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Transcription Initiation-Defective Forms of σ^{54} That Differ in Ability To Function with a Heteroduplex DNA Template

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Transcription by σ^{54}-RNA polymerase holoenzyme requires an activator that catalyzes isomerization of the closed promoter complex to an open complex. We examined mutant forms of *Salmonella enterica* serovar Typhimurium σ^{54} that were defective in transcription initiation but retained core RNA polymerase- and promoter-binding activities. Four of the mutant proteins allowed activator-independent transcription from a heteroduplex DNA template. One of these mutant proteins, L124P V148A, had substitutions in a sequence that had not been shown previously to participate in the prevention of activator-independent transcription. The remaining mutants did not allow efficient activator-independent transcription from the heteroduplex DNA template and had substitutions within a conserved 20-amino-acid segment (Leu-179 to Leu-199), suggesting a role for this sequence in transcription initiation.

Bacterial RNA polymerase holoenzyme consists of a core enzyme ($\alpha_2\beta\beta'$) and a dissociable σ subunit. Bacteria often contain multiple σ factors with individual sequence specificities that direct holoenzyme to different classes of promoters (22). σ^{54} is a distinctive bacterial sigma factor that does not share significant sequence homology with other sigma factors. Transcription initiation by σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme) occurs through a distinct mechanism that involves an activator or enhancer-binding protein (3). σ^{54}-Holoenzyme binds to promoters to form stable closed complexes. Isomerization of these closed complexes to transcriptionally active open complexes requires an enhancer-binding protein, which generally binds to sites upstream of the promoter and contacts σ^{54}-holoenzyme through DNA looping. This isomerization requires nucleoside triphosphate hydrolysis by the enhancer-binding protein.

Deletion analysis of σ^{54} has revealed that the protein consists of at least three functional regions (7, 16, 26, 32, 33). The 50 amino-terminal residues of σ^{54} constitute region I, which is rich in glutamine and leucine residues and plays a direct role in transcriptional activation (16, 17). Region II of σ^{54} from enteric bacteria, which extends from residue 50 to residue 120 and is highly acidic, appears to influence the rate of open complex formation, as well as suppress nonspecific DNA binding by holoenzyme (4, 32). Region III consists of 360 carboxy-terminal residues and contains determinants for binding of both core RNA polymerase and promoter DNA (7, 27, 28, 33). Additional functions for region III are likely, as some substitutions in this region disrupt one or more steps in transcription initiation following initial promoter recognition (11, 18).

When region I of σ^{54} is deleted, the resulting holoenzyme assumes a conformation believed to reflect polymerase isomerization (6). This isomerization is inhibited by a peptide containing the region I sequence, indicating that region I can elicit its effects on holoenzyme in *trans* (6, 14). Holoenzyme containing σ^{54} with region I deleted fails to initiate transcription under normal conditions but can initiate transcription independently of enhancer-binding protein from a transiently melted or premelted DNA template (6, 29). These studies suggest that region I inhibits isomerization of the holoenzyme to a form that can stably associate with single-stranded DNA (4). Productive interaction with the enhancer-binding protein apparently relieves this inhibition and permits transcription initiation in a reaction that requires nucleotide hydrolysis by the enhancer-binding protein (14).

We previously isolated several mutant forms of *Salmonella enterica* serovar Typhimurium σ^{54} that were defective in transcription initiation but retained core- and DNA-binding activities (18). Further analysis of these mutant σ^{54} proteins, the results of which are presented here, revealed that certain amino acid substitutions in regions I and III allowed the holoenzyme to initiate transcription from a heteroduplex DNA template in the absence of enhancer-binding protein, suggesting that sequences within these regions influence isomerization of the holoenzyme. The remaining σ^{54} mutant proteins had amino acid substitutions within region III and represented a second class of mutants that did not allow efficient transcription from the heteroduplex template in the absence of enhancer-binding protein. The strains and plasmids used in this study are described in Table 1.

