Influence of a mutation in the putative nucleotide binding site of the nitrogen regulatory protein NTRC on its positive control function

Sara Austin, Craig Kundrot¹ and Ray Dixon*

AFRC IPSR Nitrogen Fixation Laboratory, University of Sussex, Brighton BN1 9RQ, UK and

¹Department of Chemistry and Biochemistry, University of Colorado, Campus Box 215, Boulder, CO 80309-0215, USA

Received February 21, 1991; Revised and Accepted April 9, 1991

ABSTRACT

A mutation, serine 170 to alanine, in the proposed ATP binding site of the activator protein NTRC prevents transcriptional activation at σ^{54} -dependent promoters both *in vivo* and *in vitro*. The rate of phosphorylation of the mutant protein by NTRB and the stability of mutant NTRC-phosphate were similar to those of wild-type NTRC. The phosphorylated mutant protein shows only a slight decrease in affinity (around 2-fold) for tandem NTRC binding sites in the *Klebsiella pneumoniae nifL* promoter suggesting that the mutation primarily influences the positive control function of NTRC. Moreover the mutant protein is *trans* dominant to the wild-type protein with respect to transcriptional activation at both the *glnAp2* and *nifL* promoters. *In vitro* footprinting experiments reveal that the mutant protein is unable to catalyse isomerisation of closed promoter complexes between σ^{54} -RNA polymerase and the *nifL* promoter to open promoter complexes. However, the mutant protein retains the ability to increase the occupancy of the -24, -12 region by σ^{54} -RNA polymerase, forming closed complexes at the *nifL* promoter, which are not detectable in the absence of NTRC. These data support a model in which the activator influences the formation of closed complexes at the *nifL* promoter in addition to its role in catalysing open complex formation.

INTRODUCTION

The nitrogen regulatory protein NTRC is a member of a family of proteins which activate transcription at promoters recognised by core RNA polymerase associated with the alternative sigma factor, σ^{54} (1). The activity of NTRC is regulated through phosphorylation and dephosphorylation of its N-terminal domain by the histidine protein kinase NTRB (2,3). Only the phosphorylated form of NTRC is competent in transcriptional activation. Using a mutant form of *Salmonella typhimurium* NTRC, which is active in the absence of NTRB, Popham *et al.*

(4) found that ATP is required for NTRC to catalyse isomerisation of closed complexes between σ^{54} -RNA polymerase ($E\sigma^{54}$) and the *glnAp2* promoter to open promoter complexes in which the DNA surrounding the transcription initiation site is locally denatured. It was assumed that ATP hydrolysis is required for the isomerisation step since non-hydrolysable ATP analogues were inactive in the formation of stable open promoter complexes (4). The ATP requirement for open complex formation is consistent with the presence of a putative nucleotide binding site in the conserved central domain of σ^{54} -dependent activator proteins (5,6). This domain is required for positive control and is presumed to interact with $E\sigma^{54}$ during transcription initiation. The proposed nucleotide binding site is characterised by homology to the glycine-rich phosphate-binding loop found in several ATP and GTP-binding proteins of known structure (7). The presence of this motif in all known σ^{54} -dependent activators suggests a common mechanism in which ATP hydrolysis is necessary the catalysis of open promoter complexes by these activator proteins.

In *Klebsiella pneumoniae*, NTRC activates transcription of the *nifLA* operon as well as other nitrogen-regulated systems such as the glutamine synthetase promoter *glnAp2* (8). These σ^{54} -dependent promoters are characterised by highly conserved GG and GC dinucleotides located at -24 and -12 within the consensus sequence 5'TGGCAC-N₅-TTGCA (9,10). Whereas a closed promoter complex with $E\sigma^{54}$ is formed at the *glnAp2* promoter in the absence of the activator, close contacts between $E\sigma^{54}$ and the -24, -12 region of *nifL* promoter are not observed in the absence of NTRC (11). Similarly closed complexes between $E\sigma^{54}$ and the σ^{54} -dependent *nifH* promoter are not observed *in vivo* in the absence of the nitrogen fixation activator protein NIFA (10). Both the *nifL* and *nifH* promoters deviate from the σ^{54} -promoter consensus. Mutations in the *nifH* promoter which restore the consensus allow occupancy by $E\sigma^{54}$ in the absence of NIFA (12). These observations suggest that at promoters such as *nifL* and *nifH* where the affinity of $E\sigma^{54}$ for the promoter is weak, the activator may play a role in stabilising the closed complex in addition to its role in catalysing

* To whom correspondence should be addressed

open complex formation. An alternative possibility is that these promoters form weak transient closed complexes with $E\sigma^{54}$ which are undetectable in steady state experiments but are nevertheless sufficient to allow open complex formation by the activator. According to the former argument one might expect that certain mutant forms of the activator which are defective in positive control would allow visualisation of the closed complex whereas in the latter case closed complexes would remain undetected.

