

# 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 sensing 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).<sup>5</sup> 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 ( $\sim 150$ – $200$  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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<sup>5</sup> The abbreviations used are: RNAP, RNA polymerase; L1, loop 1; X-gal, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside.

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  $\sigma^{54}$  (see Fig. 1, B and C) and that these contacts form during the energy-coupling process that transmits a conformational change around the  $\gamma$ -phosphate of the ATP-binding site on PspF to changes in the  $E\sigma^{54}$  closed complex conformation, which subsequently triggers open complex formation. Specifically, an invariant threonine residue in L1 (Thr<sup>86</sup> in PspF) (see Fig. 1C) is thought to engage with  $\sigma^{54}$  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  $\sigma^{54}$  Region I will exist for open complex formation to occur (10). To elucidate the role of  $\sigma^{54}$  Region I and the invariant Thr<sup>86</sup> residue in the energy-coupling process, we have sought altered forms of  $\sigma^{54}$  that can compensate for defects displayed by a partially functional PspF variant containing a serine substitution at Thr<sup>86</sup> in L1 (3, 11). In a previous study, we established that the T86S form of L1 of PspF is defective in interaction with the  $E\sigma^{54}$  closed complex in the presence of the ATP hydrolysis transition state analog ADP·AlF<sub>x</sub> (11). The ADP·AlF<sub>x</sub>-dependent complex between PspF and the  $E\sigma^{54}$  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  $\sigma^{54}$ ,  $\sigma^{54}$ (G4L) (see Fig. 1A), capable of suppressing the defects of PspF(T86S) evident in ternary complex formation with the  $E\sigma^{54}$  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  $E\sigma^{54}$  closed complex. The properties of PspF(T86S) and  $\sigma^{54}$ (G4L) imply a role for the conserved GAFTGA motif in PspF and, by extension, in other AAA activators of  $E\sigma^{54}$  in “sensing” the conformation adopted by the DNA downstream of the −12 promoter region and coupling this information to  $E\sigma^{54}$  via Region I to allow open complex formation.

## EXPERIMENTAL PROCEDURES

**Design and Construction of a  $\sigma^{54}$  Region I Fragment 2–14 Mutant Library**—*Klebsiella pneumoniae*  $\sigma^{54}$  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  $\sigma^{54}$  variants with few amino acids substitutions (14). A detailed background of this mutagenesis method is described by Gaytán *et al.* (14). This method is based on the use of five dinucleotides designated B<sub>2</sub>B<sub>3</sub> (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 B<sub>2</sub>B<sub>3</sub> 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′-tattggccctgcatATG-AAG/B<sub>1</sub>B<sub>2</sub>B<sub>3</sub>-CAA/B<sub>1</sub>B<sub>2</sub>B<sub>3</sub>-GGT/B<sub>1</sub>B<sub>2</sub>B<sub>3</sub>-TTG/B<sub>1</sub>B<sub>2</sub>B<sub>3</sub>-CAA/B<sub>1</sub>B<sub>2</sub>B<sub>3</sub>-TTA/B<sub>1</sub>B<sub>2</sub>B<sub>3</sub>-AGG/B<sub>1</sub>B<sub>2</sub>B<sub>3</sub>-CTA/B<sub>1</sub>B<sub>2</sub>B<sub>3</sub>-AGC/B<sub>1</sub>B<sub>2</sub>B<sub>3</sub>-CAA/B<sub>1</sub>B<sub>2</sub>B<sub>3</sub>-CAG/B<sub>1</sub>B<sub>2</sub>B<sub>3</sub>-CTT/B<sub>1</sub>B<sub>2</sub>B<sub>3</sub>-GCC/B<sub>1</sub>B<sub>2</sub>B<sub>3</sub>-**atgacgccacaactg**-3′ was assembled. The wild-type  $\sigma^{54}$  codons subjected to mutagenesis are in uppercase letters. A second oligonucleotide, called oligonucleotide B (63-mer), with sequence 5′-ttgctggagtctagatgg-acagctgcagtagacgaatcgctctg**cagttgtggcgtcat**-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  $\sigma^{54}$ . 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′ exo<sup>−</sup>) 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 MgSO<sub>4</sub>, 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  $\mu$ l of Klenow fragment (5 units/ $\mu$ l) 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  $\sigma^{54}$  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 ( $\Delta$ *rpoN*)) and selected on X-gal (20  $\mu$ g/ $\mu$ l) containing nitrogen-free medium.

**In Vivo Activity Assays**—The *in vivo*  $\beta$ -galactosidase assays were conducted as described previously (3, 15) in *E. coli* strain TH1 ( $\Delta$ *rpoN*) containing plasmids pRT22 (chloramphenicol resistance) and pWKS130 (kanamycin resistance). pRT22 is a reporter plasmid and contains the  $\sigma^{54}$ -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  $\sigma^{54}$ .