L179P retains core- and promoter-binding activities. Several mutant forms of σ^{54} were isolated previously that failed to function normally at the σ^{54}-dependent *glnA* promoter (*glnA*p2) but retained promoter-binding activities (20). We included here a new σ^{54} mutant protein, L179P, that was identified using the same screening procedure following random mutagenesis of a plasmid-borne copy of *ntrA* (encodes σ^{54}) with the Epicurian Coli XL1-Red strain (Stratagene, La Jolla, Calif.). We previously used a gel mobility shift assay with labeled oligonucleotides that corresponded to the -9 to -29 region of the *Sinorhizobium meliloti nifH* promoter to examine interactions between the mutant forms of σ^{54} and core RNA polymerase, as well as the binding of the mutant proteins to promoter sequences (20). One oligonucleotide was double stranded over its entire length (double-stranded probe), while in the second oligonucleotide residues -11 to -9 of the template strand were single stranded (fork junction probe). Guo and Gralla first demonstrated fork junction DNA-binding ac-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
S. enterica serovar Typhimurium		
TRH107	Δ(*prt proAB*)-*ataP*::[(P22 *int3 c2-ts29*) *sieA44 mnt*::Kn9 P_{nifH} *arc*(Am) *H1605 ant′-′lacZYA* Δ*9-al*] *ntrA209*::Tn*10*	1
TRH134	*leu-414*(Am) *hsdL*(r^-m^+) $Fels^-$ Δ*ntrA8455*	18
E. coli YMC11	*endA1 thi-1 hsdK17 supR44* Δ*lac-169 hutC$_K$* Δ*glnALG2000 ntrA*::Tn*10*	2
Plasmids		
pALTER-1	Ap^s Tc^r	Promega
pJES534	pTZ19U *S. enterica* serovar Typhimurium *glnA* promoter regulatory region	24
pL143	pUC13 P_{lac}-*S. meliloti dctD*$_{(\Delta 1–142)}$	19
pRKRMAZ:+UAS	pRK290 *S. meliloti dctA′-′lacZYA*	19
pTRH17	pACYC184 P_{lac}-*dctD*$_{(\Delta 1–142)}$	This study
pMK8	pACYC184 *dctA′-′lacZYA* P_{lac}-*dctD*$_{(\Delta 1–142)}$	This study
pSA4	pCyt-3 P_{lac}-*ntrA lacI^q*	1
pMK11	pALTER-1 Ap^r Tc^r P_{lac}-*ntrA*	18
pNTRA1	pSA4 *ntrA* allele (L37P)	18
pNTRA2	pSA4 *ntrA* allele (L46P)	18
pNTRA4	pSA4 *ntrA* allele (L333P)	18
pNTRA5	pSA4 *ntrA* allele (E32K G189V)	18
pNTRA6	pSA4 *ntrA* allele (L124P V148A)	18
pNTRA18	pSA4 *ntrA* allele (L199P D231G)	18
pNTRA30	pSA4 *ntrA* allele (L179P)	This study
pWA328	pMK11 *ntrA* allele (W328A)	This study
pKA331	pMK11 *ntrA* allele (K331A)	This study
pRA336	pMK11 *ntrA* allele (R336A)	This study
pRA342	pMK11 *ntrA* allele (R342A)	This study
pQA351	pMK11 *ntrA* allele (Q351A)	This study

tivity of σ^{54} using these probes (15), and we subsequently found that the σ^{54}-holoenzyme bound the fork junction probe significantly better than it did the double-stranded probe (18).

We examined the binding of the L179P-holoenzyme to the fork junction and double-stranded probes. As observed with the wild-type holoenzyme, the L179P-holoenzyme shifted the fork junction probe more efficiently than the double-stranded probe (Fig. 1). L179P produced less of the holoenzyme-shifted species than wild-type σ^{54}, suggesting that this mutant protein had reduced affinity for either core RNA polymerase or fork junction DNA. These gel mobility shift data, however, demonstrate that like the other mutant forms of σ^{54} described previously (20), L179P still binds the core and directs the holoenzyme to promoter sequences.

