rpoN* gene, encoding α54, and contains plasmids pRT22 (3) and pWKS130 (18). The transformed *E. coli* TH1 cells were plated onto X-gal-containing agar, and blue-white screening was used to identify α54 mutants capable of recovering the NifA(T308S) activity. In this experimental system, *E. coli* cells containing α54 mutants capable of recovering the NifA(T308S) activity will produce colonies displaying a deep blue color. We isolated six colonies (*rpoN-sup1–6*) that displayed a deep blue color. An *in vivo* β-galactosidase activity assay was used to quantitatively assess the ability of these six mutant clones (*rpoN-sup1–6*) to recover the activity of the NifA(T308S) protein. As shown in Table 1, the NifA(T308S)-dependent expression of lacZ was ~6–20-fold higher in the presence of the six mutant α54 clones than in the presence of wild-type α54. However, we

### Table 1

| α54 Region I amino acids 1–14 | β-galactosidase activity1 (Miller Units) |
|-------------------------------|------------------------------------------|
|                               | NifA-T308S | NifA-wild-type |
| rpoN-sup1                     | 160 +/- 13 | 21 125 +/- 1678 |
| rpoN-sup2                     | 1102 +/- 52 | 26 561 +/- 1655 |
| rpoN-sup3                     | 999 +/- 30 | 24 396 +/- 2231 |
| rpoN-sup4                     | 3636 +/- 407 | 22 292 +/- 1768 |
| rpoN-sup5                     | 2247 +/- 8 | 25 904 +/- 540 |
| rpoN-sup6                     | 1358 +/- 158 | 22 009 +/- 685 |

1 Assays were conducted in *E. coli* TH1 (Ap*res*) strain containing plasmid pRT22 which carries the α54-dependent *K. pneumoniae nifH* promoter fused to the lacZ gene. See text. Values represent the average from at least three different data sets.
The level of transcriptional activity of the rpoN-sup clones with NifA(T308S) represented ~15–20% of the transcriptional activity obtained with wild-type NifA. Thus, this result suggests that all six mutant $\sigma^{54}$ clones, at least in the context of this assay, are able to specifically recover the activity of NifA(T308S).

Sequence analyses of clones rpoN-sup1–6 revealed that all, except clone rpoN-sup4, contained three to five amino acid substitutions that were widely distributed within the $\sigma^{54}$ Region I segment (amino acids 2–14) that was subjected to mutagenesis. Analysis of randomly chosen clones from the library that did not display a deep blue color also revealed multiple amino acid substitutions within this $\sigma^{54}$ Region I segment. In line with the key objective of this study, we did obtain six mutant rpoN clones capable of suppressing the activation defect displayed by the NifA(T308S) protein, but failed to obtain rpoN clones with single amino acid substitutions.

Interestingly, a common feature in all mutant clones that rescued NifA(T308S) was the high occurrence of hydrophobic amino acids at the mutated positions. Notably, all mutant clones had a mutation of the non-conserved glycine codon at position 4 (Gly$^4$), and in most cases (rpoN-sup2 and rpoN-sup4–6), Gly$^4$ was changed to a hydrophobic residue (Table 1). In most rpoN genes sequenced so far, position 4 is represented by a hydrophobic residue. From Table 1, it seems that the substitution of the conformationally flexible glycine residue at position 4 of K. pneumoniae $\sigma^{54}$ Region I is a requirement for recovering the activity of NifA(T308S). Because, unlike the other clones, clone rpoN-sup4 contained substitutions only at Gly$^4$ and Gln$^{11}$ and displayed the second highest activity in the $\beta$-galactosidase assays (Table 1), we separated the G4L/Q11F mutations to further investigate the role of G4L and Q11F substitutions in recovering the activity of NifA(T308S). Initially, we used the in vivo $\beta$-galactosidase activity assay (as described above) to establish whether the single mutants $\sigma^{54}(G4L)$ and $\sigma^{54}(Q11F)$ are able to express lacZ in the presence of wild-type NifA. As shown in Table 2, $\sigma^{54}(G4L)$ was ~20% more active than wild-type $\sigma^{54}$. In contrast, $\sigma^{54}(Q11F)$ was ~3–4-fold less active than either $\sigma^{54}(G4L)$ or wild-type $\sigma^{54}$. Notably, the double mutant $\sigma^{54}(G4L/Q11F)$ was ~20% more active than wild-type $\sigma^{54}$ in expressing lacZ in a wild-type NifA-dependent manner. E. coli TH1 cell extracts were prepared and probed with anti-$\sigma^{54}$ antibodies to confirm that the stability of $\sigma^{54}$ was not affected by the Q11F mutation. The results showed that wild-type $\sigma^{54}$, $\sigma^{54}(G4L)$, $\sigma^{54}(Q11F)$, and $\sigma^{54}(G4L/Q11F)$ were present at equal amounts under the assay conditions (data not shown). Thus, it appears that the Q11F single substitution in $\sigma^{54}$ is unfavorable for wild-type NifA-dependent activation of transcription, but that $\sigma^{54}(Q11F)$ is expressed as a stable protein.

