Oligomerization of NTRC at the *glnA* enhancer is required for transcriptional activation

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To activate transcription of the *glnA* gene, the dimeric NTRC protein (nitrogen regulatory protein C) of enteric bacteria binds to an enhancer located ~100 bp upstream of the promoter. The enhancer is composed of two binding sites for NTRC that are three turns of the DNA helix apart. One role of the enhancer is to tether NTRC in high local concentration near the promoter to allow for its frequent interaction with σ^{54} holoenzyme by DNA looping. We have found that a second role of the enhancer is to ensure oligomerization of NTRC into a complex of at least two dimers that is required for transcriptional activation. Formation of this complex is greatly facilitated by a protein-protein interaction between NTRC dimers that is increased when the protein is phosphorylated.

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The nitrogen regulatory protein C (NTRC) of enteric bacteria [also called NRI; Reitzer and Magasanik 1983] is a prokaryotic enhancer-binding protein that activates transcription by the σ^{54} holoenzyme form of RNA polymerase [Reitzer and Magasanik 1987; D.S. Weiss et al. 1992]. The mechanism of activation has been studied most intensively at the major promoter for the *glnA* gene, which encodes glutamine synthetase. To activate transcription from the *glnA* promoter, NTRC binds to an enhancer that is composed of two binding sites centered at -108 and -140 with respect to the start site of transcription [Reitzer and Magasanik 1986; positions for binding sites in *Salmonella typhimurium*]. These two binding sites, which are approximately three turns of the DNA helix apart, behave like a eukaryotic transcriptional enhancer in that they function efficiently at distances of kilobases from the promoter and downstream as well as upstream of it [Reitzer and Magasanik 1986; Ninfa et al. 1987]. We have shown previously that NTRC bound to the enhancer interacts directly with σ^{54} holoenzyme at the promoter by means of DNA loop formation [Su et al. 1990; Wedel et al. 1990]. NTRC activates transcription by catalyzing isomerization of closed complexes between polymerase and the promoter to open complexes in a reaction that requires hydrolysis of ATP [Sasse-Dwight and Gralla 1988; Popham et al. 1989]. To hydrolyze ATP and catalyze open complex formation, NTRC must be phosphorylated on an aspartate residue in its amino-terminal regulatory domain [Ninfa and Magasanik 1986; Keener and Kustu 1988; Weiss and Ma-

gasanik 1988; Weiss et al. 1991; Sanders et al. 1992]. This phosphorylation, which proceeds via the autokinase NTRB, is increased when combined nitrogen is limiting, conditions under which *glnA* transcription is high. Phosphorylation of NTRC is not required for its specific binding to DNA, which is mediated by a helix-turn-helix motif in its carboxy-terminal region [Contreras and Drummond 1987].

One function of the *glnA* enhancer is to tether NTRC in high local concentration near the promoter and thereby increase its frequency of contact with σ^{54} holoenzyme [Su et al. 1990; Wedel et al. 1990]. A tethering function has also been demonstrated for several eukaryotic enhancers, including the rRNA enhancer of *Xenopus laevis* [Dunaway and Droge 1989] and the SV40 enhancer [Mueller-Sturm et al. 1989]. We now show that a second role of the *glnA* enhancer is to facilitate formation of a complex containing at least two dimers of NTRC that is required for transcriptional activation. Formation of this complex depends on protein-protein interaction between NTRC dimers, an interaction that is increased upon phosphorylation of NTRC and is mediated by residues outside its carboxy-terminal DNA-binding region.

Results

*Unphosphorylated NTRC binds a dyad-symmetrical site as a dimer and binds the *glnA* enhancer as two dimers*

We have studied three natural binding sites for NTRC:

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sites 1 and 2 in the *glnA* promoter regulatory region of *S. typhimurium*, which compose the enhancer, and a site in the *ntrBC* promoter region, which we call the strong site (Fig. 1). Each is composed of dyad-symmetrical half-sites (imperfect) of 6 bp and a 5-bp AT-rich center. To determine the oligomeric nature of specifically bound NTRC, we analyzed protein–DNA complexes formed between NTRC and these sites with a gel mobility-shift assay (Fried and Crothers 1981; Garner and Revzin 1981; see Materials and methods). A single shifted species was formed between NTRC and a fragment carrying the single strong site (Fig. 2B, lanes 2–5). We demonstrated that a dimer of NTRC from *S. typhimurium* bound a single site by making mixed dimers of the full-length protein and a 90-amino-acid carboxy-terminal fragment of NTRC, which contains its helix–turn–helix DNA-binding motif. Characteristic of a single bound dimer, there were three shifted bands, one for each homodimer complex with DNA and a band of intermediate mobility containing the heterodimer complex (Fig. 2A, lane 4). [We had determined previously that NTRC was a dimer at high concentrations in solution ($\sim 1 \mu\text{M}$) by gel-filtration chromatography at 4°C (J. Keener and S. Kustu, unpubl.), and Reitzer and Magasanik (1983) had established the same for NTRC from *Escherichia coli*.]

Surprisingly, there was only a single shifted species with NTRC and a fragment carrying the *glnA* enhancer (sites 1 and 2 together; Fig. 2B, lanes 7–10) or a fragment

carrying all five binding sites from the *glnA* promoter regulatory region (not shown). We determined the number of NTRC dimers bound to these multisite fragments relative to the number bound to a single-site fragment by using ^{35}S -labeled NTRC and ^{32}P -labeled DNA, calculating the ratio of $^{35}\text{S}/\text{pmole}$ of DNA in the shifted bands and normalizing this ratio to one for the single-site probe (Table 1). Each of the multisite probes formed only a single complex, which contained two dimers of NTRC (Table 1). As expected, if two dimers were bound, there were five shifted species in the gel assay with a mixture of NTRC protein and a larger, maltose-binding protein fusion to NTRC (not shown).

NTRC dimers bind to the enhancer cooperatively

Because we obtained only a single shifted species in the gel mobility-shift assay with probes carrying the *glnA* enhancer (sites 1 and 2 together), we tested the possibility that NTRC bound cooperatively to the enhancer by separating the sites that constitute it. Probes carrying either site alone did not yield stable shifted complexes, although the probe carrying site 2 showed smearing characteristic of the formation of an unstable complex (not shown). We therefore concluded that NTRC bound cooperatively to the two sites that constitute the *glnA* enhancer. Although the carboxy-terminal fragment of NTRC bound stably to the single strong site, it did not

Figure 1. The natural *glnA* enhancer and variants of it. (A) The *glnA* promoter regulatory region in pJES463. The restriction sites downstream of -195 were introduced into the *glnA* promoter regulatory region of *S. typhimurium* by site-directed mutagenesis as described in Materials and methods. In addition to NTRC-binding sites 1 and 2 (denoted 1 and 2), which constitute the *glnA* enhancer, pJES463 carries binding sites 3, 4, and 5 (between positions -95 and -37), which are not shown. (B) The sequence of the natural *glnA* enhancer. Binding sites 1 and 2 are shown in bold, and half-sites are underlined. (C) The sequence of the strong enhancer in pJES500 and pJES534 (used for transcription). Natural binding sites 1 and 2 for NTRC were replaced with the same strong site (from the *ntrBC* promoter region); the spacing between the sites was preserved, and binding sites 3, 4, and 5 were removed. (D) The sequence of the weak enhancer in pJES635. The site at position 2 of the *glnA* enhancer was replaced with a site that lacked dyad symmetry, and binding sites 3, 4, and 5 were removed. (E) The sequence of the symmetrical site in pJES601 and

pJES640 (used for transcription). This site has 6-bp half-sites with perfect dyad symmetry. It differs from the strong site by 1 bp (*). Transcription templates carrying the above NTRC-binding sites were constructed by inserting 403 bp of DNA in place of the *BclII*–*NcoI* fragment to separate NTRC-binding sites from the *glnA* promoter and adding a synthetic transcription module and termination sequence at the *BstXI* site, as described in Materials and methods.