**Site-directed Mutagenesis**— $\sigma^{54}$  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  $\sigma^{54}$  and PspF(1–275) mutagenesis reaction were pVB009 and pPB1 (16), respectively.

TABLE 1

Amino acid sequences of  $\sigma^{54}$  Region I segment 1–14 from clones *rpoN-sup1*–6The transcriptional activities of clones *rpoN-sup1*–6 in response to activation by NifA(T308S) or wild-type NifA are given.

$\sigma^{54}$	$\sigma^{54}$ Region I amino acids 1–14														$\beta$ -galactosidase activity <sup>1</sup> (Miller Units)	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	NifA-T308S	NifA-wild-type
	M	K	Q	G	L	Q	L	R	L	S	Q	Q	L	A		
<i>rpoN-wild-type</i>															160 +/- 13	21 125 +/- 1678
<i>rpoN-sup1</i>				N				Y		F		S		V	1102 +/- 52	26 561 +/- 1655
<i>rpoN-sup2</i>				I					I		F	N		F	999 +/- 50	24 396 +/- 2231
<i>rpoN-sup3</i>		N		Q				L	F					L	3636 +/- 407	22 292 +/- 1768
<i>rpoN-sup4</i>				L							F				2247 +/- 8	25 984 +/- 540
<i>rpoN-sup5</i>				Y				Q			Y	Y	F		1358 +/- 158	22 009 +/- 685
<i>rpoN-sup6</i>				L					Y					I	1013 +/- 22	27 865 +/- 289

<sup>1</sup> Assays were conducted in *E. coli* TH1 ( $\Delta rpoN$ ) strain containing plasmid pRT22 which carries the  $\sigma^{54}$ -dependent *K. pneumoniae* *nifH* promoter fused to the *lacZ* gene. See text. Values represent the average from at least three different data sets.

**Proteins**—For protein overproduction, NdeI-HindIII fragments containing wild-type and mutant (G4L) *rpoN* genes were cloned into pET28b<sup>+</sup> (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 <sup>32</sup>P-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  $\pm 6\%$ .

**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  $\pm 5\%$ .

## RESULTS

**In Vivo Isolation and Characterization of  $\sigma^{54}$  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*  $\sigma^{54}$  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  $\sigma^{54}$  Region I amino acids 6–14 are important for binding to the AAA activator (11). Thus, we subjected  $\sigma^{54}$  amino acids 2–14 to a codon-based mutagenesis approach (14) (see "Experimental Procedures"). The mutant  $\sigma^{54}$  library was transformed into *E. coli* strain TH1, which has a deletion of the *rpoN* gene, encoding  $\sigma^{54}$ , and contains plasmids pRT22 (3) and pWKS130 (18). pRT22 is a reporter plasmid and contains the  $\sigma^{54}$ -dependent *K. pneumoniae* *nifH* promoter fused to the *lacZ* gene. pWKS130 constitutively encodes the NifA(T308S) activator protein. The transformed *E. coli* TH1 cells were plated onto X-gal-containing agar, and blue-white screening was used to identify  $\sigma^{54}$  mutants capable of recovering NifA(T308S) activity. In this experimental system, *E. coli* cells containing  $\sigma^{54}$  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*  $\beta$ -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  $\sim 6$ –20-fold higher in the presence of the six mutant  $\sigma^{54}$  clones than in the presence of wild-type  $\sigma^{54}$ . However, we



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

Assays were conducted in *E. coli* strain TH1 ( $\Delta rpoN$ ) containing plasmid pRT22, which carries the  $\sigma^{54}$ -dependent *K. pneumoniae* *nifH* promoter fused to the *lacZ* gene (see "Experimental Procedures"). Values represent the average from at least three different data sets.

$\sigma^{54}$	$\beta$ -Galactosidase activity		
	NifA(T308S)	NifA(T308V)	Wild-type NifA
	Miller units		
Wild-type	75 $\pm$ 20	61.4 $\pm$ 5	34 801 $\pm$ 139
G4L	460 $\pm$ 27	71 $\pm$ 10	46 887 $\pm$ 296
Q11F	97 $\pm$ 3	55.6 $\pm$ 4	12 331 $\pm$ 156
G4L/Q11F	1356 $\pm$ 46	59.1 $\pm$ 8	47 432 $\pm$ 1366

noted that 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<sup>4</sup>), and in most cases (*rpoN-sup2* and *rpoN-sup4*–6), Gly<sup>4</sup> was changed to a hydrophobic residue (Table 1). In most *rpoN* genes sequenced so far, position 4 is represented by a hydrophilic 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<sup>4</sup> and Gln<sup>11</sup> 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<sup>11</sup> 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.

