# A bacterial ATP-dependent, enhancer binding protein that activates the housekeeping RNA polymerase

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**A commonly accepted view of gene regulation in bacteria that has emerged over the last decade is that promoters are transcriptionally activated by one of two general mechanisms. The major type involves activator proteins that bind to DNA adjacent to where the RNA polymerase (RNAP) holoenzyme binds, usually assisting in recruitment of the RNAP to the promoter. This holoenzyme uses the housekeeping $\sigma^{70}$ or a related factor, which directs the core RNAP to the promoter and assists in melting the DNA near the RNA start site. A second type of mechanism involves the alternative sigma factor (called $\sigma^{54}$ or $\sigma^{N}$) that directs RNAP to highly conserved promoters. In these cases, an activator protein with an ATPase function oligomerizes at tandem sites far upstream from the promoter. The nitrogen regulatory protein (NtrC) from enteric bacteria has been the model for this family of activators. Activation of the RNAP/$\sigma^{54}$ holoenzyme to form the open complex is mediated by the activator, which is tethered upstream. Hence, this class of protein is sometimes called the enhancer binding protein family or the NtrC class. We describe here a third system that has properties of each of these two types. The NtrC enhancer binding protein from the photosynthetic bacterium, *Rhodobacter capsulatus,* is shown in vitro to activate the housekeeping RNAP/$\sigma^{70}$ holoenzyme. Transcriptional activation by this NtrC requires ATP binding but not hydrolysis. Oligomerization at distant tandem binding sites on a supercoiled template is also necessary. Mechanistic and evolutionary questions of these systems are discussed.**

[*Key Words:* Gene regulation; enhancer; ATP-dependent; housekeeping holoenzyme]

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Compilations of data from studies on nearly 150 regulated promoters in enteric bacteria have led to the view that a bacterial promoter is activated by use of one of two systems (for review, see Collado-Vides et al. 1991; Gralla and Collado-Vides 1996). These systems are denoted by where the activator protein binds on the DNA and which sigma factor is used, but a key feature is that the mechanism of activation is quite different for each. The first system involves the RNA polymerase (RNAP) containing the $\sigma^{70}$ factor. All bacterial sigma factors are related to this housekeeping sigma (except sigma 54, see below) (Lonetto et al. 1992). In these cases, the activator proteins bind between 30 and 80-bp upstream of the transcription start site, adjacent to the RNAP holoenzyme. These promoters are usually poorly recognized by RNAP without the aided recruitment by the activator protein (Ishihama 1993). Many elegant studies over the last decade have defined the exact residues of specific activators and of the RNAP subunits that make contact with each other. So far, interactions with either the α and/or σ subunits of RNAP have been characterized (for review, see Busby and Ebright 1994). On the basis of these studies, certain rules are beginning to emerge with respect to which RNAP contact is made according to where activator binding is centered (between −30 and −80 ).

A second general type of bacterial activation system involves RNAP containing the *rpoN*-encoded sigma factor called $\sigma^{54}$ ($\sigma^{N}$) (for review, see Merrick 1993; North et al. 1993; Magasanik 1996). This is the only sigma factor that is unrelated to $\sigma^{70}$ and it is responsible for directing RNAP to very highly conserved promoters that have the consensus GG-$N_{10}$-GC (at −24 and −12 bp). In these cases the activator proteins bind distantly upstream of the promoters and via DNA looping, activate the RNAP/$\sigma^{54}$ holoenzyme. A paradigm for this class of activator, for which there are now at least 30 cases in many genera of bacteria, is the nitrogen regulatory protein called NtrC (or NRI). Members of the NtrC class typically bind to DNA at tandem sites centered >100-bp upstream of the start site and these activators are therefore referred to as enhancer-binding proteins (EBPs). In addition to the $\sigma^{54}$ and enhancer characteristics, all EBPs contain a nucleotide binding fold for which its ATPase function is required for transcriptional activation (Weiss et al. 1991; Austin and Dixon 1992). For some of the EBP-activated

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promoters, RNAP/$\sigma^{54}$ has been shown to bind the promoter in a stable closed complex, independent of the activator, indicating that recruitment to the promoter is not necessary (Ninfa et al. 1987; Sasse-Dwight and Gralla 1988; Buck and Cannon 1992; Cannon et al. 1993; Syed and Gralla 1997). For NtrC, an unusual multimerization occurs whereby two dimers bind at the tandem sites and then two more dimers bind to these by protein–protein interactions (Wyman et al. 1997). This DNA-mediated oligomerization is necessary for ATPase activity and activation, perhaps prompting an interaction with both the $\sigma^{54}$ and β subunits of RNAP (Lee and Hoover 1995).

Here, we describe a third bacterial activation system with properties of both systems. Like the enteric counterpart (Ninfa and Magasanik 1986), the *Rhodobacter capsulatus* NtrC protein (RcNtrC) is phosphorylated by its cognate kinase, NtrB, in response to nitrogen deprivation (Cullen et al. 1996). This phosphorylation in enterics converts NtrC into the activator with the aforementioned characteristics. The RcNtrC also contains the nucleotide binding fold, which, by genetic analysis, is required for its activation function in vivo (Foster-Hartnett et al. 1994). Two tandem sites that are centered >100-bp upstream of the transcription start site bind purified RcNtrC in each of the five known members of the RcNtrC regulon (*nifA1*, *nifA2*, *glnB*, *anfA*, and *mopA*). The upstream tandem sites are necessary for activation in vivo, as demonstrated with the *nifA1* and *nifA2* promoters (Foster-Hartnett et al. 1994). However, RcNtrC-activated promoters are still expressed in a nitrogen-dependent manner in *rpoN* mutants and they have no sequence similarity to typical $\sigma^{54}$-activated promoters (Foster-Hartnett and Kranz 1992, 1994; Preker et al. 1992; Kutsche et al. 1996). Thus, the RNAP holoenzyme that is activated by the RcNtrC protein has remained an enigma. Here, we show by use of purified components that the *R. capsulatus* RNAP (RcRNAP)/$\sigma^{70}$ holoenzyme is activated by RcNtrC in an NtrB-dependent manner. In vitro transcriptional activation requires a supercoiled template, the upstream tandem binding sites for RcNtrC, and ATP. Our results suggest that this third type of system contains elements of each of the first two and could represent an evolutionary transition between $\sigma^{54}$ and $\sigma^{70}$ systems that were retained by this α proteobacterial species of photosynthetic bacterium.

