"Switch I" mutant forms of the bacterial enhancer-binding protein NtrC that perturb the response to DNA

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NtrC (nitrogen regulatory protein C) is a bacterial enhancer-binding protein of 469 residues that activates transcription by σ^{54}-holoenzyme. A region of its transcriptional activation (central) domain that is highly conserved among homologous activators of σ^{54}-holoenzyme—residues 206–220—is essential for interaction with this RNA polymerase: it is required for contact with the polymerase and/or for coupling the energy from ATP hydrolysis to a change in the conformation of the polymerase that allows it to form transcriptionally productive open complexes. Several mutant NtrC proteins with amino acid substitutions in this region, including $NtrC^{A216V}$ and $NtrC^{G219K}$, have normal ATPase activity but fail in transcriptional activation. We now report that other mutant forms carrying amino acid substitutions at these same positions, $NtrC^{A216C}$ and $NtrC^{G219C}$, are capable of activating transcription when they are not bound to a DNA template (non-DNA-binding derivatives with an altered helix–turn–helix DNA-binding motif at the C terminus of the protein) but are unable to do so when they are bound to a DNA template, whether or not it carries a specific enhancer. Enhancer DNA remains a positive allosteric effector of ATP hydrolysis, as it is for wild-type NtrC but, surprisingly, appears to have become a negative allosteric effector for some aspect of interaction with σ^{54}-holoenzyme. The conserved region in which these amino acid substitutions occur (206–220) is equivalent to the Switch I region of a large group of purine nucleotide-binding proteins. Interesting analogies can be drawn between the Switch I region of NtrC and that of $p21^{ras}$.

The bacterial enhancer-binding protein NtrC (nitrogen regulatory protein C) activates transcription by the σ^{54}-holoenzyme form of RNA polymerase (1–3). To do so it catalyzes the isomerization of closed complexes between this polymerase and a promoter to transcriptionally productive open complexes in a reaction that depends on hydrolysis of a purine nucleoside triphosphate. Because formation of open complexes is thermodynamically as well as kinetically unfavorable, NtrC and other activators of σ^{54}-holoenzyme must function as molecular machines. To form the large oligomers that are required for ATP hydrolysis and, hence, transcriptional activation, NtrC must be phosphorylated at aspartate 54. Binding of the phosphorylated protein to an enhancer stimulates oligomer formation and, hence, stimulates ATPase activity and transcriptional activation (4, 5). Phosphorylated NtrC oligomers bound to an enhancer contact σ^{54}-holoenzyme by means of DNA loops (1) and apparently catalyze an energy-dependent change in the conformation of the polymerase that allows it to form open complexes (6–10).

NtrC is composed of three functional domains (11–13). The N-terminal domain is the regulatory domain, which contains the site of phosphorylation. The C-terminal domain contains a helix–turn–helix DNA-binding motif and the major dimerization determinants of the protein. The central domain is the transcriptional activation domain—the domain responsible for biological output. It is composed of about 240 aa residues and shows a high degree of sequence conservation with all known transcriptional activators of σ^{54}-holoenzyme. Secondary structure predictions coupled with the use of recognition algorithms for protein folds indicated that the central domain of NtrC and the corresponding domains of other activators adopt a mononucleotide-binding fold similar to those of $p21^{ras}$ and the G domain of the bacterial polypeptide elongation factor EF-Tu (14).

Mutations causing specific loss of transcriptional activation by the NtrC protein of *Salmonella typhimurium* affect residues in the seven conserved motifs within its central domain (14–17). Many result in decreased binding or hydrolysis of ATP. Perhaps the most interesting mutations causing loss of transcriptional activation affect what appears to be the Switch I region of NtrC (residues 206–220), which lies between its putative Walker A and B motifs. These lesions have little if any effect on the ATPase activity of NtrC but essentially eliminate biological output. By inference, they must affect some aspect of the interaction with σ^{54} holoenzyme—contact with the polymerase and/or energy coupling. Two amino acid substitutions in the corresponding region of the homologous activator DctD decrease its cross-linking to σ^{54} and the β-subunit of RNA polymerase (18).