Next, we investigated whether the single mutants $\sigma^{54}(G4L)$ and $\sigma^{54}(Q11F)$ are able to recover NifA(T308S) activity as effectively as $\sigma^{54}(G4L/Q11F)$. As expected, wild-type $\sigma^{54}$ was unable to express lacZ when NifA(T308S) was used for activation. Notably, only $\sigma^{54}(G4L)$ was able to recover the activity of NifA(T308S) and expressed lacZ at an ~6-fold higher level compared with wild-type $\sigma^{54}$ (Table 2). $\sigma^{54}(Q11F)$ was unable to recover NifA(T308S) activity (Table 2). Thus, it seems that the G4L substitution is the significant mutation in $\sigma^{54}$ Region I that allows recovery of NifA(T308S) activity. However, the level of lacZ expression by $\sigma^{54}(G4L)$ was ~3-fold reduced compared with $\sigma^{54}(G4L/Q11F)$. Thus, it seems that, in the context of $\sigma^{54}(G4L/Q11F)$, the hydrophobic substitution at Gln$^{11}$ facilitates an improved interaction between Region I of $\sigma^{54}$ and L1 of NifA(T308S).

We assessed whether the $\sigma^{54}$ mutants are able to suppress the NifA(T308S) mutant in vivo in an allele-specific manner by using another NifA mutant carrying a different substitution at the same L1 position (NifA(T308V)). Like NifA(T308S), this mutant is impaired in transcription activation, but contains an amino acid at position 308 that is geometrically and spatially very similar to a threonine residue. The in vivo activation assay showed that neither $\sigma^{54}(G4L)$ nor $\sigma^{54}(G4L/Q11F)$ was able to recover the activity of the activation-defective NifA variant containing the T308V substitution (Table 2). Hence, it appears that $\sigma^{54}(G4L)$ specifically recovers NifA(T308S) activity.

### Table 2

In vivo transcriptional activities of wild-type $\sigma^{54}$, $\sigma^{54}(G4L)$, $\sigma^{54}(Q11F)$, and $\sigma^{54}(G4L/Q11F)$ in response to activation by NifA(T308S), NifA(T308V), or wild-type NifA

| $\sigma^{54}$ | $\beta$-Galactosidase activity |
|--------------|--------------------------------|
|              | NifA(T308S) | NifA(T308V) | Wild-type NifA |
| Wild-type    | 75 ± 0.70   | 6.1 ± 0.5   | 34.8 ± 1.39   |
| G4L          | 460 ± 27    | 71.5 ± 10   | 46.8 ± 296    |
| Q11F         | 97 ± 3.0    | 55.6 ± 4.4  | 12.3 ± 1.56   |
| G4L/Q11F     | 1356 ± 46   | 59.1 ± 8    | 47.4 ± 6.66   |

*Note: Values represent the average from at least three different data sets.*
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initiate transcription strictly relied upon the AAA activator and ATP hydrolysis (data not shown; see below). We used a dinucleotide-primed abortive initiation transcription assay to recapitulate in vitro with PspF-(1–275) the changes in transcription activation properties measured in vivo with NifA. As indicated in the reaction schematic in Fig. 2, open complex formation was stimulated by exposing the Er54 closed complex to wild-type PspF-(1–275) or PspF(T86S)-(1–275). Closed complexes were formed on an 88-bp-long linear DNA fragment containing the σ54-dependent S. meliloti nifH promoter (Fig. 1D, native). Control gel shift experiments indicated that approximately equal amounts of closed complexes were formed by wild-type and mutant Er54 under the experimental conditions (data not shown). Following activation, the promoter complexes were challenged with the polyanion heparin. Heparin is a commonly used DNA competitor that disrupts closed complexes or promoter complexes that have not isomerized properly and/or in which DNA opening is not stable. Thus, only transcriptionally proficient stable open complexes are relatively heparin-resistant. As shown in Fig. 2 (lanes 1 and 2), ~90% less abortive transcripts were formed by wild-type Er54 when open complex formation was stimulated by PspF(T86S)-(1–275) (−275) than by wild-type PspF-(1–275). In contrast, in reactions containing Er54(G4L), equal amounts of abortive transcripts were formed (Fig. 2, lanes 3 and 4), regardless of whether wild-type PspF-(1–275) or PspF(T86S)-(1–275) was used to stimulate open complex formation. Similar results were obtained when the assay was done using a supercoiled plasmid harboring the S. meliloti nifH promoter in which the synthesis of full-length transcripts was measured (data not shown). Overall, these results clearly recapitulate the in vivo observations (see above) and show that the G4L substitution in Region I of σ54 is able to recover, both in vivo and in vitro, the activation defect conferred by mutation of the invariant threonine residue in L1 of PspF and NifA in a transcription assay.