## Results

### *RcNtrC activates the RNAP $\sigma^{70}$ holoenzyme in an NtrB-dependent manner*

To investigate whether the RcRNAP $\sigma^{70}$ holoenzyme is activated by the RcNtrC system, we have combined the use of a set of wild-type and mutant *nifA1* promoters (Fig. 1A) and hyperactive constitutive RcNtrC mutant proteins. These RcNtrC proteins show increased in vivo activation in response to limiting or sufficient nitrogen. It is known that they are phosphorylated in vitro by NtrB

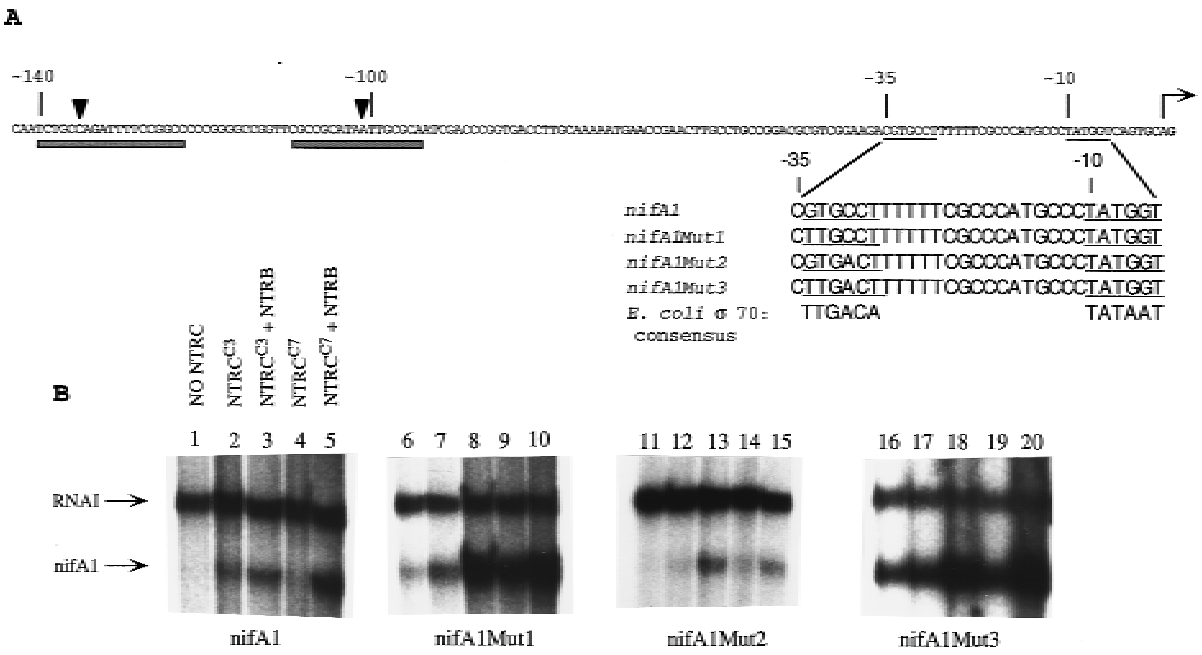


**Figure 1.** (*A*) Sequence of the *nifA1* promoter region and engineered promoters. (Shaded bars) RcNtrC binding sites, (arrowheads) major DNase I hypersensitive sites. The −35 and −10 hexamers are underlined and the transcriptional start site is marked by a horizontal arrow. The sequences of the *nifA1*, *nifA1Mut1*, *nifA1Mut2*, and *nifA1Mut3* promoters are aligned for comparison with the *E. coli* $\sigma^{70}$ consensus. (*B*) In vitro transcriptional activation of *nifA1* wild-type and mutant promoters by RcNtrC. Templates are noted below each set of transcription reactions. (Lanes *1,6,11,16*) No activators; (lanes *2,7,12,17*) 260 nM RcNtrC$^{C3}$; (lanes *3,8,13,18*) 260 nM RcNtrC$^{C3}$ and 270 nM MBP–NTRB; (lanes *4,9,14,19*) 350 nM RcNtrC$^{C7}$; (lanes *5,10,15,20*) 350 nM RcNtrC$^{C7}$ and 270 nM MBP–NtrB.

to the same level and bind to the upstream tandem sites with the same affinity as the wild-type RcNtrC (W.C. Bowman, P.J. Cullen, and R.G. Kranz, unpubl.). For in vitro transcription studies, we have recently purified the *R. capsulatus* housekeeping RNAP containing the major $\sigma^{70}$ subunit. This preparation is ~98% pure by SDS-PAGE and contains only the housekeeping $\sigma^{70}$ as determined by Western blot analysis with the 2G10 monoclonal antibody (Cullen et al. 1997) (Fig. 2A,B). We chose the *nifA1* promoter because of the in vivo information already known about its upstream DNA including its RcNtrC tandem binding sites (Foster-Hartnett et al. 1994). Moreover, we previously converted the nucleotides in the −35 region of *nifA1* toward an ideal −35 hexamer (*nifA1Mut3*) with the result of high-level basal transcription by the $\sigma^{70}$ RNAP (Cullen et al. 1997). Thus, it was known that with only two nucleotide changes, the RcRNAP $\sigma^{70}$ could melt this promoter by an RcNtrC-independent process.

