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Mutations in the RNA polymerase recognition sequence of the *Klebsiella pneumoniae nifH* promoter permitting transcriptional activation in the absence of NifA binding to upstream activator sequences

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ABSTRACT

Positive control of the wild-type Klebsiella pneumoniae nifH promoter by the NifA protein requires that NifA is bound at the upstream activator sequence (UAS). By introducing base substitutions at -15 to -17 in the RNA polymerase recognition sequence of the nifH promoter, positive control by a form of NifA unable to bind to the UAS was greatly increased when compared to the wild-type promoter. Transcriptional activation still required the rpoN encoded sigma factor and was initiated at the same nucleotide as in the wild-type promoter. Mutations at -15 to -17 suppressed the requirement that the UAS should be located on the correct face of the DNA helix with respect to the RNA polymerase recognition sequence in order that titration of NifA and efficient activation occur. This result supports the suggestion that upstream bound NifA interacts with the RNA polymerase-RpoN complex. To examine the minimal carboxy terminal sequences required for the positive control function of NifA a series of carboxy terminal deletions were constructed. Efficient positive control at a UAS-independent promoter was only observed in deletions which did not extend beyond the proposed boundary separating the carboxy terminal NifA DNA-binding domain from its central domain.

INTRODUCTION

The nitrogen fixation (nif) promoters of Klebsiella pneumoniae are amongst a class of prokaryotic promoters which require the rpoN encoded sigma factor (also called ntrA, glnF) for their recognition (1,2). The nucleotide sequence CTGG-N_8-TTGCA located 11bp from the transcription start characterises rpoN-dependent promoters, with the dinucleotides GG at -24 and GC at -12 representing the most invariant features of the promoter. Closed promoter complexes between glnAp2 and RNA polymerase-RpoN have directly demonstrated recognition of the -12,-24 sequences (3,4) and mutational analysis of the nifH and nifL promoters has demonstrated the requirement of this sequence for promoter activity (5,6). All known rpoN-dependent promoters require an additional positive control protein in order

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that transcription is activated. In the case of the K. pneumoniae nif promoters, other than nifLA, this is the NifA protein (1,2). The nifLA operon directs the synthesis of NifA and like glnAp2, is positively controlled by the phosphorylated form of the NtrC protein (7). NtrC-P accumulates under conditions of nitrogen limitation (8), thus regulating nifLA expression in response to nitrogen status. Autogenous activation of the nifLA promoter by NifA can also occur (9,10).

The NifA and NtrC proteins are DNA-binding proteins and recognise sequences usually located >100bp upstream of the transcription starts of promoters which they activate (11-15). Their consensus binding sites are distinct and are TGT-N_{10}-ACA and TGCAC-N_5-TGGTGCA respectively. The DNA binding activities of NifA and NtrC lie in their carboxy terminal regions and the DNA binding function of NifA can be separated from its positive control function (16,17). Current evidence suggests that upstream bound NifA closely approaches the downstream promoter complex via the formation of a DNA loop between the UAS and -12,-24 promoter element (18). Activation of the nifLA promoter by NtrC-P co-operatively bound to two adjacent upstream sites also involves a DNA looping mechanism (10).

The K. pneumoniae nif promoters show a strict requirement for a UAS, and therefore for NifA bound upstream, in order to be efficiently activated (11,17). We have suggested that the role of the UAS is to increase the local concentration of NifA at the promoter and to orientate NifA appropriately by topologically constraining it at the UAS (18). As anticipated a mutant form of NifA deleted of its DNA-binding domain, and therefore unable to bind the UAS, could not activate the K. pneumoniae nifH, B or U promoters efficiently (17). However activation of the nifF and nifL promoters by this mutant NifA protein, albeit weak, was observed as was activation of the glnAp2 promoter (17). In this context, activation of the R. meliloti nifH promoter by the K. pneumoniae NifA protein is reported to occur largely independently of the UAS, implying that activation at this nif promoter does not absolutely require DNA-bound NifA, at least under the conditions tested (19).

