

Mutations Affecting Motifs of Unknown Function in the Central Domain of Nitrogen Regulatory Protein C

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The positive control function of the bacterial enhancer-binding protein NtrC resides in its central domain, which is highly conserved among activators of σ^{54} holoenzyme. Previous studies of a small set of mutant forms specifically defective in transcriptional activation, called NtrC repressor [NtrC(Rep)] proteins, had enabled us to locate various functional determinants in the central domain. In this more comprehensive survey, the DNA encoding a major portion of the central domain was randomly mutagenized and mutated *ntrC* genes were introduced into the cell via multicopy expression plasmids. DNA sequencing of 95 isolates identified by a preliminary phenotypic screen revealed that the lesions in them caused 55 distinct single amino acid substitutions at 44 different positions. Assays of *glnA* transcription in vivo and in vitro yielded two conclusions. First, of the 41 mutant proteins that could be purified, 17 (1 known, 16 new) showed no detectable activity in either assay, thus qualifying them as true NtrC(Rep) proteins. These contained residue changes in six of the seven highly conserved regions in the central domain, including two never studied before. Second, some mutant proteins were inactive in vivo but were either marginally or fully active in vitro. Their surprising lack of activity in vivo may be accounted for by high levels of expression, which apparently decreased activation by these mutant proteins but not by wild-type NtrC (NtrC^{WT}). Of particular interest were a subset of these proteins that exhibited greater transcriptional activation than NtrC^{WT} at low concentrations. Their elevated activation capacities remain to be explained.

A prominent class of prokaryotic enhancer-binding proteins activates transcription by the σ^{54} holoenzyme form of RNA polymerase (23, 26, 30, 50). One such protein is nitrogen regulatory protein C (NtrC), which activates transcription in response to limitation of combined nitrogen in the medium. The NtrC protein from enteric bacteria has been well studied as an activator for the *glnA* gene; this gene encodes glutamine synthetase, an enzyme with a major role in assimilation of ammonia.

NtrC can function both as an activator and as a repressor of *glnA* transcription, depending on the nutritional status of the cell (41, 56). Positive and negative controls of expression are achieved at two different promoters: activation at a downstream σ^{54} -dependent promoter and repression at an upstream σ^{70} -dependent promoter (Fig. 1A). Under nitrogen-limiting conditions, NtrC is phosphorylated by the protein kinase NtrB (17, 32). Phosphorylated NtrC forms an unusual hexamer or octamer at the two sites that constitute the *glnA* enhancer (61). To activate transcription, this oligomer contacts σ^{54} holoenzyme at the *glnA* promoter by means of a DNA conformational change (55). It catalyzes isomerization of closed complexes between σ^{54} holoenzyme and the promoter to transcriptionally productive open complexes (29, 39, 46) in a manner that depends upon hydrolysis of ATP and an energy-coupling mechanism (37, 39, 55, 57). When NtrC is bound to the enhancer, it

represses transcription from a secondary σ^{70} -dependent promoter that lies in the enhancer region (41). Phosphorylation and oligomerization, which are essential for ATP hydrolysis and therefore for transcriptional activation (1, 40, 59, 60), are not required for repression of transcription (20).

Each monomer of the dimeric NtrC protein is comprised of three domains (Fig. 1B). The N-terminal domain contains the site of phosphorylation, D54 (17, 32). The C-terminal domain is responsible for binding to the enhancer (7, 40). The central domain of NtrC, amino acid residues 141 to 376 (Fig. 2), is directly responsible for transcriptional activation (2, 10, 14, 15, 37), which appears to entail oligomerization, nucleotide binding and hydrolysis, and coupling of energy to a thermodynamically unfavorable change in the conformation of σ^{54} holoenzyme. The functional significance of the central domain of NtrC is underscored by the high degree of sequence similarity shared by all members of the σ^{54} activator family (4, 6, 44). Sequence comparisons among these homologous proteins have revealed seven highly conserved amino acid segments (designated C1 to C7) along the central domain (30). When such comparisons were combined with genetic and biochemical analyses and were expanded to include a broader range of purine nucleotide-binding proteins (38, 47), interesting parallels that provided insights into the distribution of the subfunctions of positive control within the central domain emerged. The C1 and C4 regions, respectively, were identified as likely Walker A and Walker B motifs, which are involved in ATP binding and hydrolysis (37, 38, 43, 44, 52, 57). The C3 region appears to be analogous to the “switch I” region of other purine nucleotide-binding proteins (37, 38), a region that lies between the Walker A and B motifs and plays an important role in coupling nucleotide hydrolysis to biological output (e.g., see references 3, 13, 18, and 48). The C7 region is required for nucleotide binding and is probably involved in binding the nucleotide base (38, 43). Finally, recent secondary structure

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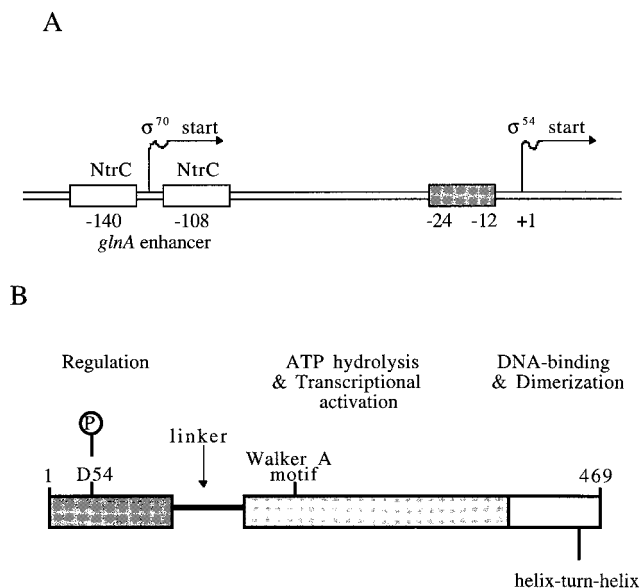


FIG. 1. (A) Diagram of the *glnA* promoter-regulatory region from *S. typhimurium* (not to scale). The upstream binding sites for NtrC, which are centered at -140 and -108 with respect to the major σ^{54} -dependent transcriptional start site at $+1$, function as a transcriptional enhancer (33, 42). Conserved promoter sequences recognized by the σ^{54} holoenzyme lie at -24 and -12 , as indicated (23, 50). When phosphorylated, NtrC forms an oligomer at the enhancer (see text) and activates transcription by σ^{54} holoenzyme. Both phosphorylated and unphosphorylated NtrC can bind to the enhancer and repress transcription from the secondary σ^{70} -dependent promoter that lies in the enhancer region (20, 41). (B) Domain structure of NtrC (not to scale) (reviewed by Kustu et al. [22], Weiss et al. [58], and Morett and Segovia [30]). An NtrC monomer (52,238 Da) contains 469 amino acid residues and is composed of three domains. The N-terminal receiver domain (~ 120 residues) contains the site of phosphorylation, D54. Under nitrogen-limiting conditions, this aspartate residue receives a phosphate from the phosphorylated NtrB protein, a physiological signal that is necessary for NtrC-mediated transcriptional activation (1, 32, 57). NtrB and NtrC constitute a sensory kinase-response regulator pair in a "two-component" signal transduction system (21, 34). The N-terminal domain is linked by a glutamine-rich flexible linker ("Q-linker") to the central domain (~ 240 residues), which appears to be directly responsible for ATP hydrolysis and transcriptional activation by σ^{54} holoenzyme (see text). This domain is highly conserved among activators of σ^{54} holoenzyme (Fig. 2). The C-terminal domain (~ 90 residues) contains a helix-turn-helix DNA-binding motif (40, 58) and the major dimerization determinants of the protein (19, 35).

predictions coupled with the use of recognition algorithms for protein folds indicated that the central domain of activators of σ^{54} holoenzyme adopts a mononucleotide-binding fold similar to those of the eukaryotic signaling protein p21^{ras} and the G domain of the bacterial polypeptide elongation factor Tu (38).

Genetic studies of the NtrC protein of *Salmonella typhimurium* were facilitated by the isolation of a class of mutant proteins known as NtrC repressor [NtrC(Rep)] forms (37, 43, 56, 57). NtrC(Rep) proteins, which have been called "positive control" or "PC" forms in other systems, were defined as those that had lost the ability to activate transcription by σ^{54} holoenzyme but retained the ability to repress transcription by σ^{70} holoenzyme (37). Prior to this study, 13 NtrC(Rep) proteins carrying single residue changes in five of the seven conserved regions of the central domain (30) had been characterized. Their properties in vitro allowed us to distinguish between those regions involved specifically in Mg-ATP binding and those responsible for ATP hydrolysis per se (43) and to identify one region (C3 or switch I) required for transcriptional activation but not for nucleotide hydrolysis (37); by inference, the C3 region appears to be involved in interaction with σ^{54} holoenzyme, that is, in contact with the polymerase, and/or cou-

pling of energy to a change in its conformation that allows it to form open complexes. Further advances in functional mapping were limited by the size of our mutant pool.

To characterize new functional determinants of NtrC, we performed random mutagenesis to scan most of the central domain of the *S. typhimurium* NtrC protein (residues 172 to 322) for new amino acid residues and regions important for positive control. We determined transcriptional activities of mutant proteins both in vivo and in vitro. As expected, these studies yielded additional NtrC(Rep) proteins. Unexpectedly, they also yielded a new and interesting class of mutant proteins that were more active than wild-type NtrC (NtrC^{WT}) at low concentrations but had greatly decreased transcriptional activation capacities at high concentrations.

