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Mutant Forms of *Salmonella typhimurium* σ^{54} Defective in Transcription Initiation but Not Promoter Binding Activity

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Transcription initiation with σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme) has absolute requirements for an activator protein and ATP hydrolysis. σ^{54}'s binding to core RNA polymerase and promoter DNA has been well studied, but little is known about its role in the subsequent steps of transcription initiation. Following random mutagenesis, we isolated eight mutant forms of *Salmonella typhimurium* σ^{54} that were deficient in transcription initiation but still directed σ^{54}-holoenzyme to the promoter to form a closed complex. Four of these mutant proteins had amino acid substitutions in region I, which had been shown previously to be required for σ^{54}-holoenzyme to respond to the activator. From the remaining mutants, we identified four residues in region III which when altered affect the function of σ^{54} at some point after closed-complex formation. These results suggest that in addition to its role in core and DNA binding, region III participates in one or more steps of transcription initiation that follow closed-complex formation.

Association of the σ subunit with core RNA polymerase results in a holoenzyme that recognizes specific promoter sequences. Multiple σ factors within a bacterial cell allow the holoenzyme to recognize different classes of promoters (17, 20). Some σ factors are primary σ factors that are responsible for transcription of most of the genes in the cell (e.g., *Escherichia coli* σ^{70}), while others are alternative σ factors that are required for the expression of specific genes (20). In addition to binding core RNA polymerase and the promoter, σ factors have also been implicated in DNA melting, transcription pausing, and in some cases interactions with activator proteins (17–19, 22, 30). The majority of σ factors exhibit homology to σ^{70}, the only exception being an alternative σ factor, σ^{54} (23).

σ^{54}-RNA polymerase holoenzyme (σ^{54}-holoenzyme) is responsible for the expression of genes whose products are involved in diverse metabolic processes, such as nitrogen assimilation and fixation, dicarboxylic acid transport, pilin and flagellin synthesis, toluene and xylene catabolism, and hydrogen metabolism (23). σ^{54}-Holoenzyme binds to promoter elements in the −12 and −24 regions to form a closed complex but is unable to form a transcriptionally competent open complex in the absence of an activator protein (25, 28, 32). The activator binds to specific sites upstream of the promoter and makes transient contact with σ^{54}-holoenzyme through DNA looping (31, 34). Protein cross-linking studies suggest that the activator contacts σ^{54} and the β subunit of σ^{54}-holoenzyme during open-complex formation (19, 40). In addition to making productive contact with σ^{54}-holoenzyme, the activator must also hydrolyze ATP to activate transcription (28, 41).

The role of σ^{54} in transcriptional initiation following formation of the closed promoter complex is poorly understood. Previous mutational studies of σ^{54} that were performed to help resolve this issue focused on specific regions of the protein (11, 15, 16, 26, 35–37). In this study, we mutagenized the entire *ntrA* gene (which encodes σ^{54}) and isolated mutant forms of *Salmonella typhimurium* σ^{54} that were defective in transcription initiation but still directed holoenzyme to the promoter. We used a unique genetic screen to assess the ability of σ^{54} mutants to direct holoenzyme to a promoter that overlapped the phage P22 *ant* promoter and thereby repress transcription of an *ant′-′lacZ* reporter gene. Mutant forms of σ^{54} that retained promoter binding activity were very rare. After screening nearly 1,200 σ^{54} mutants that were defective in transcription initiation, we found only 8 mutants that repressed transcription of the *ant′-′lacZ* reporter gene.

MATERIALS AND METHODS

Media and chemicals. Luria-Bertani broth was used for routine culture growth unless otherwise noted. For a minimal medium, we used either E minimal medium (38) supplemented with 1 mg of acid-hydrolyzed Casamino Acids/liter or M9 minimal medium (24) that contained 10 mM L-arginine as the primary nitrogen source and 50 μM leucine (M9-arginine medium). MacConkey agar was obtained from Difco Laboratories. When L-glutamine was added, it was filter sterilized and then added to autoclaved medium to a final concentration of 5 mM. Ampicillin, chloramphenicol, kanamycin, and tetracycline were added to final concentrations of 200, 20, 50, and 6.5 μg/ml, respectively. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 100 μM.

Bacterial strains. BL21 (λDE3) [F^- *ompT* (*lon*) *hsdS*$_B$ *gal* λDE3:*lacI lacUV5*-gene *1* (T7 polymerase)] carrying plasmid pLysE, which bears the gene encoding T7 lysozyme, was used for overexpression of histidine-tagged σ^{54} proteins. TRH107 {Δ(*prt proAB*)*ataP*::[(*P22 int3 c2-ts29*) *sieA44 mnt*::*Kn9* P_{nifH} *arc*(Am) *H1605 ant′-′lacZYA Δ9-a1*] *ntrA209*::Tn*10*} is a deletion prophage that carries the *Sinorhizobium meliloti* (formerly *Rhizobium meliloti*) *nifH* promoter overlapping the promoter of an *ant′-′lacZ* fusion (1). TRH107 also carries a Tn*10* insertion in *ntrA*. TRH134 [*leu414*(Am) *hsdL*(r^- m^+) $Fels^-$ Δ*ntrA8455*] is a *ntrA* deletion strain that lacks codons 8 through 455 of *ntrA* and was constructed as follows.