*E $\sigma^{54}$ (G4L) Recovers the Activation-defective Property of PspF(T86S)-(1–275) in an in Vitro Transcription Assay*—The *in vivo* analysis revealed that a single amino acid substitution at position 4 (G4L) in Region I of *K. pneumoniae*  $\sigma^{54}$  is sufficient to specifically recover the activity of NifA(T308S) and to explain the molecular basis for the activation defect of the NifA(T308S) mutant. To determine the properties of  $\sigma^{54}$ (G4L) responsible for recovering the activity of NifA(T308S), we conducted a series of *in vitro* experiments that measured activator-dependent output by E $\sigma^{54}$  at several steps leading to productive transcription initiation. For the *in vitro* assays, we used the AAA domain of the AAA activator *E. coli* PspF (PspF-(1–275)) (11) containing the T86S substitution (the equivalent of the T308S mutation in NifA) because, in contrast to the NifA protein, (i) PspF-(1–275) is far better suited for *in vitro* studies and (ii) well established experimental assays exist to study PspF-(1–275)-dependent activation of E $\sigma^{54}$  transcription (3, 17). Furthermore, PspF-(1–275) lacks the domain containing the helix-turn-helix motif and is thus able to efficiently activate transcription (*in vivo* and *in vitro*) from solution without the need to bind to upstream activating sequences, a property that significantly simplifies *in vitro* experimental design (19).

$\sigma^{54}$  containing the G4L mutation was purified as an N-terminally hexahistidine-tagged fusion protein. Initially, we determined that  $\sigma^{54}$ (G4L) bound core RNAP as well as wild-type  $\sigma^{54}$  (using a simple native gel assembly assay) and that its ability to

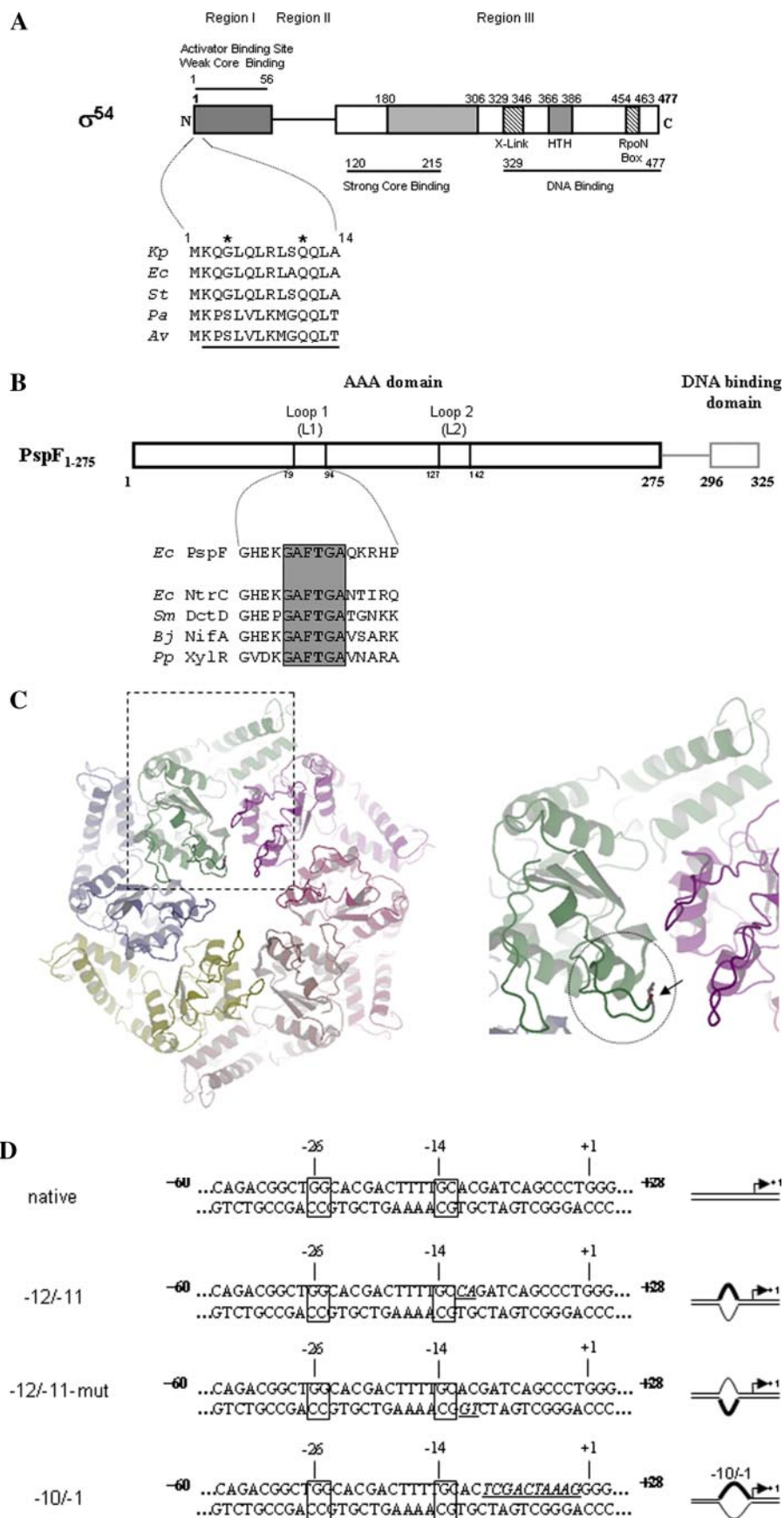
initiate transcription strictly relied upon the AAA activator and ATP hydrolysis (data not shown; see below). We used a dinucleotide-primed abortive initiation transcription assay to recapitulate *in vitro* with PspF-(1–275) the changes in transcription activation properties measured *in vivo* with NifA. As indicated in the reaction schematic in Fig. 2, open complex formation was stimulated by exposing the  $E\sigma^{54}$  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  $\sigma^{54}$ -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  $E\sigma^{54}$  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  $E\sigma^{54}$  when open complex formation was stimulated by PspF(T86S)-(1–275) than by wild-type PspF-(1–275). In contrast, in reactions containing  $E\sigma^{54}$ (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  $\sigma^{54}$  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  $\sigma^{54}$  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  $\sigma^{54}$ -promoter DNA or  $E\sigma^{54}$ -promoter DNA complex in the presence of the ATP hydrolysis transition state analog ADP·AlF<sub>x</sub> (11). We now wished to determine whether PspF(T86S)-(1–275) can stably bind to *S. meliloti nifH* promoter complexes reconstituted with  $\sigma^{54}$ (G4L) and  $E\sigma^{54}$ (G4L) in the presence of ADP·AlF<sub>x</sub>. 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  $\sigma^{54}$ (G4L) or  $E\sigma^{54}$ (G4L) as well as wild-type PspF-(1–275). Control assays established that ternary complex formation between  $\sigma^{54}$ (G4L) and PspF(T86S)-(1–275) was dependent on ADP·AlF<sub>x</sub> (data not shown). As expected, PspF(T86S)-(1–275) did not form the ternary complex when promoter complexes