MATERIALS AND METHODS

Media. Complex media were nutrient broth (NB), Luria broth (LB), and green indicator plates (5), which were supplemented with glutamine (2 mM) and/or ampicillin (100 μ g/ml) when appropriate. The minimal medium, N⁻C⁻ (12), was supplemented with glucose (0.4%) as the carbon source, glutamine (5 mM) as the nitrogen source, and L-histidine (0.2 mM) to satisfy an auxotrophic requirement. Ampicillin (50 μ g/ml) was added for plasmid-carrying strains to prevent loss of plasmids.

Random mutagenesis with PCR. Most of the DNA sequence encoding the central domain of NtrC (amino acids 141 to 376 or nucleotides 421 to 1128, where $+1$ designates the A of the ATG start codon for NtrC; Fig. 2) was subjected to random mutagenesis with PCR. The two oligonucleotide primers used to amplify this segment were 5'-ATTGGTCGGCTGTCGCGTTC-3' (nucleotides 460 to 479; upstream primer) and 5'-ATTGGCTTCACGCCTAA T-3' (nucleotides 1015 to 996; downstream primer). PCR conditions were chosen to exploit the inherent infidelity of DNA synthesis by *Thermus aquaticus* (Taq) DNA polymerase (24, 62). The template was pJES559 (Table 1), which carries *malE-ntrC* in an overexpression vector. The Mg²⁺ concentration was elevated to provide a large excess over the total deoxynucleoside triphosphates (dNTPs) present, a condition that further reduces the copying accuracy of the Taq polymerase (8). Reaction mixtures contained 5 ng of template, 100 ng of each primer, 50 μ M (each) dNTP, 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 4 mM MgCl₂ and 5 units of Taq DNA polymerase (AmpliTaq) in a total volume of 50 μ l. PCRs were carried out on a Perkin-Elmer Cetus DNA Thermal Cycler with the following cycles programmed: 30 cycles of 1 min at 94°C, 1 min at 48°C, and 1 min at 70°C.

The PCR products were digested by a pair of restriction endonucleases, *AgeI* and *AscI*, which cut close to the ends of the fragments (Fig. 2). The resulting small fragments (nucleotides 514 to 963) were gel purified with Spinex columns (Amicon) and cloned into the NtrC overexpression plasmid pJES311, which had also been digested with *AgeI* and *AscI*. Ligation mixtures (with molar ratios of insert to vector of 3:1 and 4:1) were transformed into Gibco BRL DH5 α competent cells according to the manufacturer's specifications and were incubated overnight at 37°C on LB glutamine (Gln) ampicillin (Amp) selective plates.

Genetic screen. A newly developed plate test was employed to discriminate between transformants carrying *ntrC*(Rep) alleles and those carrying the wild-type allele or *ntrC* null alleles. The screening medium was NB, which lacks sufficient glutamine to allow growth of glutamine auxotrophs. Because DH5 α has an intact chromosomal *ntrC* gene, the introduction of a *ntrC* mutant allele on a multicopy expression plasmid (strain NCM1237 [Table 1]) did not affect glutamine-independent growth of the host strain. By contrast, when an *ntrC*(Rep) allele was introduced (strain NCM1946), there was no growth on NB because both *glnA* promoters on the host chromosome were inactivated. Interestingly, the control strain NCM1099, which expressed NtrC^{WT} from pJES311, did not grow as well as the host strain on NB (phenotype intermediate between those of NCM1237 and NCM1946). This "semirepressed" phenotype is probably explained by the fact that there is only a single chromosomal copy of *ntrB*, and hence NtrC is not phosphorylated well enough to drive adequate expression from the downstream promoter.

To identify strains carrying NtrC(Rep) proteins, single colonies of 1,234 transformants were screened for growth on NB, as discussed above (all grew normally on NB Gln). One group was able to grow as well as NCM1237 (*ntrC* mutant allele). Another group grew noticeably worse, with a phenotype similar to NCM1099 (*ntrC*⁺ allele). The remainder of the transformants failed to grow on NB, a phenotype resembling that exhibited by NCM1946 (*ntrC*(Rep) allele). They were stored in glycerol at -80°C for further analysis.

DNA sequencing. Strains were streaked out fresh from glycerol stocks, and plasmids were extracted with QIAGEN Miniprep kits. DNA sequencing of the entire mutagenized region was performed on an ABI373 automated sequencer using the upstream primer 5'-TATCGAGGTTAACGGCCCGA-3' (nucleotides 390 to 409). For each sample, a second sequencing run was also performed to confirm the mutation from the reverse direction. The downstream primer was the same as that used previously for PCR mutagenesis.

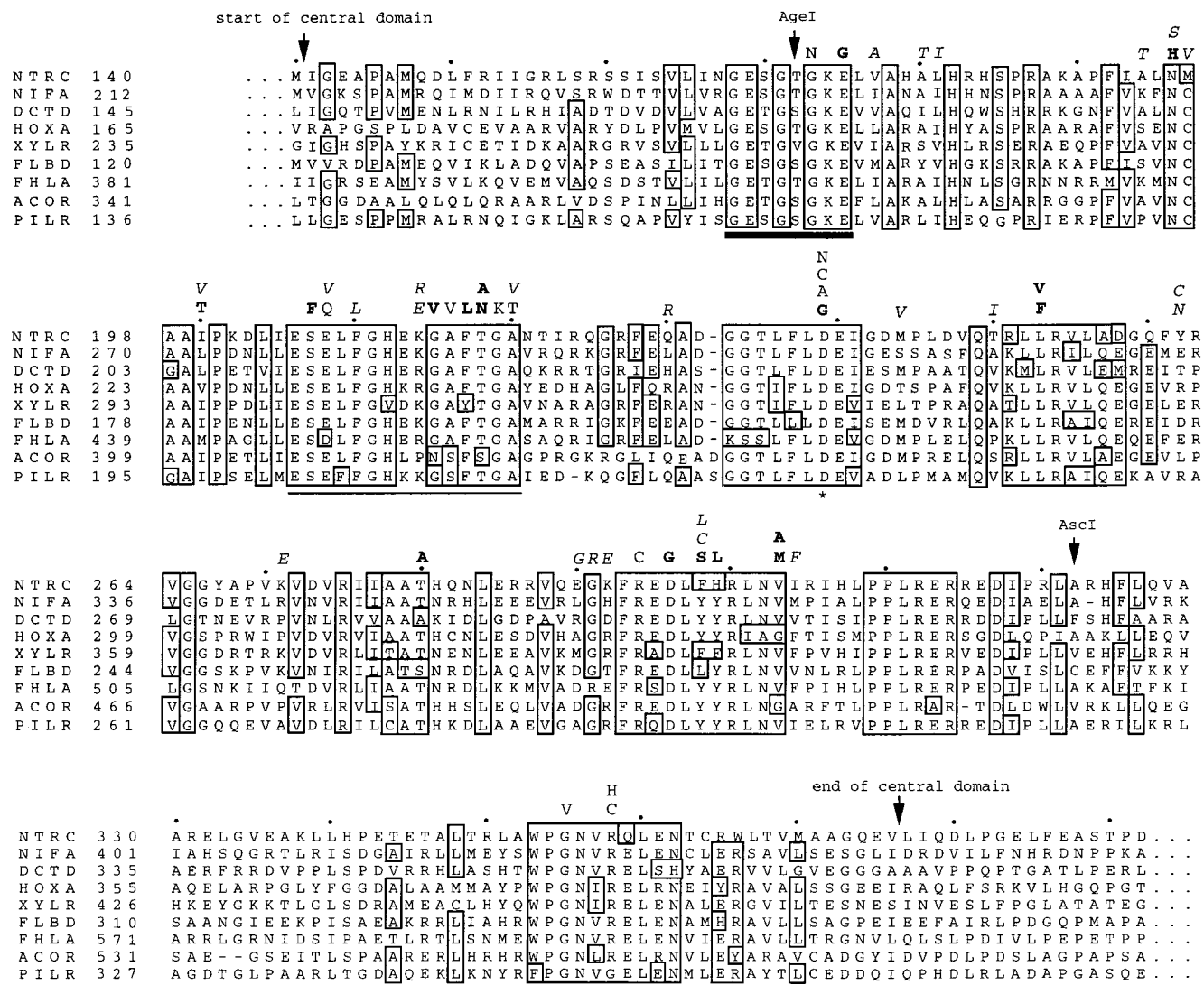


FIG. 2. Alignment of the central domains of nine activators of σ^{54} holoenzyme and the locations of amino acid substitutions in NtrC mutant proteins. The activators are chosen from members of the α , β , and δ subgroups of the purple bacteria. Respectively, the sequences belong to *S. typhimurium* NtrC, *Klebsiella pneumoniae* NIFA, *R. leguminosarum* DCTD, *Alcaligenes eutrophus* HOXA, *Pseudomonas putida* XYLR, *Caulobacter crescentus* FLBD, *E. coli* FHLA, *A. eutrophus* ACOR, and *Pseudomonas aeruginosa* PILR (original references given in reference 35). Residues that are identical in at least 6 activators are boxed. The common boundaries of their central domains are indicated by arrows, and their exact positions are according to Morett and Segovia (30). Another pair of arrows identify the locations of the *AgeI* and *AscI* restriction sites that correspond to the start and the end of the PCR mutagenesis target region (amino acids 173 to 321 in *S. typhimurium* NtrC). Four functional determinants are highlighted by symbols directly beneath their positions in the sequence alignment: the heavy black line denotes the glycine-rich segment that has been proposed to correspond to the Walker A motif of purine nucleotide-binding proteins (37, 38, 43, 44, 52, 57); the asterisk denotes the highly conserved aspartate at the end of the Walker B motif; the plain line denotes the putative switch I (effector) region (18, 37, 38); and the dashed line marks four residues believed to be involved in binding of the nucleotide base (38, 43). Letters above the sequence alignment designate all single amino acid substitutions for which *in vitro* measurements were made in this study and known substitutions resulting in NtrC(Rep) proteins. In cases where more than one such substitution was found for a given residue, the letters are arranged according to the severity of the effect, such that those resulting in the largest defects are placed closest to the alignment. Plain letters denote known NtrC(Rep) substitutions that had been described previously (37, 43, 56–58). Boldface letters indicate amino acid substitutions found in this study to yield NtrC(Rep) proteins. Like known NtrC(Rep) proteins, these new proteins were characterized by the absence of detectable transcriptional activities *in vivo* or *in vitro*. Italic letters represent other mutational substitutions generated in this study which resulted in little transcriptional activation and glutamine auxotrophy *in vivo* but readily detectable and different amounts of transcriptional activation *in vitro* (see Results).