Mutant forms of σ^{54} can be cross-linked to DctD. *S. enterica* serovar Typhimurium σ^{54} can be chemically cross-linked to the enhancer-binding protein *S. meliloti* C_4-dicarboxylic acid transport protein D (DctD), suggesting that σ^{54} is a primary target for the enhancer-binding protein (20). Consistent with this hypothesis, mutant forms of DctD that failed to activate transcription and cross-linked poorly to σ^{54} have been isolated (31). Further support of this hypothesis comes from the observation that σ^{54} bound to heteroduplex DNA in the absence of core RNA polymerase undergoes a conformational change in a reaction that requires enhancer-binding protein and nucleotide hydrolysis (8).

We wished to determine if any of the σ^{54} mutant proteins were deficient in cross-linking to DctD and therefore altered in the ability to make productive contact with the enhancer-binding protein. Since the σ^{54} mutant proteins were isolated originally based on their failure to function with nitrogen regulatory protein C (NtrC) at *glnA*p2 in vivo (18), we first verified that these mutant proteins were also defective in functioning with DctD in vivo. For these assays, we used DctD$_{(\Delta 1–142)}$, which is a truncated, constitutively active form of the protein that lacks the first 142 amino-terminal residues (21). We examined the ability of the mutant forms of σ^{54} to function with DctD$_{(\Delta 1–142)}$ in activating transcription from a plasmid-borne

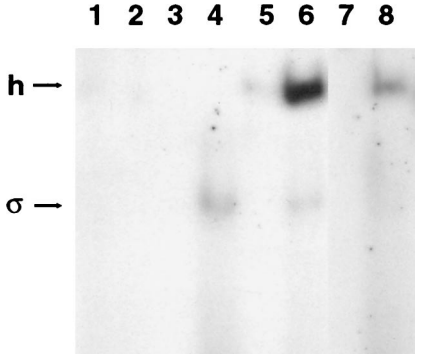

FIG. 1. Gel mobility shift assay with double-stranded and fork junction probes. Binding of mutant forms of σ^{54}-holoenzyme to the *S. meliloti nifH* promoter was analyzed by a gel mobility shift assay as previously described (18). Two different DNA probes were used for the gel mobility shift assays. One probe contained 21 bp of double-stranded DNA that included residues −9 through −29 of the *nifH* promoter. The second probe had 18 bp of double-stranded DNA, which included residues −12 through −29 of the *nifH* promoter plus a three-base 5′ overhang of the template strand that corresponded to residues −9 through −11. Binding reaction mixtures contained 5 nM DNA probe and 6 μg of sonicated calf thymus DNA/ml along with 300 nM core RNA polymerase (lanes 1 and 2), 600 nM hexahistidine-tagged σ^{54} (lanes 3 and 4), 300 nM core plus 600 nM hexahistidine-tagged σ^{54} (lanes 5 and 6), and 300 nM core plus 600 nM hexahistidine-tagged L179P (lanes 7 and 8). Core RNA polymerase and histidine-tagged σ^{54} proteins were purified as previously described (20, 23). The double-stranded probe was used in odd-numbered lanes, while the fork junction probe was used in even-numbered lanes. The holoenzyme-shifted (h) and σ^{54}-shifted (σ) species are indicated. Unbound probes are not shown.