Plasmid pMK10 is a derivative of pMAK705 (13) which carries the chloramphenicol acetyltransferase (*cat*) gene and is temperature sensitive for replication. It carries 250 and 750 bp of DNA that flank the 5′ and 3′ ends, respectively, of a truncated version of *S. typhimurium ntrA* lacking codons 8 through 455. Plasmid pMK10 was introduced into *S. typhimurium* MS1868 [*leuA414*(Am) *hsdSB*(r^- m^+) $Fels^-$] (10) by electroporation and maintained by growing the resulting transformant at 30°C. Allelic exchange between the partially deleted *ntrA* gene on pMK10 and the chromosomal copy of *ntrA* was carried out as described previously (13). A strain with a deletion in the chromosomal *ntrA* gene (TRH134) was identified by chloramphenicol sensitivity, indicating the loss of the plasmid, and glutamine auxotrophy, indicating the loss of a functional *ntrA*. To verify that the deletion had been introduced into the chromosomal copy of *ntrA*, oligonucleotide primers that flanked *ntrA* were used in a PCR to amplify this region from the chromosomal DNA of strain TRH134. The resulting PCR product was the predicted size for the deletion.

Plasmids. Plasmid pSA4 carries the *S. typhimurium ntrA* gene under the control of the *E. coli lac* promoter and operator along with *lacI*^{q} (1). Plasmid pMK10, which was used to construct TRH134, was made as follows. Plasmid pJES82 (27) carries the *S. typhimurium ntrA* gene along with 250 and 750 bp of flanking DNA at the 5′ and 3′ ends of *ntrA*, respectively. Plasmid pJES82 was

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digested with *Blp*I and *Asc*I, filled in with T4 DNA polymerase, and then religated to form pMK9. This resulted in the deletion of codons 8 through 455 of *ntrA*. A 1.2-kb *Eco*RI-*Hin*dIII fragment from pMK9, which had been filled in at the *Eco*RI site with T4 DNA polymerase, was cloned into the *Hin*cII and *Hin*dIII sites of pMAK705, resulting in plasmid pMK10. Plasmid pMK11, a derivative of pALTER-1 (Promega), was used for site-directed mutagenesis experiments. Plasmid pMK11 was constructed by cloning a 2.5-kb *Eco*RI-*Hin*dIII fragment from pSA4 that carried *lacI*^{q} and *ntrA* under the control of the *lac* promoter and operator into the *Eco*RI and *Hin*dIII sites of pALTER-1. Plasmid pJES937 (supplied by S. Kustu) is a derivative of pET-28a(+) (Novagen) and carries the *S. typhimurium ntrA* gene fused at the 5′ end to a sequence encoding six histidine residues. Expression of this fusion protein was under the control of a T7 promoter. Selected *ntrA* alleles were subcloned as 1.3-kb *Nde*I-*Asc*I fragments into the same sites of the *ntrA* allele on pJES937.

Generation and isolation of σ^{54} mutants. Random mutagenesis of *ntrA* was carried out by either PCR or spontaneous mutagenesis in Epicurian Coli XL1-Red competent cells (Stratagene). For PCR mutagenesis, we used pJES82, which carries *ntrA*, as the template and the same primers and cloning strategy as described for the construction of pSA4 (1). We relied on the inherent error frequency of *Taq* DNA polymerase (Promega) for the introduction of mutations into *ntrA*. Thirty cycles were carried out, using a regime that included a denaturation temperature of 94°C, an annealing temperature of 45°C, and an elongation temperature of 72°C. Reaction mixtures contained the buffer provided with the enzyme, supplemented with 2 mM $MgCl_2$. For generating spontaneous mutations, the Epicurian Coli XL1-Red strain was used as described by the supplier. Plasmid pSA4 was transformed into the mutator strain, and the resulting transformants were grown in Luria-Bertani broth or E minimal medium supplemented with ampicillin and L-glutamine. The cultures were subcultured twice in the same medium, and plasmid DNA was isolated.

For both methods, mutagenized plasmids were transformed into TRH107 by electroporation. The resulting transformants were plated on E minimal medium supplemented with L-glutamine and the appropriate antibiotics. Two colony sizes were observed on this medium. Approximately 10% of the smaller colonies were glutamine auxotrophs, while <0.2% of the larger colonies were glutamine auxotrophs. Glutamine auxotrophy, indicative of strains that lacked a functional σ^{54}, was identified by patching colonies onto E minimal medium. These glutamine auxotrophs were screened on MacConkey agar supplemented with appropriate antibiotics and IPTG to induce overexpression of the plasmid-borne *ntrA* alleles. Strains which produced mutant σ^{54} proteins that repressed transcription of the *ant′-′lacZ* reporter gene yielded white or pale colonies (Lac$^-$) on MacConkey agar.

Site-directed mutagenesis was done by using the Altered Sites II in vitro mutagenesis system as described by the supplier (Promega). Plasmid pMK11 was used as the template for these experiments. The *ntrA* alleles were subcloned into pSA4 so that the plasmid copy numbers were comparable to those of the other mutagenized plasmids.

Sequencing of *ntrA* alleles. The *ntrA* alleles present on derivatives of pSA4 and pMK11 were sequenced with the primers 5′-GTGTGGAATTGTGAG-3′, 5′-CATTCAGCGTTTTGAT-3′, and 5′-GCCGTAACGACACGCT-3′. The first primer is complementary to a sequence within the *lac* promoter region, while the last two primers are complementary to sequences within *S. typhimurium ntrA*. DNA sequencing was done at the Molecular Genetics Instrumentation Facility at the University of Georgia.

β-Galactosidase assays. To assess the degree to which the mutant σ^{54} proteins repressed transcription of the *ant′-′lacZ* reporter gene, β-galactosidase activities were determined in TRH107 as described previously (1). Mutant forms of σ^{54} were overexpressed by induction with IPTG. For each mutant, at least three separate assays were carried out, and activities were expressed as Miller units (24).

Glutamine synthetase assays. Glutamine synthetase activities were determined by the γ-glutamyltransferase assay as described previously (2). Cultures of strain TRH134 bearing plasmids with the various *ntrA* alleles were grown to mid-log phase in a modified E minimal medium that lacked sodium ammonium phosphate and was supplemented with acid-hydrolyzed Casamino Acids, 1 mM L-glutamine, and 100 μM IPTG to induce the expression of *ntrA*. Cells were permeabilized by including hexadecyltrimethylammonium bromide in the assay buffer as described elsewhere (2). Protein concentrations were determined from whole cells by Lowry protein assays with bovine serum albumin as a standard (21). Glutamine synthetase activities were expressed as micromoles of γ-glutamyl hydroxymate produced per minute per milligram of protein, and all assays were done at least twice.