A mutation in the potential ATP binding site of NTRC, serine 170 to alanine (S170A), abolishes its activator function *in vivo* (13). Here we report that this mutant protein is defective in open complex formation but is still able to stabilise the interaction of $E\sigma^{54}$ with the *nifL* promoter. Furthermore this mutant protein is trans-dominant to the wild-type at both the *nifL* and *glnAp2* promoters, raising the possibility that the S170A protein forms non-productive complexes with $E\sigma^{54}$ which are inaccessible to activation by wild-type NTRC.

MATERIALS AND METHODS

Bacterial strains and plasmids

These are listed in Table 1.

Assays of promoter activity

Strains were grown at 29°C in NFDM medium and assayed for β -galactosidase activity as described previously (14). When necessary 1mM isopropyl β -thiogalactopyranoside (IPTG) was added to induce expression of *ntrC* from the *tac* promoter.

Proteins

Klebsiella pneumoniae core RNA polymerase, σ^{54} , NTRB9603 and NTRC were purified according to previous procedures (8). To facilitate purification of S170A NTRC, the mutant protein was expressed from the *tac* promoter on plasmid pLB1, following induction of 1 litre cultures with IPTG. Crude cell extract was obtained by French pressure disruption and low speed centrifugation and subjected to streptomycin sulphate precipitation followed by ammonium sulphate precipitation of the supernatant. The ammonium sulphate precipitate was chromatographed on heparin agarose with a linear salt gradient from 50mM to 750mM KCl. NTRC containing fractions were further purified by FPLC chromatography on a Mono Q column. The protein was judged to be approximately 95% pure by inspection of Coomassie blue stained SDS polyacrylamide gels.

Protein concentrations were determined using both Coomassie blue G-250 reagent (Pierce) and by measurement of absorbance at 280nm, using $A^{1\%} = 9.1$ for NTRC (3). For comparative purposes protein concentrations were also estimated from stained bands on SDS polyacrylamide gels.

DNase 1 footprinting

This was carried out as described previously (14).

Dimethyl sulphate footprinting

The accessibility of promoter DNA to dimethyl sulphate (DMS) in the presence and absence of protein was assayed as described previously (11). Supercoiled DNA (5nM) was incubated in binding buffer (14) with the appropriate proteins, treated with DMS (10mM) for 3 min, then the reaction was terminated with DMS stop solution. Following phenol extraction and ethanol precipitation methylated DNA was cleaved with *Bam*H1 and *Pst*I

Table 1. Bacterial Strains and Plasmids

Description		Reference or Source
<i>Strains</i>		
<i>Escherichia coli</i>	$\Delta(rhaA\ glnA\ ntrB\ ntrC)$ 1703, <i>rbs</i> , <i>gyrA</i> , <i>hut</i> C_k^+ , <i>lacZ</i> ::151 Mu <i>cts62</i> .	(25)
MC1061	<i>araD</i> 139, $\Delta(ara, leu)$ 7697 $\Delta lac74$, <i>galU</i> , <i>galK</i> <i>hsdR</i> .	(26)
<i>Plasmids</i>		
pSA2	wild-type NTRC expressed from the <i>tac</i> promoter in pMM40 (27), Cb^R .	This paper.
pLB1	S170A NTRC expressed from the <i>tac</i> promoter in pMM40 (27), Cb^R .	This paper.
pGA1	<i>glnAp1-lacZ'</i> transcriptional fusion derived from pJAC20 (21) and pRT22 (28), Cm^R .	This paper.
pGA2	<i>glnAp2-lacZ'</i> translational fusion derived from pRD573 (21) and pRT22 (28), Cm^R .	S.Screen.
pTE103	<i>in vitro</i> transcription vector containing early T7 terminator downstream of multiple cloning site.	(29)
pRD580	340 bp <i>Eco</i> R1- <i>Bam</i> H1 fragment carrying <i>glnAp1</i> and <i>glnAp2</i> promoters in pTE103.	(8)
pRD581	360 bp <i>Eco</i> R1- <i>Bam</i> H1 fragment carrying <i>nifL</i> promoter in pTE103.	(8)
pLB2	170 bp <i>Aha</i> III- <i>Bam</i> H1 fragment carrying <i>glnAp2</i> promoter without NTRC-binding sites (21) in pTE103.	This paper.