We considered that a common feature of the K. pneumoniae nifF, nifL, glnAp2 and R. meliloti nifH promoters might facilitate activation by NifA not bound to the UAS. Comparison of the sequences of these promoters in the -12 region suggested that the

nucleotide sequence 5′-TTTTGCA from -17 to -11 might be critical in determining the response of an rpoN-dependent promoter to unbound activator. Substitutions in the K. pneumoniae nifH promoter increasing its homology to this sequence have been made and were found to improve activation by NifA deleted of its DNA-binding domain and to suppress the requirement that upstream bound NifA is on the correct face of the DNA helix with respect to the RNA polymerase recognition sequence. We suggest that the sequence from -17 to -15 is involved in modulating the recognition and binding of RNA polymerase-RpoN to -12,-24 promoter elements. A role for NifA in stabilising the binding of RNA polymerase-RpoN to -12,-24 sequences is proposed.

MATERIALS AND METHODS

Plasmids

These are listed in Table 1. Plasmids pMB2101 and pMB12461, R. meliloti nifH-lacZ′ translational fusions, were constructed by isolating HindIII-BamHI promoter fragments from pMB210 and pMB1246 (19), filling in the HindIII site and ligating these fragments into the vector pMC1403 (20) previously restricted with Sma1 and BamHI. Plasmid pMB8.3$^+$ was derived from pEMBL8$^+$ by cloning the linker 5′-TGATTGATTGA into the HincII and HindIII sites of pEMBL8$^+$ to generate translation stops in all three reading frames. Carboxy-terminal deletions of K. pneumoniae NifA were constructed by linearising pMB162 DNA with HindIII and treating this with Bal31 nuclease to remove the carboxy-terminal coding region of nifA. Following restriction of Bal31 digested DNA with EcoRI, shortened nifA fragments were isolated and ligated into pMB8.3$^+$ previously restricted with EcoRI and Sma1. The precise deletion endpoints in nifA were determined by sequencing single stranded template DNA. A control nifA plasmid (pMB164) was constructed by cloning in the EcoRI-NruI nifA fragment from pMB162 (spanning amino acids 1-457 of nifA) into EcoRI-SmaI restricted pMB8.3$^+$.

Mutagenesis

Single and multiple base changes in the K. pneumoniae nifH promoter were introduced by oligonucleotide mutagenesis as described previously (17).

Promoter activity assays

Transcriptional activation of nif promoters was assayed as described previously (5,17). Inhibition of chromosomal nif

Table 1.

Plasmid	Description	Reference
pMB1	K. pneumoniae nifH-lacZ fusion with wild-type promoter CCCTGCA, high copy, Cb^R	5
pWVC88049	as pMB1 but TTTTGCA from -17 to -11	This paper
pWVC88050	as pMB1 but TCCTGCA " " " "	"
pWVC88053	as pMB1 but CTCTGCA " " " "	"
pWVC88054	as pMB1 but CCTTGCA " " " "	"
pMB88616	as pMB1 but 5bp insert at -76	18
pWVC880492	as pWVC8049 but 5bp insert at -76	"
pMB880494	as pWVC88049 but deleted for UAS	"
pJMW6	low copy K. pneumoniae nifH-lacZ fusion, Cb^R	"
pWVC8617	as pJMW6 but 5bp insert at -76	"
pWVC880491	low copy derivative of pWVC88049	This paper
pWVC880493	low copy derivative of pWVC880492	"
pMB880495	low copy derivative of pMB880494	"
pMB210	R. meliloti nifH-lacZ fusion, Tc^R	19
pMB1246	as pMB210 but deleted for UAS, Tc^R	"
pMB2101	as pMB210 but based on pMC1403, Cb^R	This paper
pMB12461	as pMB12461 but based on pMC1403, Cb^R	"
pMB8.3⁺	Translation termination cassette cloned into pEMBL8⁺, Cb^R	"
pMJ220	nifA expressed from lac promoter, Cm^R, pACYC184 based plasmid	17
pMB162	carboxy terminal deletion of nifA expressed from lac promoter aa's 1-458, Cb^R, pEMBL8⁺ based plasmid	This paper
pMB163	carboxy terminal deletion of nifA expressed from lac promoter aa's 1-458, Cm^R, pACYC184 based plasmid	17
pMB164	carboxy terminal deletion of nifA expressed from lac promoter aa's 1-457, Cb^R, pEMBL8⁺ based plasmid	This paper
pMC71A	nifA expressed from tet promoter, Cm^R	31

expression by multiple copies of nif promoters (multicopy inhibition) was measured by assaying for nitrogenase activity (C_2H_2 reduction) as before (5).