To study the Switch I region of NtrC further we used *in vitro* mutagenesis to generate proteins with single cysteine substitutions in this region. Two of these, $NtrC^{A216C}$ and $NtrC^{G219C}$, have more than sufficient ATPase activity for transcriptional activation and bind the enhancer normally. However, unlike the previously studied forms with amino acid substitutions at these positions—$NtrC^{A216V}$ and $NtrC^{G219K}$—which fail to activate transcription, the new forms retain the ability to activate transcription poorly only at very high concentrations. Further studies of $NtrC^{A216C}$ indicated that DNA has become a negative allosteric effector: binding to an enhancer or "nonspecific" DNA inhibits transcriptional activation by this protein, although the enhancer remains a positive allosteric effector for ATP hydrolysis, as it is for wild-type NtrC ($NtrC^{WT}$). By inference, inhibitory effects are exerted on some aspect of interaction with the polymerase.

Materials and Methods

Mutagenesis and Construction of Plasmids for NtrC Overproduction. Plasmid pJES990 (19), a pTZ18U derivative that contains the cysteine-free (C30V and C364A) version of the *ntrC* coding region, was mutated with the following oligonucleotides: CATGAGAAAGGCTGCTTTACCGGGGCG (introduces the A216C substitution and a *Bbv*I site) and GGCGCTTTTACCTGCGCAAATACCATCCGGC (introduces the G219C substitution and an *Fsp*I site). Mutagenesis was performed with the Muta-Gene phagemid *in vitro* mutagenesis kit (Bio-Rad). The sequences of 369-bp *Age*I-*Bbs*I fragments of the resulting plas-

Abbreviations: GAPS, G activating proteins; MBP, maltose-binding protein; NtrC, nitrogen regulatory protein C; $NtrC^{WT}$, wild-type NtrC.

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mids, which cover the mutations, were confirmed. The *Age*I-*Bbs*I fragments were ligated into pJES990, digested similarly, to ensure that other regions of *ntrC* were error-free. The resulting plasmids were pJES1042 (A216C) and pJES1045 (G219C), respectively. The 1.9-kb *Kpn*I-*Eco*RI fragments from pJES1042 and pJES1045 then were ligated into similarly digested pJES559 (20), which is the overexpression plasmid for maltose-binding protein (MBP) fusions to NtrC. The resulting plasmids were pJES1049 (MBP-NtrC^{A216C}) and pJES1052 (MBP-NtrC^{G219C}), respectively. To construct non-DNA-binding derivatives of MBP-NtrC, the 0.6-kb *Csp*I-*Eco*RI fragments of pJES994 (cysteine-free version of MBP-NtrC) (19), pJES1049 and pJES1052 were replaced with the *Csp*I-*Eco*RI fragment from pJES641 (overexpression plasmid for NtrC^{3ala}) (21), resulting in pJES1135 (MBP-NtrC^{3ala}), pJES1137 (MBP-NtrC$^{A216C,\ 3ala}$), and pJES1138 (MBP-NtrC$^{G219C,\ 3ala}$), respectively.

Protein Purification. MBP-NtrC and its derivatives were purified essentially as described (20). Briefly, isopropyl β-D-thiogalactoside-induced cells were harvested and disrupted by passage through a French Pressure Cell. MBP-NtrC was precipitated with ammonium sulfate (35–70%) and then was chromatographed on Amylose (New England Biolabs), heparin agarose (Bethesda Research Laboratories), and Resource-Q (Pharmacia). Core RNA polymerase and σ^{54} were purified as described (22).

Transcription Assays. The ability of NtrC proteins to catalyze open complex formation by σ^{54}-holoenzyme was assessed in a single-cycle transcription assay as described (8, 23, 24). Templates were supercoiled plasmid pJES534 (carries a strong enhancer composed of two strong NtrC-binding sites) or pJES535 (lacks specific NtrC-binding site) (21) at a concentration of l nM. Final concentrations of reagents during open complex formation were: 30 nM core RNA polymerase, 50 nM σ^{54}, 10 mM carbamoyl phosphate, 4 mM ATP, and 2–1,000 nM NtrC (always reported as dimer concentration). Open complex formation was terminated by addition of heparin to a final concentration of 0.1 mg/ml, and synthesis of transcripts was achieved by addition of GTP and CTP to final concentrations of 400 μM and 100 μM, respectively, together with 5 μCi [α-^{32}P]CTP (1 Ci = 37 GBq) in each reaction.