Mutation G4L in σ54 Enables PspF(T86S)-(1–275) to Stably Interact with the Closed Complex in a Nucleotide-dependent Manner—Previously, we demonstrated that PspF(T86S)-(1–275) is unable to form the ternary complex with a binary σ54-promoter DNA or Er54-promoter DNA complex in the presence of the ATP hydrolysis transition state analog ADP-AlF6. We now wished to determine whether PspF(T86S)-(1–275) can stably bind to S. meliloti nifH promoter complexes reconstituted with σ54(G4L) and Er54(G4L) in the presence of ADP-AlF6. Promoter complexes were formed on a DNA probe that mimicked the conformation of the promoter DNA adopted within the closed complex. This promoter probe, referred to as the −12/−11 probe, contained a 2-base heteroduplex segment immediately downstream of the consensus GC sequence (Fig. 1D). As shown in Fig. 3 (A and B, compare lanes 5 and 6), PspF(T86S)-(1–275) was able to form the ternary complex with promoter complexes reconstituted with σ54(G4L) or Er54(G4L) as well as wild-type PspF-(1–275). Control assays established that ternary complex formation between σ54(G4L) and PspF(T86S)-(1–275) was dependent on ADP-AlF6 (data not shown). As expected, PspF(T86S)-(1–275) did not form the ternary complex when promoter complexes were reconstituted with wild-type σ54 or Er54 (Fig. 3, A and B, lanes 3) (11).

We extended the ternary complex formation assay to determine whether the G4L mutation in σ54 specifically recovers the activation defect of PspF(T86S)-(1–275). Four different PspF-(1–275) variants (PspF(T86A)-(1–275), PspF(R55A)-(1–275), PspF(F87L)-(1–275), and PspF(F85W)-(1–275)) that were defective in ternary complex formation with promoter complexes reconstituted with wild-type σ54 (Fig. 3C, lanes 2–6) were used for this purpose to help determine the specific defects in PspF(T86S)-(1–275). σ54(G4L) specifically formed the ternary complex only with PspF(T86S)-(1–275) (Fig. 3C, lanes 8–12). Identical results were obtained in experiments conducted in the presence of core RNA polymerase (data not shown). Overall, the results suggest that the in vivo properties of σ54(G4L) with NifA can be reproduced in vitro with PspF-(1–275). Furthermore, in full agreement with the in vivo data (Table 1), the results from the in vitro experiments suggest that the G4L substitution in Region I of σ54 specifically allows the recovery of the activity of AAA activators containing only the Thr-to-Ser substitution in L1.

The G4L Substitution in σ54 Region I Allows Remodeling of Er54 by PspF(T86S)-(1–275)—In the in vitro transcription assays (Fig. 2) suggest that the G4L substitution does not merely contribute to an improved binding interaction between σ54 and the AAA activator in the presence of promoter DNA, but also allows the latter to couple ATP hydrolysis-dependent conformational changes in PspF-(1–275) to Er54. We conducted the next set of experiments to directly investigate this. Previously, we described a simple native gel assembly assay to monitor ADP-AlF6-dependent and PspF-(1–275)-induced remodeling of Er54 (13). This assay measures the ability of PspF-(1–275) to confer upon Er54 the ability to bind a mutant heteroduplex promoter probe, referred to as the −12/−11-mut promoter probe (Fig. 1D), in an ADP-AlF6-dependent manner. The −12/−11-mut promoter probe differed from the −12/−11 promoter probe in that it contained a non-wild-type template strand sequence in the heteroduplex region (Fig. 1D). Er54 (and σ54) bound poorly to the −12/−11-mut promoter probe (Fig. 3D, lane 1). However, in the presence of PspF-(1–275) and ADP-AlF6, Er54 (and σ54) formed a stable complex on the −12/−11-mut promoter probe (Fig. 3D, lane 2). In the presence of ADP-AlF6, PspF(T86S)-(1–275) did not confer upon wild-type Er54 the ability to bind the −12/−11-mut promoter probe (Fig. 3D, lane 3), revealing a marked defect in PspF(T86S)-(1–275). We wished to investigate whether the G4L substitution allows Er54 to be remodeled by PspF(T86S)-(1–275) in the presence of ADP-AlF6 so that it can then bind to the −12/−11-mut promoter probe. As shown in Fig. 3D, Er54(G4L) bound equally well to the −12/−11-mut promoter probe in reactions containing wild-type PspF-(1–275) (lane 5) and PspF(T86S)-(1–275) (lane 6) in an ADP-AlF6-dependent manner. Thus, it appears that the G4L substitution in Region I of σ54 not only simply facilitates an interaction between Er54 and PspF(T86S)-(1–275), but also allows Er54 to undergo PspF(T86S)-(1–275)-induced conformational changes in an ADP-AlF6-dependent manner.
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A

\[ \sigma_{54} \]