were reconstituted with wild-type  $\sigma^{54}$  or  $E\sigma^{54}$  (Fig. 3, A and B, *lanes 3*) (11).

We extended the ternary complex formation assay to determine whether the G4L mutation in  $\sigma^{54}$  specifically recovers the activation defect of PspF(T86S)-(1–275). Four different PspF-(1–275) variants (PspF(T86A)-(1–275), PspF(F85A)-(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  $\sigma^{54}$  (Fig. 3C, *lanes 2–6*) were used for this purpose to help determine the specific defects in PspF(T86S)-(1–275).  $\sigma^{54}$ (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 RNAP (data not shown). Overall, the results suggest that the *in vivo* properties of  $\sigma^{54}$ (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  $\sigma^{54}$  specifically allows the recovery of the activity of AAA activators containing only the Thr-to-Ser substitution in L1.

**The G4L Substitution in  $\sigma^{54}$  Region I Allows Remodeling of  $E\sigma^{54}$  by PspF(T86S)-(1–275)**—The *in vitro* transcription assays (Fig. 2) suggest that the G4L substitution does not merely contribute to an improved binding interaction between  $\sigma^{54}$  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  $E\sigma^{54}$ . We conducted the next set of experiments to directly investigate this. Previously, we described a simple native gel assembly assay to monitor ADP·AlF<sub>x</sub>-dependent and PspF-(1–275)-induced remodeling of  $E\sigma^{54}$  (13). This assay measures the ability of PspF-(1–275) to confer upon  $E\sigma^{54}$  the ability to bind a mutant heteroduplex promoter probe, referred to as the –12/–11-mut promoter probe (Fig. 1D), in an ADP·AlF<sub>x</sub>-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).  $E\sigma^{54}$  (and  $\sigma^{54}$ ) bound poorly to the –12/–11-mut promoter probe (Fig. 3D, *lane 1*). However, in the presence of PspF-(1–275) and ADP·AlF<sub>x</sub>,  $E\sigma^{54}$  (and  $\sigma^{54}$ ) formed a stable complex on the –12/–11-mut promoter probe (Fig. 3D, *lane 2*). In the presence of ADP·AlF<sub>x</sub>, PspF(T86S)-(1–275) did not confer upon wild-type  $E\sigma^{54}$  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  $E\sigma^{54}$  to be remodeled by PspF(T86S)-(1–275) in the presence of ADP·AlF<sub>x</sub> so that it can then bind to the –12/–11-mut promoter probe. As shown in Fig. 3D,  $E\sigma^{54}$ (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·AlF<sub>x</sub>-dependent manner. Thus, it appears that the G4L substitution in Region I of  $\sigma^{54}$  not only simply facilitates an interaction between  $E\sigma^{54}$  and PspF(T86S)-(1–275), but also allows  $E\sigma^{54}$  to undergo PspF(T86S)-(1–275)-induced conformational changes in an ADP·AlF<sub>x</sub>-dependent manner.