ATPase Assays. The release of Pi from [γ-^{32}P]ATP was monitored as described (15, 24) by using essentially the same assay buffer as the one used in transcription assays (23). Phosphorylation of NtrC was achieved by incubating NtrC with 10 mM carbamoyl phosphate at 37°C for 10 min before ATP was added. A synthetic DNA oligonucleotide (69 bp) carrying the strong enhancer (24) was used to assess enhancer-stimulated ATPase activity. A 69-bp oligonucleotide lacking specific NtrC-binding sites was used as a control to demonstrate the absence of contaminating DNA-dependent ATPase activity.

DNA-Binding Assays. Gel mobility-shift assays were performed as described (21), using an amplified PCR fragment (106 bp) from pJES464, which carries the natural *glnA* enhancer (25).

Gel Filtration. Protein samples (100 μl) were sieved on a Bio-Silect Sec 400-5 column (300 × 7.8 mm; Bio-Rad) with a Bio-Silect 400 guard column (50 × 7.8 mm; Bio-Rad). The column equilibration and protein dilution buffer contained 10 mM Tris·HCl (pH 7.4), 50 mM KCl, 0.1 mM EDTA, 8 mM $MgCl_2$ and 5% glycerol, with or without 10 mM carbamoyl phosphate. Phosphorylation of NtrC was achieved by incubating NtrC with 10 mM carbamoyl phosphate at room temperature for 10 min before sample injection. The column was run at 0.8 ml/min on a Gold Nouveau HPLC system (Beckman). Elution of proteins was detected at 214 nm, which gives a better signal/noise ratio than detection at 280 nm, with a bandwidth of 4 nm.

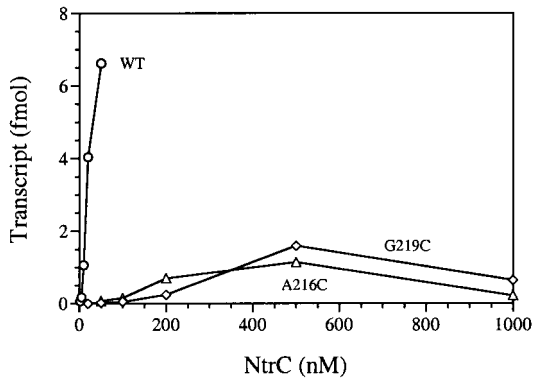

Fig. 1. Transcriptional activation at the *glnA* promoter by Switch I mutant forms of NtrC. Single-cycle transcription assays were performed as described in *Materials and Methods*. The template was supercoiled plasmid pJES534 (1 nM), which carries a strong enhancer ≈460 bp upstream of the *glnA* promoter (21). Relevant NtrC proteins are indicated next to the curves: WT, MBP-NtrC^{WT}; A216C, MBP-NtrC^{A216C}; G219C, MBP-NtrC^{G219C}.

Results

Some Switch I Mutant Forms of NtrC Retain Residual Ability to Activate Transcription. On a template carrying a strong enhancer upstream of the *glnA* promoter, phosphorylated mutant proteins (MBP-)NtrC^{A216C} and NtrC^{G219C} could activate transcription only at very high concentrations (Fig. 1). Whereas phosphorylated NtrC^{WT} activates well at 20 nM, NtrC^{A216C} and NtrC^{G219C} yielded only trace amounts of transcripts at a concentration 10-fold higher (200 nM) and showed very poor template utilization even at best (at 500 nM). As discussed below, we considered two obvious explanations for why NtrC^{A216C} and NtrC^{G219C} could activate transcription only at very high concentrations: first, they had a defect in DNA binding, which is known to cause such a requirement (25), and second, they had a defect in oligomerization or enhancer-stimulated oligomerization and, hence, in ATPase activity.