Figure 2. Stoichiometry of NTRC–DNA complexes. (A) The oligomerization state of NTRC bound to a single site was evaluated in a gel mobility-shift assay (described in Materials and methods) with a mixture of full-length NTRC and the 90-amino-acid carboxy-terminal domain. Full-length NTRC (lane 2), the carboxy-terminal domain (lane 3), or a mixture of the two (lane 4) was incubated with a 73-bp ^{32}P -labeled DNA fragment (lane 1) carrying the symmetrical binding site from pJES601 (PCR product using primers 1 and 2); a portion of each reaction mixture was then run on a polyacrylamide gel. Before incubation with the DNA fragment, full-length NTRC and carboxy-terminal domain were incubated together for 20 min at 37°C to promote rapid subunit exchange. Positions of each homodimer and of the heterodimer between full-length protein and the carboxy-terminal domain are noted. (B) The stoichiometry of NTRC-binding to the *glnA* enhancer was evaluated in a gel mobility-shift assay using ^{35}S -labeled NTRC and the following ^{32}P -labeled DNA fragments: (1) a 411-bp *Bam*HI–*Nru*I fragment from pJES132 carrying the strong NTRC-binding site (lanes 1–5) and (2) a 117-bp *Bcl*II–*Eco*RI fragment from pJES463 carrying the *glnA* enhancer (NTRC-binding sites 1 and 2; lanes 6–10). Enough NTRC was added to give ~50% shift of the DNA probe (reaction mixtures in lanes 4, 5, 7, and 8 contained ~1.3-fold more protein than the others). Shifted bands were excised from the gel, and both isotopes were counted as described in Materials and methods (data in Table 1). No protein was present in reaction mixtures in lanes 1 or 6.

form a stable shifted species with a probe carrying the *glnA* enhancer (not shown), indicating that unlike full-length NTRC, it does not bind to the enhancer cooperatively. We therefore conclude that cooperative binding is not the result of a conformational change in the DNA upon binding of a single dimer of NTRC, but is the result of protein–protein interaction between dimers of NTRC, which is mediated primarily outside the carboxy-terminal 90 amino acids.

To examine cooperative binding more quantitatively, we precisely replaced both sites 1 and 2 at *glnA* with the strong site to create what we call the strong enhancer (Fig. 1C). As assessed by competition experiments, NTRC has an ~13-fold higher affinity for the strong site than site 1 (data not shown, but see Materials and methods). We verified that NTRC had the same affinity for the strong site at positions 1 and 2 of our new construct by separating the two sites at these positions with restriction cleavage and demonstrating that they competed equally against a labeled probe carrying a single binding site (data not shown, but see Materials and methods). With the strong enhancer we were able to quantitate the intermediate species carrying a single dimer of NTRC in a gel-mobility shift assay and thereby quantitate the degree of cooperative binding of two dimers of NTRC using calculations that are independent of the concentration of the protein and its affinity for the site. When NTRC was titrated on a probe carrying the strong enhancer, the fractional maximum of the intermediate species carrying a single dimer was ~0.18 [Figs. 3A and 4A at 6 pM probe and two additional experiments at 0.6 and 300 pM probes, respectively (not shown)], indicating that the presence of one bound dimer of NTRC stimulates the binding of a second dimer to the adjacent site by ~20-fold (Tsai et al. 1989; see Materials and methods for equation). (For noncooperative binding the fractional maximum of the intermediate species is theoretically 0.5.) At NTRC concentrations around that yielding the fractional maximum of the intermediate species, the relative amounts of the three species—free probe, intermediate, and fully shifted species carrying two dimers—indicated an average value for the cooperativity factor of 20 ± 6 (Hudson and Fried 1990, 1991; see Materials and methods for equation).

The carboxy-terminal domain of NTRC showed little cooperative binding to a probe carrying the strong enhancer: The fractional maximum of the intermediate species carrying a single dimer was 0.4, corresponding to a cooperativity factor of 2.2 [Figs. 3D and 4C at 0.3 nM DNA and an additional experiment at the same DNA concentration (not shown)]. Relative amounts of the three species at concentrations of carboxy-terminal domain around that yielding the fractional maximum of the intermediate species indicated an average value of 2.8 ± 1.2 . Although the cooperativity factor was different from the theoretical value of 1, we think it indicates noncooperative binding, because it was unchanged when the sites constituting the strong enhancer were placed on opposite sides of the DNA helix at ~2.5 turns apart (not shown).

Phosphorylation of NTRC increases interaction between dimers

In the presence of ATP and the NTRB protein, NTRC is phosphorylated to a transcriptionally active form (NTRC-P). Phosphorylated NTRC binds at least 12-fold

Table 1. Stoichiometry of NTRC–DNA complexes

NTRC-binding sites	DNA shifted (pmole)	Protein shifted (³⁵ S)	Average ratio protein/DNA (³⁵ S/pmole)	Mol NTRC dimer (mol DNA)
Single strong site	0.24	1244	5409 ± 459	{1.0}
	0.21	1048		
	0.36	1949		
	0.33	1996		
<i>glnA</i> enhancer	0.15	1499	9906 ± 1778	1.8
	0.12	1353		
	0.08	879		
	0.10	737		
<i>glnA</i> promoter–regulatory region	0.11	1187	10,483 ± 700	1.9
	0.12	1188		
	0.13	1289		
	0.11	1246		

Data for the single strong site and the *glnA* enhancer were taken from the experiment shown in Fig. 2B (lanes 2–5 and 7–10, respectively) and quantitated as described in Materials and methods. As was the case for the *glnA* enhancer, only a single shifted species was observed with a DNA fragment (a 249-bp *HindIII*–*EcoRI* fragment from pJES125) carrying the entire *glnA* promoter regulatory region (five binding sites as assessed by DNase I footprinting or methylation protection; not shown). ³⁵S/pmole DNA was normalized to 1.0 for the shifted species obtained with the fragment carrying a single strong NTRC-binding site. This corresponds to one dimer of NTRC (Fig. 2A). Results similar to those shown were obtained in three additional experiments.

more cooperatively to the strong enhancer than does unmodified NTRC: For NTRC-P the fractional maximum of the intermediate species was 0.06 [Figs. 3B and 4A at 6 pM DNA and an additional experiment at this DNA concentration (not shown)], which corresponds to a cooperativity factor of 245. Analysis of data at NTRC concentrations around that yielding the fractional maximum of the intermediate species indicated an average cooperativity factor of 230 ± 137 . We give a 12-fold increase in cooperativity as a minimum estimate because NTRC has an autophosphatase activity; hence, NTRC-P is actually a mixed population of phosphorylated and unphosphorylated forms. [Because of the low concentrations of NTRC used in these experiments, we were unable to measure the degree of phosphorylation directly.] We attempted to increase the degree of phosphorylation of NTRC by increasing the concentration of NTRB from 20 nM (Figs. 3B and 4A) to 200 nM (not shown). In addition, we used several concentrations of probe [0.6 pM (not shown), 6 pM (Figs. 3B and 4A), 30 pM (not shown), and 300 pM (not shown)], anticipating that low amounts of probe could be bound exclusively by NTRC-P. Neither change in protocol yielded a further increase in the apparent cooperativity.

To demonstrate in an independent way that phosphorylation of NTRC increased its cooperativity of binding to the strong enhancer, we showed that it decreased the dissociation rate of the protein from this configuration of sites. The half-life of a complex containing two unphosphorylated dimers of NTRC and a probe carrying the strong enhancer was 1.1 min at 37°C (Fig. 4D). In the presence of NTRB under conditions in which the amount of NTRC-P would have reached steady state, the half-life increased to 13 min. (ATP was present in both cases.)

A constitutive mutant form of NTRC shows increased interaction between dimers

The NTRC^{S160F} protein, a “constitutive” form that has some ability to activate transcription and hydrolyze ATP without being phosphorylated (Weiss et al. 1991; Austin and Dixon 1992) is often employed to study transcriptional activation because, unlike NTRC-P, it is composed of a homogeneous population of active molecules. Unphosphorylated NTRC^{S160F} protein binds approximately twofold more cooperatively to the strong enhancer than does unphosphorylated wild-type NTRC. For NTRC^{S160F} the fractional maximum of the intermediate species carrying a single dimer was 0.12, which corresponds to a cooperativity factor of 54; analysis of data at several concentrations of NTRC^{S160F} around that which yielded the fractional maximum indicated an average value of 52 ± 20 [Figs. 3C and 4B at 6 pM DNA and two additional experiments (not shown)]. In accordance with its increased cooperativity of binding, the unphosphorylated mutant protein showed about a twofold slower rate of dissociation from the strong enhancer at 37°C ($t_{1/2} = 1.8$ min) than did unphosphorylated wild-type NTRC (Fig. 4D).