Fig. 1. Domain structure of NTRC and sequence of the proposed glycine-rich phosphate binding loop within the central domain. The sequence of this loop in nucleotide-binding proteins of known structure (7, 18) is shown beneath the S170A sequence. The N-terminal domain of NTRC is phosphorylated by NTRB (2, 3). A helix-turn-helix (HTH) motif within the C-terminal domain is required for DNA binding (30).

and 3' end-labelled at the *Bam*H1 site (located 125 nucleotides downstream of the *nifL* transcription start site). The digestion and labelling procedure resulted in the generation of two radiolabelled fragments of 3.3 Kb and <20 bp, the smaller of which did not interfere with subsequent electrophoretic analysis. After removal of unincorporated nucleotides by Sephadex G50

Fig. 2. DNase 1 footprinting of sites 1 and 2 in the *nifL* promoter by wild-type and mutant NTRC. A 338 bp *MluI*-*Bam*HI fragment (14) 3' end-labelled at the *MluI* end with [α^{32} -P]dCTP, was incubated with the appropriate proteins and then treated with DNase 1. The figure shows an autoradiogram of a 9% polyacrylamide sequencing gel on which the resultant fragments were analysed. The gel was calibrated with a G-specific chemical cleavage of the same end-labelled fragment (lanes marked G). The fragment was incubated with wild-type NTRC (lanes 2–4) and S170A NTRC (lanes 6–8 and 10–12). The final NTRC concentration was: 100nM (lanes 2, 6); 200nM (lanes 3, 7, 10); 500nM (lanes 4, 8, 11); 1 μ M (lane 12). In order to phosphorylate NTRC, NTRB (final concentration 30nM) and ATP (1 mM) were also added (lanes 1–8 only). NTRC was not added to reactions analysed in lanes 1, 5 and 9. The vertical bars show the position of the two NTRC-binding sites.

chromatography the methylated residues were cleaved with piperidine and the reaction products were analysed on 6% (w/v) polyacrylamide sequencing gels containing 7M urea which were subject to autoradiography.

Probing open complexes with KMnO_4

The procedure was the same as described above for DMS footprinting except that the reactions were treated with 8mM KMnO_4 for 4 min instead of DMS and then quenched with KMnO_4 stop solution (15). The DNA was 3' end-labelled and the oxidation products were cleaved with piperidine and analysed on sequencing gels as described above.

Phosphorylation of NTRC

NTRC (1 μ M) was incubated at 37° with NTRB9603 (70nM) in binding buffer (14). The reaction was initiated by addition of [γ^{32} -P] ATP (final concentration 0.3mM, 1 Ci/mmol). At the times indicated 10 μ l samples were withdrawn and spotted on

Fig. 3. Rate of phosphorylation of the mutant protein by NTRB and stability of the phosphate group. (a) NTRC was incubated with NTRB and [γ^{32} -P]ATP as described in Materials and Methods. 10 μ l samples were withdrawn at the indicated time intervals, and precipitated with trichloroacetic acid (TCA). (b) NTRC- 32 P was synthesised in a standard phosphorylation reaction as above. After a 5 min incubation at 37° the reaction mixture was diluted with unlabelled ATP in transcription buffer to give a final ATP concentration of 8.3 mM (zero time). Samples (10 μ l) were then taken at 1 min intervals for TCA precipitation. Open circles indicate wild-type NTRC, closed circles S170A NTRC. The experimental error for each point was within $\pm 10\%$.

Whatman 3 MM CHR filters (1.5 cm square) which were washed with trichloroacetic acid, dried, and their radioactivities measured in a Beckman liquid scintillation counter.