For the present study, we converted the wild-type

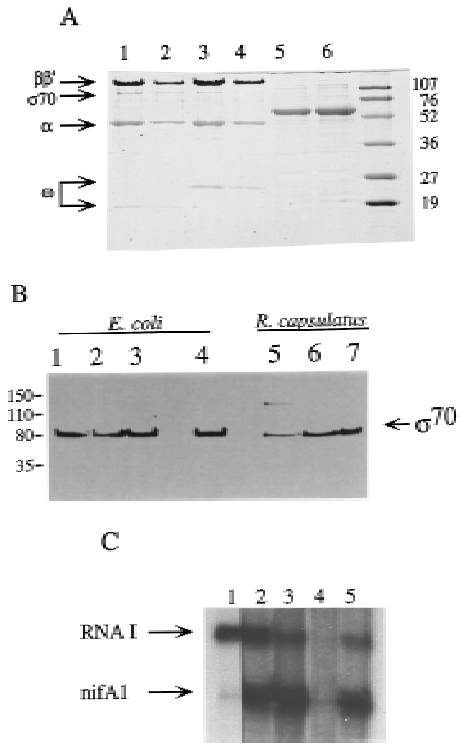


**Figure 2.** Characterization of the holoenzyme and activator preparations and transcription inhibition by monoclonal antibody 2G10. (*A*) SDS-PAGE of the *E. coli* RNAP (lanes *1,2*); RcRNAP (lanes *3,4*); RcNtrC$^{C3}$ (lane *5*) and RcNtrC$^{C7}$ (lane *6*); standards with sizes indicated in kD. (*B*) Western analysis of the RcRNAP and EcRNAP purification by use of mAb 2G10, specific for *E. coli* $\sigma^{70}$. Fractions are from *E. coli* and *R. capsulatus* as follows: (Lanes *1,5*) PEG precipitation fraction; (lanes *2,6*) heparin agarose fraction; (lanes *3,7*) DEAE–Sepharose fraction; (lane *4*) HPLC-purified EcRNAP/$\sigma^{70}$ holoenzyme. (*C*) RcNtrC activated transcription is abolished by $\sigma^{70}$ mAb 2G10. The *nifA1Mut1* promoter was used. (Lane *1*) no activator; (lanes *2–5*) 350 nM RcNtrC$^{C7}$; (lanes *3–5*) 270 nM MBP–NtrB; (lane *4*) 1 μl of mAb 2G10; (lane *5*) 1 μl of control mAb.

*nifA1* promoter toward a consensus −35 hexamer by one (*nifA1Mut1, nifA1Mut2*) or two nucleotides (nifA1Mut3) and studied transcription in the presence of purified maltose binding protein-NtrB (MBP–NtrB) and two RcNtrC constitutive mutant proteins, designated RcNtrC$^{C3}$ and RcNtrC$^{C7}$. These proteins were purified as described previously (Cullen et al. 1996) to >95% homogeneity (Fig. 2A). All transcription reactions shown in Figure 1 were with supercoiled templates in which the indicated *nifA1* DNA was cloned upstream of a transcriptional terminator. *nifA1* transcription yields an mRNA product of 92 nucleotides that migrates below the 108-nucleotide RNAI transcript. The RNAI transcript is from a well-recognized $\sigma^{70}$-type promoter (Scott 1984) that functions with both the *Escherichia coli* and RcRNAP holoenzymes (Cullen et al. 1997). RNAI transcripts serve as internal controls for each template and all *nifA1* transcripts detected in the present study were quantified relative to the RNAI transcript. This corrected for loading differences or degradation and facilitated comparisons with different templates or conditions. In the absence of activator the *nifA1Mut3* template exhibited high transcription (Fig. 1B, lane 16). Whereas *nifA1Mut1* (Fig. 1B, lane 6) gave a very low basal level of transcription, the wild-type (Fig. 1B, lane 1) and *nifA1Mut2* (Fig. 1B, lane 11) exhibited no detectable transcription without activation. Addition of the RcNtrC proteins resulted in at least a 4- to 10-fold increase in transcription at the *nifA1* wild-type, *nifA1Mut1*, and *nifA1Mut2* promoters (Fig. 1B). Because no basal level of transcription could be detected with the wild-type or *nifA1Mut2* promoters, the fold increase could not be determined. However, at least 10-fold less product from the activated sample could have been detected, indicating at minimum, a 10-fold activation. Because basal levels are so high, activated transcription of the *nifA1Mut3* promoter is obscured (Fig. 1B, lanes 16–20). No increase in transcription was observed at the RNAI promoter on addition of the activators, indicating that the activation was specific for *nifA1* and the two mutant promoters. The addition of MBP–NtrB to reactions containing each of the three promoters (wild type, *nifA1Mut1*, and *nifA1Mut2*) and RcNtrC increased the activation another two- to fourfold, depending on the promoter or RcNtrC allele. However, total activation levels were always higher when the *nifA1Mut1* promoter was used, at least fivefold higher than observed with the wild-type and *nifA1Mut2* promoters. To define the activation requirements for this system, results with the *nifA1Mut1* promoter are presented here, although most activation requirements were also demonstrated with the wild-type *nifA1* promoter.

With *nifA1Mut1*, the RcNtrC$^{C7}$ protein gives the greatest activation without MBP–NtrB (Fig. 1B, lane 9) and total activated transcription with MBP–NtrB (Figs. 1B, lane 10, and 2C, lanes 2,3). Previously, we have used the $\sigma^{70}$ monoclonal antibody 2G10 to inhibit transcription of RcRNAP $\sigma^{70}$ promoters as have other groups with *E. coli* $\sigma^{70}$ promoters (for review, see Breyer et al. 1997 and references therein). In vitro transcription of both the

RNAI promoter and the activated *nifA1Mut1* promoter is inhibited >98% by mAb 2G10 (Fig. 2C, lane 4) but not significantly by a control mAb (lane 5). The 2G10 antibody was also found to inhibit RcNtrC-dependent transcriptional activation of the wild-type *nifA1* promoter (data not shown). The only sigma factor in the RcRNAP preparation that reacts with 2G10 is $\sigma^{70}$ (Cullen et al. 1997; Fig. 2B, lane 7). The epitope for 2G10 has been mapped to residues 470–486 of the *E. coli* $\sigma^{70}$ factor (Breyer et al. 1997). As expected, the *R. capsulatus* $\sigma^{70}$ is highly conserved in this region (Pasternak et al. 1996), but no $\sigma^{54}$ factor, including *R. capsulatus* $\sigma^{54}$ (Jones and Haselkorn 1989), contain this epitope.

To further demonstrate that RcNtrC activates the *R. capsulatus* $\sigma^{70}$ holoenzyme, we constructed two plasmids containing the *R. capsulatus rpoD* gene that result in overexpression of the *R. capsulatus* $\sigma^{70}$ subunit in *E. coli*. The $\sigma^{70}$ protein expressed from pRGK300 was purified from an SDS gel and renatured by use of the method of Gross et al. (1978). A hexahistidine-tagged $\sigma^{70}$ polypeptide was overexpressed from pRGK301 and purified with a nickel affinity column. The purified $\sigma^{70}$ proteins were added to in vitro transcription reactions containing *R. capsulatus* core RNA polymerase that has been shown previously to contain very low levels of $\sigma^{70}$ (Cullen et al. 1997). This core preparation gave very low levels of RNAI transcript and no *nifA1Mut1* transcript with or without RcNtr proteins (Fig. 3, lanes 1, 2). Addition of either the histidine-tagged (Fig. 3, lanes 3,4) or the renatured $\sigma^{70}$ (Fig. 3, lane 5) resulted in a >10-fold increase in the RNAI transcript. When NtrB and NtrC was added to either reaction containing $\sigma^{70}$ (Fig. 3, lane 4,5) a significant increase in activation is observed compared with the reaction with core RNAP and $\sigma^{70}$ only (Fig. 3, lane 3). These results confirm that it is the *R. capsulatus* $\sigma^{70}$ RNAP that is activated by RcNtrC.