NtrC^{A216C} and NtrC^{G219C} Bind the Enhancer Normally and Have Sufficient ATPase Activity to Activate Transcription at Low Concentrations. Studies of the NtrC^{G219K} protein, which carries an extra positive charge in the Switch I region, provide evidence that this region, although far removed from the helix–turn–helix DNA-binding motif of NtrC in the linear sequence, is nonetheless near the DNA. NtrC^{G219K} bound better to the *glnA* enhancer than NtrC^{WT} in a gel mobility-shift assay (16). Whereas NtrC^{WT} gives only a single shifted species, which requires cooperative binding of two NtrC dimers to the two sites that constitute the enhancer, NtrC^{G219K} yielded two shifted species. Unlike NtrC^{WT}, NtrC^{G219K} also could bind separately to the stronger of the two sites when they were separated. Subsequent studies of DNA-binding by using affinity coelectrophoresis indicated that the affinity of NtrC^{G219K} for nonspecific DNA was ≈10-fold higher than that of NtrC^{WT} (25) (A. K. North and S.K., unpublished data), which probably accounts for the improved binding of NtrC^{G219K} to the *glnA* enhancer. By contrast to the case for NtrC^{G219K}, binding of NtrC^{A216C} and NtrC^{G219C} to the *glnA* enhancer appeared to be normal. Like NtrC^{WT}, both proteins gave a single shifted species in a gel mobility-shift assay and they did so at concentrations similar to those for NtrC^{WT} (data not shown).

Because the oligomerization determinants of NtrC have not been localized within the central domain, it was possible that the

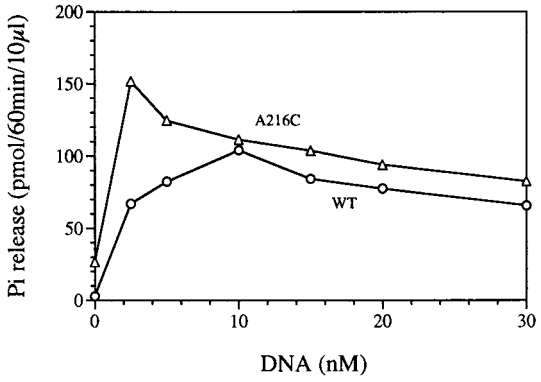

Fig. 2. Stimulation of the ATPase activity of NtrC^{A216C} by an enhancer. ATPase activity of phosphorylated NtrC^{A216C} (20 nM) was measured in the absence or presence of a synthetic DNA oligonucleotide that carries a strong enhancer (16, 24). It was compared with the ATPase activity of phosphorylated NtrC^{WT} (20 nM). Pi release was measured at 60 min. Relevant NtrC proteins are indicated next to the curves: WT, MBP-NtrC^{WT}; A216C, MBP-NtrC^{A216C}.