Phosphorylation does not affect the protein–DNA interaction

We infer that phosphorylation does not change the affinity of a dimer of NTRC for a single binding site because the rate of dissociation of NTRC from a probe carrying the strong site was the same in the presence or absence of the NTRB protein. (ATP was present in both cases.) Because the rate of dissociation of complexes between

Figure 3. NTRC binds cooperatively to the strong enhancer. Cooperativity of NTRC binding to the strong enhancer at *glnA* (two identical strong binding sites for NTRC in positions 1 and 2; Fig. 1C) was determined in a gel mobility-shift assay with a 106-bp double end-labeled probe from pJES500 (PCR product using primers 1 and 2). All reaction mixtures contained 1.5 ng of poly[d(I-C)]/ μ l and 2 mM ATP. Reaction mixtures with NTRC, NTRC-P, and NTRC^{S160F} (A, B, and C, respectively) contained 6 pM DNA probe, and reaction mixtures with the carboxy-terminal domain of NTRC (D) contained $\sim$ 0.3 nM probe. Concentrations of NTRC are indicated above the lanes. Reactions in A and B were run in a single experiment in which 20 nM NTRB was present (B) or absent (A). The shifted species carrying one or two dimers of NTRC are indicated. The ³²P label in each shifted species in a given lane was quantitated using a Molecular Dynamics PhosphorImager, and the amount of the shifted species was determined as a fraction of total counts loaded. These data are presented in the graphs in Fig. 4. An additional higher-order species (not indicated) was apparent at high concentrations of full-length NTRC proteins (higher than those used for the data in Fig. 4) but not with the carboxy-terminal domain.

NTRC and a single strong site was too rapid to be measured accurately at 37°C, we phosphorylated the protein at 37°C, allowing time for the amount of NTRC-P to reach steady state at this temperature, and then lowered the temperature to 4°C to measure the dissociation rate of complexes. [Lowering the temperature from 37°C to 4°C had the added advantage that the autophosphatase activity of NTRC-P was slowed approximately eightfold (Keener and Kustu 1988)]. Decay of complexes was first order and occurred with a $t_{1/2}$ of 1 min for both phosphorylated NTRC and unmodified protein (not shown). Even if phosphorylation was not complete, we assume that the decay rate for phosphorylated NTRC would have

been biphasic if NTRC-P did have a different affinity for DNA. Hence, we conclude that phosphorylation of NTRC does not change its interaction with DNA and probably does not increase monomer–monomer interactions. The latter is also indicated by the fact that phosphorylation does not affect the rate of monomer exchange between NTRC dimers (K. Klose and S. Kustu, unpubl.).

Activation of transcription requires more than one dimer of NTRC

Transcriptional activation at the *glnA* promoter was

Figure 4. Quantitation of cooperative binding to the strong enhancer (A–C) and rates of dissociation from this enhancer (D). Data for A–C were taken from Fig. 3. (A) The fraction of DNA carrying one dimer of NTRC (○), two dimers of NTRC (Δ), one dimer of NTRC-P (●), or two dimers of NTRC-P (▲) is plotted as a function of NTRC concentration. Data for the two lowest concentrations of NTRC (and NTRC-P) were taken from lanes not shown. The fractional maximum of DNA carrying one dimer of NTRC was 0.18, and the fractional maximum of DNA carrying one dimer of NTRC-P was 0.06. (B) The fraction of DNA carrying one dimer of NTRC^{S160F} (○) or two dimers (Δ) is plotted as a function of concentration of this protein. The fractional maximum of DNA carrying one dimer of NTRC^{S160F} was 0.12. (C) The fraction of DNA carrying one dimer of the carboxy-terminal domain of NTRC (○) or two dimers (Δ) is plotted as a function of the concentration of this domain. The fractional maximum of DNA carrying one dimer of the carboxy-terminal domain was 0.4. (D) The rates of dissociation of NTRC (□), NTRC-P (▲), and NTRC^{S160F} (○) from the strong enhancer at *glnA* were determined and quantitated as described in Materials and methods, and the natural logarithm (ln) of the shifted species carrying two dimers of NTRC [ln (% doubly-bound species)] is plotted.

much higher from the *glnA* enhancer (sites 1 and 2 together) than from a single strong binding site (not shown). Each was placed at a distance of ~400 bp upstream of the promoter so that its orientation on the DNA helix with respect to the promoter was not a concern. We considered that the observed synergy of two binding sites indicated that more than one dimer of NTRC was required for activation. However, because occupancy of the *glnA* enhancer by NTRC^{S160F}, the protein employed for these studies, was slightly better than that of the single strong site, we could not exclude the possibility that the observed synergistic effect of two binding sites, although of great magnitude, was due solely to better tethering of NTRC.

To demonstrate rigorously that synergistic activation from an enhancer consisting of two binding sites for

NTRC could not be accounted for by occupancy of the sites, we constructed a “weak enhancer” (Fig. 1D) that would specifically bind two dimers of NTRC poorly relative to the binding of a single dimer to a “symmetrical site” (Fig. 1E). [As assessed by rates of dissociation at 4°C, NTRC has an approximately threefold higher affinity for the symmetrical site, our highest affinity site, than for the strong site (not shown, but see Materials and methods).] We then compared occupancy of the weak enhancer and the single symmetrical site with their ability to stimulate transcriptional activation. Preliminary gel mobility-shift assays indicated that the NTRC^{S160F} protein formed complexes with the symmetrical site at 0.2 nM dimer, the lowest concentration tested, whereas it formed fewer complexes with the weak enhancer even at 1.6 nM (Fig. 5A). (As was the case for the natural *glnA*

Figure 5. NTRC binding to the weak enhancer at *glnA* and a single symmetrical binding site (A–C) and NTRC-mediated activation of transcription from each (D). (A) Affinity of NTRC for the weak enhancer (Fig. 1D) and the single symmetrical site (Fig. 1E) was assessed at low DNA concentrations by a gel mobility-shift assay. Binding of NTRC^{S160F} (concentrations indicated above the lanes) to the weak enhancer (lanes 6–14) and the single symmetrical site (lanes 1–5) was carried out as described in Materials and methods. The weak enhancer was carried on a 111-bp labeled probe (~0.3 nM) made by PCR amplification from pJES635 using primers 1 and 5. The shifted species formed on this probe carries two NTRC dimers (see Results). The single symmetrical site was carried on a 73-bp labeled probe (~0.3 nM) made by PCR amplification from pJES601 using primers 1 and 2. (B) Occupancy of the weak enhancer and the single symmetrical site by NTRC^{S160F} (concentrations indicated above the lanes) was assessed by DNase I footprinting on the top (nontemplate) strand. Reaction mixtures contained all components present for open complex formation in D, and the DNA concentration was 2 nM (75% of this was the appropriate fragment used in D and 25% was probe made by PCR amplification with primers 3 and 4). Protected regions are indicated by brackets to the right. Lanes labeled G and GA are Maxam–Gilbert sequencing lanes. (C) Occupancy of the sites at positions 1 (■) and 2 (●) of the weak enhancer and of the symmetrical site (○) was assessed from data in B. The ³²P label in several contiguous bands in a protected region was determined with a PhosphorImager and normalized to the label in bands outside protected regions to correct for differences in loading of different lanes. Normalized data were then expressed as a percentage of the ³²P label in the corresponding group of bands in the absence of NTRC^{S160F} (percent residual intensity). It is plotted as percent occupancy, which is the same as percent protection (100% – percent residual intensity). (D) The rate of open complex formation (fmole/10 min) was assessed in a single-cycle transcription assay at 37°C on two linear templates (2 nM): (1) an ~700-bp *KpnI*–*PstI* fragment from pJES635 that carries the weak enhancer 455 bp upstream of the *glnA* promoter (●), and (2) an ~700-bp *KpnI*–*PstI* fragment from pJES640 that carries a single symmetrical NTRC-binding site 455 bp upstream of the *glnA* promoter (○). The NTRC^{S160F} protein was used at the concentrations indicated, and reactions were started by addition of ATP to 2 mM. Formation of open complexes was stopped after 10 min by addition of heparin (Popham et al. 1989). The rate of open complex formation (transcriptional activation) from the template carrying the weak enhancer was maximal at 55 nM NTRC^{S160F}. Under the conditions employed [i.e., in the absence of polyethylene glycol], transcription from a linear template carrying the *glnA* promoter but lacking NTRC-binding sites (from pJES535) was undetectable below 100 nM NTRC^{S160F} (not shown).