It is worth noting the presence of a subunit of the RcRNAP preparation, which migrates at ~23 kD in this SDS-PAGE system (Fig. 2A, lane 3). The *E. coli* holoenzyme preparation contains a polypeptide that migrates ~4 kD smaller then this (Fig 2A, lane 1), which is similar in size to the previously noted ω subunit of unknown function (Gentry and Burgess 1986). Amino-terminal sequencing of the *R. capsulatus* 23-kD polypeptide has demonstrated that it is the ω subunit. The *R. capsulatus* gene (*rpoZ*) encoding the ω subunit has been sequenced (*R. capsulatus* sequencing project at the University of Chicago, URL http://capsulapedia.uchicago.edu). The *rpoZ* gene sequence indicates a size that is 28 residues larger than the *E. coli* counterpart, consistent with the size exhibited by SDS-PAGE.

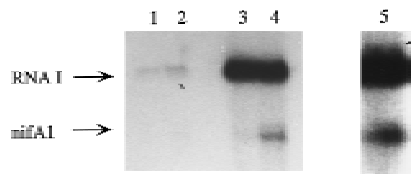


**Figure 3.** Purified $\sigma^{70}$ added to *R. capsulatus* core RNAP stimulates transcriptional activation by RcNtrC. In vitro transcription reactions using the supercoiled *nifA1Mut1* template with the *R. capsulatus* core RNAP (lanes *1*,*2*) or core RNAP with the addition of His-tagged $\sigma^{70}$ subunit (lanes *3*,*4*) or SDS-gel purified $\sigma^{70}$ subunit (lane *5*). (Lanes *1*,*3*) no activator; (lanes *2*,*4*,*5*) 270 nM MBP–NtrB and 260 nM RcNtrC$^{C3}$.

### *Supercoiled DNA and both upstream tandem sites are required for RcNtrC-mediated activation*

To determine if DNA binding by RcNtrC is required for activation, and, if so, whether one dimer site or two are necessary, we constructed plasmids in which either one or both of the RcNtrC binding sites have been removed from the *nifA1Mut1* promoter region. The single binding site that was removed was site I, the furthest upstream. This site has been shown to be necessary for in vivo activation (Foster-Hartnett et al. 1994) and deletion of it maintains the sequence/structure of DNA between site II and the promoter. Even on supercoiled templates, removal of one or both RcNtrC binding sites completely abolishes transcriptional activation of the *nifA1Mut1* promoter by the RcNtrC proteins (Fig. 4). Basal transcription from the supercoiled templates is unaffected by deletion of the binding sites. No activation was observed with either of the binding site deletion templates when the RcNtrC concentration was increased to 875 nM (data not shown). These data show that the RcNtrC protein must bind DNA to activate transcription and suggest that there is a cooperative interaction between the bound RcNtrC dimers that is also necessary.

Previously, we have shown that phosphorylation of the RcNtrC protein leads to a fourfold increase in binding to the tandem sites located upstream from the *glnB* promoter (Cullen et al. 1996). We have also shown that unphosphorylated RcNtrC still binds upstream from the *nifA1* promoter when one of the two tandem sites has been removed, although approximately fourfold more RcNtrC is required (compared with the binding at tandem sites) (Foster-Hartnett et al. 1994). To determine whether phosphorylation increases the affinity of RcNtrC for a single site, DNase I footprinting was performed with RcNtrC$^{C3}$ in the presence and absence of MBP–NtrB by use of the *Eco*RI–*Hin*dIII fragment from pALB1, the template that contains a single RcNtrC binding site, as a probe. The concentration of unphosphorylated and phosphorylated RcNtrC$^{C3}$ that protected from DNase I digestion was found to be 160 nM and 80 nM, respectively (data not shown). These results support the idea that transcriptional activation is dependent on a cooperative interaction that occurs between RcNtrC dimers when they are bound to the two tandem sites (see Discussion).

When the *nifA1Mut1* template with intact tandem binding sites was linearized, no transcriptional activation by RcNtrC ± MBP–NtrB was detected (Fig. 4, lanes 16–20). Under these conditions, much less *nifA1Mut1* and RNAI basal transcription was observed. Cut and recircularized template also showed no transcriptional activation by RcNtrC, which is consistent with the obser-

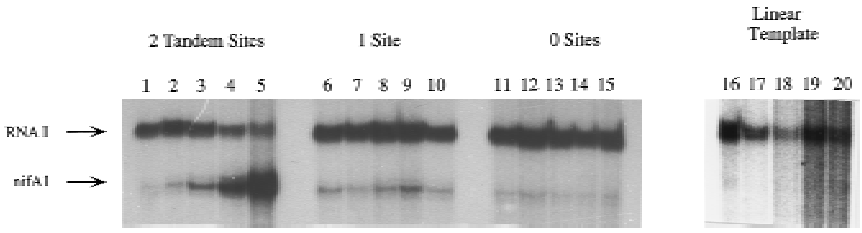


**Figure 4.** RcNtrC-activated transcription is only observed on supercoiled templates with intact tandem binding sites. In vitro transcription reactions were performed using supercoiled (lanes *1–15*) or linear templates (lanes *16–20*) that contained 0 (lanes *11–15*), 1 (lanes *6–10*), or both (lanes *1–5; 16–20*) tandem RcNtrC dimer binding sites. (Lanes *1,6,11,16*) No activators; (lanes*2,7,12,17*) 260 nM $RcNtrC^{C3}$; (lanes *3,8,13,18*) 260 nM $RcNtrC^{C3}$ and 270 nM MBP–NTRB; (lanes *4,9,14,19*) 350 nM $RcNtrC^{C7}$; (lanes *5,10,15,20*) 350 nM $RcNtrC^{C7}$ and 270 nM MBP–NtrB.

vation that an increase in nicked template results in poorer transcriptional activation by RcNtrC (data not shown).

### *ATP binding, but not hydrolysis, is necessary for RcNtrC to activate transcription*

To determine whether ATP hydrolysis is necessary for transcriptional activation by RcNtrC, in vitro transcriptional activation reactions were performed by use of the β–γ nonhydrolyzable ATP analog adenylyl imidodiphosphate (AMP–PNP). Because transcription requires hydrolysis of the α–β bond, AMP–PNP can be utilized by the RNA polymerase, but not by RcNtrC for an ATPase activity. This analog has been used previously to investigate the ATPase function for activation by enteric NtrC (e.g., Wang et al. 1995; Syed and Gralla 1997). The $RcNtrC^{C7}$ protein was used because of its high level of activation that is independent of MBP–NtrB and phosphorylation. With ATP, $RcNtrC^{C7}$ stimulated transcription at the *nifA1Mut1* promoter ~10-fold over basal levels (Fig. 5A, lanes 1,2). When AMP–PNP was substituted for ATP in the transcription reactions, no activation of the *nifA1Mut1* promoter over the basal levels was observed (Fig. 5, lanes 3,4).