TABLE 2. Abilities of mutant forms of σ^{54} to function with $DctD_{(\Delta 1-142)}$ at a *dctA'-'lacZ* reporter gene in vivo

σ^{54} protein	Transcriptional activation from *dctA'-'lacZ* reporter gene	
	β-Galactosidase activity (Miller units)[a]	% Wild-type activity
L37P	1,670 ± 23	10
L46P	3,130 ± 210	19
L179P	301 ± 33	1.8
L333P	310 ± 26	1.9
E32K G189V	555 ± 16	3.4
L124P V148A	136 ± 2.4	0.8
L199P D231G	294 ± 10	1.8
Wild type	16,450 ± 370	100

[a] β-Galactosidase activities were determined in *E. coli* strain YMC11 containing the *dctA'-'lacZ* reporter plasmid pMK8 along with plasmids carrying the mutant *ntrA* alleles.

dctA'-'lacZ reporter gene in *Escherichia coli* strain YMC11, which otherwise lacks σ^{54}. DctD-mediated transcriptional activation with the mutant forms of σ^{54} ranged from 1 to 19% of the activity achieved with wild-type σ^{54} (Table 2). These values were consistent with the activities observed for the mutant σ^{54} proteins with NtrC at *glnA*p2 in vivo (18).

Each of the mutant forms of σ^{54} was purified and examined for the ability to cross-link to $DctD_{(\Delta 1-142)}$ using succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate as the cross-linking reagent as described previously (20). All of the mutant σ^{54} proteins cross-linked efficiently to $DctD_{(\Delta 1-142)}$ (data not shown), indicating that none of them were defective in gross interactions with this enhancer-binding protein. Since the cross-linking assay provides only a qualitative assessment of interactions between σ^{54} and DctD, we cannot rule out the possibility that some of the mutant proteins had reduced affinities for DctD or that some of the substitutions in these mutant proteins interfered with specific contacts between σ^{54} and DctD required for transcriptional activation.

Certain mutant forms of σ^{54} allow activator-independent transcription. We initially used an in vitro transcription assay to examine the abilities of the σ^{54} mutant proteins to support transcription from a supercoiled template that carried the *S. enterica* serovar Typhimurium *glnA* promoter region. In this transcription assay, heparin was added immediately before the nucleotides to prevent reinitiation but allow transcription from stable open complexes. None of the σ^{54} mutant proteins supported transcription under these conditions, except the L46P mutant protein, which had given the highest activity in vivo (Table 2). The level of transcript generated with the L46P mutant protein was ~5% of that generated with wild-type σ^{54} (Fig. 2). Addition of heparin to the transcription assay reaction after addition of the nucleotides did not alter the results of the assays with the mutant proteins (data not shown). These data confirmed our earlier in vivo findings that these mutant σ^{54} proteins were defective in their function at *glnA*p2 (18).

Previous studies demonstrated that when region I of σ^{54} is deleted, the resulting holoenzyme can initiate transcription in the absence of enhancer-binding protein from a premelted, heteroduplex DNA template (6). We used this linear, heteroduplex template to determine if any of the mutant σ^{54} proteins would allow activator-independent transcription. The template spanned nucleotides −60 to +28 of the *S. meliloti nifH* promoter, and the DNA strands from −10 to −1 were noncomplementary (6).

The wild-type σ^{54}-holoenzyme was only able to initiate transcription from the heteroduplex template weakly (Fig. 3, lane 1). Likewise, holoenzymes formed with the L179P mutant protein, the L199P D231G double-mutant protein, or the E32K G189V double-mutant protein initiated transcription from the heteroduplex template poorly. In contrast, holoenzymes containing L37P, L46P, L333P, or the double mutation L124P V148A initiated transcription efficiently from the heteroduplex template (Fig. 3, lanes 2 to 4 and 8). Amino acid substitutions in σ^{54} that result in activator-independent transcription have been described previously (10, 11, 26, 29), with the substitutions in these mutant proteins occurring primarily within a leucine-rich patch of region I and a very limited number of sites in region III. In general, these previous activator-independent mutant proteins retained significant activity in vivo. In contrast, holoenzymes with L37P, L333P, and the double mutation L124P V148A were severely impaired in the ability to function in vivo and in that regard are more like region I deletion mutant enzymes which do not support transcription initiation from homoduplex DNA templates (8). This result is not surprising, however, given that the mutant proteins in this study were chosen based on their failure to function at *glnA*p2 in vivo (18).