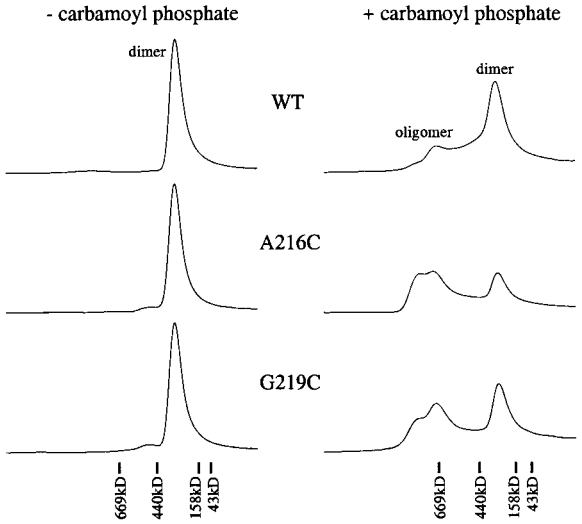

Fig. 3. Gel-filtration chromatography of unphosphorylated (− carbamoyl phosphate, *Left*) or phosphorylated (+ carbamoyl phosphate, *Right*) MBP-NtrC^{WT} (WT), MBP-NtrC^{A216C} (A216C), and MBP-NtrC^{G219C} (G219C). The column was calibrated with a set of molecular mass standards: thyroglobulin, 669 kDa; ferritin, 440 kDa; aldolase, 158 kDa; ovalbumin, 43 kDa; whose positions of elution are shown as vertical bars. Proteins (100 μl at a concentration of 1 μM) were injected into the sieving column, and eluted proteins were detected by relative absorbance at 214 nm. Traces show elution profiles from 6 to 16 min after injection. Peak positions of dimer (≈200 kDa) and oligomer (≈600–800 kDa) of MBP-NtrC are marked.

NtrC^{A216C} and NtrC^{G219C} proteins failed to oligomerize normally or to show normal enhancer stimulation of oligomerization. To test this functionally, we measured the ATPase activities of these proteins in the absence and presence of a strong enhancer (Fig. 2 and Table 1). The activity of both phosphorylated NtrC^{A216} and NtrC^{WT} (20 nM) was low in the absence of the enhancer (y intercepts), but the activity of NtrC^{A216C} was higher than that of NtrC^{WT}. For both proteins, the enhancer functioned as a positive allosteric effector. The ATPase activity of NtrC^{A216C} was greater than that of NtrC^{WT} at low enhancer concentrations (<10 nM DNA) but still as good as that of NtrC^{WT} at high enhancer concentrations. A similar result was obtained for NtrC^{G219C} (100 nM; Table 1). Thus, phosphorylation and oligomerization of these proteins appear to be intact and their ATPase activities are sufficient for transcriptional activation at low protein concentrations.

Increased ATPase Activities of NtrC^{A216C} and NtrC^{G219C} Appear to Be Due to Increased Oligomerization. To determine whether the higher ATPase activities of NtrC^{A216C} and NtrC^{G219C} might be accounted for by an increased tendency to oligomerize, we examined the behavior of the phosphorylated proteins (1 μM) on an analytical gel-filtration column (Fig. 3). Whereas all proteins eluted as dimers (188 kDa for MBP-NtrC) when unphosphorylated, both mutant proteins yielded more oligomer than NtrC^{WT} when phosphorylated with carbamoyl phosphate (10 mM) (>50% oligomer for mutant proteins and <20% for NtrC^{WT}). Although oligomeric species eluted with standards of 600–800 kDa, it is not clear whether they are hexamers, octamers, or a mixture of the two. Functional studies are most easily rationalized if the active oligomer for NtrC^{WT} is an octamer (1).

Table 1. ATPase activities of mutant NtrC proteins*

Protein	Without enhancer DNA	With enhancer DNA†	With nonspecific DNA†
NtrC^{WT}	17	615	18
NtrC^{A216C}	320	505	290
NtrC^{G219C}	280	450	260

*ATPase activity (pmol/60 min per 10 μl) was measured at 100 nM NtrC protein at 37°C. Proteins were phosphorylated as described in *Materials and Methods*.

†See *Materials and Methods*.

The Defect in Transcriptional Activation by NtrC^{A216C} Is Caused Solely by Its DNA Binding. Because the enhancer-stimulated ATPase activity of NtrC^{A216C} was intact, we suspected that the enhancer might prevent its interaction with σ^{54}-holoenzyme and that its residual ability to activate transcription only at high protein concentrations might be due to the activity of a small number of molecules in solution rather than those tethered to the template. To assess inhibitory effects of binding to the enhancer, we tested a derivative of NtrC^{A216C} that was incapable of DNA binding for its ability to activate transcription. Stable, soluble derivatives with this property carry three alanine substitutions in their helix–turn–helix DNA-binding motif and must activate transcription from solution (25). Without the stimulatory effects of the enhancer on nucleating oligomer formation and raising the local concentration of NtrC, the NtrC^{3ala} protein activates transcription only at much higher concentrations (200 nM) than the NtrC^{WT} protein from which it was derived (detectable activation at 5 nM) (Fig. 4). But at a high enough concentration (1000 nM), NtrC^{3ala} yields as high a level of template utilization as NtrC^{WT}. Surprisingly, NtrC$^{A216C,\ 3ala}$ activated transcription at lower concentrations (50 nM) than NtrC^{3ala} itself and reached the same maximum template utilization as NtrC^{3ala} at a concentration of only 200 nM. Better transcriptional activation by NtrC$^{A216C,\ 3ala}$ than NtrC^{3ala} at low concentrations is commensurate with the increased ability of NtrC^{A216C} to oligomerize and with its increased ATPase activity in solution (Figs. 2 and 3). The control protein NtrC^{A216C} yielded barely detectable amounts of transcripts in this experiment even at high protein concentrations (Fig. 4). Lack of reproducibility of transcriptional activation by this DNA-binding form of the protein may be accounted for by the fact that it, too, must activate transcription from solution and can do so only when binding to the template is saturated.