Strain constructions. Preparation of lysates of phage P22, transductional crosses, and the use of green indicator plates to purify transductants free of phage were as described previously (5). Strain SK3424 (*glnAp381 glnAp131 ntrC352::Tn10 Δ hisF645 putPA::[Kan^r- Φ (glnA'-lacZ)]*), which allowed us to assess the effects of putative *ntrC*(Rep) alleles on *glnA* transcription *in vivo*, was constructed in two steps as described below. In step one, a phage lysate was prepared on strain SK3044, which carries the *ntrC::Tn10* allele and was used to transduce SK1490 (*glnAp381 glnAp131 Δ hisF645*) to tetracycline resistance. Large transductant colonies were selected because their phenotype indicated that they had retained the promoter mutations (27). In step two, the same phage lysate was used to transduce the intermediate strain, SK3423, to kanamycin resistance to select for transfer of the Φ (*glnA'-lacZ*) fusion.

The *glnA* promoter(Up) mutations in SK3424 were introduced because a strain carrying the *ntrC352::Tn10* allele alone grows poorly on glutamine as the sole nitrogen source (doubling time of ~170 min versus ~130 min for a *ntrC*⁺ strain) and such a strain transformed with plasmid pJES412 (an *ntrC*(Rep) allele) grows worse (doubling time, >220 min). The presence of the promoter(Up) mutations in SK3424 alleviated growth problems. Use of glutamine as the sole nitrogen source was chosen because it is a derepressing condition for *glnA* expression in a *ntrC*⁺ strain. The construction of strain SK3482 (*glnAp381 glnAp131 ntrC352::Tn10 ntrA75 hisF645 putPA::[Kan^r- Φ (glnA'-lacZ)]*), which carries an *ntrA* null allele, was similarly accomplished in two cycles of P22-mediated transduction. In the first cycle, the *ntrC352::Tn10* allele from strain SK3424 was introduced into strain

TABLE 1. Bacterial strains^a and plasmids used in this study

Strain or plasmid	Genotype and/or characteristic(s)	Construction, ^b reference, and/or source
<i>E. coli</i> strains		
DH5 α	F ⁻ ϕ 80dlacZ Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (r_{κ}^{-} m_{κ}^{+}) <i>phoA supE44 λ^{-} thi-1 gyrA96 relA1</i>	Purchased as Subcloning Efficiency DH5 α Competent Cells from Gibco BRL (cat. no. 18265-017)
NCM724	HfrC(λ) <i>ntrA6 argG::Tn10</i>	Weiss et al. (57)
NCM1099	DH5 α /pJES311	Transformation; this work
NCM1237	DH5 α /pJES504	Transformation; this work
NCM1946	DH5 α /pJES1065	Transformation; this work
<i>S. typhimurium</i> strains		
SK1489	<i>glnAp381 glnAp131 ntrA75 ΔhisF645</i>	McCarter et al. (27)
SK1490	<i>glnAp381 glnAp131 ΔhisF645</i>	McCarter et al. (27)
SK3044	<i>ntrC352::Tn10 putPA::[Kan^r-Φ(<i>glnA'</i>-<i>lacZ</i>)]</i>	Ikeda et al. (16)
SK3423	<i>glnAp381 glnAp131 ntrC352::Tn10 ΔhisF645</i>	SK1490, SK3044 ^{c,d} ; this work
SK3424	<i>glnAp381 glnAp131 ntrC352::Tn10 ΔhisF645 putPA::[Kan^r-Φ(<i>glnA'</i>-<i>lacZ</i>)]</i>	SK3423, SK3044 ^c ; this work
SK3481	<i>glnAp381 glnAp131 ntrC352::Tn10 ntrA75 ΔhisF645</i>	SK1489, SK3424 ^c ; this work
SK3482	<i>glnAp381 glnAp131 ntrC352::Tn10 ntrA75 ΔhisF645 putPA::[Kan^r-Φ(<i>glnA'</i>-<i>lacZ</i>)]</i>	SK3481, SK3424 ^c ; this work
SK3489	<i>glnAp381 glnAp131 ntrA75 ΔhisF645 putPA::[Kan^r-Φ(<i>glnA'</i>-<i>lacZ</i>)]</i>	SK1489, SK3424 ^c ; this work
Plasmids		
pJES311	Overexpression vector for NtrC ^{WT} under control of a T7 promoter ^f	Weiss et al. (57)
pJES412	Overexpression vector for NtrC ^{S207F,g} derived from pJES311	Weiss et al. (57)
pJES504	Overexpression vector for NtrC ^{Δ412-469} , derived from pJES311	North and Kustu (36)
pJES534	pJES436 derivative which contains a strong enhancer situated ~460 bp from the <i>glnA</i> promoter	Porter et al. (40)
pJES559	Overexpression vector for MBP-NtrC fusion protein under control of the <i>tac</i> promoter	Klose et al. (19)
pJES1065	Overexpression vector for NtrC ^{D239N,g} derived from pJES311	Rombel et al. (43)

^a This table lists all strains used in this study except those listed in Table 2, which have the same chromosomal genotype as SK3424 (*ntrC*), SK3482 (*ntrC ntrA*), or NCM724 (*E. coli*).

^b For each phage P22-mediated transductional cross, we list the recipient and the donor.

^c Selected for tetracycline resistance.

^d Large colonies were selected to ensure that the transductants had not lost the promoter(Up) mutations (27).

^e Selected for kanamycin resistance.

^f ϕ 10 promoter of phage T7 (dependent upon T7 RNA polymerase).

^g A known NtrC(Rep) mutant protein (37, 43).

SK1489, which carries a frameshift mutation in the *ntrA* gene (*ntrA75*) to yield strain SK3481 (Table 1). In the second cycle, the Φ (*glnA'*-*lacZ*) fusion at the *put* locus of strain SK3424 was transferred to strain SK3481 to yield strain SK3482. Strain SK3489 {*glnAp381 glnAp131 ntrA75 hisF645 putPA::[Kan^r- Φ (*glnA'*-*lacZ*)]*}, which carries an *ntrC*⁺ allele in the same background as SK3482, was constructed by introducing the Φ (*glnA'*-*lacZ*) fusion from SK3424 into SK1489.

All plasmids were electroporated into the host strain SK3424. Selected plasmids were electroporated into strain SK3482.

Growth conditions and β -galactosidase assay. Cultures of strains carrying putative *ntrC*(Rep) alleles in the SK3424 background were adapted to growth on glutamine as the sole nitrogen source by overnight growth in minimal medium containing 5 mM glutamine and 2 mM NH₄Cl. These precultures were subjected to centrifugation at room temperature. Cells were washed once with fresh medium containing 5 mM glutamine as the sole nitrogen source and were inoculated into 10 ml of this medium at an initial optical density at 650 nm (OD₆₅₀) of 0.05. Cultures were incubated in baffled culture tubes on a New Brunswick model G76 water bath shaker at 37°C and 240 rpm. Samples in the exponential phase of growth were harvested in duplicate between OD₆₅₀s of 0.2 and 0.6 and were immediately frozen on dry ice. Cultures of control strains SK3424 and SK3425 were grown along with each set of strains carrying putative *ntrC*(Rep) alleles in the overexpression vector. β -Galactosidase assays were carried out, and units of activity were calculated according to Miller (28) (see Fig. 3).

To assess the effect of an *ntrA* mutant allele on *glnA'*-*lacZ* expression for plasmids that yielded activity in the *ntrA*⁺ background, strains carrying such plasmids in strain SK3482 were grown as described above. β -Galactosidase assays were conducted as described above. Strain SK3483, which carries plasmid pJES311 (*ntrC*⁺ allele), and strain SK3489, which contains a single chromosomal *ntrC*⁺ gene, were used as controls.

Western blotting. Cultures grown under the same conditions as those used for β -galactosidase assays (500 μ l at an OD₆₅₀ of 0.5) were subjected to centrifugation, and cell pellets were suspended in 20 μ l of sodium dodecyl sulfate (SDS) gel-loading buffer (~25-fold concentration). In each case, the entire sample was subjected to electrophoresis in an SDS-10% polyacrylamide gel and proteins were transferred to nitrocellulose membranes as described previously (45). Membranes were exposed to polyclonal mouse antiserum directed against the

carboxy-terminal domain of the NtrC protein from *S. typhimurium*, and bands were detected with secondary antibodies directed against mouse immunoglobulin G and coupled to alkaline phosphatase (Bio-Rad). Purified NtrC and pre-stained markers (Broad Range; New England Biolabs) were used as standards.