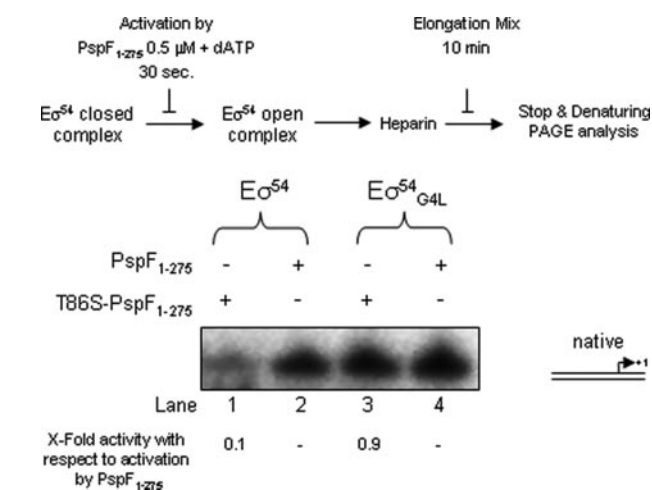


FIGURE 2. Autoradiograph of a denaturing gel showing the abortive transcription from open complexes formed by  $E\sigma^{54}$  (lanes 1 and 2) and  $E\sigma^{54}(G4L)$  (lanes 3 and 4) in response to activation by wild-type PspF(1–275) (lanes 2 and 4) and PspF(T86S)-(1–275) (lanes 1 and 3). Open complexes were formed on the native promoter probe. The amount of abortive transcripts synthesized in reactions containing PspF(T86S)-(1–275) with respect to reactions containing wild-type PspF(1–275) is given at underneath the gel. A reaction schematic is shown on top of the gel. The elongation mixture contained 100  $\mu$ g/ml heparin, 4  $\mu$ Ci of [ $\alpha$ - $^{32}$ P]GTP, and 0.5 mM GTP. The reaction components in each lane are indicated.

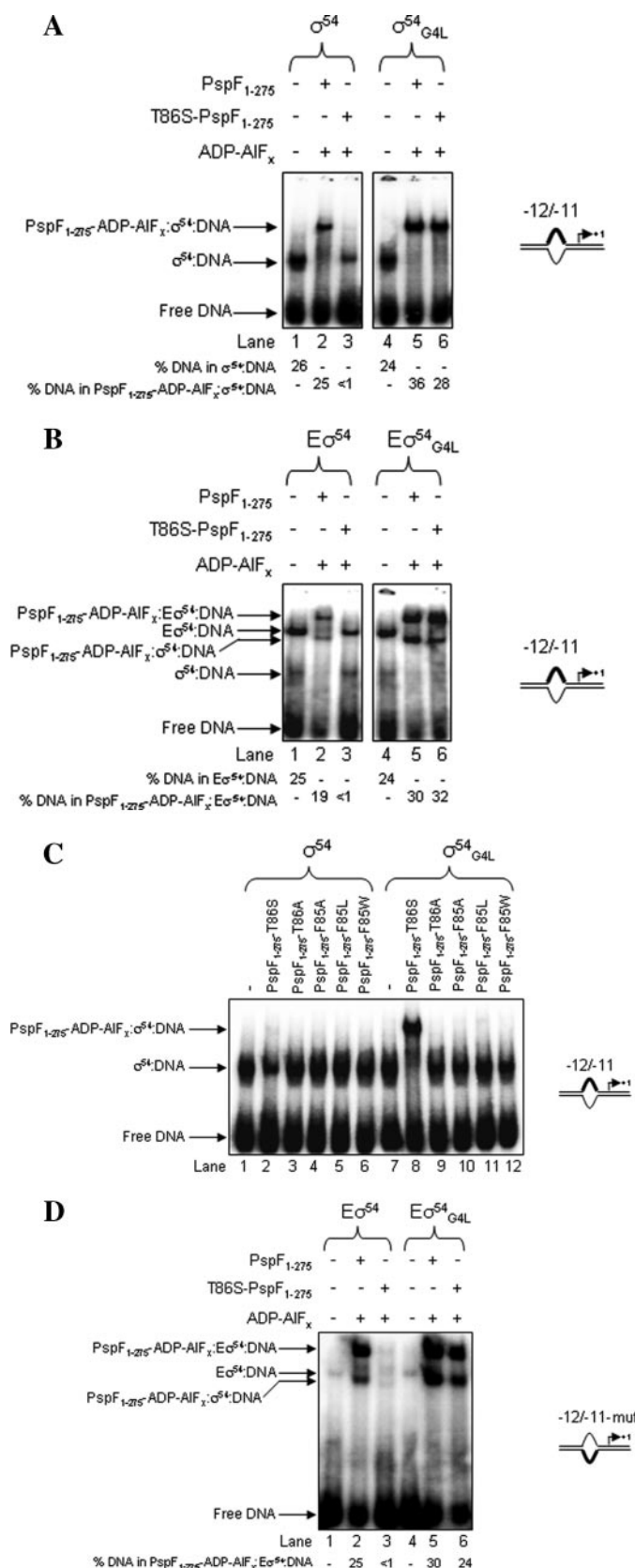
**Recovery of the Transcription Activity of PspF(T86S)-(1–275) by  $\sigma^{54}(G4L)$  Is Dependent on Promoter DNA Structure**—To determine the molecular basis by which the G4L substitution in  $\sigma^{54}$  Region I recovers the activity of PspF(T86S)-(1–275), we repeated the abortive transcription initiation assay using a heteroduplex promoter DNA probe. This heteroduplex probe contained a heteroduplex segment between positions –10 and –1 with respect to the transcription start site at +1 and so stably mimicked the conformation of the promoter DNA in the open complex (Fig. 1D). Our aim was to investigate whether the T86S substitution negatively influences stable DNA opening by  $E\sigma^{54}$ . As shown in Fig. 4 (lanes 2 and 4), the use of the –10/–1 probe did not allow PspF(T86S)-(1–275) to significantly overcome its activation defect, suggesting that the molecular basis for its activation defect involves steps prior to engagement of  $E\sigma^{54}$  with melted DNA. Interestingly, however, the recovery of PspF(T86S)-(1–275) activity by  $\sigma^{54}(G4L)$ , as seen on the native promoter probe (Fig. 4, compare lanes 2 and 6), was not readily evident on the –10/–1 promoter probe. As shown in Fig. 4, regardless of the type of  $E\sigma^{54}$  used, ~60–70% less abortive transcripts were synthesized when abortive initiation was stimulated by PspF(T86S)-(1–275) in reactions containing the –10/–1 promoter probe (compare lanes 4 and 8). We next investigated whether PspF(T86S)-(1–275) can form the ternary complex (in the presence of ADP·AlF<sub>x</sub>) with  $E\sigma^{54}$  and