- Region I
- Region II
- Region III

- Activator Binding Site
- Weak Core Binding
- DNA Binding

Ec: MKQGLQRLOQQLA
Eco: MKQGLQRLOQQLA
St: MKQGLQRLOQQLA
Pa: MKPSVLRNMQCQLT
Av: MKP8LQLMRNQLT

B

\[ \text{AAA domain} \]

- DNA binding domain

PspF, L-275

- Loop 1 (L1)
- Loop 2 (L2)

Ec: PspF GHRKQAFTGQKRRDP
Eco: PspF GHRKQAFTGQKRRDP
Sm: oct A GHRKQAFTGQKRRDP
St: NifA GHRKQAFTGQKRRDP
Pp: XyIA GHRKQAFTGQKRRDP

C

- Protein structure

D

- native
- -12/-11
- -12/-11-mut
- -10/-1

- Nucleotide sequences

...CATACGCGTCGACGACTTTGACGATCGACCTGGGAGGG... 
...CTCTGCGGAGCTGCTGAAAAGCTGCTAGTGGGACCAC...
...CATACGCGTCGACGACTTTGACGATCGACCTGGGAGGG... 
...CTCTGCGGAGCTGCTGAAAAGCTGCTAGTGGGACCAC...
...CATACGCGTCGACGACTTTGACGATCGACCTGGGAGGG... 
...CTCTGCGGAGCTGCTGAAAAGCTGCTAGTGGGACCAC... 
...CATACGCGTCGACGACTTTGACGATCGACCTGGGAGGG... 
...CTCTGCGGAGCTGCTGAAAAGCTGCTAGTGGGACCAC...
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A β4(G4L) bound to the −10/−1 probe. As expected, PspF(T86S)-(1–275) was able to form ternary complexes in reactions containing only β4(G4L) (data not shown). Thus, it seems that (i) stably pre-opening the DNA does not allow PspF(T86S)-(1–275) to overcome its activation defect and that (ii) the conformation adopted by the melted out promoter DNA region has a potential role in the recovery of PspF(T86S)-(1–275) activity by β4(G4L) in a transcription assay (recall that recovery with homoduplex DNA was better than with the −10/−1 probe) (Fig. 2). However, the conformation of the promoter DNA does not seem to influence the ability of β4(G4L) to recover the activity of PspF(T86S)-(1–275) to form the ADP-AIF−-dependent ternary complex.

PspF(T86S)-(1–275) Can Interact with β4 in the Absence of Promoter DNA—The results so far demonstrate that the G4L mutation in Region I of β4 allows the recovery of the defective property of PspF(T86S)-(1–275), but only in a restricted range of functional assays. Notably, experiments with heteroduplex DNA (Fig. 4) suggested that the conformation of the promoter DNA has a role in the ability of PspF(T86S)-(1–275) to be recovered by β4(G4L) in a transcription assay. To investigate the role of promoter DNA in the recovery process, we conducted a simple native gel assembly assay to measure the ADP-AIF−-dependent interaction between β4(G4L) bound to the −10/−1 probe and PspF-(1–275) in the absence of promoter DNA. As shown in Fig. 5 (compare lanes 3 and 4 and lanes 6 and 7), in contrast to the results obtained in experiments with promoter DNA (Fig. 3), wild-type β4 was able to interact with wild-type PspF-(1–275) and PspF(T86S)-(1–275) as well as β4(G4L). Because β4 Region I adopts different conformations in the presence (i.e. within promoter complexes) and absence of promoter DNA (20, 21), it seems that the conformation adopted by β4 Region I in the presence of certain promoter DNA conformations is unfavorable for interaction with PspF(T86S)-(1–275). Clearly, the G4L substitution allows PspF(T86S)-(1–275) to overcome this defect. However, on heteroduplex promoter DNA (−10/−1 probe), it seems that the conformation adopted by β4(G4L) Region I is unable to recover the defect displayed by PspF(T86S)-(1–275) in transcription (Fig. 4).

