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Mutational analysis of upstream sequences required for transcriptional activation of the *Klebsiella pneumoniae nifH* promoter

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ABSTRACT

Upstream sequences of the Klebsiella pneumoniae nifH promoter were mutagenised and activation of the mutated promoters by the nif-specific transcriptional activator protein NifA examined in vivo. Of the sixteen mutations analysed, only those within the nifH upstream activator sequence (UAS), characterised by a TGT-N_{10}-ACA motif, influenced nifH promoter activity. Mutations altering the two-fold rotational symmetry of the UAS or the spacing between the TGT and ACA motifs reduced promoter activity, consistent with the UAS functioning as a NifA binding site. The bases flanking the TGT-ACA motif of the UAS also appear to influence activation by NifA. Substituting the nifH UAS with a binding site for the transcriptional activator NtrC resulted in improved NtrC-dependent activation of the nifH promoter demonstrating that the activator specificity of the nifH promoter is dependent upon the presence of the appropriate upstream sequences to which the activator binds.

INTRODUCTION

Enteric bacteria respond to nitrogen deficiency by activating transcripton of genes whose products are involved in the assimilation of various forms of combined or free nitrogen (reviewed in 1 and 2). Examples of nitrogen regulated genes include the nitrogen fixation (nif) genes and glnA, which encodes glutamine synthetase (3). Transcriptional activation of these (and other) genes is dependent upon the ntrA gene product (NtrA) functioning as an alternative sigma factor when complexed with core RNA polymerase (4-7). It appears that NtrA-RNA polymerase recognises sequences around -12 and -24bp from the transcription start which characterise NtrA-dependent promoters such as the nif promoters (3). In addition to NtrA, transcription is dependent upon a positive activator protein, either the general nitrogen regulatory protein NtrC or the nif-specific activator protein NifA. These two proteins are functionally and structurally

homologous and can substitute for one another with various degrees of efficiency (2,3). NtrC is most active when phosphorylated (8); whether NifA activity is similarly controlled is not yet known.

Activation of transcription by NtrC and NifA occurs at a distance. Specific sequences required for activation reside ≈110-270 bp upstream of the transcription start, the exact location depending upon the promoter examined (9,10). Recently, evidence was obtained to suggest that activation of the Klebsiella pneumoniae nifH promoter by NifA involves DNA loop formation between upstream and downstream promoter sequences (11). In the loop model it is envisaged that NifA is bound to the upstream activator sequence (UAS) found in the nifH and other NifA-activated nif promoters (ref. 9 and Figures 1 and 2). Support for this comes from the observation that (i) like many sites on DNA where proteins bind, the UAS is a sequence with dyad symmetry (9), and (ii) NifA is predicted to have a helix-turn-helix super secondary structure, a structural motif characteristic of many DNA-binding proteins which recognise and bind to sequences with two-fold rotational symmetry (12). Thus it is this structure in NifA which is predicted to interact with the UAS. In this paper we report results which identify the nifH UAS as the major upstream sequence required for NifA-dependent activation of the nifH promoter, and demonstrate experimentally that its two-fold symmetry is critical for activity, supporting the suggestion that NifA binds to the UAS.

MATERIALS AND METHODS

Plasmids. These are listed in Table 1.

Mutagenesis. Oligonucleotide directed mutagenesis of the K. pneumoniae nifH promoter (Figure 1) was conducted on the M13-nifHp clone as described before (11). Bisulphite mutagenesis was carried out essentially as described previously (13). Single stranded DNA (ssDNA) for chemical mutagenesis was obtained from the pEMBL8$^+$ based plasmid pSMM82 which carries nucleotides -80 through to -156 of the nifH promoter (9). Following bisulphite treatment of ssDNA, the complementary DNA strand was synthesised in vitro with the Klenow fragment of DNA polymerase. The resulting double stranded nifH sequence was excised as a PstI - BamHI restriction fragment for cloning into M13mp9 and subsequent

DNA sequencing to determine the mutated sites. Mutated nifH upstream sequences were recovered as EcoRI fragments from replicative form DNA and ligated into the EcoRI site of plasmid pSMM4 which lacks the nifH UAS (9). Thus the altered upstream sequences were cloned in front of the wild type nifH -12,-24 promoter sequences. Synthetic UAS's (with EcoRI ends) were also cloned into the EcoRI site of pSMM4 and their orientation determined by sequencing.