Dephosphorylation of NTRC- 32 P was measured after addition of a 27-fold excess of unlabelled ATP. Samples (10 μ l) were removed from the reaction mixture at time intervals and precipitated with trichloroacetic acid as described above.

In vitro transcription

DNA templates were prepared and transcription assays were performed as described previously (16).

RESULTS

Molecular modelling of the glycine-rich phosphate binding loop

Many nucleotide-binding proteins contain a homologous region which is glycine-rich and by comparison with nucleotide-binding proteins of known structure, is predicted to form a loop which connects a β -strand with an α -helix (17,18). The central domain of NTRC shows primary sequence homology with this glycine-rich region between residues 168 and 175 (Fig. 1) and secondary structure predictions indicate that this region also connects a β -sheet with an α -helix. Using the atomic coordinates for the elongation factor EF-tu, for which a 2.9Å resolution structure is available (19,20), we have examined the effects of replacing the EF-tu residues in the phosphate-binding loop with the corresponding residues in NTRC. The modelling predicts that this sequence in NTRC would not disrupt the structure of the loop since NTRC residues E169, S170, and T172 have side chains which point towards the solvent. It is possible that these side chains can interact with another domain of NTRC or with another protein. The S170A mutation is not predicted to prevent ligand binding but may corrupt the domain interface.

Fig. 4. Influence of wild-type and mutant NTRC proteins on closed and open complex formation by $E\sigma^{54}$ at the *nifL* promoter. (a) Dimethyl sulphate probing of interactions with supercoiled pRD581 DNA. Reactions contained: DNA only (lane 1); $E\sigma^{54}$ only (lane 2); $E\sigma^{54}$ with wild-type NTRC (lane 3); $E\sigma^{54}$ and S170A NTRC (lane 4). Protein concentrations were: E, 500 nM; σ^{54} , 750nM; NTRC, 900nM. Reactions analysed in lanes 3 and 4 also contained NTRB, 75nM and ATP, 1mM. (b) $KMnO_4$ probing of open complexes. Reactions contained the same additions as in (a) except that the concentrations were: E, 150nM; σ^{54} , 200nM; NTRC, 500 μ M; NTRB, 70nM and ATP, 1mM.

In vivo phenotype of the S170A mutation

When expressed from the *tac* promoter S170A NTRC, like the wild-type protein, repressed transcription from the *glnAp1* promoter even when the *tac* promoter was not induced with IPTG, indicating that the mutant protein retains DNA-binding ability. Activation at the *glnAp2* promoter (which requires a higher concentration of NTRC) was not observed with the S170A mutant (data not shown). These results confirm previous observations with other constructs (13).

Properties of the mutant protein *in vitro*

(a) *DNA binding.* Phosphorylation of NTRC has only a limited influence on its affinity for binding sites which show strong homology to the symmetrical consensus sequence GC-AC-N₇-GTGC (21) but increases its affinity for non-symmetrical 'weak' binding sites such as those found upstream of the *K. pneumoniae nifL* promoter (14). Binding of the mutant NTRC to sites 1 and 2 in the *nifL* promoter was examined by DNase 1 footprinting. Titration of phosphorylated wild-type and mutant proteins indicated that the S170A mutation decreased the affinity for both sites approximately two-fold (Fig. 2 lanes 1–8). This decrease may not be significant since it could reflect differences in the activity of the two protein preparations. In the absence of phosphorylation the occupancy of these sites by the mutant protein was considerably reduced (Fig. 2 lanes 9–12) as has been observed with the wild-type protein (14).

(b) *Phosphorylation.* The inability of the mutant protein to activate transcription could result from a decreased rate of

phosphorylation of its N-terminal domain or from increased lability of the phosphate moiety on the mutant protein. We compared the rate of phosphorylation of the wild-type and mutant proteins in the reaction catalysed by NTRB and [γ^{32} -P] ATP. The initial rate and the extent of phosphorylation of both proteins as determined by TCA precipitation was similar (Fig. 3a). In order to measure the rate of autodephosphorylation we performed a cold-chase with excess unlabelled ATP (Fig. 3b). The stability of the phosphate group on both proteins was similar and the half-life in each case was approximately 3.5 min.