Like NtrC$^{A216C,\ 3ala}$, NtrC$^{G219C,\ 3ala}$ activated transcription better than its DNA-binding form, NtrC^{G219C}, but, unlike the case for NtrC$^{A216C,\ 3ala}$, NtrC$^{G219C,\ 3ala}$ activated far less well than

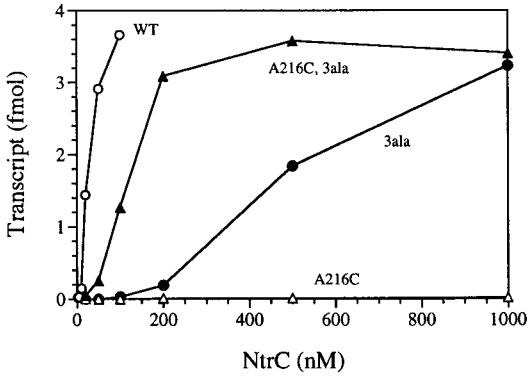

Fig. 4. Transcriptional activation at the *glnA* promoter by DNA-binding or non-DNA-binding forms (3ala) of NtrC. Single-cycle transcription assays were performed as described in *Materials and Methods*. The template was supercoiled plasmid pJES534 (1 nM). Non-DNA-binding forms of NtrC carry three alanine substitutions in the helix–turn–helix motif and decrease binding affinity for the strong enhancer by >5,000-fold (25). Relevant NtrC proteins are indicated next to the curves: WT, MBP-NtrC^{WT}; A216C, MBP-NtrC^{A216C}; 3ala, MBP-NtrC^{3ala}; A216C, 3ala, MBP-NtrC$^{A216C,\ 3ala}$.

NtrC^{3ala} itself (data not shown). Thus, the G219C substitution alone appears to cause a defect in the interaction with σ^{54}-holoenzyme and the enhancer further inhibits this interaction.

Inhibitory Effects of DNA Binding on Transcriptional Activation by NtrC^{A216C} Are Not Enhancer-Specific. To determine whether inhibitory effects of DNA binding were enhancer-specific, we compared transcriptional activation by NtrC^{A216C} on a supercoiled template that lacked an enhancer with that on one which carried an enhancer. For the NtrC^{WT} control, a higher protein concentration (50 nM) was required to reach the threshold for transcriptional activation on a template without the enhancer than on one with the enhancer (Fig. 5*A*). It should be noted that the concentration to reach this threshold (50 nM) was nonetheless lower than that for NtrC^{3ala} (200 nM) (Fig. 4), presumably because of residual tethering effects of DNA binding in the former case (25). By contrast, there was no difference between the concentration of NtrC^{A216C} required to reach the threshold for transcriptional activation on templates with or without the enhancer (Fig. 5*B*; note differences in scale from Fig. 5*A*). Moreover, NtrC^{A216C} gave many fewer transcripts on either template than did its non-DNA-binding derivative, NtrC$^{A216C,\ 3ala}$, which must activate from solution (Fig. 4). This indicates that both "nonspecific" DNA binding and enhancer binding inhibit transcriptional activation by NtrC^{A216C}.