$E\sigma^{54}(G4L)$  bound to the –10/–1 probe. As expected, PspF(T86S)-(1–275) was able to form ternary complexes in reactions containing only  $E\sigma^{54}(G4L)$  (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  $\sigma^{54}(G4L)$  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  $\sigma^{54}(G4L)$  to recover the activity of PspF(T86S)-(1–275) to form the ADP·AlF<sub>x</sub>-dependent ternary complex.

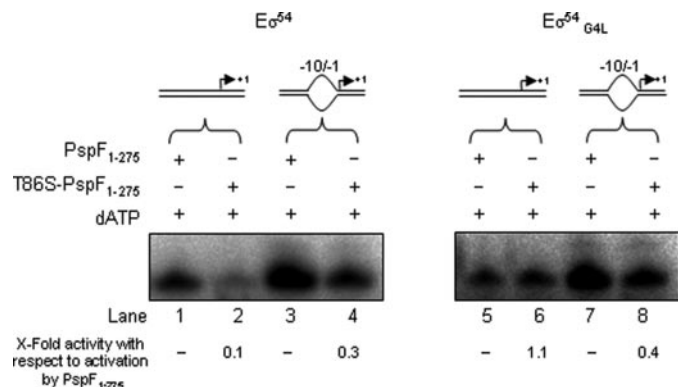
**PspF(T86S)-(1–275) Can Interact with  $E\sigma^{54}$  in the Absence of Promoter DNA**—The results so far demonstrate that the G4L mutation in Region I of  $\sigma^{54}$  allows the recovery of the defective property of PspF(T86S)-(1–275), but only in a restricted range of functional assays. Notably, 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  $E\sigma^{54}(G4L)$  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·AlF<sub>x</sub>-dependent interaction between  $E\sigma^{54}$  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  $E\sigma^{54}$  was able to interact with wild-type PspF(1–275) and PspF(T86S)-(1–275) as well as  $E\sigma^{54}(G4L)$ . Because  $\sigma^{54}$  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  $\sigma^{54}$  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  $\sigma^{54}(G4L)$  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  $\sigma^{54}$  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)