enhancer, only complexes carrying two dimers of NTRC gave a stable shifted species with a probe carrying the weak enhancer.) The NTRC concentration required for half-maximal binding of the symmetrical site by a dimer

was ~0.4 nM, whereas that required for half-maximal binding of the weak enhancer by a pair of dimers was between 6 and 13 nM. Thus, the affinity of the symmetrical site for a dimer was 15- to 30-fold higher than that

of the weak enhancer for a pair of dimers. [Unphosphorylated wild-type NTRC, which binds less cooperatively than NTRC^{S160F} (see above) failed to shift the weak enhancer even at a concentration of 25 nM (not shown).] DNase I protection assays performed at the same high DNA concentration used for transcriptional activation (2 nM rather than the 0.3 nM used for gel mobility-shift assays) indicated that NTRC^{S160F} yielded full occupancy of the single symmetrical site at 3.5 nM (Fig. 5B,C). In contrast, higher concentrations of NTRC were required for occupancy of the weak enhancer: Half-maximal occupancy of the sites at positions 1 and 2 occurred at ~3 and 10 nM, respectively (Fig. 5B,C).

Transcriptional activation (the rate of open complex formation) from the weak enhancer tracked its occupancy by two dimers (Fig. 5C,D). Moreover, activation from the weak enhancer was more than twice that from the single symmetrical site at all concentrations of NTRC^{S160F} ≤ 30 nM, indicating that activation from the enhancer was synergistic with respect to that from a single site. For example, at 7 nM NTRC^{S160F}—a concentration at which the symmetrical site was fully occupied by a dimer but the enhancer was not yet fully occupied by a pair of dimers (a tetramer)—activation from the enhancer was nevertheless 24-fold higher than that from the symmetrical site. At 7 nM, activation from the symmetrical site was only twofold above background, indicating that occupancy of this site by a dimer was not sufficient for transcriptional activation.

Demonstration that activation from a single binding site also requires more than one dimer of NTRC

At concentrations of NTRC^{S160F} > 7 nM, there was increasing transcriptional activation from the single symmetrical site. To demonstrate that this activation also required formation of a tetramer or higher-order oligomer of NTRC [formed by interaction of an additional dimer (or dimers) with the one that was specifically bound], we demonstrated that forms of NTRC that have little, if any, residual ability to bind to DNA can nevertheless activate transcription synergistically in combination with low concentrations of DNA-binding forms (Fig. 6).

The nonbinding forms of NTRC that we employed contain alanines in place of three hydrophilic residues in the second ("recognition") helix of the DNA-binding motif (see Materials and methods) and are designated NTRC^{3ala} and NTRC^{S160F, 3ala}. They fail to yield a footprint at the symmetrical site, fail to bind both the strong enhancer and nonspecific DNA in an affinity coelectrophoresis assay that detects weak DNA-protein interactions (Lim et al. 1991), and behave in transcription assays as if they are incapable of DNA binding (A. North and S. Kustu, unpubl.). These mutant proteins activate transcription only at very high concentrations, presumably by interacting with σ^{54} holoenzyme from solution.

On a supercoiled template carrying the symmetrical site, activation by the DNA-binding form NTRC^{S160F} at 5 nM was very low (only twofold above background).

Figure 6. Transcriptional activation from a single NTRC-binding site is stimulated by NTRC^{3ala} proteins, which cannot bind to DNA. Formation of open complexes at the *glnA* promoter was assessed in a single-cycle transcription assay using as template plasmid pJES640 (20 nM, supercoiled), which carries a single symmetrical binding site for NTRC. The concentrations of σ^{54} and RNA polymerase core were 180 and 90 nM, respectively. (A) Stimulation of open complex formation by the NTRC^{S160F, 3ala} protein (concentrations indicated on the x axis) at 5 nM NTRC^{S160F} (●) or in the absence of NTRC^{S160F} (○). Reactions were performed as described in Materials and methods under conditions that minimized subunit exchange between dimers. (B) Stimulation of open complex formation by the NTRC^{3ala} protein (concentrations indicated on the x axis) at 5 nM wild-type NTRC (●) or in the absence of wild-type NTRC (○). NTRB was present at 100 nM to phosphorylate the NTRC proteins. (Note the differences in scale with respect to A.)

Likewise, at concentrations < 320 nM, activation by the NTRC^{S160F, 3ala} protein, which cannot contribute to occupancy of the symmetrical site, was slight. In contrast, activation by a mixture of NTRC^{S160F} at 5 nM and the NTRC^{S160F, 3ala} protein at different concentrations was considerably greater than the sum of the activities of the individual proteins (i.e., it was synergistic; Fig. 6A). The maximum measurable degree of synergy was sixfold (observed at 240 nM NTRC^{S160F, 3ala}, the lowest concentration at which activation by this protein alone could be detected). Presumably, synergistic activation is the result of the formation of mixed oligomers by means of

protein-protein interaction between dimers, with the DNA-binding form (NTRC^{S160F}) serving as tether.

The results were even more striking for phosphorylated wild-type NTRC. Again, activation by 5 nM NTRC-P on a template carrying the symmetrical site was only twofold above background, and activation by phosphorylated NTRC^{3ala} was slight at concentrations below 75 nM. Activation by a mixture of phosphorylated wild-type NTRC at 5 nM and phosphorylated NTRC^{3ala} at different concentrations was synergistic (Fig. 6B). The maximum measurable degree of synergy was 40-fold (at 40 nM phosphorylated NTRC^{3ala}). As expected, synergistic activation was greatly decreased under conditions that facilitated subunit exchange between dimers (not shown), presumably because the ability of the DNA-binding form (NTRC-P) to serve as tether was diminished in heterodimers.

In agreement with data indicating that activation of transcription from a single NTRC-binding site requires a complex of more than one dimer of active NTRC, phosphorylated NTRC forms a large structure at a single strong binding site in electron micrographs. In the absence of ATP, NTRC^{S160F} (105,000 m.w. dimer) formed a small structure at a single strong site (Fig. 7b,c), whereas when ATP was added to allow phosphorylation, it formed a larger structure (Fig. 7d; NTRB was present in both cases). Comparison of the size of the larger structure with that of σ^{54} holoenzyme (~450,000 m.w.) suggests that it contains at least two and possibly more dimers of NTRC. σ^{54} Holoenzyme, which shadows darker with tungsten than NTRC (Su et al. 1990), is visible in Figure 7c bound to the ends of the DNA fragment and in Figure 7d at the *glnA* promoter in open complexes.

Discussion

The glnA enhancer facilitates formation of an active oligomer of NTRC-P

The enhancer upstream of the *glnA* gene of enteric bacteria maintains the NTRC activator protein in high local concentration to increase the frequency with which it contacts σ^{54} holoenzyme bound at the promoter. We have now shown that a second role of the enhancer is to organize an oligomer of NTRC-P that is required for transcriptional activation.