RNA isolation and transcript mapping

Bacteria (5-10 ml) were grown to an A_{600} of 0.5-0.7, harvested by centrifugation and lysed in the presence of hot phenol (60°C) as described (21). RNA was re-extracted twice with hot phenol, precipitated and dissolved in H_2O. Transcription starts were determined by primer extension analysis using the 5'-^{32}P labelled oligonucleotide 5'-TTACCGTAAATAGCGCATT (1,500 Ci/mMole) complementary to nucleotides +43 to +62 of the K. pneumoniae nifH promoter. Extensions were carried out in 12 μl of 50 mM Tris-HCl, pH 8.0, 6 mM

$MgCl_2$, 40 mM KCl, 0.5 mM deoxynucleotide triphosphates with 3 units of reverse transcriptase for 30 minutes at 37°C. Reactions were terminated by the addition of formamide dye mix and products analysed by gel electrophoresis.

RESULTS

Transcriptional activation by NifA deleted of its DNA-binding domain

a. Activation of the R. meliloti nifH promoter. Initially the supposition that activation of the *R. meliloti nifH* promoter would occur with a form of NifA unable to bind the UAS of this promoter was examined. Results shown in Table 2 clearly demonstrate that activation of the *R. meliloti nifH* promoter lacking a UAS is roughly equivalent whether NifA is present in its wild-type form (expressed from pJM220) or in its truncated form lacking amino acids after Asp458 (expressed from pMB162, Figure 2). The presence of the UAS results in a *ca*. 3-fold increase in activation of the *R. meliloti nifH* promoter by the wild-type NifA, but little increase by the form of NifA which is unable to bind the UAS. A similar 3-fold increase in activation due to the presence of the UAS was also observed when activation by the chromosomally encoded *nifA* was examined (Table 2).

b. Activation of mutant K. pneumoniae nifH promoters bearing transitions from -17 to -15. The proposal that activation by unbound NifA could be facilitated by the sequence 5'-TTT between -17 to -15 was examined by introducing via oligonucleotide directed mutagenesis single transitions at -17 (pWVC88050), -16 (pWVC88053) and -15 (pWVC88054) and transitions in all three positions (pWVC88049) into the *K. pneumoniae nifH* promoter (Table 1). Activation of these promoters by NifA with and without its DNA-binding domain was examined (Table 3). In all cases single transitions at -17, -16 and -15 improved activation by the truncated NifA, with the transition at -15 bettering that at -17 and the transition at -16 resulting in the least increase in activation. The triply mutated promoter was activated the most efficiently by the truncated NifA. This promoter did however show a somewhat reduced activation by the wild-type NifA as did the promoter bearing the transition at -15.

c. Transcription starts of mutant K. pneumoniae nifH promoters. Activation of the four mutant *nifH* promoters by the truncated NifA

Table 2. Activation of the R. meliloti nifH promoter

Promoter plasmid	Activator in trans	ß-Gal activity
pMB210 (+UAS)	pMJ220, wt NifA	44,000[1]
pMB1246 (-UAS)	pMJ220, wt NifA	11,500[1]
pMB210 (+UAS)	pMB162, COOH deleted NifA	15,000[1]
pMB1246 (-UAS)	pMB162, COOH deleted NifA	11,100[1]
pMB2101 (+UAS)	none	120[1]
pMB12461 (-UAS)	none	130[1]
pMB2101 (+UAS)	NifA, NtrC (UNF932, $-NH_4^+$)	46,000[2]
pMB12461 (-UAS)	NifA, NtrC (UNF932, $-NH_4^+$)	13,000[2]
pMB2101 (+UAS)	repressed ($+NH_4^+$) UNF932	160[2]
pMB12461 (-UAS)	repressed ($+NH_4^+$) UNF932	300[2]