Alanine scanning mutagenesis of the region around Leu-333. The similarities between the L37P and L333P mutant proteins in the ability to support activator-independent tran-

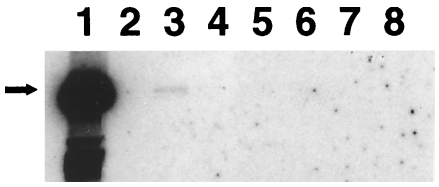

FIG. 2. In vitro transcription with mutant forms of σ^{54} from the *glnA* promoter regulatory region on a supercoiled DNA template. Transcription assays were carried out as described previously (24, 31). Assay reaction mixtures contained 400 nM maltose-binding protein–NtrC, 20 mM carbamoyl phosphate, 100 nM core RNA polymerase, and a 300 nM concentration of either hexahistidine-tagged σ^{54} (lane 1), L37P (lane 2), L46P (lane 3), E32K G189V (lane 4), L124P V148A (lane 5), L179P (lane 6), L199P D231G (lane 7), or L333P (lane 8). The arrow indicates the transcript from the *glnA* promoter.

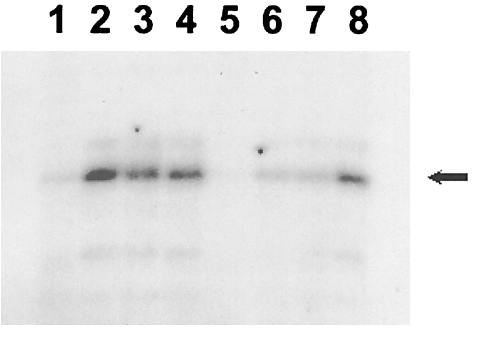

FIG. 3. In vitro transcription assays using a heteroduplex DNA template. Transcription assays were carried out using a heteroduplex DNA template that spanned residues −60 to +28 of the *S. meliloti nifH* promoter and was noncomplementary between residues −10 and −1. The heteroduplex template was generated using two oligonucleotides as previously described (6). Transcription assays were conducted as previously described (6), except that 7.5 μCi of [α-^{32}P]CTP (3,000 Ci/mmol) was used to label the transcripts. Reaction mixtures contained 100 nM core RNA polymerase, 300 nM hexahistidine-tagged σ^{54} proteins, and 30 nM heteroduplex DNA template. The σ^{54} proteins assayed were the wild type (lane 1) and the L37P (lane 2), L46P (lane 3), L333P (lane 4), E32K G189A (lane 5), L179P (lane 6), L199P D231G (lane 7), and L124P V148A (lane 8) mutant proteins. The arrow indicates the 28-base transcript.

scription (Fig. 3) and the reduced affinity for fork junction DNA (18) prompted us to examine conserved residues in the vicinity of Leu-333. Leu-333 lies within a well-conserved region of σ^{54} that was shown previously to be cross-linked to DNA upon UV irradiation of σ^{54}-promoter complexes (5). It is also close to a putative helix-turn-helix motif that has been implicated in recognition of the −12 region of the promoter (12, 23). When we compared the sequences of this portion of σ^{54} proteins from 25 different bacteria, we noted that it was similar to a motif from single-stranded DNA-binding proteins (Fig. 4A). This motif from single-stranded DNA-binding proteins, (R/K)-N_{4-5}-(K/R)-N_{4-6}-(Y/F)-N_{5-8}-(Y/F/N)-N_{6-9}-(Y/C)-N_9-(E/D)-N_{7-9}-(Y/W)-N_{4-8}-(Y/F/E)-N_{11-12}-(R/K), is thought to stabilize the binding of single-stranded DNA by stacking interactions between aromatic residues and bases and electrostatic interactions between basic residues and phosphate groups of single-stranded DNA (25).