NTRC dimers bound at the *glnA* enhancer interact to form an oligomer at low solution concentrations of the protein. NTRC dimers bind cooperatively to the enhancer (Figs. 3A and 4A), which consists of two specific binding sites approximately three turns of the DNA helix apart. We have demonstrated that cooperative binding results from interaction between NTRC dimers rather than conformational change of the DNA by showing that the carboxy-terminal domain of NTRC, its DNA-binding domain, does not bind cooperatively to the enhancer (Figs. 3D and 4C). This also demonstrates that determinants of the cooperative interaction lie outside the DNA-binding domain. As expected for interacting

Figure 7. Visualization of NTRC and NTRC-P bound to a single site. Electron microscopy was used to examine the binding of NTRC and NTRC-P to a single strong binding site. A 411-bp *Bam*HI–*Nru*I fragment from pJES132, which carries a single strong NTRC-binding site, is shown after incubation in the absence of protein (a) or in the presence of wild-type NTRC (500 nM; b). In c, a 392-bp *Eco*RI–*Pvu*II fragment from pJES483 (Santero et al. 1992), which carries the same strong NTRC-binding site upstream of the *glnA* promoter, is shown after incubation with NTRC^{S160F} (500 nM), NTRB (100 nM), and σ^{54} holoenzyme (100 nM), and in d, after incubation with the above proteins and ATP (2 mM). In the presence of ATP, NTRC becomes phosphorylated and σ^{54} holoenzyme is bound at the promoter in open complexes. Arrows indicate NTRC or NTRC-P at the binding site. NTRC-P forms larger structures.

dimers, the cooperativity of binding of NTRC was influenced by the spacing between the sites that constitute the enhancer, being decreased when these sites were placed on opposite sides of the helix (~2.5, 3.5, or 4.5 turns apart) and partly restored when they were returned to the same side (~4 turns apart) (S. Porter and S. Kustu, unpubl.).

Phosphorylation of NTRC, which is absolutely required for its function as a transcriptional activator, increases the cooperativity of binding to the *glnA* enhancer at least 12-fold (Figs. 3B and 4A) but does not affect the affinity of a dimer for a single DNA-binding site. Congruent with its effect on cooperativity, phosphorylation decreases the dissociation rate of NTRC from the enhancer by approximately the same factor (Fig. 4D). Thus, under activating conditions, the combination of strong protein-protein interaction between dimers and the ar-

range of binding sites in the *glnA* enhancer ensures formation of at least a specifically bound tetramer of NTRC-P. As yet, we are unable to determine whether this specifically bound tetramer is sufficient for transcriptional activation or whether a higher-order oligomer is required.

We present two compelling lines of evidence that multimerization of NTRC is required for transcriptional activation. (1) Activation from an enhancer—two binding sites for NTRC—that was engineered to be a worse tether than a single symmetrical site was greater than that from the single site at all NTRC concentrations (Fig. 5). At NTRC concentrations below 30 nM, activation from the weak enhancer was synergistic with respect to that from the single site, that is, >2-fold greater than that from the single site, even though the affinity of a dimer for the single site is at least 15-fold better than the affinity of a tetramer for the enhancer (Fig. 5). Synergism was most apparent between 4 and 14 nM NTRC, concentrations at which activation from the enhancer was as much as 24-fold better than that from the single site, even though the single site was filled at 4 nM and the enhancer was not fully occupied even at 14 nM. These results indicate that a single dimer is not sufficient for activation and that an important role of the enhancer is to organize an active multimer of NTRC. (2) Activation of transcription from a single site requires higher concentrations of NTRC than are needed for occupancy, and mutant forms of NTRC that are unable to bind the site or to activate well by themselves dramatically stimulate activation at low concentrations of DNA-binding forms (Fig. 6). Because the mutant forms cannot contribute to occupancy, they must stimulate by means of protein–protein interaction with specifically bound dimers to form active oligomeric species at the single site.

As expected, if the active species of NTRC-P is an oligomer, the rate of activation varies with the spacing between the two binding sites that constitute an enhancer (S. Porter and S. Kustu, unpubl.). At saturating concentrations of NTRC, activation is poor when sites are located on opposite sides of the helix (similar to activation from a single site); this is congruent with the view that bound dimers of NTRC must interact and that simply tethering two dimers near a promoter is not sufficient for activation. Moreover, activation is two- to threefold less rapid when sites are separated by four turns of the DNA helix rather than three, even though NTRC binds cooperatively in both cases. It is our working hypothesis that the decrease in rate of activation at four turns results from reduced ability of bound dimers of NTRC to interact with one another appropriately.

While this work was in progress, V. Weiss et al. (1992) provided evidence that NTRC binds cooperatively to the *glnA* enhancer of *E. coli* and that cooperativity is greatly increased upon phosphorylation. On the basis of results of filter-binding assays, they reported that phosphorylation of NTRC increased its cooperativity constant for binding to the *glnA* enhancer by 50,000-fold, whereas we observed an increase of ~12-fold for binding to the strong enhancer that we constructed. Factors that may contrib-

ute to the difference include use of a lower temperature (20°C) and lower salt by V. Weiss et al. (1992) and uncertainties about the degree of phosphorylation of NTRC in the two cases. We have found that lower temperature dramatically increases both the interaction between dimers of NTRC^{S160F} (S. Porter and S. Kustu, unpubl.) and the interaction between monomers within a dimer of NTRC (K. Klose and S. Kustu, unpubl.). We studied binding at 37°C because this is the temperature at which transcriptional activation is studied.

V. Weiss et al. (1992) also presented evidence that multimerization of NTRC is required for transcriptional activation by showing that activation from the *glnA* enhancer was greater than that from a single strong binding site. We obtained more rigorous proof that oligomerization is required for activation by using an enhancer engineered to be a considerably worse tether for tetramers (15- to 30-fold) than our single symmetrical site is for a dimer and showing that transcriptional activation (the rate of open complex formation) paralleled occupancy of the enhancer by tetramers (Fig. 5D). In addition, we showed directly that activation from the single symmetrical site at high concentrations of NTRC also depended on oligomerization by demonstrating that forms of NTRC unable to bind DNA could activate transcription synergistically with low concentrations of DNA-binding forms (Fig. 6).

Dual requirement for oligomerization and phosphorylation

To activate transcription, NTRC must oligomerize in addition to being phosphorylated. What is the physiological significance of the dual requirement and what are possible physiological advantages of the requirement for oligomerization? Phosphorylation is known to activate NTRC at the correct time, that is, under nitrogen-limiting conditions (Reitzer and Magasanik 1987). It appears to alter the protein so that it has the potential to oligomerize appropriately. The enhancer—the DNA ligand—mediates association between dimers of NTRC-P (and NTRC) at low solution concentrations of the protein. The enhancer thereby facilitates activation only at the correct places on the chromosome, that is, at nitrogen-regulated promoters. Preventing spurious activation from other σ^{54} -dependent promoters is important because other activators of this polymerase—homologs of NTRC—activate transcription in response to a variety of physiological signals (Kustu et al. 1989; Thony and Hennecke 1989). Although NTRC is likely to bind at least transiently to pseudosites in the vicinity of these other promoters, a requirement for oligomerization would prevent promiscuous activation by single dimers of NTRC-P, and the probability that an active oligomer would form at a pseudosite or that two such sites would occur at the correct spacing to function as an enhancer would be low. [Note that limiting activation to the right place is a general problem for enhancer-binding proteins because they can function efficiently from many different

positions in the vicinity of a promoter and possibly from solution (Magasanik 1989).]

In addition to restricting activation of transcription to the correct promoters, a second possible advantage of a requirement for oligomerization of NTRC-P is that different enhancers for this protein can facilitate the formation of oligomers with different catalytic efficiencies. As mentioned above, the NTRC multimer formed when two strong binding sites are separated by four turns of the DNA helix rather than three activates transcription from the *glnA* promoter two- to threefold less rapidly than the multimer formed when these sites are three turns apart. As noted by V. Weiss et al. (1992), the three best studied natural enhancers for NTRC are all composed of two binding sites. Interestingly, the spacing between these sites is different in each case; three turns of the helix at *glnA*, two turns at *nifLA* [the nitrogen fixation regulatory operon (Wong et al. 1987; Minchin et al. 1988)], and approximately one turn at *glnH* [the glutamine transport operon (Claverie-Martin and Magasanik 1991)].