Activities were measured in anaerobically-grown (1), E. coli ET8894, a glnA ntrBC deletion strain grown with 200 μg/ml^{-1} glutamine or (2), K. pneumoniae UNF932, a nif^+ntr^+ strain grown under derepressing ($-NH_4$) or repressing conditions ($+NH_4$) as described previously (5,17).

was shown to be dependent upon RpoN (Table 3). Mapping of the transcription start points of nifH mRNA resulting from activation by either the wild-type NifA or the truncated NifA (figure 1) confirmed the same start point was used in each case (22). Activation of the wild-type promoter by the truncated NifA was too weak to detect a transcript (1B, lane 2). A transcript was only just detectable with the mutant bearing the transition at -16 (1B, lane 5). The band of the sequencing ladder just below to the transcription start points (open arrow in figure 1) corresponds to an extension product of 61bp. Therefore the major transcript corresponds to an extension product of 62bp in the primer extension assay, consistent with the previously reported rpoN-dependent transcription start indicated in the legend to figure 2 (22). A second start 1bp downstream of the major rpoN-dependent start was also evident particularly when levels of transcription were high. In figure 1A a minor transcript starting 29bp further upstream than the major was detected and is indicated with the closed arrow. This transcript was not detected when the levels of transcription from the rpoN-dependent promoter were lower (Fig. 1B, lanes 2-6) or under derepressing conditions in K. pneumoniae UNF932 (data not shown also ref. 22). It is possible that the transcript from the rpoN-dependent promoter stabilises the

Table 3. Activation of mutant *K. pneumoniae nifH* promoters by NifA deleted of its DNA-binding domain

Promoter sequence, from -17 to -11 (plasmid)	Activation (β-gal U) wt NifA(pMJ220)	COOH deleted NifA(pMB163)	none
CCCTGCA (pMB1,wt)	75,000	90 (15)	20
TTTTGCA (pWVC88049)	34,000	9,000 (11)	50
TCCTGCA (pWVC88050)	79,000	1,200 (20)	20
CTCTGCA (pWVC88053)	59,000	400 (7)	20
CCTTGCA (pWVC88054)	18,000	2,300 (10)	20

Assays were conducted in *E. coli* ET8894 as described previously (17). Control experiments in *E. coli* ET8045, an *rpoN::Tn10* background, with pMB163 demonstrated activation required the *rpoN* gene product (data in parenthesis).

minor upstream transcript, thus permitting its detection. There is no sequence upstream of the minor transcript to suggest that the promoter responsible for its synthesis is *rpoN*-dependent. Rather, a potential *rpoD*-dependent promoter has previously been identified in this region although the transcript we have detected appears *ca.* 10bp shorter than predicted (23). Nonetheless a plausible -10 region exists before the transcription start identified in figure 1a, although this weak putative *rpoD* dependent promoter does not appear to be as active in *K. pneumoniae* as in *E. coli*.

Suppression of the face-of-the-helix-dependency for NifA activation

We have previously shown that activation of the *nifH* promoter by NifA requires that NifA is bound to a UAS located on the correct face of the DNA helix with respect to the downstream RNA polymerase recognition sequence (18). The effect of the base substitutions in the -15 to -17 region of the *nifH* promoter is to suppress this requirement (Table 4). The level of activation of the *nifH* promoter (pWVC 880493) bearing the three transitions from -15 to -17 but with the UAS located on the opposite face of the helix with respect to the wild-type promoter approached that of the wild-type promoter (pJMW6). Appropriate controls demonstrated that the majority of the