We introduced alanine substitutions at five conserved positions near Leu-333 by site-directed mutagenesis as described previously (18). Substitutions were introduced at Trp-328, Lys-331, Arg-336, Arg-342, and Gln-351. Two of these residues, Arg-336 and Arg-342, are located within the region of the protein that can be cross-linked to promoter DNA and are also part of the sequence that resembles the single-stranded DNA-binding motif. Gln-351 occurred in all 25 of the σ^{54} protein sequences that we compared, while Trp-328 occurred in 20 of these sequences and lysine or arginine occurred at position 331 in 20 of these sequences. During the course of our experiments, Chaney and Buck (11) introduced alanine substitutions at Trp-328, Arg-336, and Arg-342 in *Klebsiella pneumoniae* σ^{54} and reported that the R336A mutation allowed activator-independent transcription.

The alanine substitution mutant proteins that we generated were overexpressed at comparable levels, and each conferred glutamine prototrophy on an *S. enterica* serovar Typhimurium strain that lacked σ^{54} (strain TRH134), indicating that these mutant forms of σ^{54} could function with NtrC at *glnA*p2 (data not shown). Glutamine prototrophy is not a quantitative indicator of the functionality of σ^{54} mutant proteins, so we examined the abilities of these mutant proteins to initiate transcription from *glnA*p2 by measuring glutamine synthetase activities. Strains expressing the alanine substitution mutant proteins had glutamine synthetase activities that were comparable to that of a strain that expresses wild-type σ^{54} (Fig. 4B).

We also assessed the DNA-binding activities of the alanine substitution mutant proteins by examining their abilities to repress the transcription of an *ant'-'lacZ* reporter gene in vivo. For these assays, we used an *S. enterica* serovar Typhimurium strain (TRH107) that contained a partially deleted P22 prophage bearing an *ant'-'lacZ* reporter gene in which the *S. meliloti nifH* promoter overlapped the *ant* promoter (1). Binding of the σ^{54}-holoenzyme to the *nifH* promoter in the prophage represses transcription from the *ant'-'lacZ* reporter gene (1, 18). Overexpression of wild-type σ^{54} repressed transcription from the *ant'-'lacZ* reporter gene about sevenfold (Fig. 4C). With the exception of R336A, the mutant σ^{54} proteins repressed transcription from the *ant'-'lacZ* reporter gene as well as or better than wild-type σ^{54}. Interestingly, the R336A mutant protein, which functioned as well as wild-type σ^{54} from *glnA*p2, only repressed transcription from the *ant'-'lacZ* reporter by ~25%, suggesting that the R336A-holoenzyme had reduced affinity for promoter sequences. Chaney and Buck (11) similarly observed that the *K. pneumoniae* σ^{54} R336A mutant protein had reduced affinity for the *nifH* promoter but supported ~70% of the level of expression from a *glnA*p2-*lacZ* reporter gene that was achieved with wild-type σ^{54}. Arg-336 is in a region of the protein that can be cross-linked to DNA following UV irradiation of σ^{54}-promoter complexes (5), and it has been proposed that Arg-336 participates in a σ^{54}-DNA interaction that helps keep the closed complex from undergo-