To estimate the intracellular concentration of NtrC when the protein was expressed from a single chromosomal copy of the *ntrC* gene or from plasmid pJES311, we made the following assumptions: for *S. typhimurium* growing exponentially on minimal medium under nitrogen-limiting conditions, an OD₆₅₀ of 1 corresponds to 0.37 mg (dry weight) per milliliter of culture (16) and cell volume is 2.0 μ l/mg (dry weight) (16). Values of cell water content for enteric bacteria range from 1.9 to 2.5 μ l/mg (dry weight) (25, 31, 53).

Overexpression and small-scale purification of NtrC proteins. Plasmids for overproduction of NtrC proteins could not be maintained in *Escherichia coli* strains that contained T7 RNA polymerase (57); therefore, all of the 55 plasmids carrying putative *ntrC*(Rep) alleles were transformed into an Hfr strain of *E. coli* (NCM724), and induction was accomplished by infection with an M13 phage that carries T7 RNA polymerase under control of the *lac* promoter (M13mGP1-2). Cultures were grown in 50 ml of LB medium containing 2 mM glutamine and 100 μ g of ampicillin per ml at 37°C with vigorous shaking. At an OD₆₀₀ of ~0.5, M13mGP1-2 was added to a multiplicity of infection of about 10 (49). IPTG (isopropyl- β -D-thiogalactoside) was also added, to 0.5 mM, to allow induction of T7 RNA polymerase. After 2 h, cultures were harvested by centrifugation. Cells were suspended in 5 ml of breakage buffer (50 mM Tris-acetate [pH 8.2], 200 mM KCl, 1 mM EDTA, 1 mM dithiothreitol [DTT]) and were lysed by two passages through a French pressure cell (SLM-Aminco) at 8,000 lb/in². The resulting lysate was subjected to centrifugation at 27,000 \times g for 30 min at 4°C, and ammonium sulfate (35% final concentration) was added to the supernatant at room temperature. The ammonium sulfate pellet was dissolved in 10 ml of B-50 buffer (10 mM Tris-acetate [pH 8.2], 50 mM KCl, 0.1 mM EDTA, 5% glycerol, 1 mM DTT) and loaded on DEAE Sephacel (Pharmacia Biotech) (1 ml of resin packed into Poly-Prep chromatography columns [Bio-Rad]) at room temperature. Columns were washed twice with 5 ml of B-50 buffer, and then the bottom of each column was closed and 2 ml of B-500 buffer (10 mM Tris-acetate [pH 8.2], 500 mM KCl, 0.1 mM EDTA, 5% glycerol, 1 mM DTT) was added. After 10 min at room temperature, the proteins were eluted by centrifugation at

2,500 × g for 5 min at 4°C. To each sample (2 ml), 18 ml of B-0 buffer (10 mM Tris-acetate [pH 8.2], 0.1 mM EDTA, 5% glycerol, 1 mM DTT) was added and the diluted samples were loaded on heparin Sepharose CL-6B (Pharmacia Biotech) (0.5 ml of resin packed in the columns described above) at room temperature. The columns were washed with 5 ml of B-50 buffer, and then the bottoms were closed and 2 ml of B-500 buffer was added. After 10 min of incubation at room temperature, the proteins were eluted and dialyzed into B-50 buffer overnight at 4°C. Proteins were assessed to be at least 90% pure by visual inspection of SDS-10% polyacrylamide gels stained with Coomassie blue (Sigma). NtrC concentrations were determined from absorption at 280 nm in the presence of 6 M guanidine hydrochloride (Pierce) by using an extinction coefficient of 44,907.8 M⁻¹ cm⁻¹ for NtrC (9). Because none of the amino acid substitutions in mutant proteins involved W residues, all mutant proteins had approximately the same extinction coefficient as NtrC^{WT}.

The above procedure allowed us to purify all 41 mutant NtrC proteins that were soluble. SDS-polyacrylamide gels of various fractions indicated that purification was obtained by ammonium sulfate fractionation, because most proteins in crude cell supernatants remained in the ammonium sulfate supernatant, and upon binding to heparin Sepharose, because most of the remaining contaminating proteins did not bind (data not shown).

Assay for open complex formation. The ability of NtrC proteins to catalyze the formation of open complexes by σ^{54} holoenzyme was assessed on a supercoiled plasmid template by using a single-cycle transcription assay, as described previously (10, 39, 55, 57). The template was plasmid pJES534 (1 nM) (40), which carries a "strong enhancer" situated ~460 bp from the *glnA* promoter and directs synthesis of a 155-nucleotide transcript that contains no uracil (57). Final concentrations of reagents added to the buffer were as follows: core RNA polymerase, 30 nM; σ^{54} , 50 nM; carbamyl phosphate, 10 mM; ATP, 4 mM; GTP, 400 μ M; CTP, 100 μ M containing 5 μ Ci of [α -³²P]CTP. The NtrC concentration was varied. After allowing phosphorylation of NtrC by carbamyl phosphate for 10 min at 37°C, open complex formation was initiated by adding ATP to the reaction mixture. After 10 min, synthesis of transcripts was initiated by adding a mixture of heparin (100 μ g/ml) and the remaining two nucleotides. After an additional 10 min, transcripts were precipitated, electrophoresed on 6% sequencing gels, and quantified with a Molecular Dynamics PhosphorImager. To test the transcriptional ability of the NtrC mutant proteins in the absence of phosphorylation, carbamyl phosphate was omitted from the reaction mixture. The detection limit with this assay was 0.05 fmol.

RESULTS

Random PCR mutagenesis yielded 55 different single amino acid substitutions in the central domain of NtrC. To generate new mutant forms of NtrC defective in positive control, we performed PCR mutagenesis of the DNA encoding most of its central domain (see Materials and Methods). PCR-amplified DNA fragments were then substituted for the corresponding fragment of pJES311, which expresses wild-type NtrC from a strong T7 promoter and translational start. The resulting plasmids were transformed into *E. coli* DH5 α to generate a random mutant library.

When subjected to the genetic screen described in Materials and Methods, 95 of 1,234 transformants in the mutant library (8%) were unable to grow on the selective medium NB in the absence of glutamine, indicating that the plasmids they carried encoded putative *ntrC*(Rep) alleles. Plasmid DNA was extracted from each of these transformants, and sequencing revealed that 75 plasmids carried base substitutions in the targeted region of *ntrC* that resulted in single amino acid substitutions in the NtrC protein (Table 2). Among these, 14 plasmids carried an additional silent mutation. The plasmids from these 75 transformants implicated 55 different amino acid substitutions (at 44 amino acid positions) in producing a "repressor-like" phenotype. Plasmids from the remaining 20 transformants were found to carry either two (19 plasmids) or three (1 plasmid) point mutations. They were not analyzed further. Tabulations of the frequency of each of the 12 possible base pair substitutions showed a clear predominance of A · T → G · C transition mutations (63% [data not shown]), a distinct feature of the base substitution specificity of *Taq* DNA polymerase (51).

Assays of *glnA'*-*lacZ* expression indicated that all 55 new mutant forms of NtrC were deficient in transcriptional activation in vivo. We measured levels of expression of a Φ (*glnA'*-

lacZ) fusion at the *put* locus of *S. typhimurium* for all 55 mutant NtrC proteins containing single residue changes to assess their residual ability to activate transcription (Table 2 and Fig. 3). The β -galactosidase activity of the host strain (SK3424 *ntrC*) was ~850 U/ml/OD₆₅₀ and is attributable to transcription from the upstream σ^{70} -dependent promoter. When a multicopy expression vector encoding a known NtrC (Rep) protein, NtrC^{S207F} (that is, NtrC with a change from serine to phenylalanine at position 207), was introduced into SK3424, the β -galactosidase activity dropped to 16 U/ml/OD₆₅₀ because NtrC^{S207F} represses transcription from the upstream promoter and is unable to activate transcription from the σ^{54} -dependent promoter. By contrast, when the multicopy expression vector encoding the NtrC^{WT} protein was introduced, expression increased to ~1,350 U/ml/OD₆₅₀ under the derepressing growth conditions employed (see Materials and Methods). This increase in *glnA'*-*lacZ* expression was attributed to NtrC-dependent transcriptional activation at the downstream σ^{54} -dependent promoter because transcription from the upstream σ^{70} -dependent promoter was presumably repressed. When similar experiments were done with plasmids encoding all 55 new mutant forms of NtrC, they allowed us to divide these forms into two classes. One class (40 of 55) yielded β -galactosidase values comparable to those of known NtrC (Rep) proteins, i.e., ≤ 70 U/ml/OD₆₅₀. The remainder yielded values ranging from ~90 to ~500 U/ml/OD₆₅₀, less than that of the *ntrC* mutant host strain.

Residual *glnA'*-*lacZ* expression in strains expressing mutant NtrC proteins is σ^{54} dependent. For mutant NtrC proteins that yielded the highest residual *glnA'*-*lacZ* expression (90 to 500 U/ml/OD₆₅₀), we wanted to determine whether this activity was due to residual transcriptional activation from the downstream σ^{54} -dependent promoter or to loss of repression at the upstream σ^{70} -dependent promoter. To do so we performed two sorts of experiments: first, we ascertained whether residual expression was dependent on function of the *ntrA* gene product, σ^{54} ; second, we assessed immunologically whether any of the mutant proteins were significantly degraded.

When plasmids encoding all of the mutant NtrC proteins that yielded 90 to 500 U/ml/OD₆₅₀ of β -galactosidase activity (*glnA'*-*lacZ* expression) in an *ntrA*⁺ background were introduced into an *ntrA* mutant background (SK3482 host strain [see Materials and Methods and Table 1]), β -galactosidase activities dropped to values lower than 75 U/ml/OD₆₅₀ in all cases (Table 2). Control strain SK3489, which contains a single chromosomal *ntrC*⁺ allele in the same background as SK3482, had activities ranging between 40 and 80 U/ml/OD₆₅₀ in five independent experiments, and control strain SK3483, which carries plasmid pJES311 encoding NtrC^{WT}, gave β -galactosidase values between 10 and 70 U/ml/OD₆₅₀ in six independent experiments (Table 2).