Assays of Promoter Activity. Transcriptional activation of the nifH promoter was assessed by assaying β-galactosidase activity of nifH-lacZ transcriptional fusions constructed in the low copy number pJEL126 vector (see Figure 1 and ref. 14). The ability of

Table 1. nifH promoter mutations studied

Mutation/method		high copy number plasmid	low copy number plasmid	Reference
none	-	pMB101	pWVC101	(11)
G→T-136	spontaneous	pMB753	pWVC752	(11,26)
C→A-123	oligo	pMB86221	pWVC86221	This paper
C→T-125	oligo	pMB86231	pWVC86231	"
C→T-126	oligo	pMB8625	pWVC8625	"
ΔG-131 (-1bp)	oligo	pMB132	pWVC132	"
G→T-131	oligo	pWVC052	-	"
G→A-131	oligo	pWVC054	-	"
G→C-131	oligo	pWVC056	-	"
ΔG-131, T-134(-2bp)	oligo	pMB372	pWVC372	"
∇G-128,-127(+1bp)	oligo	pMB8657	pWVC8657	"
G→C-96	oligo	pWVC042	-	"
G→A-96	oligo	pWVC044	-	"
G→A-114	HSO_3	pJMW144	-	"
G→A-142,G→A-149	HSO_3	pJMW142149	-	"
G→A-149	HSO_3	pJMW149	-	"
G→A-154	HSO_3	pJMW154	-	"
UAS deletion		pSMM4	pJMW5	(9,18)
UAS at -156		pJMW80	-	This paper

Legend to Table 1

Each nifH promoter plasmid carries upstream nucleotides through to position -156 or -153 (depending upon whether the 5' end point was created by BglI or HaeIII restriction (11)) and 17aa of nifH coding sequence (Figure 1). The bisulphite produced mutants have the UAS at -156 as a result of the sequences introduced during the reconstitution of the nifH promoter from pSMM4 (see materials and methods) but numbering of mutated sites refers to the wild type nifH sequence. A plasmid, pJMW80, was constructed with the UAS at -156 as a control for these plasmids.

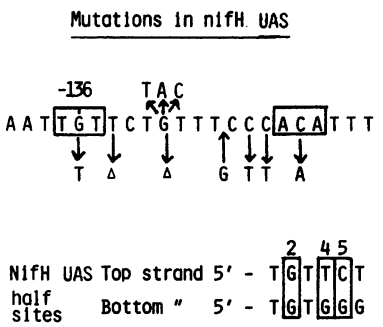
Legend to Figure 1. Features of the nifH promoter. The downstream -12,-24 promoter element is characterised by the dinucleotides GC and GG respectively. The UAS is located at -136 (the G of the TGT-ACA motif is at position -136). Assays of promoter activity were made using nifH-lacZ transcriptional fusion plasmids. 17aa of nifH coding sequence were present on the nifH promoters assayed.

the nifH promoter to titrate NifA (multicopy inhibition) was determined by assaying for nitrogenase (C_2H_2 reduction) as a measure of chromosomal nif gene expression (15). In multicopy inhibition assays the nifH promoter was present in the high copy number vector pMC1403 (16). For assays, K. pneumoniae UNF932 a nif$^+$ ntr$^+$ strain or UNF926, a his-nif deletion strain, was grown under repressing (+2 mg/ml $(NH_4)_2SO_4$) or derepressing (+100 μg/ml aspartic acid) conditions as described previously (17).