NTRC as a molecular machine

NTRC is an example of a simple molecular machine; it must hydrolyze ATP to activate transcription by σ^{54} holoenzyme. As is true for other molecular machines, the gross aspects of NTRC function are understood and several laboratories are working out what have been called "the details" (Alberts and Miake-Lye 1992). We consider the requirement for oligomerization of NTRC-P in view of what is currently known about its function in transcriptional activation.

It is known that NTRC-P must hydrolyze a nucleotide to allow isomerization of closed complexes between σ^{54} holoenzyme and a promoter to open complexes; we infer that ATP hydrolysis is required after NTRC has contacted the polymerase by means of DNA loop formation. Presumably, successful formation of open complexes depends on two functions of NTRC in addition to its ability to hydrolyze ATP: the ability to engage σ^{54} holoenzyme and the ability to couple ATP hydrolysis to a change in conformation of this polymerase at a promoter. Nothing is known about the latter two functions. Because oligomerization of NTRC is required for transcriptional activation, it is presumably required for one or more of these steps. As discussed below, oligomerization has been shown to stimulate ATP hydrolysis.

We demonstrated previously that phosphorylation of NTRC was required for its ATPase activity and proposed that oligomerization might also be required because the ATPase activity of the protein in solution showed a steep dependence on the concentration of phosphorylated NTRC (Weiss et al. 1991). Austin and Dixon (1992) demonstrated that the ATPase activity of NTRC-P in solution was stimulated by its binding to an enhancer (the *nifLA* enhancer), but much less so by binding to a single site, again indicative of the need for oligomerization. We have extended their study by showing that stimulation of ATPase activity by a DNA fragment carrying a single

binding site is also the result of oligomerization of NTRC rather than allosteric activation of a single dimer (K. Klose and S. Kustu, unpubl.). Moreover, it appears that all dimers of NTRC within an oligomer must have a functional ATPase for transcriptional activation to occur: Stimulation of activation by non-DNA-binding forms of NTRC does not occur if the form specifically bound to a single site on DNA carries the G173N lesion in its nucleotide-binding motif ("glycine-rich" or "phosphate" loop) and is therefore incapable of ATP hydrolysis (Weiss et al. 1991; S. Porter and S. Kustu, unpubl.). We speculate that a requirement for multiple active dimers of NTRC may be due to the need for simultaneous hydrolysis of at least two ATP molecules to allow a change in configuration of polymerase-promoter complexes. The sensitivity of transcriptional activation to low concentrations of the competitive inhibitor ATP γ S is accommodated by this view (Weiss et al. 1991).

Synergistic activation by eukaryotic enhancer-binding proteins

Many eukaryotic enhancer-binding proteins show synergistic effects on activation of transcription by RNA polymerase II (B). As is the case for NTRC, the ability of these proteins to activate from multiple binding sites is greater than additive and cannot be accounted for by occupancy of the sites (e.g., Courey et al. 1989; Carey et al. 1990, 1992). Moreover, activation by DNA-binding forms of the Sp1 protein from a single site can be stimulated by nonbinding forms, which themselves have no capacity to activate (Courey and Tjian 1988; Courey et al. 1989; Pascal and Tjian 1991). This phenomenon, referred to as superactivation, indicates that oligomeric forms of the enhancer-binding protein are more active than the monomeric form.

Unlike NTRC, eukaryotic enhancer-binding proteins are not known to have an enzymatic activity that is required for their function. Synergistic activation by several molecules of one such protein, or by two or more different enhancer-binding proteins, is thought to be mediated by multiple contacts with downstream targets (e.g., Lin et al. 1990; examples cited in Herschlag and Johnson 1993), which have not yet been precisely defined. As discussed above, appropriate oligomerization of NTRC-P greatly stimulates its required ATPase activity. We do not know whether oligomerization also stimulates the ability of NTRC-P to contact σ^{54} holoenzyme or to couple ATP hydrolysis to a change in configuration of this polymerase at a promoter.

Materials and methods

Construction of plasmids for studies of DNA binding and transcription (open complex formation)

All plasmids constructed for this study were derived from pJES463 (Fig. 1A,B), which was made by cutting pTZ 19U (Mead et al. 1986) with *Bam*HI, inserting a 219-bp *Sau*3A–*Sau*3A fragment from pJES125 (P. Wong and S. Kustu, unpubl.) carrying the *glnA* promoter regulatory region from *S. typhimurium*, and

then introducing restriction sites into this fragment as described below. The *glnA* promoter fragment carries the *glnA* enhancer, which is composed of binding sites 1 and 2 for NTRC; the center of the enhancer is at position -124 and lies 107 bp from the center of the *glnA* promoter at -17. On the basis of DNase I footprinting and methylation protection studies, the fragment also carries three additional binding sites for NTRC, which lie between -95 and -37 (Hirschman et al. 1985), but these sites are not involved in transcriptional activation (Reitzer and Magasanik 1986; Reitzer et al. 1989; A. North and S. Kustu, unpubl.). Oligonucleotide site-directed mutagenesis (Kunkel 1985; Bio-Rad) was used to create the following restriction sites within the *glnA* promoter regulatory region (Fig. 1A): (1) a *Bgl*II site at position -155 with respect to the start site of transcription (17 bp upstream from the center of binding site 1 for NTRC); (2) a *Hpa*I site at -124 (17 bp upstream from the center of binding site 2 for NTRC); (3) a *Bcl*II site at -95 (12 bp downstream from the center of site 2); (4) an *Nco*I site at -37; and (5) a *Bst*XI site at +28. The oligonucleotide used to create the *Bst*XI site also changed additional bases downstream to T's to create a T-rich region that would function in termination of transcription (see below). The presence of restriction sites was confirmed by DNA sequencing, as was the correctness of all changes introduced into the *glnA* promoter regulatory region in other constructs.

The following plasmids were used to make probes to study DNA binding. pJES499 was made from pJES463 by using site-directed mutagenesis to replace binding site 1 and binding site 2 for NTRC with the same strong binding site from the *ntrBC* promoter region to create the strong enhancer (refer to Fig. 1 for sequences). pJES500 (Fig. 1C) was made by digesting pJES499 with *Bcl*II and *Nco*I, filling both ends with the Klenow fragment of DNA polymerase I, and religating to eliminate NTRC-binding sites 3, 4, and 5 (the *Nco*I site is recreated at this junction).

Plasmid pJES523, which has a single strong binding site for NTRC at position 1, was made by removing the strong binding site at position 2 from pJES500 on a 33-bp *Hpa*I-*Nco*I fragment. Plasmid pJES601, which has a single symmetrical NTRC-binding site at position 1 (Fig. 1E), was constructed from pJES523 by using a PCR primer that overlapped position 1 and changed it to a symmetrical site.

Transcription templates, which carried a 403-bp spacer between NTRC-binding sites and the *glnA* promoter and yielded a synthetic transcript lacking U residues, were constructed from the plasmids described above as follows. Plasmid pJES534 was made by replacing the 58-bp *Bcl*II-*Nco*I fragment of pJES499 with a 403-bp *Bcl*II-*Nco*I fragment from pACYC184 (Chang and Cohen 1978; Rose 1988) to move the enhancer farther from the promoter. In addition, a synthetic module of six directly repeated double-stranded oligonucleotides lacking T residues (21 bp each; AGACACCACAGACCACACACAAGAC) was inserted into the *Bst*XI site, which is located at +28 and is immediately followed by a T-rich sequence that functions in termination of transcription (TTGGTTTTGTTTACCTGCAGGCATG). Plasmid pJES534 yields an ~155-base transcript from the *glnA* promoter that contains no uracil. Plasmid pJES635 carries a weak enhancer (Fig. 1D) in place of the strong enhancer (Fig. 1C) in pJES534 and has an *Eco*RV site 7 bp upstream of the *Bcl*II site.

Plasmid pJES520 is a transcription plasmid with a single strong NTRC-binding site; it is identical to pJES534 except that it lacks the strong NTRC-binding site at position 1, which was removed on a 68-bp *Sma*I-*Hpa*I fragment. Plasmid pJES640, a transcription plasmid with a single symmetrical binding site at position 1, was constructed in the following steps: (1) PCR was used to amplify a 180-bp region of pJES500. One of the primers,

which overlapped the strong binding site for NTRC at position 1, made a single base change to create a symmetrical binding site and simultaneously created a *Bgl*II site 15 bp upstream (5') from the center of position 1; (2) the PCR product was digested with *Bgl*II and *Hpa*I to produce a 30-bp fragment carrying the symmetrical site, which was used to replace the *Bgl*II-*Eco*RV fragment of pJES635.