To assess the integrity of all 55 mutant NtrC proteins, we performed Western blotting experiments with cells carrying plasmids encoding these proteins in the SK3424 background (*ntrC glnA'*-*lacZ*). Cells were grown under the same derepressing conditions used for β -galactosidase assays (Materials and Methods). Of these, 54 gave rise to only one prominent band which had the same mobility as NtrC^{WT} (encoded by pJES311). No secondary bands or bands of higher mobility were observed, and intensities of staining appeared to be comparable in all cases (data not shown). The host strain SK3424 (*ntrC*) yielded no band. In only one case, NtrC^{L297P} (SK3477), did we fail to detect any Western blot signal. Further analysis showed that strain SK3477 had lost its plasmid.

Taken together, the above results allowed us to conclude that the residual *glnA'*-*lacZ* expression in an *ntrA*⁺ back-

TABLE 2. Summary of DNA sequence changes and transcriptional activation at *glnA* in vivo and in vitro

Amino acid substitution ^a	Codon change	<i>S. typhimurium</i> strain ^b		In vivo activity ^c (U/ml/OD ₆₅₀)		<i>E. coli</i> strain ^d	In vitro activity ^e	Region of NtrC central domain ^f
		<i>ntrC</i>	<i>ntrC ntrA</i>	<i>ntrC</i>	<i>ntrC ntrA</i>			
E175G	GAA→GGA	SK3431		9		NCM3264	—	C1
V177A*	GTC→GCC	SK3430	SK3496	130	7	NCM3265	+	C1-C2
A180T	GCG→ACG	SK3427		13		NCM3216	+/-	C1-C2
L181I	CTT→ATT	SK3432	SK3485	86 ^g	73	NCM3208	+	C1-C2
A190V	GCG→GTG	SK3472	SK3527	105	64	NCM3266	ppt	C1-C2
A194T*	GCA→ACA	SK3438		20		NCM3078	+	C2
N196H	AAT→CAT	SK3473		6		NCM3267	—	C2
N196S	AAT→AGT	SK3436		17		NCM3048	+/-	C2
M197V	ATG→GTG	SK3439		21		NCM3079	+/-	C2
I200T	ATC→ACC	SK3440		8		NCM3080	—	C2
I200V	ATC→GTC	SK3441	SK3497	196	44	NCM3081	+	C2
S207F	TCC→TTC	SK3426		16		NCM3268	—	C3
E208V	GAA→GTA	SK3460		37		NCM3269	+/-	C3
F210L	TTT→TTG	SK3461		15		NCM3217	+/-	C3
K214E	AAA→GAA	SK3463		23		NCM3270	+/-	C3
K214R	AAA→AGA	SK3462	SK3498	216	20	NCM3271	+/-	C3
G215V*	GGC→GTC	SK3464		7		NCM3218	—	C3
F217L	TTT→CTT	SK3466		10		NCM3272	—	C3
T218A	ACC→GCC	SK3465		13		NCM3219	—	C3
T218N	ACC→AAC	SK3467		7		NCM3273	—	C3
A220V	GCG→GTG	SK3468		11		NCM3274	+	C3
Q230R	CAG→CGG	SK3442		66		NCM3204	+	C3-C4
F237S	TTT→TCT	SK3443		15		NCM3220	ppt	C4
L238P	CTG→CCG	SK3475		32		NCM3262	ppt	C4
D239G	GAC→GGC	SK3457		18		NCM3261	—	C4
M244V	ATG→GTG	SK3458		61		NCM3260	+	C4-C5
Q249R	CAG→CGG	SK3437		23		NCM3123	ppt	C4-C5
T250I	ACT→ATT	SK3433	SK3486	128	10	NCM3121	+	C4-C5
L253F	TTA→TTT	SK3450		18		NCM3259	—	C5
L253V	TTA→GTA	SK3434		18		NCM3122	—	C5
L256P	CTG→CCG	SK3476	SK3528	279	2	NCM3206	ppt	C5
Y262C	TAC→TGC	SK3435		20		NCM3253	+/-	C5-C6
Y262N*	TAC→AAC	SK3446		13		NCM3252	+/-	C5-C6
K271E	AAA→GAA	SK3451	SK3501	333	13	NCM3221	+	C5-C6
V274E	GTG→GAG	SK3444	SK3529	192	15	NCM3245	ppt	C6
T280A	ACC→GCC	SK3469		7		NCM3213	—	C6
L284H	CTC→CAC	SK3470		23		NCM3209	ppt	C6
E290G*	GAA→GGA	SK3452	SK3502	295	36	NCM3246	+	C6
G291R	GGG→AGG	SK3447	SK3503	127	7	NCM3207	+	C6
K292E	AAA→GAA	SK3471	SK3530	325	17	NCM3205	+	C6
D296G	GAC→GGC	SK3474		6		NCM3210	—	C6
L297P*	CTG→CCG	SK3477	SK3488	467	40	NCM3251	ppt	C6
F298C	TTC→TGC	SK3428		14		NCM3254	+/-	C6
F298L	TTC→CTC	SK3453	SK3531	268	3	NCM3250	+	C6
F298S	TTC→TCC	SK3454		29		NCM3255	—	C6
H299L	CAC→CTC	SK3448		17		NCM3249	—	C6
L301P*	CTG→CCG	SK3478		37		NCM3211	ppt	C6
V303A	GTG→GCG	SK3429		31		NCM3215	—	C6
V303M	GTG→ATG	SK3459		21		NCM3256	—	C6
I304F*	ATT→TTT	SK3455		21		NCM3247	+/-	C6
I304N	ATT→AAT	SK3449		26		NCM3257	ppt	C6
P309L*	CCG→CTG	SK3479		15		NCM3214	ppt	C6
I318T	ATT→ACT	SK3445	SK3505	98	6	NCM3248	ppt	C6-C7
L321P	CTG→CCG	SK3480		38		NCM3258	ppt	C6-C7
L321Q	CTG→CAG	SK3456		39		NCM3212	ppt	C6-C7
None		SK3425	SK3483	1,349 ± 343 [†]	30 ± 22 [‡]	NCM3120		
—		SK3424	SK3482	838 ± 90 [‡]	664 ± 206 [§]	NCM724		

^a Eleven of the mutational substitutions in this column occurred multiple times (number in parentheses): E175G (3), N196S (6), M197V (2), I200T (2), S207F (5), F217L (2), L256P (2), Y262C (3), V274E (2), L301P (2), and I318T (2). In each case, one isolate was chosen for further analysis. *, in addition to the mutation causing the amino acid substitution indicated, the plasmid we used contained one "silent mutation" in the mutagenized region of *ntrC*; —, no plasmid in these strains.

^b Strains listed in this column were constructed by electroporating multicopy plasmids into SK3424 (*ntrC* mutant host strain) or SK3482 (*ntrC ntrA* mutant host strain). The plasmid carried in each strain was either pJES311 or a pJES311 derivative expressing mutant NtrC proteins with the specific amino acid substitution indicated in the first column.

^c Values presented in this column were calculated from the slopes of plots of β-galactosidase activities (U/ml) expressed from a *glnA'*-*lacZ* fusion versus the OD₆₅₀ of the cultures. Where means ± standard deviations are given, values were calculated from 10 (†), 6 (‡), or 7 (§) independent experiments.

^d Strains listed in this column are NCM724 derivatives obtained by transforming this overexpression host strain with the plasmids described in footnote b.

^e The "+", "+/-", and "-" designations indicate that the mutant protein had, respectively, no detectable activity, less than the maximum activity of NtrC^{WT} (observed at 100 nM dimer) at any concentration, or at least as much as the maximum activity of NtrC^{WT} at some concentration; "ppt" means that these proteins were in the pellet after cells were broken with the French pressure cell and therefore could not be analyzed by in vitro transcription assays. Refer to Table 3 and Fig. 4 for more complete data.

^f Conserved regions of the NtrC central domain according to Morett and Segovia (30).

^g This strain gave two different values (447 and 697) when assayed in two other independent experiments.