RESULTS

Phenotypes of Mutations in Upstream Sequences of the nifH Promoter.

Mutations outside the nifH UAS were obtained by random chemical mutagenesis with bisulphite, mutations within the UAS were constructed using oligonucleotide-directed mutagenesis.



Legend to Figure 2. Mutations introduced into the NifH UAS. Based on the known interactions of a number of DNA-binding proteins with their target sites on DNA, residues which may make specific contacts with NifA (bases, 2,4,5 of the half-site) are indicated (23).

Table 2. Phenotypes of mutated nifH promoters

a) Mutations outside the UAS	C_2H_2 reduction	
none, UAS at -136	0.4%	
G→C-96	3%	
G→A-96	3%	
none, UAS at -156	2%	
G→A-114	3%	
G→A-149	2%	
G→A-149,-142	2%	
G→A-154	3%	
b) Mutations within the UAS	**C_2H_2 reduction**	**Transcriptional Activation by NifA (β-gal U)**
wild type	0.4%	36,000
G→T-136	75%	1,700
C→A-123	68%	2,000
C→T-125	0.04%	60,000
C→T-126	0.1%	25,000
G→T-131	0.4%	-
G→A-131	0.4%	-
G→C-131	0.4%	-
ΔG-131	30%	2,200
ΔG-131, T-134	100%	1,100
VG-128,-127	39%	2,000
UAS deletion	100%	500
Vector plasmid	100%	400

Legend to Table 2

Transcriptional activation of the nifH promoter cloned into the low copy vector pJEL126 was measured in K. pneumoniae UNF932 grown under derepressing conditions for 18 h (17). The titration of NifA was assessed by measuring the level of nitrogenase in UNF932 harbouring multicopy nifH promoter clones in the vector pMC1403 and is expressed as a % of the C_2H_2 reduction that was obtained with pMC1403.

Sixteen mutant nifH promoters (see Table 1 and Figures 1 and 2) bearing upstream mutations were examined for their ability to interact with NifA by measuring (i) the degree to which they titrated NifA (multicopy inhibition) and (ii) their level of transcriptional activity in response to NifA-mediated activation. Results of the multicopy inhibition assays (C_2H_2 reduction data) and transcriptional activation assays (β-galactosidase data) are shown in Table 2. Unless the upstream mutations relieved multicopy inhibition, indicating a diminished interaction with NifA, the mutated promoters were not usually assayed for

Table 3. Restoration of activation by synthetic UAS sequences

	Plasmid	Sequence cloned into EcoRI site of pSMM4	Transcriptional activation by NifA (β-gal U)
1.	pMB0102	5'-AATTCTGTTCTGTTTCCCACAG	4,400
2.	pMB0304	5'-AATTCAATTGTTCTGTTTCCCACATTG	52,000
3.	pMB1617	5'-AATTCGGCTGTTCTGTTTCCCACACCG	3,500
4.	pMB5354	5'-AATTCTTCTGTTCTGTTTCCCACATTG	11,400
5.	pSMM4	none	1,200

Legend to Table 3

Transcriptional activation was measured in K. pneumoniae UNF932 as for Table 2. The G of the TGT motif in plasmid pMB0102 is at -98, in plasmids pMB0304, pMB1617 and pMB5354 it is at position -100. The UAS sequence is in each case identical to the wild type nifHUAS, but differs in flanking sequences. Overlined sequences are identical to sequences which flank the UAS in the wild type nifH promoter.

transcriptional activation in the low copy number pJEL126 vector (11). Of the sixteen mutations examined five relieved multicopy inhibition and reduced transcriptional activation by NifA markedly. All of these five mutations lie within the proposed NifA binding site, the UAS (see Figure 2). Several mutations, C to T transitions at -125 and -126 and mutations at -131, within the UAS were silent. In contrast transversions in the UAS at -136 and -123 displayed strong promoter down phenotypes. The probable basis for the phenotypes of these and other mutations within the UAS is discussed below.