(c) *Formation of the closed complex.* Previous work has shown that $E\sigma^{54}$ does not protect guanine residues in the -24, -12 region of the *nifL* promoter from methylation by dimethylsulphate in the absence of phosphorylated NTRC, indicating that $E\sigma^{54}$ does not make close contacts with the promoter in the absence of the activator (11). We were interested to determine whether the S170A mutant NTRC would stabilise the interaction of $E\sigma^{54}$ with this promoter, even though the mutant protein is defective in transcriptional activation. It is essential to use supercoiled DNA templates for these experiments since transcriptional activation from the *nifL* promoter *in vitro* is dependent on DNA topology (16). This requirement also necessitates the use of a reagent such as dimethylsulphate which does not cleave the DNA backbone. In order to maximise the yield of closed complexes we used saturating concentrations of $E\sigma^{54}$ and NTRC-phosphate; under these conditions activation of transcription is not dependent on the binding of NTRC to the upstream binding sites (11), even though these were present on the template. In agreement with previous results we found that $E\sigma^{54}$ alone did not protect any

Fig. 5. *In vivo* trans-dominance of the S170A mutation. The strain background was MC1061 (pGA2). Strains were grown in NFD medium + yeast extract (500 $\mu\text{g ml}^{-1}$) with the following additions: none (-N, open bars); 1mM $(\text{NH}_4)_2\text{SO}_4$ (+N, left-hatched bars); 1mM IPTG (-N + IPTG, closed bars); 1mM $(\text{NH}_4)_2\text{SO}_4$ plus 1mM IPTG (+N + IPTG, right-hatched bars). Each histogram plots β -galactosidase activity in Miller units on the y axis (note the logarithmic scale) and the x axis indicates the presence or absence of *ntrC* on a plasmid: w.t. NTRC indicates plasmid pSA2; S170A NTRC indicates plasmid pLB1. The vector plasmid, pMM40, from which these constructs are derived had no influence on the level of expression from pGA2 (data not shown).

of the G residues in the -24, -12 region from methylation (Fig. 4a, lane 2). The pattern of methylation in the presence of $E\sigma^{54}$ alone did not alter when the concentration of σ^{54} holoenzyme was further increased (data not shown), apart from a slight increase in methylation of the residue at -26, which we have detected previously (11). Addition of wild-type NTRC-phosphate resulted in protection of guanine residues at -25, -24 and -13 (Fig. 4a, lane 3). These residues are predicted to lie in adjacent major grooves on the same face of the helix and may be critical for $E\sigma^{54}$ recognition. We also detected hyper-reactivity of the -26, -8 and -9 residues as found previously (11). Hypermethylation at -9 and -8 may be a consequence of open complex formation since these two bases lie close to the melted region (11) (Fig. 4b). The presence of the mutant NTRC-phosphate also resulted in protection of the -25, -24, and -13 residues as well as influencing hypermethylation at -26 (Fig. 4a, lane 4). However, in this case the -9 and -8 residues were not hyper-reactive. The pattern of methylation with the S170A mutant protein did not differ when its concentration was varied between 450nM and 1.5 μM (data not shown). Therefore the mutant protein, like the wild-type is able to stabilise occupancy of $E\sigma^{54}$ at the *nifL* promoter.

(d) *Open complex formation.* Potassium permanganate (KMnO_4) was used as a probe to compare the ability of the wild-type and S170A proteins to catalyse local melting of the DNA by bound $E\sigma^{54}$. The enhanced reactivity of KMnO_4 for single-stranded compared with double-stranded DNA was utilised to detect open complexes (31). These experiments were again performed on supercoiled *nifL* promoter DNA. The presence of the phosphorylated mutant protein did not enhance the KMnO_4 reactivity of T residues from -7 to +4 which is a characteristic of open complex formation by the wild-type protein (11) (Fig. 4b compare lanes 3 and 4). Neither the mutant nor wild-type