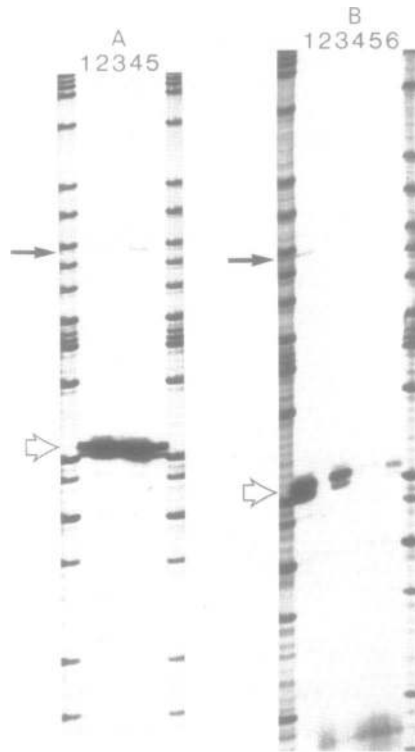

Figure 1 legend. Transcription start points of the K. pneumoniae nifH promoter analysed by primer extension. A: activation by the wild-type NifA (pMJ220), with lane 1) wild-type promoter (pMB1), lanes 2)-5) mutant promoters present on pWVC88049, 88050, 88053, 88054 respectively. B: activation by the carboxy terminal deleted NifA (pMB163). Lane 1 is a control with RNA isolated from activation of pWVC88049 by wild-type NifA (i.e. as A lane 2), lanes 2)-6) are the wild-type nifH promoter (pMB1) and mutant promoters present on pWVC88049, 88050, 88053 and 88054 respectively. RNA was isolated in all cases from E. coli ET8894 with the appropriate plasmids. The sequencing tracks run as size markers were obtained as dideoxy adenosine termination products of reactions primed on single stranded M13mp8 DNA, carrying the EcoRI-BamHI nifH promoter fragment from pMB1 using the 17bp universal sequencing primer. The open arrow indicates the major rpoN-dependent transcription start, the closed arrow a minor transcription start. Neither transcript was detected in the absence of RpoN or NifA.

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          -24           -12            **
CGCACGGCTGGTATGTTCCCTGCACTTCTCTGCTGG
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The stars correspond to the rpoN-dependent starts indicated by the open arrow in figure 1. The sequence shown is from the wild-type nifH promoter.

Table 4. Activation of K. pneumoniae nifH promoter mutants with the UAS on the incorrect face of the DNA helix.

Activation(β-galU)[1]		Relevant promoter features	Acetylene reduction	
33,000	(pJMW6)	UAS -17 CCC -15	0.2%	(pMB1)
1,200	(pWVC8617)	CCC	60%	(pMB8616)
39,000	(pWVC880491)	TTT	3%	(pWVC88049)
24,000	(pWVC880493)	TTT	1%	(pWVC880492)
3,100	(pMB880495)	TTT	100%	(pMB880494)

1. Transcriptional activation of nifH promoters was measured in E. coli ET8894 with pMC71A providing nifA in trans. Low copy nifH-lacZ fusions were assayed (see Table 1). In the absence of pMC71A 60-150U of activity were obtained. 2) Multicopy inhibition (C_2H_2 reduction) was measured in K. pneumoniae UNF932 derepressed as described previously (17). Plasmids used were high copy nifH-lacZ fusions described in Table 1. Whole cell acetylene reduction activities are expressed as a percentage of that obtained with the vector pMC1403. Relevant promoter features are shown indicating the wild-type (CCCTGCA) or mutant (TTTTGCA) downstream promoter sequences from -17 to -11 and the relative face of the DNA helix upon which the UAS is located. The solid triangle indicates the 5bp insert placing the UAS on the opposite face of the DNA helix (18). The UAS of the wild-type nifH promoter is located at position -136.

level of activation measured from pWVC880493 was UAS dependent, that is required upstream-bound NifA (compare pMB880495 to pWVC880493).