Significantly, the results imply overall that the conserved threonine residue in the GAFTGA motif of AAA activators has a role in sensing the conformation of β4 Region I within promoter complexes as directed by the DNA conformation.

DNA Sequences Downstream of the −12 Consensus Promoter Region Prevent PspF(T86S)-(1–275) from Interacting with the Closed Complex—Previously, we showed that, during open complex formation, AAA activators can be cross-linked to DNA sequences downstream (termed here to-be-melted DNA)
of the −12 consensus promoter sequence (22). Our results implied that AAA activators potentially interact with the to-be-melted DNA at some step during open complex formation (22). In light of the present observations suggesting that PspF(T86S)-(1–275) is defective in efficiently and productively interacting with the E54 closed complex, but not with E54 per se (i.e. in the absence of DNA) (Fig. 5), we considered whether the to-be-melted DNA sequences downstream of the −12 consensus region might somehow interfere with PspF(T86S)-(1–275) and prevent it from interacting with the E54 closed complex. We therefore constructed promoter probes (probes A–F) in which we systematically moved the downstream DNA end points (Fig. 6).

FIGURE 3. E54(G4L) mutant enables PspF(T86S)-(1–275) mutant activator to interact with the closed promoter complex in an ADP-AlFx-dependent manner. A, autoradiograph of a native gel showing ADP-AlFx-dependent binding of wild-type PspF-(1–275) and PspF(T86S)-(1–275) to promoter complexes reconstituted with either wild-type σ54 or σ54(G4L) using the −12/−11 probe. The migration positions of ADP-AlFx-dependent PspF-(1–275) and PspF-(1–275) DNA, E54 DNA, and unbound (free) DNA are indicated. The reaction components in each lane are indicated. Lane 1, 2, 5, and 6). The reaction components in each lane are indicated.

FIGURE 4. Recovery of the transcription activity of PspF(T86S)-(1–275) by E54(G4L) is dependent on promoter DNA structure. The conditions were as described in the legend to Fig. 2, but abortive transcription was also measured from open complexes formed on the −10/−1 probe (lanes 3, 4, 7, and 8). Abortive transcription from the native probe is shown for comparison (lanes 1, 2, 5, and 6). The reaction components in each lane are indicated.

FIGURE 5. Native gel (stained with Coomassie Brilliant Blue) showing ADP-AlFx-dependent complex formation between E54 and PspF-(1–275). The reaction components in each lane are indicated. Unbound PspF-(1–275) ran off the gel under the experimental conditions used here. The migration positions of ADP-AlFx-dependent PspF-(1–275):E54, σ54, and ADP-AlFx-dependent PspF-(1–275)-oligomer are indicated.

PspF-(1–275)σ54-DNA, σ54-DNA, and unbound (free) DNA are indicated. The percent promoter probe in the ADP-AlFx-dependent PspF-(1–275):σ54-DNA and σ54-DNA complexes is given underneath the gel. The reaction components in each lane are indicated. B, same as described for A, but assays were conducted in the presence of core RNA. The protein-DNA complexes formed are indicated. C, same as described for A, but different PspF variants (as indicated) were used. D, the gel is as shown in B, but the assay was conducted using the −12/−11-mut probe (see “Results” for details).
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PspF(T86S)-(1–275) to form the ternary complex on probes A–E (Fig. 6C, lanes 19–23). Strikingly, however, PspF(T86S)-(1–275) was able to form the ternary complex on probe F as efficiently as wild-type PspF-(1–275) (Fig. 6C, compare lanes 15, 6, and 18). This implies that DNA sequence downstream of position –10 has a negative effect upon interactions between Ea(54) and PspF(T86S)-(1–275). Interestingly, we also noted that ternary complex formation by both wild-type PspF-(1–275) and PspF(T86S)-(1–275) was relatively weaker on probe E than on all other probes in reactions with Ea(54)(G4L).

We extended the assay to investigate whether it is the non-template or template strand DNA that prevents PspF(T86S)-(1–275) from interacting with the closed complex. To do so, we constructed promoter DNA probes in which we shortened either only the non-template or template strand DNA. In each case, the end points were as described above (Fig. 6A). The results revealed that PspF(T86S)-(1–275) was unable to form the ternary complex if either the non-template or template strand DNA extended beyond position –10 (data not shown). Thus, it seems that the to-be-melted DNA sequences downstream of position –10, in either the single-stranded (template and non-template strands) or double-stranded conformation, somehow interfere with the ability of PspF-(1–275) to interact with the closed complex when the integrity of L1 is compromised by the T86S mutation.