Minimal UAS sequences required for activation

A number of short DNA fragments whose sequences consisted of the nifH UAS with various 5′ and 3′ flanking residues (Table 3) were synthesised and cloned into the UAS deletion plasmid pSMM4 to determine whether these minimal sequences retaining the UAS would restore NifA-dependent activation. Previous work had shown sequences -156 through to -80 restored transcriptional activation, and that sequences -79 through to -28 were not essential for activation (9). From the results shown in Table 3 it is clear that the greatest degree of activation was observed with plasmid pMB0304 which has short sequences flanking the UAS which are present in the wild type nifH promoter (specifically

5'-CAAT and 5'-TT, the sequences overlined in Table 3). The level of activation of pMB0304 is comparable to nifH promoter clones which retain all sequences upstream from position -80 (ref. 9, plasmids pMB42 and 43). Least activation was observed with plasmids lacking this flanking homology, indicating bases immediately 3' and 5' to the TGT-ACA motifs of the UAS influence the activity of the nifH promoter. None of the four plasmids constructed with synthetic UAS sequences caused significant levels of multicopy inhibition in K. pneumoniae UNF932. Since multicopy inhibition diminishes as the U~AS is brought towards the downstream promoter element, the failure of the constructs with synthetic UAS's to cause multicopy inhibition is likely to reflect the proximity of the synthetic UAS to the downstream promoter element (11).

Influence of NtrC Binding Sites upon Activation of the nifH Promoter

Transcriptional activation of the glnA and nifLA promoters by NtrC requires an upstream binding site for NtrC (10,7), whereas efficient transcriptional activation by NifA requires the nif UAS, the proposed binding site for NifA (9). The specificity of activation of a promoter by the functionally and structurally homologous activators NtrC and NifA is therefore determined, at least in part, by the presence of the appropriate upstream binding site(s). However some mutations in the downstream -12,-24 element of the nifH promoter were found to increase activation by NtrC (19), suggesting activator specificity may also reside in downstream sequences. By introducing an NtrC binding site upstream of the nifH -12,-24 promoter element we were able to increase NtrC-dependent activation of the nifH promoter (Table 4). The NtrC binding site used corresponds to the high affinity site of the K. pneumoniae glnA promoter (10,20,21). Transcriptional activation of the glnAp-nifH promoter by NtrC was not as great as that observed with the wild type nifH promoter and NifA, indicating perhaps that the downstream promoter sequences do confer some activator specificity. Interestingly the glnAp-nifH hybrid promoter was also activated by NifA (Table 4) despite lacking a nif UAS, suggesting NifA may recognise a nucleotide sequence within the glnAp NtrC binding site.

Table 4. Transcriptional activation of the glnAp-nifH hybrid promoter.

Plasmid	Binding sites present	Transcriptional Activation by	
		NtrC	NifA
pMB1	UAS	800	25,000
pSMM4	none	920	650
pMB2326	NtrC binding site	8,500	2,200

Legend to Table 4

Transcriptional activation by NtrC was assayed in UNF926, a nif deletion mutant (17), grown in the presence of 100 μg/ml aspartic acid. Activation by NifA was assayed in UNF926 with pMC71A (22) providing nifA in trans, and with growth media supplemented with 2 mg/ml $(NH_4)_2SO_4$. Plasmid pMB2326 has the sequence 5'-AATTCACAAATGCACTATATTGGTGCAATGCA TTCATTG cloned into the EcoRI site of pSMM4 to place the underlined NtrC binding site normally present in glnAp upstream of the nifH promoter -12,-24 element and the same distance upstream as in glnAp (20). Without NtrC or NifA, 20-40U of β-galactosidase activity were recorded for pMB1, pSMM4 and pMB2326. Similar relative levels of activation by NtrC and NifA for pMB1, pSMM4 and pMB2326 were obtained in the ntrBC deletion strains ET8894 (data not shown) with pMM14 and pMC71A providing ntrC and nifA in trans (13).