Purification of N-terminally histidine-tagged σ^{54} proteins. Selected histidine-tagged σ^{54} proteins were overexpressed in BL21 (DE3) by induction with 1 mM IPTG. Cells were harvested after a 3-h induction period, resuspended in 50 mM Tris-acetate (pH 8.2)–200 mM KCl–1 mM EDTA–1 mM dithiothreitol (breakage buffer), and lysed in a French press cell at 12,000 lb/in^2. Following centrifugation at 12,400 × *g* for 40 min, a majority of each histidine-tagged protein was in the insoluble fraction. Pellets were washed with a solution containing 1 M NaCl and 1% Triton X-100, after which the histidine-tagged σ^{54} proteins were solubilized in 50 mM Tris-HCl (pH 8.0)–50 mM NaCl–0.1 mM EDTA–1 mM dithiothreitol–5% glycerol–1% sarkosyl as described previously (7). Solubilized proteins were loaded onto an Ni-nitrilotriacetic acid resin column (Qiagen) and eluted with 250 mM imidazole. For storage, purified proteins were dialyzed against 50 mM Tris-HCl (pH 8.0)–0.5 M NaCl–0.1 mM EDTA–1 mM dithiothreitol–50% glycerol.

Gel mobility shift assays. The binding of mutant forms of σ^{54}-holoenzyme to the *Sinorhizobium meliloti nifH* promoter was assessed by a modification of the method described by Guo and Gralla (12). Oligonucleotides that covered the −9 through −29 region of the *Sinorhizobium meliloti nifH* promoter (5′-GGCTGG CACGACTTTTGCACG-3′, 5′-GGCTGGCACGACTTTTGC-3′, and 5′-CGT GCAAAAGTCGTGCCAGCC-3′) were used. Two different DNA probes were generated from these oligonucleotides for the binding assays. One probe consisted of 21 bp of double-stranded DNA. The second probe had 18 bp of double-stranded DNA plus a 3-base 5′ overhang of the template strand which corresponded to residues −9 through −11. For each probe, the template strand was labeled with [γ-^{32}P]ATP and polynucleotide kinase before being annealed to the respective nontemplate strand. Binding reaction mixtures contained 300 nM core RNA polymerase (Epicentre Technologies), 600 nM histidine-tagged σ^{54}, and 5 nM DNA probe in a solution consisting of 50 mM HEPES-HCl (pH 7.9), 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 1 mM dithiothreitol, 0.05 μg of bovine serum albumin/ml, 2.8% polyethylene glycol 8000, and 6 μg of sonicated calf thymus DNA/ml. Reaction mixtures were incubated on ice for 30 min, loaded onto a chilled 5% native polyacrylamide gel, and then subjected to electrophoresis at 10 V/cm for 2 h. DNA bands were visualized by exposing the gels to X-ray film.

RESULTS

Isolation of mutant forms of *S. typhimurium* σ^{54}. Expression of glutamine synthetase (encoded by *glnA*) in enteric bacteria is regulated from two promoters. The major promoter, *glnAp$_2$*, is σ^{54} dependent and requires the nitrogen-regulatory protein C (NtrC) as an activator; the minor promoter, *glnAp$_1$*, is σ^{70} dependent and subject to catabolite repression (14, 29). Strains that lack σ^{54} are glutamine auxotrophs, since they are unable to initiate transcription from *glnAp$_2$*. Utilization of several forms of nitrogen, including arginine, is also dependent on σ^{54} and NtrC (29).

S. typhimurium TRH107 failed to grow on E minimal medium supplemented with acid-hydrolyzed Casamino Acids, since this medium is deficient in glutamine. Introduction of plasmid pSA4, which carries a copy of *S. typhimurium ntrA* under the control of the *lac* promoter and operator, allowed this strain to grow on E minimal and M9-arginine media. IPTG induction of *ntrA* from pSA4 was not necessary to allow growth on either of these media. The *ntrA* gene carried on pSA4 was subjected to random mutagenesis, and the resulting plasmids were introduced into TRH107. Transformants were screened for glutamine auxotrophy on E minimal medium to identify σ^{54} mutants that had reduced activity at *glnAp$_2$*. Nearly 1,200 glutamine auxotrophs were isolated in this screen.

TRH107 carries a partially deleted P22 prophage bearing an *ant′-′lacZ* reporter gene with the σ^{54}-dependent *Sinorhizobium meliloti nifH* promoter overlapping the *ant* promoter (Fig. 1A). Overexpression of σ^{54} from pSA4 in this strain allowed σ^{54}-holoenzyme to repress transcription of the *ant′-′lacZ* reporter gene (Fig. 1B). Therefore, the binding of σ^{54}-holoenzyme to the *nifH* promoter in vivo could be easily assessed by examining the Lac phenotype of the strain.

The glutamine auxotrophs isolated as described above were streaked on MacConkey agar supplemented with IPTG to induce expression of the *ntrA* alleles. This allowed us to assess the abilities of the mutant forms of σ^{54}-holoenzyme to repress transcription from the *ant′-′lacZ* reporter gene. Only eight of the σ^{54} mutants repressed transcription when overexpressed, as indicated by white or pale-colored colonies. Plasmids from these strains were isolated and designated pNTRA1 to pNTRA8.