Discussion

Roles of the Switch I Region for Activators of σ^{54}-Holoenzyme. The C-terminal portion of the Switch I region (^{215}GAFTGA^{220} for NtrC) is one of the most highly conserved regions among activators of σ^{54}-holoenzyme, and mutations affecting this small region arise very frequently upon screening for loss of transcriptional activation *in vivo* (16, 17, 26, 27). Phosphorylated forms of NtrC mutant proteins NtrC^{A216V}, NtrC^{G219K}, and NtrC^{A220T} fail in transcriptional activation despite the fact that they show little if any loss of ATPase activity, and, hence, it was inferred that they fail in productive interaction with σ^{54}-holoenzyme (16). Because the alanine and glycine residues that are altered in the mutant proteins are unlikely to be involved in specific contacts with the polymerase, it is probable that this portion of Switch I is important for energy coupling—an event triggering the conformational change in the polymerase that is essential for open complex formation. Similarly, mutant NtrC proteins with single cysteine substitutions for A216 and G219 have only a small residual capacity to activate transcription despite the fact that they have normal ATPase and enhancer-stimulated ATPase activities. In the case of NtrC^{A216C}, defects in transcriptional activation are completely relieved in a derivative, NtrC$^{A216C,\ 3ala}$, that is incapable of DNA binding. Hence, DNA binding appears to inhibit profoundly the interaction of NtrC^{A216C} with σ^{54}-holoenzyme, an interaction that is otherwise intact. The non-DNA-binding derivative of NtrC^{G219C} (NtrC$^{G219C,\ 3ala}$) had less activity than NtrC$^{A216C,\ 3ala}$, and both NtrC$^{A216V,\ 3ala}$ (1, 5) and NtrC$^{G219K,\ 3ala}$ (A. K. North and S.K., unpublished data) lacked detectable activity. Unfortunately, direct assays for conformational changes in NtrC upon ATP hydrolysis and contact with polymerase have yet to be developed. Although the homologous activator DctD can be cross-linked to the polymerase (28), this was not the case for NtrC (29). In NifA (nitrogen fixation protein A), another activator of σ^{54}-holoenzyme, the phenylalanine and threonine residues corresponding to F217 and T218 of NtrC, respectively, each were replaced by nine other amino acid residues (27). Only tyrosine and serine substitutions, respectively, yielded partially active forms of NifA (assessed *in vivo*). The strict requirement for an aromatic residue followed by a residue bearing a hydroxyl group at these two positions implicates these residues as having specific functional roles, again presumably in some aspect of the interaction with σ^{54}-holoenzyme (14, 27).

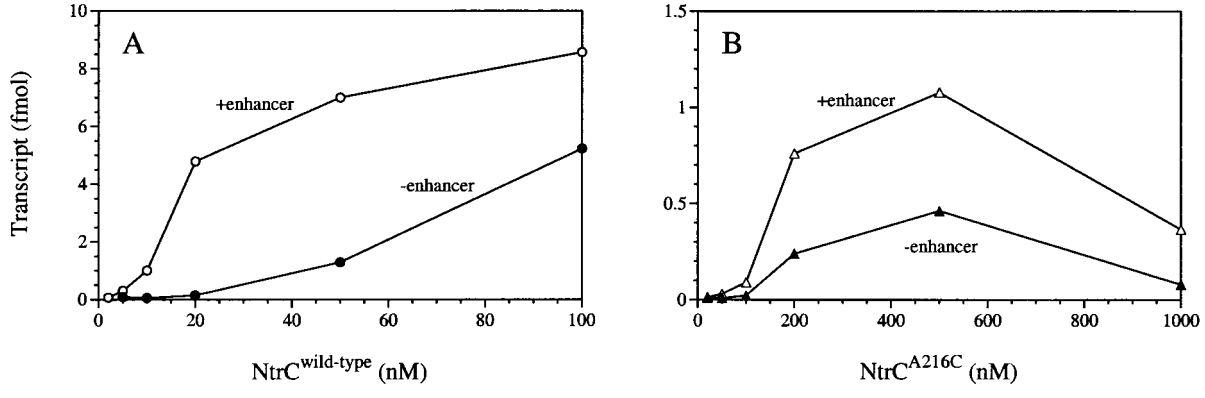

Fig. 5. Transcriptional activation by MBP-NtrC^{WT} (*A*) and MBP-NtrC^{A216C} (*B*) on supercoiled templates carrying or lacking an enhancer. Single-cycle transcription assays were performed as described in *Materials and Methods* on supercoiled plasmid pJES534 (1 nM), which carries two strong NtrC-binding sites (+enhancer), or pJES535 (1 nM), which lacks specific NtrC-binding sites (−enhancer).