Plasmid pJES535, which lacks specific binding sites for NTRC, is identical to pJES534 except that the enhancer was removed on a 60-bp *Bgl*II-*Bcl*II fragment (ends are compatible).

Construction of plasmids for overproduction of mutant NTRC proteins

The mutant protein that we designate NTRC^{3ala} contains alanine residues at three positions in the second helix of the helix-turn-helix DNA-binding motif and is fully designated NTRC^{R456A, N457A, R461A}. The plasmid used for overproduction of this protein was constructed by oligonucleotide site-directed mutagenesis as follows: A 1.7-kb *Eco*RV-*Hind*III fragment from pJES311 (Weiss et al. 1991), which is used to overproduce wild-type NTRC, was cloned into the mutagenesis vector pTZ19U, which had been digested with *Sma*I and *Hind*III. A single mutagenic oligonucleotide [5'-CGGTTGGGGC(GCC)(GCA)ACCTTAACG(GCT)AAGCTTAAAGAGCTG-3'] was then used to generate all three alanine substitutions (sequences in parentheses) and a *Hind*III site (bold), which was created without changing wild-type amino acids. The resulting plasmid, pJES591, was digested with *Rsr*II and *Eco*RI and the 527-bp fragment carrying the altered DNA-binding region of NTRC was cloned into two plasmids, which had been similarly digested, to replace the corresponding wild-type sequence: (1) pJES311 to yield pJES641 for overexpression of NTRC^{3ala} and (2) pJES312 to yield pJES642 for overexpression of the multiply altered protein NTRC^{S160F, 3ala}.

A plasmid used to overproduce the isolated carboxy-terminal domain of NTRC (a 90-amino-acid peptide that was known to bind DNA in vivo; Drummond et al. 1990) was constructed by PCR mutagenesis as follows: Plasmid pJES311 was used as a template for amplification of a fragment joining the amino-terminal methionine of NTRC to its carboxy-terminal 90 amino acids. The sequence of the amino-terminal primer was 5'-ATA-CATATGGATTACCGGGCGAACTG-3'. The first 9 bases are from the beginning of the NTRC gene in pJES311, with the *Nde*I site that overlaps the initiating methionine codon (ATG) underlined. The last 18 nucleotides correspond to codons 380-385 of NTRC. The sequence of the carboxy-terminal primer was: 5'-GGAATTCTTTACACTCGCGGTA-3'. This primer hybridizes to pJES311 downstream of a NTRC-coding sequence beginning at a position 5 nucleotides beyond the UAA stop codon, and it introduces an *Eco*RI site (underlined). The PCR product was digested with *Nde*I and *Eco*RI and was ligated into pJES311, which had been similarly digested to yield pJES580. The correctness of the construction was confirmed by sequencing.

Labeling DNA probes

Probes were prepared by end-labeling restriction fragments from plasmids with polynucleotide kinase (5' label) or the Klenow fragment of DNA polymerase I (3' label) or by using end-labeled primers for PCR amplification of fragments from plasmids (Krummel 1990). Primers for PCR amplification were primer 1, 5'-GAGCGTTAAAGGGTGGC-3', which is a top-strand primer that begins 22 bp upstream of the *Bgl*II site in Figure 1A;

primer 2, 5'-TCTGTGCCAACTTTCCCA-3', which is a bottom-strand primer that begins within the *glnA* promoter; primer 3, 5'-GTTTTCCCAGTCACGAC-3', which is a top-strand primer that begins 38 bp upstream of the *EcoRI* site in Figure 1A; primer 4, 5'-AGGGAAAGCTTGCATGCCTG-3', which is a bottom-strand primer that begins downstream of *glnA* promoter sequences; and primer 5, 5'-CCCGTGGAGGTAATAATTG-3', which is a bottom-strand primer that begins 31 bp downstream of the *BclI* site (Fig. 1A) in transcription vectors. To label probes to high specific activity, both ends were labeled and several labeled nucleotides were used to fill in 3' ends with Klenow fragment. Alternatively, PCR products were internally labeled. Labeled fragments were purified on polyacrylamide gels, eluted by diffusion, and precipitated with spermine as described (Hoopes and McClure 1981). The concentration of probes was determined by absorbance at 260 nm.

Purification of proteins

Wild-type NTRC, the NTRC^{S160F} mutant form, σ^{54} , and NTRB were purified as described (Keener and Kustu 1988; Popham et al. 1991; Weiss et al. 1991). The NTRC^{3ala} and NTRC^{S160F, 3ala} proteins were overexpressed and purified as was wild-type NTRC, except that the mutant proteins did not bind to heparin-agarose. The carboxy-terminal domain of NTRC was overexpressed in strain NCM629, which carries the gene for T7 RNA polymerase under control of a *lac* promoter; induction was with IPTG. The carboxy-terminal domain was purified as was full-length NTRC, except that the initial ammonium sulfate precipitation occurred at 35–50% (wt/vol). Core RNA polymerase from *E. coli* was a kind gift from D. Hager and R. Burgess (University of Wisconsin, Madison).

The amount of active wild-type NTRC was determined by titrating a fixed concentration of protein with a probe carrying a single symmetrical binding site for NTRC (from plasmid pJES601) in a gel mobility-shift assay (not shown). Titrating a dyad-symmetrical site shifts the equilibrium toward formation of NTRC dimers and should therefore allow us to detect all active dimers. Concentrations of wild-type NTRC are reported in terms of active binding dimer (as detected at high concentrations of the binding site). The concentrations of mutant NTRC proteins and of the carboxy-terminal domain were determined by Bradford assays (Bradford 1976).

Labeling of NTRC

NTRC was labeled with [³⁵S]methionine using a variation of the method described by Tabor (1990). (1) Cells (100 ml culture) were grown to an OD₆₅₀ of 0.5 in M9 minimal medium supplemented with all amino acids (to 0.1 mg/ml) except methionine and cysteine. (2) NTRC production was then induced by addition of IPTG and an m13 phage that carries T7 RNA polymerase under control of the *lac* promoter. NTRC was expressed from the T7-7 overexpression plasmid pJES311 in strain NCM790 (NCM724/pJES311) as described (Weiss et al. 1991). (3) Thirty minutes after induction, rifampicin (to 0.2 mg/ml) and 1.5 mCi of [³⁵S]methionine were added. (4) Sixty minutes after induction, cells were harvested and disrupted by sonication in B buffer (see below). Cell extracts were subjected to low-speed centrifugation, and NTRC protein was precipitated with ammonium sulfate (0–35%), dialyzed into B buffer, and purified on a heparin-agarose column (1 ml) using stepwise elution. NTRC eluted at 100 mM potassium chloride.

Gel mobility-shift assay for DNA binding

Mobility-shift assay and DNA binding were based on the meth-

ods of Fried and Crothers (1981) and Garner and Revzin (1981). Reaction mixtures for binding assays contained 10 mM Tris-acetate (pH 8.0 at 25°C), 100 mM potassium acetate, 8 mM magnesium acetate, 1 mM dithiothreitol (DTT), 27 mM ammonium acetate (the same buffer used for transcription); in addition, they contained 6 pM to 0.3 nM ³²P-end-labeled probe and poly[d(I-C)] as specified. NTRC was diluted at 0°C in B buffer (10 mM Tris-Cl at pH 8.0 at 25°C, 50 mM potassium chloride, 5% glycerol, 1 mM DTT, and 1 mM EDTA) containing bovine serum albumin (0.2 mg/ml) and added to reaction mixtures at the same temperature. Reactions were then started immediately by transfer to 37°C—the same temperature used for transcription—and reaction mixtures were incubated at this temperature for 20 min. Glycerol was then added to 5%, and 10 μ l of the reaction mixture was loaded on a running gel [5% polyacrylamide, 0.06% bis-acrylamide in Tris-glycine buffer (380 mM glycine, 50 mM Tris, pH 8.6 at 25°C) at 4°C and subjected to electrophoresis at 16 V/cm for 1 hr.