Given that we overexpressed the σ^{54} mutants to examine their abilities to repress transcription from the *ant′-′lacZ* reporter gene, we reexamined the phenotypes of the strains overexpressing the mutant σ^{54} proteins. Strains that carried pNTRA2, pNTRA3, or pNTRA5 grew as well as the strain that

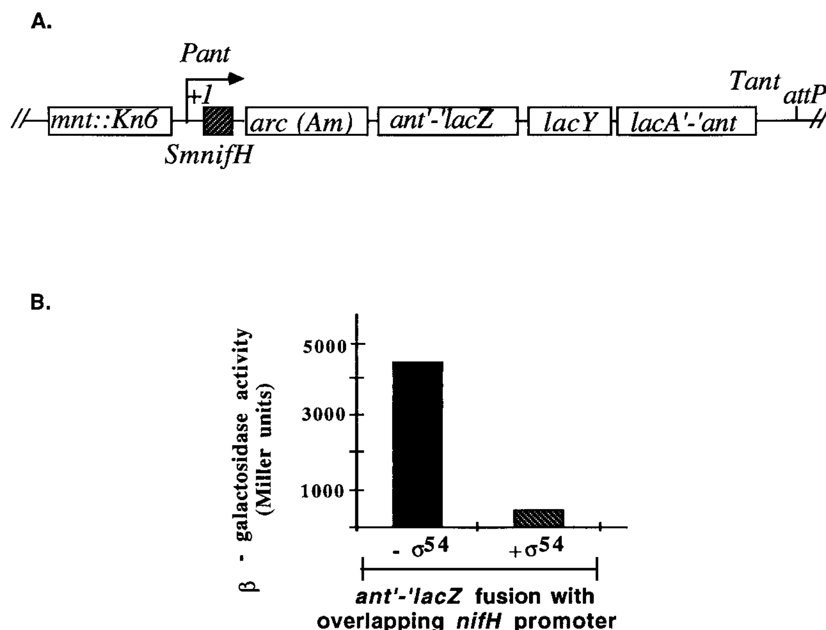

FIG. 1. Repression of the *ant'-'lacZ* fusion in *S. typhimurium* by σ^{54}. (A) The P22 prophage deletion within TRH107 carries the overlapping *Pant* and *Sinorhizobium meliloti nifH* (*SmnifH*) promoters and an *ant'-'lacZ* fusion. The GC doublet of the *nifH* promoter is at position +4 relative to the transcriptional start site of *Pant*. (B) IPTG induction of wild-type *ntrA* from pSA4 represses expression from the *ant'-'lacZ* reporter gene, indicative of σ^{54}-holoenzyme binding to the *Sinorhizobium meliloti nifH* promoter. In TRH107, overexpression of σ^{54} caused a ~10-fold repression relative to TRH107 that lacked σ^{54}.

overexpressed wild-type σ^{54} on E minimal medium supplemented with IPTG (Table 1). These three strains, however, did exhibit growth defects on M9-arginine medium supplemented with IPTG, indicating that the mutant σ^{54} proteins did indeed have reduced activities. Strains that carried pNTRA1 or pNTRA4 grew on E minimal medium supplemented with IPTG, but not as well as the strain with wild-type σ^{54}. Neither of these strains grew on M9-arginine medium supplemented with IPTG. Strains that carried pNTRA6, pNTRA7, or pNTRA8 failed to grow on either E minimal or M9-arginine medium supplemented with IPTG.

Analysis of mutant *ntrA* alleles. The degrees to which the eight σ^{54} mutants repressed transcription from the *ant'-'lacZ* reporter gene were assessed by assaying β-galactosidase (LacZ) activities. For these assays, the σ^{54} mutants were overexpressed by including IPTG in the culture medium. When wild-type σ^{54} was overexpressed from pSA4 in TRH107, there was a ~10-fold repression of the *ant'-'lacZ* reporter gene relative to a strain that lacked σ^{54} (Fig. 1 and 2). When the mutant forms of σ^{54} were overexpressed in TRH107, equivalent levels of repression of the *ant'-'lacZ* reporter gene were observed (Fig. 2).

We verified that the eight σ^{54} mutants were stably expressed in TRH107 by immunoblotting with antiserum directed against *S. typhimurium* σ^{54}. All eight σ^{54} mutant proteins were overexpressed, although the levels of some of the proteins were not as high as that of wild-type σ^{54} (Fig. 3). All of the σ^{54} mutant proteins accumulated to levels that were higher than the level of protein expressed from the chromosomal copy of *ntrA* (Fig. 3). The mutant proteins expressed from pNTRA2 and pNTRA4, however, accumulated to levels only a fewfold higher than that of σ^{54} expressed from the chromosomal copy of *ntrA*.

All eight *ntrA* alleles were sequenced to identify the mutations. Four of the *ntrA* alleles had single missense mutations, two alleles had two missense mutations, and two alleles had five missense mutations (Table 1). The deduced amino acid sequences of the *ntrA* alleles revealed that the alleles carried on plasmids pNTRA2 and pNTRA3 had the same amino acid substitution, a proline for the leucine at position 46 (L46P). These two mutant plasmids were isolated independently of each other. Of the 16 different positions with amino acid substitutions in the mutant proteins, 10 positions were either identical or similar in at least 26 of 29 σ^{54} proteins from various bacteria.

To quantitate the activities of the σ^{54} mutants at *glnAp*$_2$, we measured glutamine synthetase activities in vivo. We used the γ-glutamyltransferase assay for glutamine synthetase, which measures the conversion of glutamine and hydroxylamine to γ-glutamyl hydroxymate (2). Glutamine synthetase is regulated by adenylylation, but the γ-glutamyltransferase activities of both the adenylylated and unadenylylated forms of the enzyme are supported in the presence of Mn^{2+}. Glutamine synthetase activities were measured at pH 7.15, a pH at which all forms of the enzyme have equivalent activities in the presence of 0.3 mM Mn^{2+} (33). This allowed us to compare the total amounts of glutamine synthetase produced in the various strains. Cultures were grown under nitrogen-limited conditions in a modified E minimal medium that lacked sodium ammonium phosphate and contained 1 mM L-glutamine, the lowest concentration of glutamine which permitted growth of the glutamine auxotrophs. IPTG was included in the medium for overexpression of σ^{54}.