Fig. 6. S170A protein inhibits transcriptional activation by wild-type NTRC *in vitro*. In each case the template DNA concentration was 10nM. E , σ^{54} , NTRB and ATP were present at 78nM, 220nM, 30nM and 1mM respectively. Reaction mixtures were incubated for the times indicated to allow formation of open complexes, then heparin (final conc. 100 $\mu\text{g ml}^{-1}$) and a mixture of nucleotide triphosphates containing [$\alpha^{32}\text{P}$]UTP were added and the incubation was continued for a further 10 min to allow synthesis of transcripts (16). The following incubations were carried out prior to the addition of heparin: lane 1, All components except NTRC were preincubated for 20 min in transcription buffer, then pre-phosphorylated wild-type NTRC (400nM) was added and incubation was continued for 5 min. lane 2, $E\sigma^{54}$ was incubated with NTRB, ATP and S170A NTRC (400 nM) for 25 mins in transcription buffer. lane 3, $E\sigma^{54}$ was preincubated with NTRB, ATP and S170A NTRC (400nM) for 20 mins, then pre-phosphorylated wild-type NTRC (400nM) was added and incubation continued for 5 min. lane 4, $E\sigma^{54}$ was incubated with NTRB, ATP and wild-type NTRC (400nM) for 25 mins in transcription buffer. lane 5, All components except NTRC were preincubated for 20 mins in transcription buffer, then pre-phosphorylated S170A NTRC (400nM) was added and incubation continued for 5 min. lane 6, $E\sigma^{54}$ was preincubated with NTRB, ATP and wild-type NTRC for 20 mins, then pre-phosphorylated S170A protein (400nM) was added and incubation continued for 5 min. The figures beneath each lane are arbitrary units of transcription obtained by scanning bands corresponding to full-length transcripts and normalising the data to a percentage relative to the level of transcription in lane 4. Arrows to the right of each autoradiogram indicate the position of full-length (420 nucleotide) transcripts. DNA templates were (a) pRD581, which contains the wild-type *nifL* promoter, (b) pRD580, containing the wild-type *glnA* promoters, and (c) pLB2, containing the *glnA*p2 promoter, but without NTRC-binding sites.

NTRC enhanced KMnO_4 reactivity when incubated with the DNA template alone (data not shown). However, both proteins in the presence of $E\sigma^{54}$ enhanced the reactivity of the T residue at -10. The reactivity of this residue was also enhanced less strongly by $E\sigma^{54}$ alone (Fig. 4b, lane 2). We have previously argued that the reactivity of this residue is a consequence of DNA distortion arising from an interaction with $E\sigma^{54}$ (11). This conclusion is further supported by the observation that the KMnO_4 reactivity of this residue is more enhanced by $E\sigma^{54}$ on a mutant *nifL* promoter which forms closed complexes in the absence of the activator (S. Whitehall and R. Dixon, unpublished). We conclude from the above results that the S170A NTRC is unable to activate open complex formation by $E\sigma^{54}$.

The mutant protein is trans-dominant to the wild-type

Since the mutant NTRC protein is able to interact with $E\sigma^{54}$ to form the closed complex but is unable to catalyse open complex

formation, we considered that the mutant protein might inhibit transcriptional activation by the wild-type. This possibility was first examined *in vivo* using merodiploid strains in which plasmid located mutant or wild-type *ntrC* was maintained *in trans* to a normal chromosomal copy of *ntrC*. In these experiments, the plasmid located *ntrC* was expressed from the IPTG inducible *tac* promoter, whereas the chromosomal copy was subject to 'normal' nitrogen regulation from the *glnA* promoter. Activation of transcription at the *glnAp2* promoter was measured on a compatible plasmid carrying a *glnAp2-lacZ* fusion. The data in Fig. 5 show that in the absence of an *ntrC* plasmid *in trans*, activation by chromosomal *ntrC* is subject to nitrogen regulation and as expected is not influenced by the addition of IPTG. The presence of wild-type *ntrC* *in trans* increased the level of activation when the *tac* promoter was induced with IPTG, but had no effect in the absence of IPTG induction. In contrast, the mutant *ntrC* inhibited activation by the wild-type, even in non-inducible conditions, when only low levels of mutant protein are expressed from the *tac* promoter. These results indicate that the S170A mutation gives rise to a *trans* dominant negative phenotype even when expressed at a low level. The *trans* dominant nature of this mutation was also revealed by the inability of the *ntrC*⁺/*ntrC*⁻ merodiploid strain to utilise arginine as sole nitrogen source and its growth requirement for glutamine (Gln⁻ phenotype).