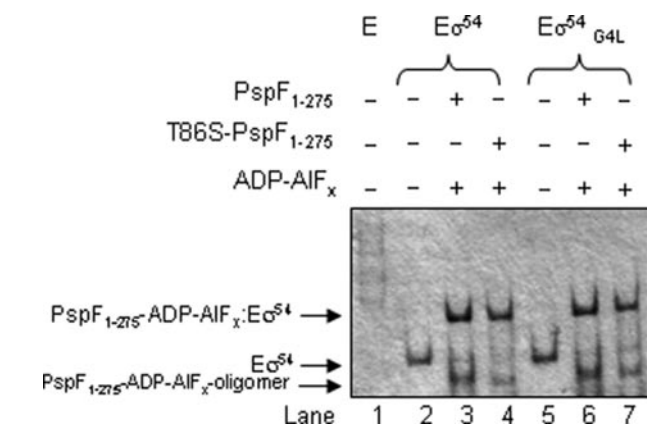
FIGURE 1. A, schematic showing the domain organization of *K. pneumoniae*  $\sigma^{54}$ . The regions associated with various activities are indicated. An alignment of amino acid sequences of the region that was subjected to mutagenesis is shown, and the residues mutated (Gly<sup>4</sup> and Gln<sup>11</sup>) are marked with asterisks. Kp, *K. pneumoniae*; Ec, *E. coli*; St, *Salmonella typhimurium*; Pa, *Pseudomonas aeruginosa*; Av, *Azotobacter vinelandii*; HTH, helix-turn-helix. B, schematic showing the domain organization of the *E. coli* PspF protein. The locations of the conserved L1 and L2 loops are shown. An alignment of amino acid sequences corresponding to L1 from five  $E\sigma^{54}$ -dependent AAA activators is shown. The conserved GAFTGA motif is boxed, and the invariant threonine residue (Thr<sup>86</sup> in PspF) is shown in boldface. Sm, *S. meliloti*; Bj, *B. japonicum*; Pp, *Pseudomonas putida*. C, crystal structure of the AAA domain of the *E. coli* PspF(1–275) hexamer (left). The boxed region of the green PspF(1–275) monomer is magnified (right). L1 is circled, and the arrow points to Thr<sup>86</sup>. D, *S. meliloti* *nifH* promoter DNA probes used in this study. The highly conserved dinucleotides at positions –14 (GC) and –26 (GG) are boxed and indicate the  $\sigma^{54}$  recognition motif. Shown in boldface and italics are non-complementary DNA sequences that were used to generate the heteroduplex promoter probes (see “Experimental Procedures”). A symbolic representation of each promoter probe is shown on the right.



**FIGURE 3.  $E\sigma^{54}$ (G4L) mutant enables PspF(T86S)-(1-275) mutant activator to interact with the closed promoter complex in an ADP-AIF<sub>x</sub>-dependent manner.** A, autoradiograph of a native gel showing ADP-AIF<sub>x</sub>-dependent binding of wild-type PspF(1-275) and PspF(T86S)-(1-275) to promoter complexes reconstituted with either wild-type  $\sigma^{54}$  or  $\sigma^{54}$ (G4L) using the -12/-11 probe. The migration positions of ADP-AIF<sub>x</sub>-dependent



**FIGURE 5. Native gel (stained with Coomassie Brilliant Blue) showing ADP-AIF<sub>x</sub>-dependent complex formation between  $E\sigma^{54}$  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-AIF<sub>x</sub>-dependent PspF(1-275)- $E\sigma^{54}$ ,  $E\sigma^{54}$ , and ADP-AIF<sub>x</sub>-dependent PspF(1-275)-oligomer are indicated.