The result with AMP–PNP could be caused by either a requirement for ATP hydrolysis or an inability of RcNtrC to bind AMP–PNP. In fact, transcriptional activation by the enteric NtrC (S. Kustu, pers. comm.) and the EBP, DctD (T.R. Hoover, pers. comm.), is not inhibited by AMP–PNP. This is in contrast to ADP and ATPγS that both inhibit activation by enteric NtrC (Popham et al. 1989; Weiss et al. 1991), suggesting that AMP–PNP may not bind these EBPs. Similarly, we tested whether AMP–PNP, ADP, and ATPγS inhibit the ATP-dependent activation property of RcNtrC. None of the analogs inhibited activation, suggesting that they do not bind RcNtrC, or alternatively, they bind and can function as coactivators (Fig. 5B shows the result with ADP). To resolve this, in place of ATP, we initially tested ATPγS, which can be incorporated into RNA but is considered nonhydrolyzable. Reactions with ATPγS resulted in at least a 10-fold increase in transcription in the presence of RcNtrC (Fig. 5C, lane 2) compared with basal levels (lane 1). Again, ADP did not inhibit this activation (Fig. 5C, cf. lane 4 with lane 3), even at ADP levels 30-fold higher then ATPγS. To confirm that ADP does not act as a coactivator, ADP was added with AMP–PNP and results identical to those shown in Figure 5A were observed (data not shown). Taken together, we conclude that, in addition to phosphorylation by NtrB, RcNtrC requires the specific binding of ATP for transcriptional activation.

### *The enteric RNAP and RcNtrC activation*

We have been unsuccessful in attempts to find condi-

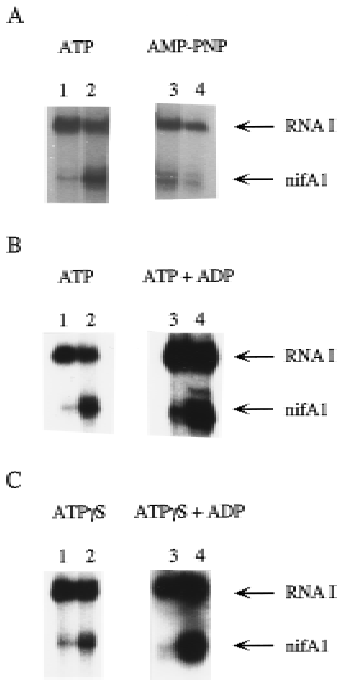


**Figure 5.** Effects of ADP and nonhydrolyzable ATP analogs on transcriptional activation by RcNtrC. For each set of reactions, lanes *1* and *3* contained no activator and lanes *2* and *4* contained 350 nM $RcNtrC^{C7}$. (*A*) In vitro transcription reactions were performed using either ATP (lanes *1,2*) or AMP–PNP (lanes *3,4*) at 1.5 mM. (*B*) In vitro transcription reactions were performed in the presence of 1.5 mM ATP (lanes *1,2*) or 0.4 mM ATP and 15 mM ADP (lanes *3,4*). (*C*) In vitro transcription reactions were performed in the presence of 1.5 mM ATPγS (lanes *1,2*) or 0.4 mM ATPγS and 15 mM ADP (lanes *3,4*).

tions in which the *E. coli* RNAP $\sigma^{70}$ is activated by RcNtrC at wild-type or *nifA1Mut1* promoters (e.g., see Fig. 6, lanes 4–6). The *E. coli* RNAP was also not activated when the purified enteric $\sigma^{54}$ subunit was added to supercoiled templates containing the wild-type or *nifA1Mut1* promoters. This later result with $\sigma^{54}$ is not unexpected because none of the RcNtrC-dependent promoters are $\sigma^{54}$-type (i.e., GG-N10-GC). The failure of RcNtrC to activate the *E. coli* $\sigma^{70}$ RNAP could be the result of a lack of interaction with RcNtrC or inability to form an open complex at this promoter (or both). To begin to address this, we used the *nifA1Mut3* promoter, which yields high basal transcription with the RcRNAP holoenzyme. Surprisingly, the *nifA1Mut3* promoter on a supercoiled template was transcribed by *E. coli* RNAP at a very low basal level, at least 20-fold less than the RNAI promoter and 50-fold less than the *R. capsulatus* housekeeping enzyme (Fig. 6, lane 7). This result suggests that the *E. coli* enzyme cannot melt the −10 region of the *nifA1* promoter as the *R. capsulatus* enzyme can. Nevertheless, a basal level of transcription with *nifA1Mut3* is observed, and this was not increased on addition of RcNtrC and MBP–NtrB relative to the RNAI transcript (Fig. 6, lanes 7–9) (see Discussion).

## Discussion

### *RcNtrC-activated promoters*

During the last 7 years, there has been considerable speculation on the promoters activated by the EBP RcNtrC, and on the holoenzyme(s) that is used (e.g., Kranz and Foster-Hartnett 1990; Morett and Segovia 1993; Foster-Hartnett et al. 1994; Kranz and Cullen 1995; Masepohl and Klipp 1996). In the present study, of the four promoters tested, the −35 hexamer of the *nifA1Mut1* promoter is the optimal RcNtrC-activated −35 region. We note that hexamers containing at least four out of six of this optimal sequence are present in each of the five natural RcNtrC-activated promoters (Fig. 7). As in *E. coli* $\sigma^{70}$-activated systems (Busby and Ebright 1994), evolution away from the optimal recognition element results in lower basal levels of expression, as demonstrated here for *nifA1*. Whereas a consensus −10 region for these promoters remains to be determined, 15- to 18-bp downstream of each of these −35 elements is a potential −10 hexamer with a second position A that is 76% conserved in *E. coli* $\sigma^{70}$ promoters (see Lisser and Margalit 1993). All five −10 regions, including the *nifA1* promoter, also contain a GC or a GG dinucleotide. Even if the −10 hexamers shown in Figure 7 have been incorrectly chosen, extended −10 regions show at least 50% GC content. Because the genomic GC content of *R. capsulatus* is 65%, the *R. capsulatus* housekeeping holoenzyme may have evolved the ability to melt high GC regions that the *E. coli* holoenzyme cannot. This ability is probably not limited to RcNtrC-activated promoters, because other natural *R. capsulatus* promoters with high GC content in the −10 region are poorly transcribed by the *E. coli* enzyme (Cullen et al. 1997). However, this capability does not reside in the *R. capsulatus* $\sigma^{70}$ regions called 2.4 or 2.5 that have been implicated in melting the −10 hexamer (see Malhotra and Severinova 1996) and extended −10 bp (Barne et al. 1997), respectively; the *R. capsulatus* sequence of $\sigma^{70}$ is identical to that of *E. coli* in these regions (Pasternak et al. 1996).