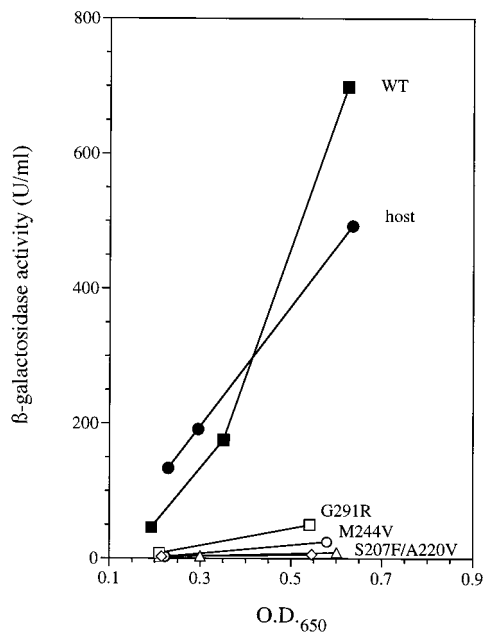


FIG. 3. In vivo expression from the *glnA* promoter in three strains carrying mutant NtrC proteins. Expression of a Φ (*glnA'*-*lacZ*) fusion at the *put* locus of *S. typhimurium* was measured as a function of the OD₆₅₀ for cultures grown under nitrogen-limiting conditions (see Materials and Methods). Curves labeled A220V (open diamonds), M244V (open circles), and G291R (open squares) give data for strains carrying plasmids that overexpress NtrC proteins with these three amino acid substitutions (strains SK3468, SK3458, and SK3447, respectively). In each case, two data points were used to determine the slope of the line, which reflects the differential rate of *glnA'*-*lacZ* expression. All three mutant NtrC proteins activated transcription better than NtrC^{WT} at low concentrations in vitro (Fig. 4B through D). Curves labeled WT (closed squares) and S207F (open triangles) give data for control strains carrying plasmids that overexpress NtrC^{WT} (strain SK3425) and NtrC^{S207F} (strain SK3426), a known NtrC(Rep) protein. The curve labeled host (closed circles) gives data for the *ntrC* mutant host strain SK3424. For control strains, three data points were taken to allow reliable comparisons between experiments.

ground was, in most instances, due to residual transcriptional activation by mutant NtrC proteins: activity was dependent on σ^{54} , the product of the *ntrA* gene (with the possible exceptions of proteins carrying the L181I [but see Table 2, footnote g] and A190V substitutions), and there was no sign of degradation of the mutant proteins. Moreover, experiments with control strains confirmed that expression of NtrC from a single chromosomal copy was sufficient for full repression (assessed in an *ntrA* mutant background), whereas expression of all proteins from plasmids was far in excess of this amount.

Forty-one of 55 mutant forms of NtrC were soluble and could be purified. To characterize their transcriptional activation capacities in vitro, we attempted to purify all 55 NtrC mutant proteins by using the rapid protocol described in Materials and Methods. Fourteen of the proteins could not be purified by this protocol because they were in the pellet after cells were broken with the French pressure cell (Table 2). The 41 proteins that were purified yielded from 2.6 to 18 nmol of dimer per 50 ml of culture (0.85 to 6 μ M), with the average yield around 6 nmol (2 μ M). The variation in yield could be due to inefficient induction during overexpression or to differences in behavior during purification. All proteins were at least 90% pure based on visual inspection of SDS-polyacrylamide gels stained with Coomassie blue, and none showed evidence of degradation (data not shown).

Seventeen of 41 soluble mutant proteins failed to activate transcription by σ^{54} holoenzyme in vitro. The 41 NtrC mutant

proteins purified were tested for the ability to catalyze the formation of open complexes by σ^{54} holoenzyme at the *glnA* promoter (Table 2; see also Materials and Methods). Phosphorylated NtrC^{WT} catalyzed the formation of open complexes at concentrations ranging from 10 nM dimer (the lowest concentration tested) up to 500 nM dimer (the highest concentration tested) (Fig. 4 and Table 3). In contrast, 17 of the 41 phosphorylated NtrC mutant proteins, which had amino acid substitutions in six of the seven highly conserved regions in the central domain, failed to catalyze open complex formation at 10, 20, 50, and 100 nM dimer (Table 2). Four of these 17 (N196H, F217L, L253V, and V303A) were also tested at 200 and 500 nM dimer and, in all cases, yielded <1% as many open complexes as the wild-type protein at the same concentrations (data not shown). The 17 mutant proteins that failed to activate transcription from the σ^{54} -dependent *glnA* promoter in vitro also showed little *glnA* transcription in vivo (<70 U/ml/OD₆₅₀ of β -galactosidase activity from the *glnA'*-*lacZ* fusion). Hence, they behaved like the NtrC(Rep) proteins characterized previously.

The remaining 24 of 41 soluble mutant proteins did activate transcription by σ^{54} holoenzyme in vitro. Unexpectedly, in vitro transcription assays revealed a large class of 24 mutant proteins that did catalyze formation of open complexes by σ^{54} holoenzyme in vitro. Among these proteins, 14 yielded essentially no *glnA* transcription in vivo (<70 U/ml/OD₆₅₀ of β -galactosidase activity from the *glnA'*-*lacZ* fusion). Ten of these proteins (A180T, N196S, M197V, E208V, F210L, K214E, Y262C, Y262N, F298C, and I304F) failed to reach the maximum activity of phosphorylated NtrC^{WT} in vitro (achieved at 100 nM dimer) at any concentration, and two of these (E208V and K214E) had very low levels of activity (Table 3 and Fig. 4D). The remaining four proteins of this subclass (A194T, A220V, Q230R, and M244V) did reach the maximum activity of NtrC^{WT} (Table 3; Fig. 3, 4B, and 4C).

Ten of the 24 mutant proteins that activated transcription by σ^{54} holoenzyme in vitro also did so in vivo (between 90 and 500 U/ml/OD₆₅₀ of β -galactosidase activity from the *glnA'*-*lacZ* fusion and dependent on an *ntrA*⁺ allele). Nine of these 10 proteins (V177A, L181I, I200V, T250I, K271E, E290G, G291R, K292E, and F298L) were fully active in vitro at a dimer concentration of 100 nM (Table 3; Fig. 3, 4A, and 4D), whereas 1 (K214R) yielded only 1% as many open complexes as NtrC^{WT} at the same concentration and had very low levels of activity generally (Table 3).

Although it is reasonably common for mutant proteins to show less activity in vitro than in vivo, the reverse is unusual; therefore, we sought a specific explanation for why a large number of our mutant proteins showed activity in vitro but not in vivo. Because preliminary Western blot experiments had indicated that all proteins were greatly overexpressed even in the absence of T7 RNA polymerase, the 24 mutant proteins described in this section were tested for transcriptional activation at high concentrations (from 200 to 500 nM dimer). The activities of at least half dropped dramatically (Table 3; Fig. 4A through D). We infer that decreases in activity were due to abnormal oligomerization or aggregation because NtrC^{WT} maintained maximal activity at 500 nM dimer (Table 3 and Fig. 4). For the 14 proteins that lacked activity in vivo, in vitro activities at 200 to 500 nM dimer dropped to values well below those for NtrC^{WT} (Table 3; Fig. 4B through D). As shown below, the estimated in vivo concentrations of NtrC proteins from the overexpression plasmid were well above 500 nM, even in the absence of T7 RNA polymerase.

Nineteen of the 24 NtrC mutant proteins that activated transcription by σ^{54} holoenzyme in vitro showed hyperactivity

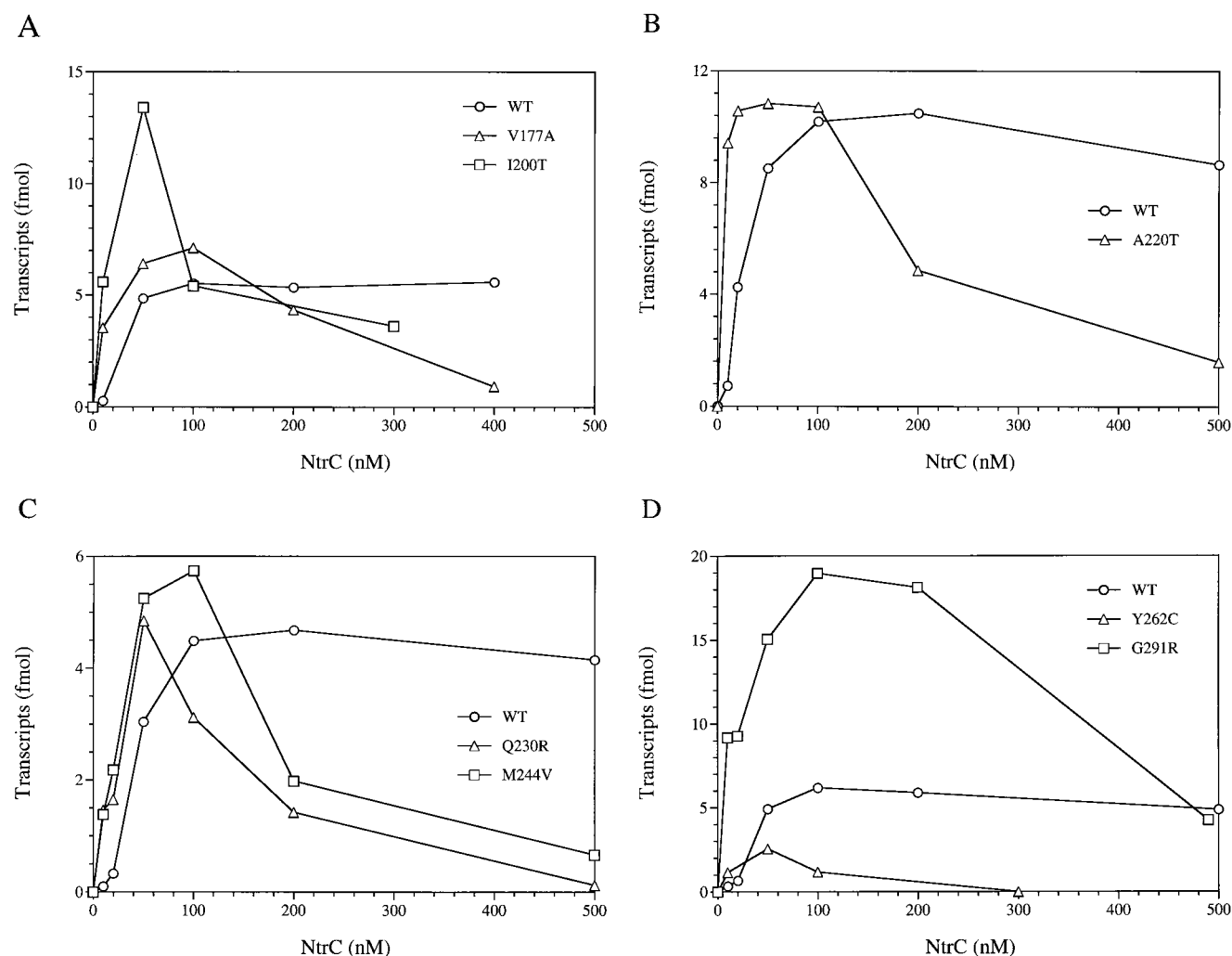


FIG. 4. Transcriptional activation by selected mutant NtrC proteins in vitro. Formation of open complexes by σ^{54} holoenzyme was assessed by a single-round transcription assay as described in Materials and Methods. NtrC proteins were present at the concentrations indicated and were phosphorylated with carbamyl phosphate (10 mM). Effects of single amino acid substitutions were assessed by comparison to NtrC^{WT} (open circles) in each panel. (A) V177A and I200V. (B) A220V. (C) Q230R and M244V. (D) Y262C and G291R. Proteins were chosen to illustrate their hyperactivity at low concentrations and the widespread locations of their amino acid substitutions within the central domain of NtrC: V177A between conserved regions 1 and 2 (C1-C2), I200V (C2), A220V (C3), Q230R (C3-C4), M244V (C4-C5), Y262C (C5), and G291R (C6). Data for NtrC^{WT} is included in each plot to illustrate the variability between experiments.