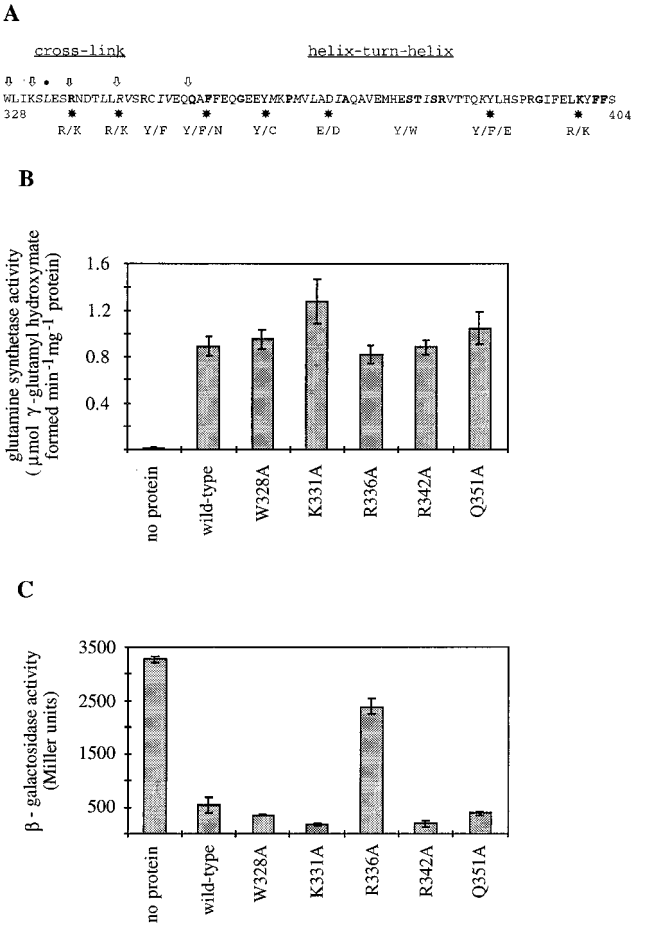

FIG. 4. Alanine scanning mutagenesis of conserved residues near Leu-333. (A) Amino acid sequence of the σ^{54} protein from *S. enterica* serovar Typhimurium near Leu-333. The PILEUP program was used to compare sequences around Leu-333 from σ^{54} proteins from 25 bacteria. The sequence shown spans residues 328 to 404. Leu-333 is marked with a dot over the sequence. Boldface letters indicate residues that are identical in at least 22 of the 25 σ^{54} protein sequences, and italic letters indicate residues that are similar in at least 22 of the σ^{54} protein sequences. Arrows indicate the residues where alanine substitutions were introduced. The stars indicate residues that align with the single-stranded DNA-binding motif, which is indicated on the bottom line. The sequence that is cross-linked to promoter DNA upon UV irradiation (5) is indicated, as is a putative helix-turn-helix motif that appears to be involved in DNA binding (23). (B) Glutamine synthetase activities observed with σ^{54} mutant proteins. Cells were grown in a modified E medium that lacked sodium ammonium phosphate and was supplemented with acid-hydrolyzed Casamino Acids at 1 mg/liter as described previously (18). Glutamine synthetase activities of strains that express the alanine substitution σ^{54} mutant proteins were carried out using the γ-glutamyl transferase assay as described previously (18). Glutamine synthetase activities were expressed as micromoles of γ-glutamyl hydroxymate per minute per milligram of protein, and all assays were done at least twice. Error bars show one standard deviation for each data set. The wild-type and mutant *ntrA* alleles, which were carried on plasmids, were under the control of the *E. coli lac* promoter-operator and were overexpressed by including 100 μM isopropyl-β-thiogalactopyranoside in the growth medium. The no-protein designation indicates the glutamine synthetase activity from *S. enterica* serovar Typhimurium strain TRH134 without a plasmid-borne *ntrA* allele. (C) Repression of *ant'-'lacZ* reporter gene in *S. enterica* serovar Typhimurium strain TRH107 by σ^{54} proteins. β-Galactosidase assays were done in triplicate with strain TRH107, carrying plasmids that encode the σ^{54} proteins indicated, as described previously (1, 18). Error bars show one standard deviation for each data set. The no-protein control shows the activity for strain TRH107 with no plasmid-borne *ntrA* allele.

ing isomerization in the absence of enhancer-binding protein (11).