The introduction of 5bp between the UAS and downstream sequences also diminished the ability of multiple copies of the nifH promoter to inhibit chromosomal nif gene expression (18). When a mutant nifH promoter bearing the three transitions from -15 to -17 but with the UAS on the incorrect face of the helix (pWVC880492) was assayed for its ability to inhibit chromosomal nif expression this was found to be unaltered with respect to the wild-type nifH promoter (Table 4). Appropriate control plasmids demonstrated that this property was UAS-dependent (compare pMB880494 to pWVC880492). Although the R. meliloti nifH promoter is reported to cause multicopy inhibition (22) we could not detect this (data not shown). It is possible that previous measurements of inhibition reflected product inhibition associated with the synthesis of incomplete nif products from the

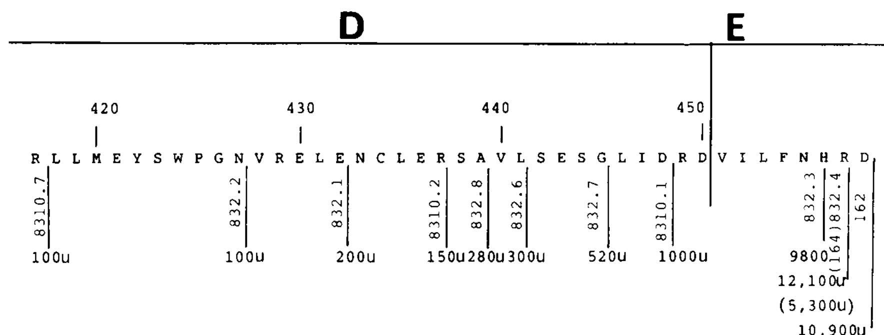

Figure 2.
Activation of the R. meliloti nifH promoter by carboxy-terminal deletion derivatives of NifA. Deletions of NifA were generated as described in materials and methods and assayed for their ability to activate the R. meliloti nifH promoter (pMB210) in E. coli ET8894. The relevant nifA deletion pMB plasmid number is indicated on the vertical bar which is aligned with the last amino acid encoded by nifA in that plasmid. Activation data (β-gal U) are presented below each deletion end point. In the absence of NifA 80-120U of activity was obtained from pMB210. Plasmid pMB832.4 gave 12,100U of activity, and plasmid pMB164 with the same deletion end point gave 5,300U (data in parenthesis). This is discussed in Results. The numbering of NifA amino acids is given above the sequence, and the boundary between the central domain (D) and DNA-binding domain (E) of NifA is indicated (24).

large R. meliloti nif gene fragment used in the experiments rather than activator titration.

Delineation of the boundary between the carboxy terminal and central domains of NifA

The ability of NifA to activate transcription of the R. meliloti nifH promoter in the absence of an interaction with the UAS (Table 2) was exploited to determine which carboxy terminal sequences were required in order that the positive control function of NifA was retained. Starting with the plasmid pMB162 (which retains amino acids 1-458 of NifA and positively activates glnAp2 and the R. meliloti nifH promoter) a series of carboxy terminal NifA deletions was constructed and assayed for the ability to activate the R. meliloti nifH promoter (Figure 2). The truncated NifA's expressed from pMB162 and all derivatives of this plasmid lack the DNA-binding domain of NifA which recognise the UAS. Activation of the R. meliloti nifH promoter diminished substantially after the deletion of amino acids 449 to 456. This region bounds the DNA-binding and

Table 5. Activation of nifH promoters by NtrC

Plasmid	Promoter sequence (origin) from -17 to -11	Activation (β-galU) $+NH_4$	$-NH_4$
pMB1	CCCTGCA (wt. Kp nifH)	27	1,900
pWVC88049	TTTTGCA (mutant Kp nifH)	700	15,000
pWVC88050	TCCTGCA (" " ")	80	2,000
pWVC88053	CTCTGCA (" " ")	15	1,000
pWVC88054	CCTTGCA (" " ")	200	4,300
pMB2101	TTTTGCA (wt. Rm nifH)	200	4,500

Activation by NtrC in UNF926, a K. pneumoniae his-nif deletion strain, was assayed as before (5).

central domains of NifA in the model of Drummond et al. (24). Deletion of residues 433 to 448 resulted in the loss of remaining NifA positive control activity and represents a deletion interval entering the central domain in the NifA model of Drummond et al. (24). Plasmids pMB832.4 and pMB164 are identical except for the sequence of amino acids beyond the 457th codon of NifA. In pMB164 the sequence is GSV, in pMB832.4 the sequence is RDPSD. It is possible this difference in carboxy terminal sequence accounts for the higher activity of pMB832.4 compared to pMB164.