DISCUSSION

Transcriptionally proficient open complex formation on bacterial promoters involves a complex and coordinated set of protein-protein and protein-DNA isomerization events. On Ea(54)-dependent promoters, such events are triggered in response to interaction of the closed complex with an AAA activator protein in an ATP hydrolysis-dependent manner. The highly conserved regulatory Region I domain of Ea(54) is responsible for preventing open complex formation in the absence of activation. Accordingly, in the closed complex, Region I is positioned in the –12 consensus promoter region, where it makes repressive interactions with a fork junction DNA structure and so prevents open complex formation in the absence of activation (4). This nucleoprotein organization in the –12 promoter region is referred to as the “regulatory center” and constitutes a major interaction target for the AAA activator (3). Activation results in the reorganization of the regulatory center and the loss of

FIGURE 6. DNA sequences downstream of the –12 consensus promoter region prevent PspF(T86S)-(1–275) to interact with the closed promoter complex. A, schematic showing the end points on the native S. meliloti nifH promoter that were used to generate probes A–F. The consensus GG and GC dinucleotide sequences and transcription start site (+1) are indicated. B, graph showing the amount of closed complexes formed on probes A–F by Ea(54) (open bars) and Ea(54)(G4L) (hatched bars). C, autoradiograph of a native gel showing ternary complex (ADP-AlF(3) dependent PspF-(1–275):Ea(54)-DNA) formation on probes A–F by Ea(54) and Ea(54)(G4L) in reactions with wild-type PspF-(1–275) (lanes 1–12) and PspF(T86S)-(1–275) (lanes 13–24). The migration positions of ADP-AlF(3) dependent PspF-(1–275):Ea(54)-DNA, Ea(54)-DNA, and unbound (free) DNA are indicated. The percent DNA in the ternary complex is shown underneath the gels.

pendent ternary complexes (22). Native PAGE analysis revealed that Ea(54) bound probes A–F equally well (Fig. 6B). Similarly, Ea(54)(G4L) bound probes A–D as well as wild-type Ea(54). However, Ea(54)(G4L) revealed ~2-fold higher affinity for probes E and F compared with wild-type Ea(54) (Fig. 6B). Next, we measured the ability of Ea(54) and Ea(54)(G4L) closed complexes formed on probes A–F to interact with wild-type PspF-(1–275) in an ADP-AlF(3)-dependent manner and to form ternary complexes. As shown in Fig. 6C, no detectable differences were seen in the ability of wild-type PspF-(1–275) to interact with Ea(54) and Ea(54)(G4L) closed complexes formed on probes A–F and to form ternary complexes. As expected (Fig. 3), PspF(T86S)-(1–275) was defective in efficiently forming the ternary complex on probes A and B in reactions containing wild-type Ea(54) (Fig. 6C, lanes 13 and 14). On probes C–E, 30–35% of the DNA was in the ternary complex in reactions containing wild-type PspF-(1–275) (Fig. 6C, lanes 3–5). In contrast, only ~13% of the DNA was in the ternary complex in reactions containing PspF(T86S)-(1–275) (Fig. 6C, lanes 15–17). Reactions containing Ea(54)(G4L) confirmed that the G4L mutation in Region I of Ea(54) is able to rescue the ability of

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interaction between $\sigma^{54}$ Region I and a repressive fork junction structure, and open complex formation then follows (1). The reorganization of the regulatory center is likely to occur via several discrete intermediate states. In a previous study, we proposed that the conserved GAFTGA motif of the AAA activator is part of an internal conformational signaling pathway and is involved in sensing and communicating the conformational variations adopted by the regulatory center en route to open complex formation (11). The substitution of the invariant threonine residue with serine in the GAFTGA motif prevents the AAA activator from sensing the conformation adopted by the regulatory center and results in a mutant AAA activator form that is incapable of efficient transcription activation both in vivo and in vitro. The key result of this study is the finding that a Gly-to-Leu substitution in Region I of $\sigma^{54}$ (position 4 in K. pneumoniae $\sigma^{54}$) is able to specifically recover the activation-defective property conferred by the Thr-to-Ser substitution in the highly conserved GAFTGA sequence of the AAA activator PspF (and NifA). Thus, the results from the set of experiments presented here clearly point toward an involvement of $\sigma^{54}$ Region I in the sensing and communicating of information relating to regulatory center conformation by the GAFTGA motif during open complex formation. The interplay between $\sigma^{54}$ Region I and the GAFTGA motif during open complex formation is of functional significance because $\sigma^{54}$ Region I also appears to determine the activities of three structurally conserved RNAP domains ($\beta'$ lobe, $\beta'$ jaw, and $\beta'$ clamp) that contribute to a DNA-binding channel in RNAP, where DNA downstream of the RNAP active center lies, and ensure that stable DNA opening near the transcription start site is maintained (7, 23). Previously, we proposed that $\sigma^{54}$ Region I acts as a “relay” domain and communicates with the downstream DNA-binding channel in RNAP in response to activation (24). In view of the present results, we suggest that $\sigma^{54}$ Region I could also be involved in relaying information regarding the regulatory center conformation to the AAA activator during transcription activation.