For unphosphorylated wild-type NTRC and NTRC^{S160F}, binding to all configurations of sites was at equilibrium after 20 min; this is at least 10 times the $t_{1/2}$ for dissociation of these forms from the strong enhancer (see Results). Even though binding of phosphorylated NTRC to the strong enhancer was not at equilibrium after 20 min ($t_{1/2}$ for dissociation of 13 min), we used this time to ensure the stability of NTRB and NTRC.

Calculations of cooperativity of binding

The degree of cooperativity of binding of NTRC to the strong enhancer at *glnA* was estimated from data obtained with the gel mobility-shift assay using equations derived by Hudson and Fried (1990, 1991) and by Tsai et al. (1989), which do not involve the concentration of NTRC or its affinity for a binding site. (The assumption that the distribution of species in the gel reflects the distribution in solution is implicit.) It was important to use calculations independent of the concentration of NTRC dimer for two reasons: (1) Comparison of the cooperativity of binding of wild-type and mutant forms of NTRC did not depend on determining the active concentration (with respect to DNA binding) for each preparation of protein; and (2) the fact that NTRC dimers undergo rapid subunit exchange at 37°C ($t_{1/2}$ of ~3 min) indicates that there may be a significant amount of monomer at low protein concentrations (K. Klose and S. Kustu, unpubl.).

In the method of Hudson and Fried (1990), the cooperativity factor K_{dl}/K_{dII} is calculated using the equation

$$\frac{K_{dl}}{K_{dII}} = 4 \left(\frac{(F)(II)}{I^2} \right)$$

where K_{dl} is the apparent dissociation constant for binding of a dimer of NTRC to one site in the strong enhancer (intrinsic dissociation constant), K_{dII} is the apparent dissociation constant for binding of a second dimer to the adjacent site, F is the amount of free DNA, II is the amount of the fully shifted species carrying two dimers of NTRC, and I is the amount of the intermediate species carrying a single dimer. The equation, which is a simplified form of that given by Hudson and Fried (1990), pertains to the special case in which the two binding sites that constitute the enhancer are equivalent. Theoretically, noncooperative binding results in a binomial distribution of the three species, whereas positive cooperative binding skews the distribution toward the species carrying two dimers.

At the concentration of NTRC at which the fraction of I , the intermediate species, reaches a maximum, the fraction of F and

the fraction of *I* are equal and a simplification of the Hudson-Fried equation pertains (Tsai et al. 1989):

$$\frac{K_{dl}}{K_{dlf}} = \left(\frac{1}{\text{fractional maximum of } I} - 1 \right)^2$$

Theoretically, noncooperative binding yields a fractional maximum of *I* = 0.5.

Relative affinities of NTRC for different binding sites

The relative affinities of NTRC for the strong site, site 1 of the natural *glnA* enhancer, and site 2 of the *glnA* enhancer were determined by using unlabeled DNA fragments carrying these sites to compete for NTRC binding to a labeled probe carrying a single symmetrical site. Different amounts of a 53-bp unlabeled DNA fragment carrying the site to be tested (site 1 or site 2 of the *glnA* enhancer or the strong binding site in position 1 or position 2 of the strong enhancer) were added to reaction mixtures containing a fixed concentration of probe and 2 nM wild-type NTRC. The ratios of concentrations of competitor fragments required to inhibit binding of NTRC to the probe by 50% are inversely proportional to the relative binding affinities of the protein for these fragments.

Stoichiometry of binding

Binding reactions contained ³⁵S-labeled NTRC and 0.5–0.8 pmole of one of the following ³²P-labeled probes: a 411-bp *Bam*HI–*Nru*I fragment from pJES132, which carries a single strong binding site for NTRC (promoter regulatory region for the *ntfBC* operon; P. Wong and S. Kustu, unpubl.); a 117-bp *Bcl*I–*Eco*RI fragment from pJES463, which carries the natural *glnA* enhancer (binding sites 1 and 2; Fig. 1B); or a 249-bp *Hin*dIII–*Eco*RI fragment from pJES125, which carries the entire *glnA* promoter regulatory region (five binding sites for NTRC as assessed by DNase I footprinting or protection of G residues from methylation; Hirschman et al. 1985; P. Wong and S. Kustu, unpubl.). Protein was incubated with DNA for 15 min at 37°C and then loaded on a running gel as described above. Bands were visualized by autoradiography, excised, and dissolved as described (Popham et al. 1989). Samples were prepared for scintillation counting with constant quench in Aquasol scintillation fluid (DuPont) and counted in two channels of a Beckman LS1801 counter. One channel counted 80% of the ³²P and the other counted ~80% of the ³⁵S. ³⁵S counts were corrected by subtracting “spilled” ³²P, which was ~9% of total ³²P in the sample. To determine the specific activity of the labeled DNA, a standard was counted with the same quench and in the same channel as the experimental samples and DNA concentration was determined by spectrophotometry at 260 nm. The specific activity of the protein was not determined. Relative ratios of protein to DNA were determined by normalizing the ratio of ³⁵S to pmoles of shifted DNA fragment to 1.0 for the fragment carrying a single NTRC-binding site.

Dissociation rates

Binding of NTRC (7 nM) to probes carrying one or two binding sites (~0.3–1 nM) was carried out as described above at 37°C in the presence of 30 ng/μl of poly[d(I-C)]. To generate NTRC-P, NTRB was present at 20 nM. After 20 min, a 500-fold molar excess of an unlabeled 30 bp fragment carrying the single strong NTRC-binding site from the *ntfBC* promoter region was added, and 10 μl samples were withdrawn at various times and loaded on a running gel as described above. For measurements of the dissociation rate at 4°C, binding was carried out at 37°C for 20

min, and reaction mixtures were then cooled to 4°C before addition of excess unlabeled 30-bp fragment.

Open complex formation

Formation of open complexes at the *glnA* promoter was carried out at 37°C in the same buffer used for binding, and open complexes were detected in a single cycle transcription assay as described (Popham et al. 1989). The concentrations of σ⁵⁴, RNA polymerase core, and NTRB were 90, 45, and 100 nM, respectively, unless specified otherwise. Transcripts were run on denaturing gels and quantitated using a PhosphorImager. In experiments for which occupancy of NTRC-binding sites was established by DNase I footprinting, 2 mM CaCl₂ was added to the other buffer components for open complex formation. The rate of open complex formation (Klose et al. 1993) was assessed on linear templates (2 nM, as for footprinting); after digestion of plasmids pJES635 and pJES640 with appropriate restriction enzymes, templates of ~700 bp were isolated on agarose gels. For studies of stimulation of activation by the NTRC^{3ala} and the NTRC^{S160F, 3ala} proteins, open complex formation was studied on supercoiled template (plasmid pJES640 at 20 nM). Wild-type NTRC or NTRC^{S160F} (5 nM) was added to the template and allowed to bind for 20 min at 0°C. The corresponding “3-alanine” protein was then added. Reactions were started with the addition of ATP to 2 mM and immediately transferred to 37°C for 5 min to allow formation of open complexes, which were detected in the single cycle transcription assay. To achieve complete subunit mixing between wild-type NTRC and NTRC^{3ala} in control experiments, these proteins were mixed and incubated for 20 min at 37°C before being added to the template DNA.

Footprinting

Footprinting was based on the method of Galas and Schmitz (1978). Reaction mixtures were identical to those used for measuring the rate of open complex formation on linear templates (they contained σ⁵⁴ holoenzyme as well as the other components; see above), except that the template (2 nM) consisted of 75% unlabeled DNA and 25% end-labeled probe. Digestion with DNase I (0.3 ng/μl for 15 sec) and analysis by denaturing gel electrophoresis were as described (Popham et al. 1989).

Electron microscopy

Binding reactions were performed as described above with the proteins specified (see legend to Fig. 7). After incubation at 37°C, reaction mixtures were diluted 20-fold into the same buffer, prepared for microscopy as described (Williams 1977), and photographed at ~30K magnification.

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