Positive control of the K. pneumoniae nifH promoter by NtrC

The wild-type nifH promoter is weakly activated by NtrC in a UAS-independent manner (11). The replacement of the nifH UAS with the high affinity NtrC binding site from the K. pneumoniae glnAp2 promoter greatly improved nifH activation by NtrC (25). Substitutions at positions -17 to -15 in the nifH promoter separately and in combination also improved activation by NtrC (Table 5). As with the truncated form of NifA lacking its DNA-binding domain (Table 3) the greatest activation by NtrC occurred with the triply substituted mutant nifH promoter (pWVC88049). Activation of the R. meliloti nifH promoter by NtrC was also significant (Table 5). We considered the possibility that other positive control factors which function in concert with rpoN-dependent promoters might also activate the mutant nifH promoters and R. meliloti nifH promoter in the absence of specific binding sites on these promoters. However no activation of these promoters by the positive control elements normally regulating the rpoN-dependent fdhF promoter was detected, although control experiments

demonstrated activation of the fdhF promoter on plasmid pBN2 by these factors (26) data not shown.

DISCUSSION

By introducing base substitutions into the downstream promoter sequences of the *K. pneumoniae* *nifH* promoter transcriptional activation by a truncated form of NifA unable to bind to the upstream activator sequence of this promoter was greatly improved. The region of the *nifH* promoter mutated in these experiments corresponds to the binding site for RNA polymerase complexed with RpoN in the *glnAp2* promoter. Although the binding of RNA polymerase-RpoN to the *K. pneumoniae* *nifH* promoter was not detected *in vivo* (12), the promoter-down phenotypes of mutations in the -12 to -24 sequences of this promoter are consistent with an interaction of polymerase with the downstream promoter element. Examination of closed and open *K. pneumoniae* *nifH* promoter complexes *in vivo* has revealed that the base substitutions described in this paper, which facilitate activation by unbound NifA, permit the detection of the closed *rpoN*-dependent *nifH* promoter complex in the absence of NifA (E. Morett, M. Buck, manuscript in preparation), an interaction which is apparently too weak to readily detect with the wild-type *K. pneumoniae* *nifH* promoter (12). The formation of the closed complex in the absence of NifA may explain the phenotypes of the mutations from -17 to -15. We have argued that the role of the UAS is to bind and topologically constrain NifA within the vicinity of the downstream-bound RNA polymerase-RpoN complex (18). By constraining the RNA polymerase-RpoN complex [rather than NifA at the UAS] through the formation of a stronger closed promoter complex, it is possible that activation of the *nifH* promoter by unbound NifA is favoured. It also seems likely that an interaction between RNA polymerase-RpoN and NifA is required to activate transcription. Evidence in support of an interaction between NifA and the downstream promoter complex comes from the observation that mutations which increase the binding of RNA polymerase-RpoN to the *nifH* downstream promoter element improve the ability of a promoter bearing a UAS on the incorrect face of the DNA helix to titrate NifA and to be activated by NifA. We suggest that the increased occupancy of the downstream promoter element by RNA polymerase-RpoN improves the probability that activator bound to the incorrect face of the DNA helix will interact with this complex, thus allowing the

activation of transcription to occur. To account for its titration, the binding of NifA at the UAS may be stabilised by this interaction or NifA may in some other way be made unavailable by virtue of an interaction with the downstream promoter complex.