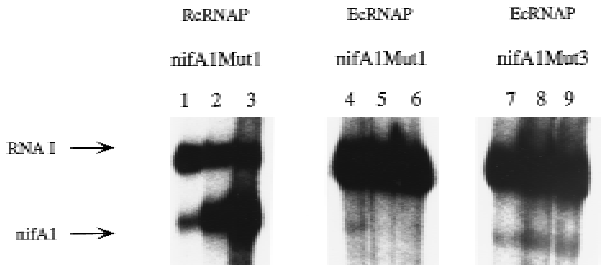


**Figure 6.** RcNtrC does not activate the *E. coli* RNAP. In vitro transcription reactions were performed with purified RNAP $\sigma^{70}$ from *R. capsulatus* and *E. coli* and the MBP–NtrB/RcNtrC$^{C7}$ transcriptional activation system. Templates: *nifA1Mut1* (lanes *1–6*) and *nifA1Mut3* (lanes *7–9*). (Lanes *1,4,7*) No activator; (lanes *2,5,8*) 350 nM RcNtrC$^{C7}$; (lanes *3,6,9*) 350 nM RcNtrC$^{C7}$ and 270 nM MBP–NtrB.

### *Requirements of upstream tandem binding sites: How does RcNtrC contact RNAP?*

We have demonstrated that two RcNtrC dimers bound to the upstream tandem sites are necessary for activation. Phosphorylated RcNtrC at 875 nM concentration was still unable to activate transcription from supercoiled templates with only one dimer binding site. This result is similar to that initially demonstrated for the *E. coli* NtrC EBP (Ninfa et al. 1987). In the *R. capsulatus* system, as low as 160 nM unphosphorylated RcNtrC (Foster-Hartnett et al. 1994) and 80 nM of phosphorylated RcNtrC was able to protect the single site from DNAse on linear DNA. Moreover, as low as 80 nM of phosphorylated RcNtrC could activate transcription of *nifA1* on the template containing tandem sites. These results suggest that oligomerization of at least two dimers is required for transcriptional activation. We also note conserved phasing of the tandem sites for all five RcNtrC-activated promoters with either 5, 6, or 15 bp separating each RcNtrC binding site (see Fig. 7). Such phasing was shown to be necessary for the EBP XylR (Perez-Martin and de Lorenzo 1996).

All five promoters contain tandem sites that bind RcNtrC located distant from the promoter. We consider three possible mechanisms by which RcNtrC might contact RNAP. (1) It is possible that the DNA between RNAP and RcNtrC binding sites loops out naturally as is the case for the *glnA* promoter and enteric NtrC (Su et al. 1990; Wedel et al. 1990). Magasanik and colleagues have reported elegant studies recently on promoters activated by the enteric NtrC, demonstrating that some contain natural curvature of the DNA that is necessary to optimize contact with RNAP (Carmona and Magasanik

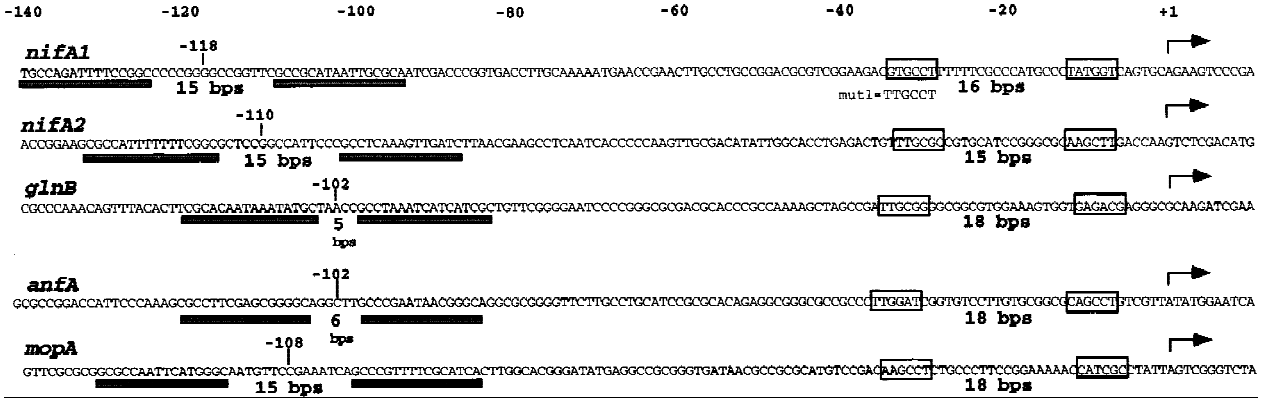


**Figure 7.** The five natural promoters activated by RcNtrC. (Arrows) In vivo mRNA start sites. Each member of the RcNtrC regulon has tandem sites containing the consensus RcNtrC binding region underlined with dark boxes (Foster-Hartnett and Kranz 1994). The *nifA1* and *nifA2* tandem sites were shown previously to bind RcNtrC in vitro (Foster-Hartnett et al. 1994) as were the *glnB* (Foster-Hartnett and Kranz 1994), *anfA*, and *mopA* sites (W.C. Bowman and R.G. Kranz, unpubl.). The RcNtrC consensus binding region has been presented previously (Foster-Hartnett and Kranz 1994). The boxed DNA on the *left* of each promoter region contains at least four of the optimal −35 hexamer (Mut1) sequence. The boxed hexamers (*right*) are putative −10 regions discussed in the text.