The *trans* dominant nature of the mutant protein was examined *in vitro*, using single-round transcription assays on supercoiled DNA templates in which the *K.pneumoniae* *nifL* and *glnAp2* promoters are cloned upstream of a strong transcriptional terminator. Clearly the S170A mutant NTRC is unable to activate transcription at either the *nifL* or *glnAp2* promoters on templates pRD581 and pRD580 (Fig. 6a, b; in each case compare lane 1 with lane 5, and lane 2 with lane 4). When phosphorylated wild-type NTRC is first allowed to form open complexes with E σ^{54} and *nifL* promoter DNA, the subsequent addition of S170A NTRC has little influence on the level of transcripts (Fig. 6a compare lanes 4 and 6). However, when the mutant form of NTRC is first incubated with E σ^{54} and DNA, a reduced level of transcription is observed on subsequent addition of wild-type NTRC (Fig. 6a compare lanes 1 and 3). Similar results were obtained at the *glnAp2* promoter and transcription was inhibited by 80% when the mutant protein was first preincubated with E σ^{54} and DNA (Fig. 6b compare lanes 1 and 3).

A simple interpretation of these results is that the mutant protein pre-binds to the NTRC binding sites upstream of these promoters and inhibits transcriptional activation by preventing subsequent access to these binding sites by the wild-type protein. Recall, however, that transcriptional activation at these promoters can occur in the absence of NTRC binding sites, provided that the NTRC is present at high concentration (4,8,11,22). When these experiments were repeated with a *glnAp2* template (pLB2) in which all 5 upstream NTRC binding sites had been deleted, a similar level of inhibition (73%) was observed (Fig. 6c). It seems likely therefore that inhibition is not solely a consequence of specific DNA binding by the mutant protein and it may occur at a subsequent stage in the process of transcriptional activation.

DISCUSSION

The S170A mutant NTRC has many of the characteristics expected of a positive control mutant. The *in vivo* and *in vitro* studies show that it binds DNA with little change in affinity

compared with the wild-type protein. The mutant protein is phosphorylated by the histidine protein kinase NTRB at the same rate as the wild-type and both proteins show a similar rate of autophosphatase activity. The phenotype of the mutant is therefore unlikely to result from a phosphorylation defect. Although the mutant protein is unable to activate transcription our results indicate that it is still able to contact σ^{54} -RNA polymerase. The presence of the S170A protein stabilises the interaction of the polymerase with the *nifL* promoter, allowing close contacts between E σ^{54} and guanine residues in the -24, -12 region, hence forming the closed complex. The negative *trans* dominant phenotype of the mutant protein also suggests that S170A NTRC contacts σ^{54} polymerase. *Trans*-dominance occurs irrespective of whether NTRC-binding sites are present, suggesting that non-productive complexes formed between the mutant protein and E σ^{54} may block activation by the wild-type protein.

Our data demonstrate that the mutant protein is unable to catalyse the isomerisation of closed promoter complexes with E σ^{54} to transcriptionally active open complexes. The location of this mutation in the potential nucleotide binding site suggests that the S170A protein is unable to couple ATP hydrolysis to catalysis of the isomerisation step. The modelling studies predict that NTRC residues 168 to 175 form a phosphate binding loop consisting of a glycine-rich region followed by a lysine residue, a conserved feature of such loops in many ATP and GTP-binding proteins (7). By analogy with nucleotide-binding proteins of known structure this loop may undergo a conformational change upon ligand binding (18,23,24). The role of ATP hydrolysis in open complex formation has not been defined. One possibility is that contact between NTRC and E σ^{54} triggers ATP hydrolysis and the free energy of this hydrolysis is utilised for DNA strand separation. The S170A protein is defective in this step, possibly because a domain interaction with the phosphate binding loop is prevented by the mutation.

We have previously suggested that NTRC cooperatively interacts with E σ^{54} to stabilise the interaction of E σ^{54} with the -24, -12 region of the *nifL* promoter (11). The observation that the mutant protein allows E σ^{54} to contact the -24, -12 region in the closed complex strongly supports this model and argues against the possibility that these contacts are only stabilised in open complexes. We therefore predict that at the *nifL* promoter, NTRC influences the initial binding of σ^{54} -RNA polymerase as well as accelerating the isomerisation of the closed promoter complex to the open complex.