Using activation of the R. meliloti nifH promoter by NifA lacking a DNA-binding domain as a measure of the positive control function of NifA we were able to delineate the minimal carboxy terminal NifA sequences required for activation. A significant loss of activator function occurred when the region which bounds the DNA-binding domain of NifA (24) was deleted. This region may be a linker region since the conserved DNA-binding and central domains of NifAs from a number of diazotrophs are joined through rather variable sequences (24,27). The loss of positive control function resulting from deletion of this non-conserved region of NifA suggests it influences the activator function of NifA in some way. The corresponding linker region of the Bradyrhizobium japonicum NifA (27) also appears necessary for NifA activity (although effects on positive control and DNA-binding were not separated). Therefore NifA may have at the carboxy terminus of its positive control domain a structure of variable primary sequence which influences activity. Deletions which extended beyond the proposed linker region completely eliminated positive control function, thus confirming the proposed boundary of the DNA-binding and central domain of NifA assigned on the basis of sequence alone (24).

The mutations from -15 to -17 in the K. pneumoniae nifH promoter improved activation by NtrC in addition to permitting activation by unbound NifA. Previous work had demonstrated that point mutations in the K. pneumoniae nifH promoter isolated on the basis of improved activation by NtrC included transitions at -17 and -15, although other base changes were also present (28). It seems likely that activation by NtrC (and unbound NifA as argued above) is facilitated by the existence of a closed promoter complex favoured by the base changes introduced, rather than by direct recognition of the altered nifH downstream promoter sequences by NtrC (binding of NtrC to the E. coli glnAp2 promoter, which shares the sequence 5'-TTTCGCT with the mutant nifH promoters, has not been observed in vivo or in vitro (31,3)). The wild-type nifH downstream promoter sequence may restrict the formation of a closed complex in the absence of the activator to ensure that activation requires that the activator

protein is bound upstream and therefore that the appropriate positive control element (NifA which binds the UAS) activates transcription. In K. pneumoniae this presumably introduces fidelity into the nif regulation process by preventing transcription of nifH under nitrogen-limiting conditions (under which NtrC accumulates) but allowing it to occur under microanaerobic nitrogen-limiting conditions (when NifA accumulates and nitrogenase is active). It is also possible that the nifL promoter may form a weak closed complex (S. Minchin, R. Dixon, manuscript in preparation) in order to ensure that it is only activated by NTRC when this activator accumulates to high levels and is phosphorylated (thus occupying the low affinity NTRC binding sites in the nifLA promoter (10,16)) i.e. under conditions of strict nitrogen limitation. In general, the levels and/or activities of positive control proteins present in the cell may also be important for ensuring that only promoters bearing specific binding sites for the appropriate activator proteins are activated. For example activation of mutant nifH promoters by the positive control protein(s) required for expression from the fdhF promoter was not observed. Similarly the xylR gene product did not activate glnAp2 (29) whereas NifA lacking its DNA binding domain does activate glnAp2 (17). The xylR gene product and fdh control factor(s) may be present at lower concentrations and/or activities compared to NtrC and the form of NifA lacking its DNA-binding domain to account for their failure to activate the mutant nifH promoters or glnAp2 in the experiments described above.

In conclusion, the nucleotides in the -17 to -15 region of rpoN-dependent promoters appear important in modulating the response of the promoter to its activator. In general the presence of the sequence 5′-TTT in this position may (1) make the promoter insensitive to the face of the DNA helix upon which the activator protein binds, at least when the occupancy of the upstream activator binding site is high, and (2) under conditions of high activator concentration permit activation by the unbound form of the activator. This latter point appears true for glnAp2 (3,17) and the R. meliloti nifH promoter (19). The formation of a closed promoter complex in the absence of DNA-bound activator may be a critical factor in determining the behaviour of the promoter (E. Morett, M. Buck, manuscript in preparation), although a role for bases in the -17 to -15 region in the transcriptional activation event, possibly the open complex formation rate, cannot be discounted. In promoters

where the closed promoter complex is apparently weak and therefore difficult to detect, such as the K. pneumoniae nifH promoter (12), it is possible to envisage that the upstream bound activator could stabilise the closed complex prior to open complex formation. In support of this suggestion we have shown that the binding of NifA to the nifH UAS does not absolutely require rpoN or downstream promoter sequences (12), and therefore could occur before an interaction of the RNA polymerase-RpoN complex with the downstream promoter sequences.

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