1996; Carmona et al. 1997). (2) Some of the RNAP/$\sigma^{54}$ systems require that IHF bind and loop the DNA between the promoter and EBP binding sites, thereby increasing the frequency of contact between EBP and RNAP (e.g., Santero et al. 1992). We have shown previously that IHF does not bind to the *nifA1* DNA (Foster-Hartnett et al. 1994) and no such potential IHF binding sites are found upstream of any of these promoters (Fig. 7). The RcNtrC and RNAP preparations used here are >95% pure, although we cannot rule out a minor contaminating factor that plays such a role. (3) It is possible that RcNtrC multimerizes on the DNA from the upstream tandem binding sites toward the RNAP. This could involve less specific binding to DNA, particularly because no RcNtrC-consensus binding sites are present in the intervening DNA between the tandem sites and promoter. We have not observed such binding on linear templates. We have engineered nine different DNA inserts of sizes from 4 to 130 bp into positions −84 and −47, relative to the transcript start site of *nifA1* (Foster-Hartnett et al. 1994). All nine inserts resulted in loss of activation in vivo by RcNtrC. This result indicates that the structure and/or sequence of the intervening DNA is important, as might be expected for mechanisms (1) or (2).

The mechanism by which RcNtrC activates open complex formation by the *R. capsulatus* holoenzyme and the protein–protein contacts that are made, remain important questions. It is intriguing that the *E. coli* enzyme is not activated at this promoter. Even with a −35 hexamer of the *nifA1* promoter (i.e., Mut3), which yields a low level of basal transcription with the *E. coli* holoenzyme, it cannot be further activated by RcNtrC. It is therefore possible that RcNtrC does not interact with the *E. coli* holoenzyme. In a similar line of experiments, the *Salmonella* NtrC$^{S160F}$ protein did not activate RcRNAP (not shown) even at concentrations in which DNA binding of NtrC$^{S160F}$ is not necessary for activation of the enteric $\sigma^{54}$ RNAP (Weiss et al. 1991). These results suggest that the RcNtrC and RcRNAP may have coevolved specific sites for interaction, although more studies will be necessary to confirm this.

### *Requirement for ATP binding but not ATP hydrolysis*

We used the in vitro activation system to determine whether ATP binding or ATP hydrolysis is required by RcNtrC. Inhibition with various ATP analogs of the ATP-dependent activation by RcNtrC was analyzed. The ability of analogs to act as coactivators was investigated, as has been carried out with the enteric EBPs (Popham et al. 1991; Weiss et al. 1991). The results indicate that AMP–PNP and ADP do not bind RcNtrC. Importantly, the nonhydrolyzable analog ATPγS supports activation by RcNtrC (Fig. 5C). Because the nonhydrolyzable AMP–PNP does not support activation when it replaces ATP in the transcription reaction, it can also be concluded that only ATP and not other nucleotide triphosphates are coactivators. We conclude that the RcNtrC protein specifically requires ATP binding for activation, but unlike the EBP activators of $\sigma^{54}$ RNAP, ATP hydrolysis is not essential. This is consistent with our observation that none of the purified RcNtrC proteins, wild-type or constitutive, exhibit a detectable ATPase activity, with or without DNA containing tandem binding sites (P.J. Cullen and R.G. Kranz, unpubl.).

Beside the EBP family of $\sigma^{54}$ RNAP activators, two other bacterial regulators contain nucleotide binding folds and activate the $\sigma^{70}$ RNAP. TyrR binds to ATP and has an ATPase activity (Cui et al. 1993). However, mutational analysis of Walker motif A indicates that ATP binding is not necessary for its activation function (Pittard and Davidson 1991; Yang et al. 1993). The TyrR protein appears to bind between −35- and −80-bp upstream of the transcription start site when it is an activator (Wilson et al. 1994; Gralla and Collado-Vides 1996). MalT binds to both maltotriose and ATP as coactivators (Richet and Raibaud 1989). MalT always binds to the promoters that it activates near −38-bp upstream

of the start site (Danot and Raibaud 1994) and this binding only occurs when the coactivators are present (Richet and Raibaud 1989). It is quite clear that the RcNtrC protein binds to its upstream tandem sites in the absence of ATP, indicating that the ATP-mediated mechanism for activation will be different from that of MalT. RcNtrC wild-type, RcNtrC with mutations in the nucleotide binding fold, and $RcNtrC^{C3}$ and $RcNtrC^{C7}$ all bind to the upstream tandem sites with similar affinities in the presence or absence of ATP (W.C. Bowman and R.G. Kranz, unpubl.). We suggest that the RcNtrC protein simultaneously senses two states of the cell. One is the nitrogen status that is mediated by the classic two-component NtrB kinase pathway (via GlnB). A second is the concentration of ATP or energy status. This may not be too surprising because it is crucial that a photosynthetic bacterium knows the levels of ATP that are available for nitrogen fixation, for example, before it induces the >36 genes necessary for this energy-intense process (Kranz and Cullen 1995).

### *Evolutionary aspects*

Two important properties that are essential for $\sigma^{54}$/EBP systems are retained by the RcNtrC system: (1) cooperative binding to tandem sites upstream to induce oligomerization and (2) nucleotide binding fold in the central domain that requires ATP for activation. It is shown that the natural holoenzyme used for activation by RcNtrC is the housekeeping RNAP and a −35 consensus region is now better defined for this unique system. Therefore, we conclude that *R. capsulatus* has three general types of activation systems: (1) the traditional $\sigma^{70}$-type in which activators bind the DNA adjacent to the RNAP (Bauer 1995; Cullen et al. 1997), (2) a bonafide $\sigma^{54}$ RNAP and EBP activators (e.g., The NifA and AnfA proteins) (Cullen et al. 1994; Kutsche et al. 1996), and (3) the RcNtrC system defined here with properties of 1 and 2. Because *R. capsulatus* is considered to be one of the most metabolically versatile microorganisms, it is possible that it has evolved these systems to add to its control repertoire (i.e., for regulatory versatility). It is worth considering that a system like the RcNtrC activator pathway evolved into the classic $\sigma^{54}$/EBP and was retained by this α proteobacterium, along with the classical system.