The glutamine synthetase activity of the strain that overexpressed wild-type σ^{54} was 0.988 μmol of γ-glutamyl hydroxymate produced/min/mg of protein (Fig. 4). The glutamine synthetase activity in TRH107 in the absence of σ^{54} was 0.034 μmol of γ-glutamyl hydroxymate produced/min/mg of protein. This background level of glutamine synthetase activity represented the level of expression from the σ^{70}-dependent *glnAp*$_1$ promoter. Strains carrying mutant plasmids that allowed growth on E minimal medium supplemented with IPTG (pNTRA1, pNTRA2, pNTRA4, and pNTRA5) had glutamine synthetase activities that ranged from 8 to 19% of that observed for the strain carrying pSA4. Strains carrying mutant plasmids that did not allow growth on E minimal medium even in the presence of IPTG (pNTRA6, pNTRA7, and pNTRA8) had glutamine

TABLE 1. σ^{54} mutants and their affects on growth

Plasmid	Growth phenotype on medium[a]:		Amino acid substitution(s)[b]
	E + IPTG	M9 + Arg + IPTG	
None	−	−	
pSA4 (wild type)	+++	+++	
Initial isolates			
pNTRA1	++	−	L37P
pNTRA2	+++	+	L46P
pNTRA3	+++	+	L46P
pNTRA4	+	−	L333P
pNTRA5	+++	+	E32K G189V
pNTRA6	−	−	L124P V148A
pNTRA7	−	−	W126R L199P D225E D231G I428V
pNTRA8	−	−	E42G I56T G189S L235W F318S
Derivatives of pNTRA5			
pNTRA9	+++	+++	E32K
pNTRA10	+++	+++	G189V
Derivatives of pNTRA6			
pNTRA11	+++	−	L124P
pNTRA12	+++	+++	V148A
Derivatives of pNTRA7			
pNTRA13	+++	+++	W126R
pNTRA14	+++	+++	D225E D231G I428V
pNTRA15	−	−	L199P D225E D231G I428V
pNTRA16	+++	+++	W126R D225E D231G I428V
pNTRA17	+++	+++	L199P
pNTRA18	−	−	L199P D231G
pNTRA19	+++	+++	D231G
Derivatives of pNTRA8			
pNTRA20	+++	+++	E42G I56T G189S
pNTRA21	+++	+++	L235W F318S
pNTRA22	+++	+++	E42G I56T L235W F318S
pNTRA23	+++	+++	G189S
pNTRA24	+++	+++	F318S
pNTRA25	+++	+++	G189S F318S

[a] Growth phenotypes are as follows: +++, wild-type growth; ++, slower growth, but colonies eventually attain wild-type size; +, poor growth with small colonies; and −, no growth.

[b] Amino acids which are identical or similar in at least 26 or 29 σ^{54} proteins from various bacteria are underlined.

synthetase activities that ranged from 2 to 7% of that observed for the strain carrying pSA4.

Analysis of *ntrA* alleles with multiple mutations. For each of the σ^{54} mutants with multiple amino acid substitutions, we wanted to determine if a single amino acid substitution or a combination of substitutions was responsible for the loss of function. For the mutants with two amino acid substitutions, E32K G189V (encoded on pNTRA5) and L124P V148A (encoded on pNTRA6), we separated the mutations by subcloning.

Separation of the two mutations on pNTRA5 by subcloning yielded plasmids pNTRA9 and pNTRA10 (Table 1). When the last two plasmids were introduced into TRH107, the growth phenotypes were identical to those of the strain with pSA4. Since the double mutant was overexpressed at a level comparable to that of the wild-type σ^{54} but allowed only poor growth on M9-arginine medium, the E32K and G189V substitutions appear to act synergistically to disrupt the function of σ^{54}.

The two mutations on pNTRA6 were similarly separated by subcloning, yielding plasmids pNTRA11 and pNTRA12 (Table 1). When pNTRA12 was introduced into TRH107, the strain

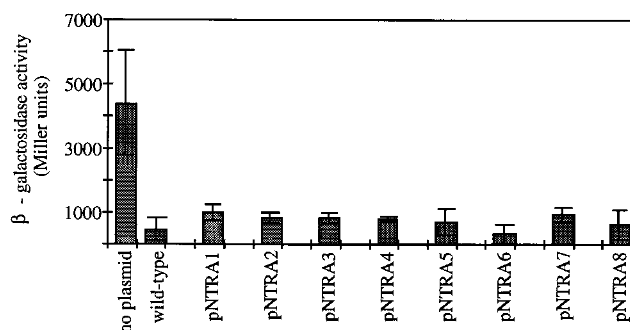

FIG. 2. Repression of the *ant'-'lacZ* fusion in *S. typhimurium* by mutant σ^{54} proteins. β-Galactosidase activities were determined in TRH107 carrying the plasmids indicated. Values are averages of at least three assays. Error bars show the standard deviations for the data sets. no plasmid, control (activity for TRH107 in the absence of a plasmid-borne *ntrA* allele); wild-type, activity for TRH107 with the wild-type *ntrA* allele on pSA4. The plasmids and the *ntrA* alleles that they carry are indicated in Table 1.

exhibited wild-type growth on both E minimal and M9-arginine media. In contrast, the growth phenotype of TRH107 carrying pNTRA11 was similar to that of the strain carrying pNTRA6. Consistent with the growth phenotypes, the glutamine synthetase activities of the strains carrying pNTRA6 or pNTRA11 were comparable (0.066 and 0.056 μmol of γ-glutamyl hydroxymate produced/min/mg of protein, respectively). Overexpression of the *ntrA* allele on pNTRA11 resulted in a level of repression of the *ant'-'lacZ* reporter gene which was comparable to that observed with pNTRA6 (103 and 305 Miller units, respectively). These data indicated that the L124P substitution was responsible for the loss of function in the original mutant.