at low concentrations. Perhaps our most interesting result was the observation that 19 of the 24 mutant proteins that catalyze the formation of open complexes by σ^{54} holoenzyme in vitro (discussed above) did so better than NtrC^{WT} at low concentrations. They yielded 3 to 20 times as many open complexes as NtrC^{WT} at 10 nM dimer (Table 3; Fig. 4A through D). Figures 4A through D show some particularly striking examples of hyperactivity (V177A, I200V, A220V, Q230R, M244V, and G291R) and a single example (Y262C) in which hyperactivity at low concentrations is not accompanied by the ability to reach the maximum activity for NtrC^{WT} at any concentration.

All 24 mutant proteins that are active in vitro must be phosphorylated. Some NtrC mutant proteins, called NtrC constitutive [NtrC(Con)], can hydrolyze ATP and activate transcription in vitro without being phosphorylated. Amino acid substitutions in NtrC(Con) proteins have been localized to both the N-terminal regulatory domain and the central domain (10). To determine whether any of the 24 NtrC mutant proteins that were active in vitro were active without being phosphorylated, i.e., had the NtrC(Con) phenotype, all were tested

for transcriptional activation in the absence of carbamyl phosphate, the in vitro phosphate donor. All 24 mutant proteins were tested at concentrations of 50 and 100 nM dimer, concentrations at which all of the phosphorylated proteins gave rise to clearly visible transcripts, as did the unphosphorylated NtrC(Con) protein NtrC^{S160E} (10), which was used as a positive control. No visible transcripts were observed for the 24 new mutant proteins, showing that all must be phosphorylated to catalyze formation of open complexes by σ^{54} holoenzyme at the *glnA* promoter.

Levels of NtrC protein from the expression plasmid in the absence of T7 polymerase are hundreds of times higher than the chromosomal level. The unexpected activities of many mutant NtrC proteins in vitro, together with the observation that their activities dropped considerably with an increase in protein concentration in many instances, led us to quantitate the overexpression of these proteins in vivo. Preliminary Western blot experiments performed with cells carrying plasmids that encoded either NtrC^{WT} (pJES311) or the mutant proteins had

TABLE 3. Transcriptional activation by NtrC mutant proteins relative to the wild-type protein and by NtrC^{WT} in different experiments

Amino acid substitution or expt	Concn ^a of NtrC (at indicated concn of dimer [nM])						
	10	20	50	100	200	300	500
Substitution ^b							
None	1.0	1.0	1.0	1.0	1.0	1.0	1.0
V177A (6)	10.0	10.5	1.3	1.1	0.7	nd	0.2 ^c
A180T (3)	6.0	0.7	0.6	0.2	0.06	nd	0.03 ^c
L181I (5)	8.7	5.6	1.2	1.0	1.0	nd	0.7
A194T (9)	12.0	nd	1.7	0.5	0.07 ^d	nd	nd
N196S (2)	1.0	0.7	0.5	0.4	0.2	nd	0.05
M197V (8)	3.5	nd	0.6	0.6	0.4	nd	nd
I200V (8)	28.0	nd	2.8	1.1	nd	0.8	nd
E208V (9)	0.5	nd	0.1	0.01	0.0	nd	nd
F210L (7)	3.0	nd	0.4	0.2	nd	0.01	nd
K214E (2)	0.1	0.05	0.03	0.06	0.06	nd	0.02
K214R (6)	6.6	2.3	0.1	0.01	0.0	nd	0.0 ^c
A220V (1)	13.4	2.5	1.3	1.0	0.5	nd	0.2
Q230R (4)	15.0	5.7	1.6	0.7	0.3	nd	0.03
M244V (4)	14.0	7.3	1.7	1.3	0.4	nd	0.2
T250I (9)	28.5	10.2	2.2	1.5	nd	nd	nd
Y262C (7)	3.0	nd	0.5	0.2	nd	0.0	nd
Y262N (3)	5.0	0.5	0.2	0.2	0.03	nd	0.0 ^c
K271E (8)	1.0	nd	0.9	1.6	nd	1.3	nd
E290G (7)	17.0	nd	2.0	1.7	nd	1.0	nd
G291R (5)	23.0	13.3	3.0	3.0	3.0	nd	0.8
K292E (7)	25.0	nd	2.0	1.6	nd	0.8	nd
F298C (9)	1.0	nd	0.7	0.4	0.2	nd	nd
F298L (8)	13.0	nd	1.5	1.4	nd	1.4	nd
I304F (1)	4.0	1.5	0.8	0.4	0.06	nd	0.05
Expt							
1	0.7	4.3	8.5	10.2	10.5	nd	8.7
2	1.2	4.2	9.7	10.7	10.2	nd	8.0
3	0.2	4.0	8.4	10.2	10.2	nd	8.6 ^c
4	0.1	0.3	3.0	4.5	4.7	nd	4.2
5	0.4	0.7	4.9	6.2	5.9	nd	4.9
6	0.4	0.6	5.0	6.2	6.4	nd	5.6 ^c
7	0.4	nd	4.8	5.8	nd	6.2	nd
8	0.2	nd	4.7	4.8	4.7	4.5	nd
9	0.2	0.6	3.0	4.2	4.0	nd	nd
Avg	0.4 ± 0.3	2.2 ± 1.9	5.3 ± 2.2	6.5 ± 2.4	7.2 ± 2.7	5.3 ± 1.0	6.5 ± 2.0

^a Data for individual amino acid substitutions are ratios of activities of mutant proteins to those of the wild-type protein. Data for the wild-type protein in individual experiments are presented in femtomoles. nd, not determined.

^b Numbers in parentheses refer to the corresponding experiment numbers (1 through 9 [below]), for which the data are wild-type values used for normalization.

^c Value at 400 nM dimer.

^d Value at 150 nM dimer.

already attracted our attention to the high levels of overexpression even in the absence of T7 RNA polymerase.

To estimate the degree of overexpression with respect to a single chromosomal *ntrC*⁺ allele, we lysed whole cells of strain SK3425, which carries pJES311, and strain SK1490, which contains a single chromosomal copy of *ntrC*, in SDS loading buffer and compared the intensity of the NtrC band in Western blots for dilutions of the lysate from SK3425 to those for undiluted lysate from SK1490. Comparable intensities were observed when the lysate of strain SK3425 was diluted on the order of 500-fold (Fig. 5A). To estimate the concentration of NtrC when it is overexpressed 500-fold, we next determined the absolute amount of NtrC in lysates of SK1490 by comparing the intensity of the NtrC band in Western blots for a lysate of this strain to those of different amounts of purified NtrC protein added to a lysate of strain SK3424 (*ntrC*) (Fig. 5B). Matched intensities were observed when 25 to 50 ng of purified NtrC protein was added to the lysate of SK3424. Assuming that the amount of NtrC expressed from a single chromosomal copy of *ntrC* is between 25 and 50 ng for the number of cells used

(see below) and using the conversion factors given in Materials and Methods, we estimate that the concentration of NtrC is between 1.0 and 2.0 μ M under nitrogen-limiting conditions (25 ng of NtrC dimer = 2.4×10^{-13} mol [the molecular mass of NtrC is 104,476 g/mol of dimer]; 0.5 ml of culture at an OD₆₅₀ of 0.5 has 9.25×10^{-2} mg [dry weight]; 9.25×10^{-2} mg [dry weight] $\times 2 \mu$ l/mg [dry weight] = 0.19 μ l $\therefore 1.9 \times 10^{-7}$ liter; [2.4×10^{-13} mol]/[1.9×10^{-7} liter] = 1.3 μ M). Because the amount of NtrC protein expressed from plasmid pJES311 and its derivatives encoding mutant NtrC proteins (see above) appears to be on the order of 500-fold higher than that expressed from a single *ntrC*⁺ allele, the concentration of NtrC in strains carrying these plasmids is in the range of 0.5 to 1.0 mM, several hundredfold above the maximum concentration tested in vitro (0.5 μ M). Thus, the apparent lack of activity of many NtrC mutant proteins in vivo appears to be accounted for by high levels of expression.