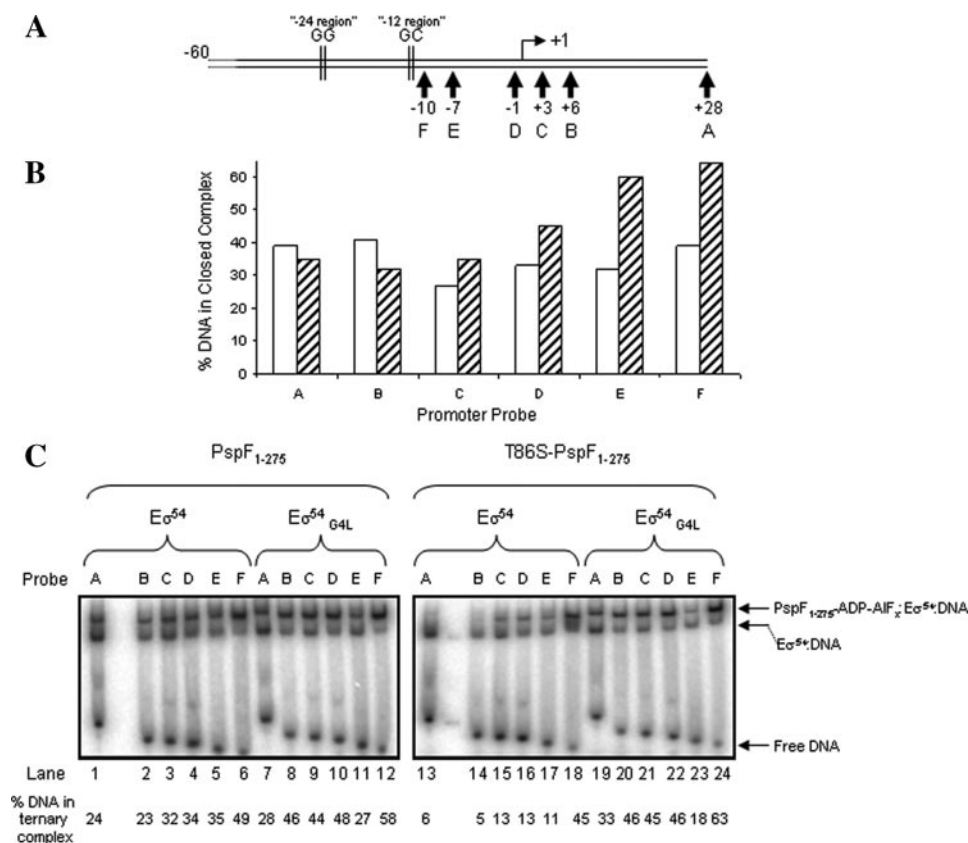


**FIGURE 5. Native gel (stained with Coomassie Brilliant Blue) showing ADP-AIF<sub>x</sub>-dependent complex formation between  $E\sigma^{54}$  and PspF(1-275).** The reaction components in each lane are indicated. Unbound PspF(1-275) ran off the gel under the experimental conditions used here. The migration positions of ADP-AIF<sub>x</sub>-dependent PspF(1-275)- $E\sigma^{54}$ ,  $E\sigma^{54}$ , and ADP-AIF<sub>x</sub>-dependent PspF(1-275)-oligomer are indicated.

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  $E\sigma^{54}$  closed complex, but not with  $E\sigma^{54}$  *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  $E\sigma^{54}$  closed complex. We therefore constructed promoter probes (probes A-F) in which we systematically moved the downstream DNA end points (Fig. 6A). The end points were chosen based on DNA positions to which PspF(1-275) can be cross-linked within ADP-AIF<sub>x</sub>-de-

PspF(1-275)- $\sigma^{54}$ -DNA,  $\sigma^{54}$ -DNA, and unbound (free) DNA are indicated. The percent promoter probe in the ADP-AIF<sub>x</sub>-dependent PspF(1-275)- $\sigma^{54}$ -DNA and  $\sigma^{54}$ -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 RNAP. 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).





**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 Eo<sup>54</sup> (open bars) and Eo<sup>54</sup>(G4L) (hatched bars). C, autoradiograph of a native gel showing ternary complex (ADP·AlF<sub>x</sub>-dependent PspF-(1-275)·Eo<sup>54</sup>·DNA) formation on probes A–F by Eo<sup>54</sup> and Eo<sup>54</sup>(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<sub>x</sub>-dependent PspF-(1-275)·Eo<sup>54</sup>·DNA, Eo<sup>54</sup>·DNA, and unbound (free) DNA are indicated. The percent DNA in the ternary complex is shown underneath the gels.

pendent ternary complexes (22).<sup>6</sup> Native PAGE analysis revealed that Eo<sup>54</sup> bound probes A–F equally well (Fig. 6B). Similarly, Eo<sup>54</sup>(G4L) bound probes A–D as well as wild-type Eo<sup>54</sup>. However, Eo<sup>54</sup>(G4L) revealed ~2-fold higher affinity for probes E and F compared with wild-type Eo<sup>54</sup> (Fig. 6B). Next, we measured the ability of Eo<sup>54</sup> and Eo<sup>54</sup>(G4L) closed complexes formed on probes A–F to interact with wild-type PspF-(1-275) in an ADP·AlF<sub>x</sub>-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 Eo<sup>54</sup> and Eo<sup>54</sup>(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 Eo<sup>54</sup> (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 Eo<sup>54</sup>(G4L) confirmed that the G4L mutation in Region I of  $\sigma^{54}$  is able to rescue the ability of

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 Eo<sup>54</sup> 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 Eo<sup>54</sup>(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  $\sigma^{54}$ -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  $\sigma^{54}$  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

<sup>6</sup> P. C. Burrows, S. R. Wigneshweraraj, and M. Buck, unpublished data.

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  $E\sigma^{54}$ -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  $E\sigma^{54}$  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 GAFTGA 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  $E\sigma^{54}$  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  $E\sigma^{54}$  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 results here suggest that the absence of DNA downstream of position –10 permits PspF(T86S)-(1–275) to directly interact with the regulatory center. The characterization of  $\sigma^{54}$ (G4L) did not reveal any obvious (activation-independent) gain of single-stranded DNA-binding properties (in the context of the holoenzyme) compared with wild-type  $\sigma^{54}$ . Thus, we propose that the G4L mutation most likely somehow alters the conformation of the regulatory center and thereby enables PspF(T86S)-(1–275) to interact and communicate with the regulatory center independently of DNA downstream of position –10 or with a binding energy that overcomes the negative effects of 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)· $\sigma^{54}$  complex suggested that at least two GAFTGA motifs interact with  $\sigma^{54}$ . 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 also sensing/communicating, via/to  $\sigma^{54}$  Region I, the different conformation adopted by the regulatory center) during successive rounds of ATP hydrolysis to allow the regulatory center to proceed through the range of conformational changes required for progression to an open complex formation. Overall, the results presented here highlight the level of regulatory precision and complexity that operates during transcription activation on  $\sigma^{54}$ -dependent promoters.

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

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