The mutant allele carried on pNTRA7 had the amino acid substitutions W126R, L199P, D225E, D231G, and I428V. It was not possible to separate all of the mutations by subcloning. Nevertheless, by subcloning it was shown that the σ^{54} mutant with the W126R substitution allowed growth on both E minimal and M9-arginine media, as did the mutant with the substitutions D225E, D231G, and I428V (Table 1). The σ^{54} mutant with the substitutions L199P, D225E, D231G, and I428V (pNTRA15), however, did not allow growth on E minimal or M9-arginine media. The glutamine synthetase activity of the

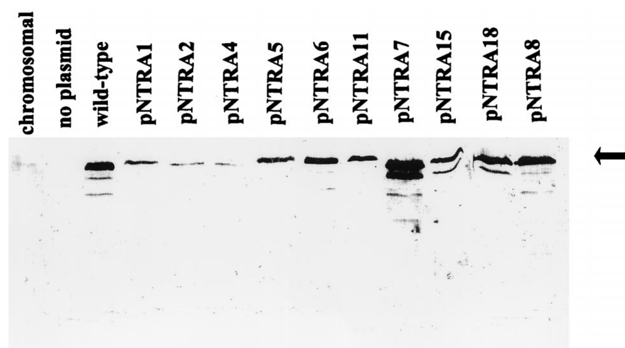

FIG. 3. Overexpression of the mutant σ^{54} proteins. The mutant and wild-type σ^{54} proteins, indicated by the arrow, were overexpressed in TRH134. Cultures were grown to mid-log phase, and then expression from *ntrA* was induced for 3 h with 100 μM IPTG. Whole-cell extracts were analyzed by immunoblotting with antiserum directed against *S. typhimurium* σ^{54}. no plasmid, TRH134 without any plasmid; wild-type, TRH134 bearing pSA4; chromosomal, *S. typhimurium* MS1868, the parental strain of TRH134, which contains a chromosomal copy of *ntrA* but no plasmid-borne copy of the gene. The plasmids and the *ntrA* alleles that they carry are indicated in Table 1.

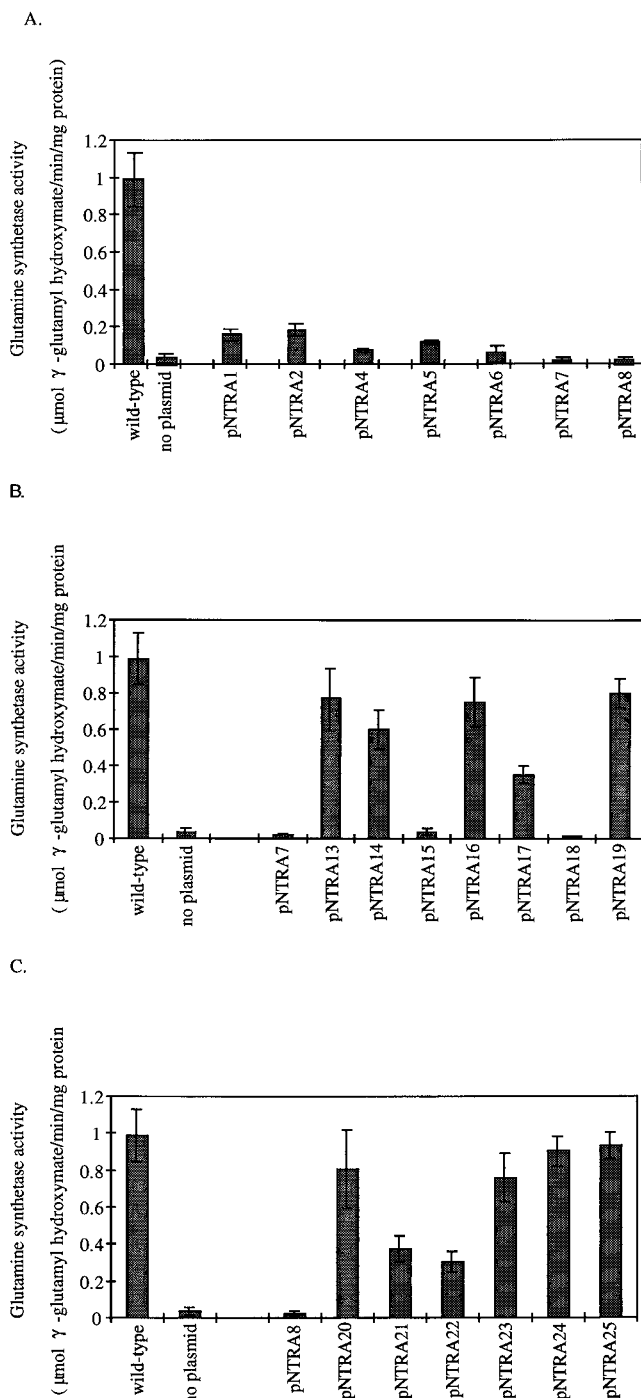

FIG. 4. Glutamine synthetase activities of selected strains that overexpress σ^{54} mutants. The plasmids and the *ntrA* alleles that they carry are indicated in Table 1. Assays were done at least twice, and error bars show the standard deviations for the data sets. (A) The *ntrA* alleles originally isolated. (B) The pNTRA7-borne allele and the derivatives of this mutant allele. (C) The pNTRA8-borne allele and the derivatives of this mutant allele. no plasmid, strain TRH134 without a plasmid-borne *ntrA* allele; wild-type, TRH134 with the wild-type *ntrA* allele on pSA4.

strain carrying pNTRA15 was comparable to that of the strain carrying pNTRA7 (Fig. 4B). These results suggested that L199P was responsible for the loss of function. Consistent with this, the proline at position 199 had reverted to the original leucine residue in four independent derivatives of pNTRA7 that complemented the growth defect of TRH107 on E minimal medium (represented by pNTRA16).