The above estimate for the concentration of NtrC in a cell carrying a single chromosomal *ntrC*⁺ allele is 10- to 20-fold higher than an estimate made previously (70 molecules/cell =

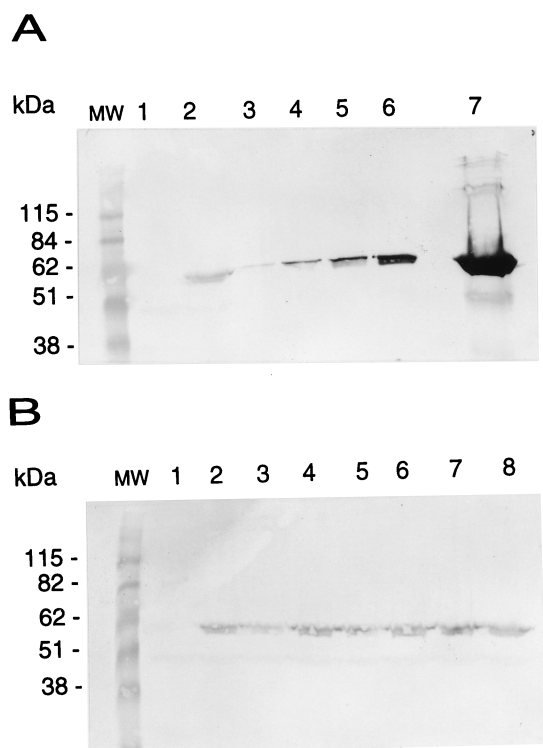


FIG. 5. Amounts of NtrC protein expressed from a single chromosomal *ntrC*⁺ allele or from plasmid pJES311, which overexpresses NtrC^{WT}. Cells were cultivated under nitrogen-limiting conditions as for assays of *glnA*'-*lacZ* expression (see Materials and Methods), and 0.5 ml of culture at an OD₆₅₀ of 0.5 was concentrated and loaded on an SDS-10% polyacrylamide gel. Amounts of NtrC protein in crude lysates were determined by Western blotting with mouse polyclonal antiserum raised against the carboxy-terminal domain of NtrC. (A) Crude lysates of SK3424 (*ntrC*) (lane 1) and SK1490 (*ntrC*⁺) (lane 2) were compared with dilutions of lysates of SK3425(pJES311): lane 3, 1:1,250; lane 4, 1:625; lane 5, 1:125; lane 6, 1:25; lane 7, undiluted. (B) Crude lysates of strain SK1490 (lanes 2, 4, 6, and 8) were compared with different amounts of purified NtrC^{WT} added to crude lysates of SK3424: lane 1, 0 ng; lane 3, 12.5 ng; lane 5, 25 ng; lane 7, 50 ng. MW, BenchMark molecular weight standards (Gibco BRL).

~70 molecules/fl = ~0.1 μ M) (41). Nonetheless, if the amount of NtrC protein expressed from pJES311 and its derivatives is 500-fold higher than that expressed from a single *ntrC*⁺ allele, the concentration of NtrC in strains carrying these plasmids would be in the range of 50 μ M, still 100-fold above the maximum concentration tested in vitro. Hence the conclusion stands.

DISCUSSION

New NtrC(Rep) proteins (positive control forms) have lesions in regions of the central domain that are not well studied. In vitro assays of transcriptional activation at the σ ⁵⁴-dependent *glnA* promoter by 41 NtrC mutant proteins with decreased activity in vivo yielded 16 new forms that failed in transcriptional activation. Together with the 13 previously studied forms, lesions in these NtrC(Rep) proteins affect residues in or, in one case, directly adjacent to all seven of the conserved motifs in the central domain (30, 38). These include two regions—C2 and C5—in which such lesions had not been found previously and the unusually long conserved region C6, in which only a single lesion had been characterized (37, 43, 57). Lesions affecting C2 and C5 have not yet been characterized for other σ ⁵⁴-dependent activators, and few lesions in C6 have been characterized (54). In only 1 of 29 cases (T280A in

the C6 region) was the amino acid residue in the inactive mutant form of NtrC found at the corresponding position of a homologous activator of σ ⁵⁴ holoenzyme (DctD of *Rhizobium leguminosarum*). Several of the residues that were altered in the inactive mutant forms of NtrC (N196, T218, D296, R358) had been predicted to be “functional” residues (38).

Unlike regions C1, C3, C4, and C7, which have obvious functional parallels in the large family of purine nucleotide-binding proteins with a mononucleotide fold (see the introduction), regions C2, C5, and C6 do not. Based on its location just downstream of the conserved aspartate of the Walker B motif, D239 in conserved region 4 of NtrC, it is possible that region C5 or the region between C4 and C5 corresponds to the switch II motif, critical in coupling of nucleotide hydrolysis to biological output. The one lesion in C6 that we have characterized previously, R294C, resulted in loss of ATP hydrolysis without effect on ATP binding or on oligomerization of NtrC (37, 43). It is possible that R294 is a catalytic residue. Oligomerization determinants of NtrC, which are thought to lie in its central domain (10), remain to be defined.

NtrC mutant forms that are hyperactive at low concentrations have lesions throughout the central domain. Unexpectedly, 24 new mutant forms of NtrC that failed to activate transcription in vivo, or activated poorly, did activate in vitro. Because the activation by many of these forms decreased at high concentrations in vitro, their apparent failure to activate in vivo is probably accounted for by high levels of overexpression (~500-fold [see Results]). Nineteen such forms were more active at low concentrations (10 to 50 nM) than NtrC^{WT}, and many of these showed decreases in activity at higher concentrations (200 to 500 nM). These mutant forms have lesions that are widespread in the central domain of NtrC. They occur in the region between C1, the Walker A motif, and C2 (e.g., V177A and L181I); in or immediately adjacent to the C2 region (e.g., A194T and I200V); in the region between C4 and C5 (e.g., M244V and T250I); and in region C6, where they affect the three adjacent residues E290 to K292 and residue E298. There is one such lesion (A220V) in C3, the switch I region, and one (Q230R) between C3 and the Walker B motif, C4. The significance of the widespread occurrence of these forms is not understood, nor is the functional basis for their unexpected phenotype in vitro. Hyperactivity at low concentrations may be due to increased oligomerization and ATPase activity, increased biological output, or both, and the basis may be different for different lesions. Lesions to hyperactivity that occurred between, rather than within, conserved regions of NtrC tended to affect residues that were tree determining for the NtrC subfamily of σ ⁵⁴-dependent activators (e.g., V177, L181, Q230, and T250) (38). These residues were postulated to participate in unique aspects of oligomerization or of the response to signals from the regulatory domain.

Interestingly, amino acid covariation analysis indicated that region C6, a region in which several changes to hyperactivity occurred in NtrC, appears to make tertiary contacts with many of the other conserved regions in activators of σ ⁵⁴ holoenzyme. For several of the lesions in this region, the amino acid substitution that results in hyperactivity of the mutant form of NtrC at low concentrations is found naturally in the wild-type form of another activator. (This is also true for lesions to hyperactivity in other regions of NtrC; for a complete list, see the legend to Fig. 2.) A particularly interesting example is the G291R substitution, because this glycine residue is highly conserved among activators of σ ⁵⁴ holoenzyme and the substitution appears to be extreme. NtrC^{G291R} has 20 times higher activity than NtrC^{WT} at a concentration of 10 nM and continues to have 3-fold-more activity than NtrC^{WT} when the wild-

type protein has reached its maximal activity at 100 nM. An arginine is found at the corresponding position for the wild-type form of the *E. coli* FhlA protein (Fig. 2). Similar observations pertain regarding the extreme substitution at the adjacent position in C6, K292E, and for the F298L substitution (Fig. 2). Moreover, in the latter case, different amino acid substitutions at the same position result in either hyperactivity (F298L) or loss of activity (F298S). The only other positions at which both phenotypes were observed were position I200 in the C2 region (I200V resulted in hyperactivity, whereas I200T resulted in loss of activity) and position 220 in the C3, or switch I, region (see below).

Comments on new lesions in the Walker A (C1), Walker B (C4), and switch I (C3) regions. The Walker A motif for activators of σ^{54} holoenzyme (C1) differs from that of other members of the purine nucleotide-binding protein family in ending with a conserved E or D, rather than the usual S or T, which is often involved in coordination of the divalent cation (38). NtrC^{E175G}, in which the conserved glutamate is altered, was inactive *in vivo* and *in vitro*. The same was true when the corresponding glutamate in the homologous activator DctD was changed to either T or A (11). The mutant DctD proteins were shown to be greatly defective in ATP hydrolysis (11). Lesions in NtrC that altered the two hydrophobic residues preceding D239, the conserved aspartate of the Walker B motif, resulted in precipitation of the corresponding proteins (NtrC^{F237S} and NtrC^{L238P}). The hydrophobic residues normally present at these positions of purine nucleotide-binding proteins are part of a β -strand (38). Like previously characterized lesions at position 239 (D239A, N, and C [43]), each of which resulted in loss of ATPase activity but not ATP binding, an additional lesion at this position, D239G, resulted in loss of transcriptional activation. Interestingly, although NtrC has a glycine at position 242, as do other "DEXG proteins," the E247G substitution at the corresponding position of *Rhizobium meliloti* DctD caused loss of transcriptional activation both *in vivo* and *in vitro* (54). This appears to be the only example in which changing a residue of a homologous activator to that found at the corresponding position of NtrC results in loss of activity.

The A220V substitution at the end of C3, the switch I region, results in hyperactivity at low concentrations, whereas the A220T substitution and an A-to-T substitution at the corresponding position of DctD (54) result in loss of transcriptional activation with little decrease in ATPase activity (37). Given that the switch I region does not appear to affect ATP hydrolysis *per se* but rather couples the energy made available to biological output, hyperactivity of NtrC^{A220V} at low concentrations may be due to an improved efficiency of interaction with σ^{54} holoenzyme.

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