Analogies and Differences Between the Switch I Regions of NtrC and p21^{ras}. The Switch I region of the G protein Ras serves as a contact interface with its target or effector proteins, such as the Raf kinase, which are responsible for biological output (30, 31). It is also involved in contact with G activating proteins (GAPS), such as p120 and the tumor suppressor protein neurofibromin, which stimulate GTP hydrolysis by Ras and appear to be regulatory (32). The target or effector protein for NtrC is σ^{54}-holoenzyme. As discussed above, the Switch I region of NtrC may be involved in contact with σ^{54}-holoenzyme because this region plays an essential role in transcriptional activation but not ATP hydrolysis. Interestingly, phosphorylation and the enhancer, though not proteins, appear to be analogous to GAPS. Phosphorylation is essential for detectable ATP hydrolysis, and binding to the enhancer greatly stimulates nucleotide hydrolysis by phosphorylated NtrC (4, 15, 33). The properties of NtrC^{A216C} and its non-DNA-binding derivative NtrC$^{A216C,\ 3ala}$ indicate that substitutions in the Switch I region of NtrC can perturb the response to the enhancer and, hence, that this region is involved in mediating effects of the enhancer. Interestingly, the properties of NtrC^{G219K} indicate directly that Switch I is near the DNA and, hence, presumably near the primary DNA-binding region of the protein (16). Some amino acid substitutions in the Switch I region of NtrC perturb the response of the protein to phosphorylation (unpublished data) and, hence, indicate that the Switch I region is also involved in mediating effects of phosphorylation. Although the Switch I region of NtrC appears to resemble that of Ras in mediating contact with its target and the response to "GAPS," there are also differences in the behavior of the two proteins. Whereas Ras has high affinity for its targets in the nucleoside triphosphate-bound form (34), neither the triphosphate-bound form nor the diphosphate-bound form of NtrC appears to have high affinity for σ^{54}-holoenzyme, and contact between the two proteins is transient (1, 29). GAPS decrease biological output by Ras because the GTP-bound form of Ras has much higher affinity for its target proteins than the GDP-bound form (34, 35). By contrast, the GAP analogs for NtrC increase biological output because ATP hydrolysis *per se* is required to change the conformation of σ^{54}-holoenzyme. In the sense that energy coupling is required for the biological function of NtrC, analogies between NtrC and other members of the purine nucleotide-binding protein family such as the F_1-ATPase and myosin are better than analogies to Ras. Despite intensive study, however, the roles of the Switch I regions of myosin and the F_1-ATPase are less well defined than those of G proteins (36–38).

DNA Context Effects on Eukaryotic Enhancer-Binding Proteins. DNA-binding context determines whether the glucocorticoid receptor (GR) and other intracellular receptors (IR) function as transcriptional activators or repressors (39). Base substitution mutations in one glucocorticoid-response element at which GR normally represses or is inert, plfG, enabled GR to activate transcription from this element (40). Analogously, change of one lysine residue (K461) in the zinc-binding region of GR enabled it to activate from wild-type plfG. We think it unlikely that specific alteration of the strong enhancer for NtrC would relieve inhibitory effects of the enhancer on the function of NtrC^{A216C} because binding to "nonspecific" DNA also inhibits transcriptional activation by this protein (Fig. 5). However, second-site amino acid substitutions in NtrC^{A216C} that relieve the inhibitory effects of the enhancer without eliminating DNA binding should be revealing with respect to a possible mechanism of communication between the C-terminal DNA-binding domain of NtrC and its Switch I region.

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