Introduction of proline at position 199 by site-directed mutagenesis, however, allowed wild-type growth on E minimal and M9-arginine media (Table 1). These data suggested that L199P in combination with D225E, D231G, or I428V was required for the loss of function of σ^{54}. The D231G substitution was the most drastic, and we guessed that it might be required for loss of function. We generated an *ntrA* allele with both the L199P and D231G substitutions by site-directed mutagenesis (pNTRA18). The strain that overexpressed this σ^{54} mutant failed to grow on E minimal or M9-arginine medium and had a level of glutamine synthetase activity comparable to that of the strain carrying pNTRA7 (Fig. 4B). The L199P D231G double mutant also repressed transcription of the *ant'-'lacZ* reporter gene as effectively as the original mutant (214 and 642 Miller units, respectively). The mutant with the single substitution D231G allowed growth on both E minimal and M9-arginine media (Table 1). These data indicated that both the L199P and D231G substitutions were required for a loss of function. We cannot exclude the possibility that other combinations of amino acid substitutions could also have resulted in a loss of function.

The remaining mutant allele with five mutations (encoded on pNTRA8) was analyzed in a similar manner. These mutations were divided into groups of three (E42G, I56T, and G189S) and two (L235W and F318S) mutations by subcloning. Both of these alleles allowed growth on E minimal and M9-arginine media (Table 1). We selected for revertants of pNTRA8 that complemented the growth defect of TRH107 on E minimal medium. Two independent isolates were sequenced, and in both cases the serine at position 189 had reverted to the original glycine (represented by pNTRA22). These data suggested that the G189S substitution was responsible for the loss of function of the original mutant. When a serine was introduced at position 189 by site-directed mutagenesis, however, the resulting mutant σ^{54} allowed growth on both E minimal and M9-arginine media. This implied that a combination of G189S and either L235W or F318S was required for loss of function. F318 had been previously identified as a functionally important residue in *E. coli* σ^{54} (11). However, the G189S F318S double mutant as well as the F318S single mutant allowed wild-type growth on E minimal and M9-arginine media (Table 1). We did not construct the G189S L235W double mutant.

In vitro analysis of core binding activities of the σ^{54} mutants. We assumed that the σ^{54} mutants were able to bind core RNA polymerase and direct the holoenzyme to the *nifH* promoter in vivo given that the affinity of free σ^{54} for promoter DNA is very low (3). To test this assumption, selected mutant σ^{54} proteins were purified and tested for their abilities to bind to the *nifH* promoter in a gel mobility shift assay in conjunction with core RNA polymerase. Histidine tags were placed at the amino termini of these proteins to facilitate their purification.

For these gel shift assays, we used labeled oligonucleotides that corresponded to the -9 to -29 region of the *Sinorhizobium meliloti nifH* promoter. One of these oligonucleotides was double stranded over its entire length (double-stranded probe), while the other oligonucleotide had a 5′ overhang of 3 bp that corresponded to residues -11 to -9 of the template strand (fork junction probe). *E. coli* σ^{54} had been shown previously to bind to this fork junction probe to form a heparin-resistant complex (12). We found that σ^{54}-holoenzyme shifted the fork junction probe much more effectively than it did the double-stranded probe (Fig. 5, lanes 1 and 2). Free σ^{54} also shifted the fork junction probe, but this species had a faster mobility than the species shifted with holoenzyme (data not shown). Unlike

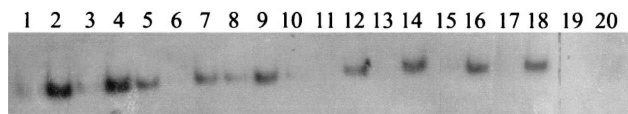

FIG. 5. Gel mobility shift assays with mutant forms of σ^{54}-holoenzyme. The double-stranded probe was used in the odd-numbered lanes, while the fork junction probe was used in the even-numbered lanes. The free probe is not shown. Binding reaction mixtures contained 300 nM core RNA polymerase plus either wild-type σ^{54} (lanes 1 and 2), histidine-tagged σ^{54} (lanes 3 and 4), the L37P mutant (lanes 5 and 6), the L46P mutant (lanes 7 and 8), the L333P mutant (lanes 9 and 10), the L199P D231G mutant (lanes 11 and 12), the E32K G189V mutant (lanes 13 and 14), the E42G I56T G189S L235W F318S mutant (lanes 15 and 16), the L124P V148A mutant (lanes 17 and 18), or no σ^{54} protein (lanes 19 and 20). For assays with wild-type σ^{54}, >50% of the fork junction probe was shifted by holoenzyme.

the complex formed with σ^{54}, the complex formed by σ^{54}-holoenzyme and the fork junction probe was heparin sensitive (data not shown). Core RNA polymerase did not bind to the fork junction probe under the assay conditions used in the gel shift assay (Fig. 5, lane 20). These data confirmed that the supershifted species was a complex of σ^{54}-holoenzyme and the fork junction probe and indicated that this gel shift assay could be used to assess the binding of the σ^{54} mutants to core RNA polymerase.

The histidine tag at the amino terminus of σ^{54} did not interfere with the ability of σ^{54}-holoenzyme to bind to either probe (Fig. 5, lanes 3 and 4). Interestingly, the holoenzymes formed with three of the σ^{54} mutants, L37P, L46P, and L333P, bound the double-stranded probe better than wild-type holoenzyme (Fig. 5, lanes 5, 7, and 9). These mutant forms of σ^{54}-holoenzyme, however, bound very poorly to the fork junction probe (Fig. 5, lanes 6, 8, and 10). The remaining mutant forms of σ^{54}-holoenzyme behaved like the wild-type holoenzyme in that they had higher affinities for the fork junction probe than for the double-stranded probe (Fig. 5, lanes 11 to 18). These data demonstrated that the mutant σ^{54} proteins retained core binding activity. The data also showed that the σ^{54} mutants could be divided into at least two classes on the basis of their affinities for the fork junction probe.