## Materials and methods

### *Plasmids*

pUct–nifA1, pA1M1, pA1M2, and pA1M3 have been described previously (Cullen et al. 1997). pALB1 was made by PCR of the *nifA1Mut1* promoter region in plasmid pA1M1 with the upstream oligonucleotide 5′-CCCGGTACCGGTTCGCCGCATAATTG-3′ and the downstream oligonucleotide 5′-TGACCGGCAGCAAAATG-3′. The 0.3-kb product was digested with *Kpn*I and *Hin*dIII and cloned into pUC118. pALB2 was made by PCR of the *nifA1Mut1* promoter region in plasmid pA1M1 by the upstream oligonucleotide 5′-CCCGGTACCCTTGCAAAAATGAACC-3′ and the same downstream oligonucleotide as pALB1. The 0.3-kb product was digested with *Kpn*I and *Hin*dIII and cloned into pUC118. pALB1 and pALB2 were sequenced to confirm the removal of 1 or both RcNtrC binding sites, respectively. Plasmid pRGK300, which allows overexpression of the *R. capsulatus* $\sigma^{70}$ protein, was made by PCR of the *rpoD* gene (Pasternak et al. 1996) from the *R. capsulatus* chromosome by the upstream oligonucleotide 5′-TGCGCAGCCCCGATGCAGCCCGACGAGGAG-3′ and the downstream oligonucleotide 5′-GCATCTTCAGATCTTCGGGGCCTTACTGGT-3′. These oligonucleotides contain *Nco*I and *Bgl*II sites that facilitated cloning the 2-kb PCR product downstream of the T7 promoter in pET15 (Novagen). Plasmid pRGK301, which allows overexpression of the *R. capsulatus* $\sigma^{70}$ protein that contains a hexahistidine tag, was made by PCR of the *rpoD* gene from the *R. capsulatus* chromosome by use of the upstream oligonucleo tide 5′-CGAGGAGCGCATATGGCCGCCAAGGACATC-3′ and the same downstream oligonucleotide that was used for pRGK300. The 2.0-kb PCR product was digested with *Nde*I and *Bgl*II and ligated in frame to the amino-terminal hexahistidine tag encoded by pET15.

### *RNAP purification*

Purification of RNAP from *E. coli* and *R. capsulatus* has been described previously (Cullen et al. 1997). Both heparin–agarose pure and DEAE–Sepharose pure RcRNAP holoenzyme were used in in vitro transcription reactions, as well as DEAE pure *R. capsulatus* core RNA polymerase. For in vitro transcription reactions that utilized the *E. coli* RNA polymerase EcRNAP, only the DEAE-Sepharose pure fractions were used.

### *$\sigma^{70}$ purification*

The $\sigma^{70}$ subunit from *R. capsulatus* without a hexahistadine tag was overexpressed in *E. coli* strain BL21–δDE3 containing pRGK300 by the addition of 1 mM IPTG for 3 hr at 37°C. Cells were sonicated in 20 mls of buffer (20 mM Tris-HCl, 2 mM EDTA, at pH 8) and cell debris was removed by centrifugation at 12,000*g* for 15 min at 4°C in a Sorvall centrifuge. The supernatant contains a major polypeptide that is not present in BL21–δDE3 containing no plasmid; this polypeptide migrates at the same size as the $\sigma^{70}$ from the *R. capsulatus* holoenzyme preparation (not shown). The supernatant (0.5 ml) was run on an 8% polyacrylamide gel and the $\sigma^{70}$ protein was purified from the gel and renatured by use of the method of Gross et al. (1978).

Hexahistidine-tagged $\sigma^{70}$ subunit from *R. capsulatus* was overexpressed in *E. coli* strain BL21–δDE3 containing pRGK301 by the addition of 1 mM IPTG for 3 hr at 37°C. Cells were sonicated in 20 mls of buffer (20 mM Tris-HCl, 2 mM EDTA at pH 8) and cell debris was removed by centrifugation at 12,000*g* for 15 min at 4°C in a Sorvall centrifuge. The supernatant contains a major polypeptide that is not present in BL21–δDE3 containing no plasmid; this polypeptide was similar in size by SDS-PAGE to the *R. capsulatus* $\sigma^{70}$ subunit in the holoenzyme preparation (not shown). The supernatant was loaded onto a His-Bind (Novagen) column and the column was washed with 10 volumes of binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl at pH 7.9), followed by 10 volumes of wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl at pH 7.9). The histidine-tagged $\sigma^{70}$ was eluted in 6 volumes of elution buffer (250 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl at pH 7.9). The protein was concentrated to ⅒ of the original volume in a Centricon 30, diluted to 1 ml with storage buffer (50 mM Tris-HCl, 0.5 mM EDTA at pH 8, 1 mM DTT, 50% glycerol), and stored at −80°C.

### *Purification of MBP–NtrB and $RcNtrC^{C}$ proteins*

Purification of the MBP–NtrB has been described previously (Cullen et al. 1996). RcNtrC constitutive mutant proteins

($RcNtrC^C$) were purified through the DEAE–Sepharose step by use of the method of Cullen et al. (1996). The isolation of genes encoding *R. capsulatus* $NtrC^C$ alleles will be described elsewhere.

*In vitro transcription*

In vitro transcription reactions were performed in transcription buffer [50 mM Tris-HCl at pH 8, 100 mM potassium acetate, 10 mM magnesium acetate, 1 mM ATP, 10 mM DTT and 0.5 µl RNAsin (Promega)] by the method of Cullen et al. (1997). The concentration of linear or supercoiled templates was ~40 nM for all reactions reported here. For in vitro transcriptional activation reactions, MBP–NtrB (270 nM) was incubated in transcription buffer for 10 min at 37°C prior to the addition of $RcNtrC^C$ (80–875 nM as noted) and RNAP (40 nM). $RcNtrC^C$ and RNAP were added simultaneously and the reactions were incubated at 24°C for 30 min. Purified $\sigma^{70}$ proteins were added to the core RNAP prior to the start of the reactions.

*DNase I footprinting*

DNase I footprinting analysis of phosphorylated and unphosphorylated $RcNtrC^C$ at the *nifA1* promoter region was performed by the method of Cullen et al. (1996). Probes were prepared by digesting plasmids (pA1M1 of pALB1) with *Eco*RI and dephosphorylating the ends with calf intestinal phosphatase. The 5′ ends were labeled with [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase, then the labeled DNA was digested with *Hin*dIII. After separation on a 4% native acrylamide gel (50 mM Tris–borate EDTA), gel sliced that contained the appropriate fragments were excised and the probes were eluted overnight at 37°C in Tris–EDTA buffer. Approximately 0.2 nM of probe (30,000–60,000 cpm) was used in each footprinting reaction.

*Other methods*

Western analysis was performed with peroxidase detection reagents from Pierce. Use of monoclonal antibody 2G10, a gift from Nancy Thompsen and Richard Burgess (University of Wisconsin, Madison) and monoclonal antibodies to β-galactosidase have been described previously (Cullen et al. 1997). Transcripts were quantitated by scanning the autoradiograms with an HP scanjet 4C and analyzing the bands by use of IP Labgel software from Data Analysis Corp (Malek et al. 1997). The scanning and software could easily distinguish differences in transcript levels twofold or above. Protein sequencing was carried out with an Applied Biosystems 470A protein sequencer.

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