On pre-melted DNA, which mimics the conformation of the promoter in the open complex, $\sigma^{54}(G4L)$ is unable to recover the activation defect of PspF(T86S)-(1–275) (Fig. 4). However, unlike wild-type $\sigma^{54}$, $\sigma^{54}(G4L)$ allows PspF(T86S)-(1–275) to interact with the Eo$^{54}$-pre-melted DNA complex. Because the pre-melted DNA does not support transcription in the absence of activation, the regulatory center of this DNA most likely adopts the repressed (non-activated) conformation (25). In contrast, the pre-melted DNA is in the “open complex” conformation. Thus, the fact that $\sigma^{54}(G4L)$ allows PspF(T86S)-(1–275) to interact with but not activate Eo$^{54}$ for abortive initiation on the pre-melted DNA further argues for a role for the GAFTGA motif in sensing and communicating DNA conformation. Notably, the results with the pre-melted DNA also imply that the leucine substitution does not simply contribute to a non-native interaction with the GAFSGA motif of PspF(T86S)-(1–275), which facilitates its interaction with the regulatory center. Rather, it seems that $\sigma^{54}(G4L)$ with the appropriate promoter DNA structure can utilize PspF(T86S)-(1–275).

PspF(T86S)-(1–275) is unable to interact stably with the closed complex, but is able to interact with Eo$^{54}$ in the absence of promoter DNA. Experiments with shortened promoter probes (Fig. 6) indicated that the DNA downstream of position −10, i.e. the to-be-melted DNA that lies downstream of the regulatory center, prevents the AAA activator from interacting with the closed complex if the integrity of the GAFTGA motif is compromised by the Thr-to-Ser substitution. In the closed complex, $\sigma^{54}$ interacts with the DNA to position −5 (26). Thus, on shortened probes, e.g. probes E and F (Fig. 6A), the lack of or an altered interaction between $\sigma^{54}$ and the DNA to position −5 could permit PspF(T86S)-(1–275) to interact with the regulatory center. The DNA downstream of position −10 is non-conserved in $\sigma^{54}$-dependent promoters, but appears to influence the functionality of the AAA activator via the GAFTGA motif. Thus, a direct interaction between PspF-(1–275) and the to-be-melted promoter DNA is possible. A previous study has reported that the AAA activator lies proximal to the to-be-melted DNA during transcription activation (22). However, the precise role of the to-be-melted DNA and the nature of the proximity of the AAA activator to this DNA segment in transcription activation remain unclear. Transcriptional silencing of the closed complex in the absence of activation strictly relies upon the interaction made predominantly by $\sigma^{54}$ Region I with a fork junction DNA structure at position −12/−11 within the regulatory center, which masks the determinants in $\sigma^{54}$ needed for binding downstream single-stranded DNA between positions −10 and −7 during early stages of open complex formation (27). ATP hydrolysis-dependent activation of the closed complex by the AAA activator appears to unmask the determinants in $\sigma^{54}$ needed for single-stranded DNA binding and to allow $\sigma^{54}$ to establish interactions with single-stranded DNA for open complex formation (27). We propose that, during activation, the AAA activator could nonspecifically sense the conformation of the DNA downstream of position −10 and couple the DNA structure per se to $\sigma^{54}$ Region I (and possibly to other determinants of $\sigma^{54}$ and RNAP) or couple information relating to its conformation to $\sigma^{54}$ via Region I to allow single-stranded DNA-binding determinants in Eo$^{54}$ to establish the interaction with the single-stranded DNA segments needed for open complex formation. The transcription activation defect in AAA activators with a Thr-to-Ser substitution in the GAFTGA motif could manifest itself through their defective and negative interactions with the DNA downstream of position −10. The functional state of PspF-(1–275) (and most AAA activators) is a hexamer (10). How many GAFTGA motifs are involved in interactions with the regulatory center during transcription activation remains unclear. Cryoelectron microscopic
analysis of the PspF-(1–275)σ54 complex suggested that at least two GAFTGA motifs interact with σ54. The results here now suggest that the GAFTGA motif of the AAA activator has multiple interrelated roles during transcription activation. In the crystal structure of PspF-(1–275) (9, 10) and A. aeolicus NtrC1 (12), the GAFTGA motif is located on the tip of a flexible loop (L1) that juts out during ATP hydrolysis and contacts the regulatory center. Thus, it is possible that more than one GAFTGA motif interacts with the regulatory center (thereby regulating the regulatory center). Thus, it is possible that more than one GAFTGA motif interacts with the regulatory center (thereby regulating the regulatory center). Thus, it is possible that more than one GAFTGA motif interacts with the regulatory center.