ACKNOWLEDGEMENTS

This work was supported by NATO grant CRG 900995. We thank Steve Screen for plasmid pGA2, Rosemary Foote for typing and Martin Buck, Mike Merrick and Barry Smith for their comments on the manuscript.

REFERENCES

1. Kustu, S., Santero, E., Popham, D. and Keener, J. (1989) *Microbiol. Rev.*, **53**, 367-376.
2. Keener, J. and Kustu, S. (1988) *Proc. Natl. Acad. Sci. USA.*, **85**, 8934-8938.
3. Weiss, V. and Magasanik, B. (1988) *Proc. Natl. Acad. Sci. USA.*, **85**, 8919-8923.
4. Popham, D., Szeto, D., Keener, J. and Kustu, S. (1989) *Science.*, **243**, 629-635.
5. Drummond, M., Whitty, P. and Wootton, J.C. (1986) *EMBO J.*, **5**, 441-447.

6. Ronson, C.W., Astwood, P.M., Nixon, B.T. and Ausubel, F.M. (1987) *Nucl. Acids Res.*, 15, 7921–7934.
7. Saraste, M., Sibbald, P.R. and Wittinghofer, A. (1990) *TIBS*, 15, 430–434.
8. Austin, S., Henderson, N. and Dixon, R. (1987) *Mol. Microbiol.*, 1, 92–100.
9. Beynon, J., Cannon, M., Buchanan-Wollaston, V. and Cannon, F. (1983) *Cell*, 34, 665–671.
10. Morett, E. and Buck, M. (1989) *J. Mol. Biol.*, 210, 65–77.
11. Minchin, S., Austin, S. and Dixon, R. (1989) *EMBO J.*, 8, 3491–3499.
12. Buck, M. and Cannon, W. (1989) *Nucl. Acids Res.*, 17, 2597–2612.
13. Drummond, M., Contreras, A. and Mitchenall, L.A. (1990) *Mol. Microbiol.*, 4, 29–38.
14. Minchin, S.D., Austin, S. and Dixon, R. (1988) *Mol. Microbiol.*, 2, 433–442.
15. Sasse-Swight, S. and Gralla, J.D. (1989) *J. Biol. Chem.*, 264, 8074–8081.
16. Dixon, R.A., Henderson, N.C. and Austin, S. (1988) *Nucl. Acids Res.*, 16, 9933–9946.
17. Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J. (1982) *EMBO J.*, 1, 945–952.
18. Fry, D.C., Kuby, S.A. and Mildvan, A.S. (1986) *Proc. Natl. Acad. Sci. USA.*, 83, 907–911.
19. Morikawa, K., La Cour, T.F.M., Nyborg, J., Rasmussen, K.M., Miller, D.L. and Clark, B.F.C. (1978) *J. Mol. Biol.*, 125, 325–358.
20. La Cour, T.F.M., Nyborg, J., Thirup, S. and Clark, B.F.C. (1985) *EMBO J.*, 4, 2385–2388.
21. Dixon, R.A. (1984) *Nucl. Acids Res.*, 20, 7811–7830.
22. Ninfa, A.J., Reitzer, L.J. and Magasanik, B. (1987) *Cell*, 50, 1039–1046.
23. Parsonage, D., Al-Shawi, M.K. and Senior, A.E. (1988) *J. Biol. Chem.*, 263, 4740–4744.
24. Dreusicke, D. and Schultz, G.E. (1988) *J. Mol. Biol.*, 203, 1021–1028.
25. MacNeil, T., Roberts, G.P., MacNeil, D. and Tayler, B. (1982) *Mol. Gen. Genet.*, 188, 325–333.
26. Casadaban, M. and Cohen, S.N. (1980) *J. Mol. Biol.*, 138, 179–207.
27. Kleiner, P., Paul, W. and Merrick, M.J. (1988) *J. Gen. Microbiol.*, 134, 1779–1784.
28. Tuli, R. and Merrick, M.J. (1988) *J. Gen. Microbiol.*, 134, 425–432.
29. Elliot, T. and Geiduschek, E.P. (1984) *Cell*, 36, 211–219.
30. Contreras, A. and Drummond, M. (1988) *Nucl. Acids Res.*, 16, 4025–4039.
31. Borowicz, J.A., Zang, L., Sasse-Dwight, S. and Gralla, J.D. (1987) *J. Mol. Biol.*, 196, 101–111.