Conclusions. Region I of σ^{54} functions as an intramolecular inhibitor that prevents isomerization of the closed complex to the open complex, but it also has a role in DNA melting in response to the enhancer-binding protein (6, 13, 29). Two of the mutants that were analyzed here, L37P and L46P, had single amino acid substitutions in region I and allowed activator-independent transcription from the heteroduplex template, indicating that these amino acid substitutions disrupt the inhibitory effect of region I on the holoenzyme. Leu-37 is within a small leucine-rich patch, residues Leu-25 through Leu-37, that was shown previously to be important for mediation of the inhibitory effect of region I on the holoenzyme (10, 26, 30). Substitution of arginine for Leu-37 in *E. coli* σ^{54} was shown previously to allow activator-independent transcription. The L37R mutant protein, however, remained responsive to enhancer-binding protein in vitro, allowing NtrC-mediated activation to about 50% of the level observed with wild-type σ^{54} (26). The L37P mutant protein described in this study appeared to be more severely impaired in the ability to respond to enhancer-binding protein, which likely reflects disruption of secondary structure in the protein by the proline substitution. Casaz and coworkers identified two other areas within region I, residues 15 to 17 and 42 to 47, that are important for inhibition of the conformational change in the holoenzyme and responsiveness to the activator (10). Consistent with these previous results, the L46P mutant enzyme was capable of activator-independent transcription from the heteroduplex template and also appeared to be impaired in the ability to respond to enhancer-binding protein, but not as severely as the L37P mutant protein.

Not all of the mutant σ^{54} proteins with amino acid substitutions in region I that we used in our study were capable of activator-independent transcription, as the E32K G189V double-mutant protein failed to activate transcription from the heteroduplex template. Since both substitutions in this double-mutant protein are needed for loss of function (18), we cannot rule out the possibility that the substitution of valine for Gly-189 interfered with the ability of the protein to initiate transcription from the heteroduplex template.

Holoenzymes formed with L37P or L333P were capable of efficient activator-independent transcription from the heteroduplex template (Fig. 3) and also had diminished fork junction DNA-binding activities (18). These observations suggest that these two σ^{54} mutant proteins stabilize the same conformation of holoenzyme, indicating that region I may function together with a sequence around Leu-333 in region III to prevent isomerization of the closed complex in the absence of enhancer-binding protein. We identified here a second sequence within region III that also appears to be important in keeping holoenzyme locked in the closed complex conformation prior to activation. Like the L333P mutant protein, the L124P V148A mutant protein allowed activator-independent transcription from the heteroduplex DNA template (Fig. 3). Unlike the L333P mutant protein, the L124P V148A mutant protein retained fork junction DNA-binding activity (18), suggesting that the L124P V148 mutant protein stabilizes a conformation of the holoenzyme that differs from that of the L333P-holoenzyme. Leu-124 and Val-148 lie within the minimal core-binding domain of σ^{54}, which spans residues 120 to 215, and Leu-124 is also located in a portion of the protein that is protected from hydroxyl radical cleavage by core RNA polymerase (9). It is possible that the sequence around Leu-124 interacts directly with core RNA polymerase to prevent isomerization of the closed complex prior to activation.

The remaining mutant forms of σ^{54} examined here, L179P, E32K G189V, and L199P D231G, did not allow efficient activator-independent transcription from the heteroduplex DNA template. Gel mobility shift assays showed that these mutant forms of σ^{54} retained core RNA polymerase and fork junction DNA-binding activities (18; Fig. 1). Taken together, these findings indicate that these mutant forms of σ^{54} represent a different class of mutant proteins. While both amino acid substitutions were required for loss of function in the two double-mutant proteins (18), all of these mutant proteins had an amino acid substitution within a well-conserved 20-amino-acid segment (Leu-179 to Leu-199) in the core-binding domain of σ^{54}. It is possible that this segment of σ^{54} participates in some function in transcription initiation following formation of the closed complex, such as mediation of signal transduction from the enhancer-binding protein.

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