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REFERENCES

1. Wigneshweraraj, S. R., Burrows, P. C., Bordes, P., Schumacher, J., Rappas, M., Finn, R. D., Cannon, W. V., Zhang, X., and Buck, M. (2005) *Prog. Nucleic Acid Res. Mol. Biol.* 79, 339–369
2. Zhang, X., Chaney, M., Wigneshweraraj, S. R., Schumacher, J., Bordes, P., Cannon, W., and Buck, M. (2002) *Mol. Microbiol.* 45, 895–903
3. Chaney, M., Grande, R., Wigneshweraraj, S. R., Cannon, W., Casaz, P., Gallegos, M. T., Schumacher, J., Jones, S., Elderkin, S., Dago, A. E., Morett, E., and Buck, M. (2001) *Genes Dev.* 15, 2282–2294
4. Wigneshweraraj, S. R., Chaney, M. K., Ishihama, A., and Buck, M. (2001) *J. Mol. Biol.* 306, 681–701
5. Wang, J. T., Syed, A., Hsieh, M., and Gralla, J. D. (1995) *Science* 270, 992–994
6. Gallegos, M. T., Cannon, W. V., and Buck, M. (1999) *J. Biol. Chem.* 274, 25285–25290
7. Wigneshweraraj, S. R., Burrows, P. C., Severinov, K., and Buck, M. (2005) *J. Biol. Chem.* 280, 36176–36184
8. Wigneshweraraj, S. R., Fujita, N., Ishihama, A., and Buck, M. (2000) *EMBO J.* 19, 3038–3048
9. Schumacher, J., Joly, N., Rappas, M., Zhang, X., and Buck, M. (2006) *J. Struct. Biol.* 156, 190–199
10. Rappas, M., Schumacher, J., Beuron, F., Niwa, H., Bordes, P., Wigneshweraraj, S., Keetch, C. A., Robinson, C. V., Buck, M., and Zhang, X. (2005) *Science* 307, 1972–1975
11. Bordes, P., Wigneshweraraj, S. R., Chaney, M., Dago, A. E., Morett, E., and Buck, M. (2004) *Mol. Microbiol.* 54, 489–506
12. De Carlo, S., Chen, B., Hoover, T. R., Kondrashkina, E., Nogales, E., and Nixon, B. T. (2006) *Genes Dev.* 20, 1485–1495
13. Cannon, W., Bordes, P., Wigneshweraraj, S. R., and Buck, M. (2003) *J. Biol. Chem.* 278, 19815–19825
14. Gaytán, P., Osuna, J., and Soberón, X. (2002) *Nucleic Acids Res.* 30, e84
15. Grande, R. A., Valderrama, B., and Morett, E. (1999) *J. Mol. Biol.* 294, 291–298
16. Bordes, P., Wigneshweraraj, S. R., Schumacher, J., Zhang, X., Chaney, M., and Buck, M. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 2278–2283
17. Wigneshweraraj, S. R., Nechaev, S., Bordes, P., Jones, S., Cannon, W., Severinov, K., and Buck, M. (2003) *Methods Enzymol.* 370, 646–657
18. González, V., Olvera, L., Soberón, X., and Morett, E. (1998) *Mol. Microbiol.* 28, 55–67
19. Jovanovic, G., Rakonjac, J., and Model, P. (1999) *J. Mol. Biol.* 285, 469–483
20. Casaz, P., and Buck, M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 12145–12150
21. Casaz, P., and Buck, M. (1999) *J. Mol. Biol.* 285, 507–514
22. Burrows, P. C., Severinov, K., Buck, M., and Wigneshweraraj, S. R. (2004) *EMBO J.* 23, 4253–4263
23. Wigneshweraraj, S. R., Savalia, D., Severinov, K., and Buck, M. (2006) *J. Mol. Biol.* 359, 1182–1195
24. Wigneshweraraj, S. R., Burrows, P. C., Nechaev, S., Zenkin, N., Severinov, K., and Buck, M. (2004) *EMBO J.* 23, 4264–4274
25. Cannon, W., Gallegos, M. T., Casaz, P., and Buck, M. (1999) *Genes Dev.* 13, 357–370
26. Cannon, W. V., Gallegos, M. T., and Buck, M. (2000) *Nat. Struct. Biol.* 7, 594–601
27. Guo, Y., Wang, L., and Gralla, J. D. (1999) *EMBO J.* 18, 3736–3745