DISCUSSION

Transcription initiation involves several discrete steps, including formation of a closed complex between polymerase and the promoter, isomerization of the closed complex to an open complex, and promoter clearance. σ^{54} could play important roles at any of these steps.

The σ^{54} protein consists of three functional regions (Fig. 6). The highly conserved region I of σ^{54} consists of approximately 50 amino-terminal residues and is rich in glutamine and leucine (23). Mutational analysis of region I has indicated that it is required for σ^{54}-holoenzyme to respond to the activator protein (15, 16, 35, 39). Region I is not required for binding to either core or DNA (6, 43). Given these properties of region I mutants, we expected our screen to yield some σ^{54} mutants with substitutions in region I. Indeed, four of our original eight σ^{54} mutants had substitutions within this region. We identified E32, L37, and L46 as potentially important residues in *S. typhimurium* σ^{54}. Previous studies had suggested that L33, E36, and L37 were functionally important in *E. coli* σ^{54} (35). Region I-deleted holoenzyme protects a larger region of the promoter from S1 nuclease cleavage than the wild-type holoenzyme, indicating that region I influences the conformation of the holoenzyme (5). The L37P and L46P mutant proteins may have affected the conformation of the holoenzyme similarly, which could explain the reduced affinities of the mutant holoenzymes formed with these σ^{54} mutants for the fork junction probe.

Region II is poorly conserved and is in fact missing in *Rhodobacter capsulatus* σ^{54} (9). In σ^{54} proteins from enteric bacteria, region II has a high proportion of acidic amino acid residues. Deletions within region II of *E. coli* σ^{54} appear to decrease the rate of open-complex formation (42). None of the residues that we identified as functionally important in *S. typhimurium* σ^{54} in this study was in region II.

Determinants for core and DNA binding are located within region III of σ^{54} (6, 11, 36, 37, 43). Our results clearly show that in addition to these roles, region III is involved in one or more subsequent steps in transcription initiation. We identified four residues within region III—L124, L199, D231, and L333—which when altered disrupted transcription initiation at some point following closed-complex formation.

Substitution of proline for leucine at position 124 resulted in the loss of function. L124 lies within the minimal core binding domain (residues 120 to 215) as defined by deletion analysis (6, 43). Core protects the region between residues 36 and 140 of σ^{54} from hydroxyl-radical cleavage (8), suggesting that L124 is in a region of the protein that contacts core. Given that the L124P mutant retains core binding activity, interactions between the region around L124 and core may be required for open-complex formation rather than binding of σ^{54} to core. The L199P and D231G mutations acted synergistically to disrupt the function of σ^{54}. L199 is within the minimal core binding domain. The region around L199, however, does not appear to closely contact core, since it is not protected from hydroxyl-radical cleavage by core (8). D231 is within a domain that modulates the DNA-binding activity of the protein (7). Like the region around L124, the regions around L199 and D231 had not been shown previously to participate in steps following closed-complex formation.

L333 is located within the DNA-binding domain of σ^{54} (residues 332 to 462) (6), and a proline substitution at this position caused a loss of function. The region around L333 had been suggested previously to play a role in later steps in transcription initiation. Deletion of residues 293 through 332 disrupts the function of *E. coli* σ^{54}, and like the L333P mutant isolated in our study, this deletion mutant retained its core and DNA-

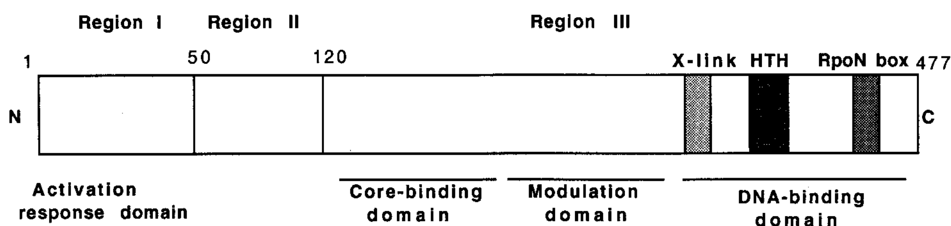

FIG. 6. Structure of σ^{54}. Regions I, II, and III are the three functional regions of σ^{54}. X-link, a region that cross-links to promoter DNA on UV irradiation (4); HTH, a putative DNA-binding helix-turn-helix motif; RpoN, the signature motif of σ^{54} proteins that has the consensus sequence ARRTVAKYRE.

binding activities (43). L333P mutations in *E. coli* σ^{54} were described previously (11), but in this earlier publication it was not specified whether these proteins had other substitutions. In addition, the core and DNA-binding activities of the *E. coli* mutants were not reported. Like the holoenzymes formed with L37P and L46P, the L333P holoenzyme had a low affinity for the fork junction probe, suggesting that the region around Leu-333 could influence the conformation of holoenzyme.

The frequency with which we isolated σ^{54} mutants that retained core and DNA-binding activities was very low. Many of these σ^{54} mutants had substitutions of proline for leucine. This may reflect the fact that such mutations require a single base change. Alternatively, this may have been due to the stringency of the genetic screen used to isolate the σ^{54} mutants. Proline substitutions could have disrupted the secondary structure and severely impaired the function of σ^{54}. Consistent with this idea, strains with as little as 8% of the wild-type glutamine synthetase activity were glutamine prototrophs, indicating that the σ^{54} mutants isolated were severely impaired in their function at the *glnAp*$_2$ promoter. Determining the biochemical basis for the failure of these mutant proteins to function will help clarify the roles of σ^{54} in transcription initiation.

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