DISCUSSION

Central to the current model of transcriptional activation of nif promoters by NifA is the binding of NifA upstream at the UAS (11). Results obtained by mutating upstream sequences in the nifH promoter indicate that the only upstream mutations which influence NifA-dependent promoter activity lie within the UAS, a result consistent with an interaction between the UAS and NifA. Transversions in the conserved G or C residues of the TGT-N_{10}-ACA motif each reduced activation by NifA, confirming that the two-fold rotational symmetry of the UAS is important to its function and supporting the suggestion that the helix-turn-helix motif of NifA is involved in binding the UAS. Presumably the G:C at -136 and C:G at -123 in the NifH promoter are base pairs which make important contacts with the second helix of the DNA binding domain of NifA. The latter conclusion is based on the knowledge that a number of DNA-binding proteins interact with their target sites on DNA by using the second helix of the binding domain as a recognition helix. Amino acids at the surface of this helix contact functional groups of bases which define (in part) the symmetry of the target DNA sequence (23,24). Assuming the stably bound form of NifA is a dimer, transversions at -136 or -123 may reduce the binding of one monomer of NifA to one UAS half-site

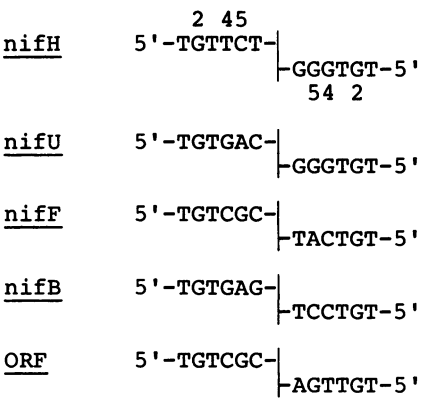


Figure 3. K. pneumoniae nif promoter UAS half-sites
Bases (2,4,5) of the half-sites which may make specific contacts with NifA (ref. 23, see also Figure 2).

and so reduce occupancy of the UAS, hence reducing activation and titration of NifA.

The DNA sequence between the TGT and ACA motifs of nif UAS's does not have an obvious consensus, but is always a 10 bp sequence (9). We determined that base substitutions at -131 were silent, and then deleted base -131, reducing the spacing to 9 bp (See Figure 2). This single base-pair deletion markedly reduced NifA-dependent activation of the NifH promoter. Presumably the 10 bp spacing configers the half-sites of the UAS into the preferred alignment within NifA, permitting optimal contacts between bound monomers of NifA and so stabilising binding. The 9, 8 or 11 bp spacings generated by mutagenesis may not allow optimal NifA monomer-monomer contacts and so reduce binding of NifA to the UAS and hence diminish activation and multicopy inhibition. Therefore the conserved 10 bp spacing found in nif UAS sequences contributes to the specificity of NifA binding and activation.

Not all of the bases of the intervening 10 bp sequence will be involved in making base specific contacts with NifA (although several are predicted to, see Figures 2 and 3, and reference 23) but may instead serve to influence the affinity of NifA for the UAS by altering the local DNA structure of the UAS, an effect observed with intervening bases in the operator of bacteriophage

434 repressor (25). By analogy, it is possible that the synthetic UAS sequences which retain 5' and 3' flanking bases homologous to the nifH promoter are better activator sequences by virtue of a favourable local DNA structure around the UAS. Some DNA-binding proteins alter DNA conformation upon binding, and it is possible that structural changes within and local to the UAS occur upon the binding of NifA, and that these changes contribute to the stability of binding.

Comparison of half-site UAS sequences of the K. pneumoniae nifH, B, U, F and ORF promoters (9,12,18) reveals that T, G or C are found adjacent to the TGT motif at base position 4 of the UAS (Figure 3). This position, by analogy with other DNA-binding proteins, may be occupied by a contact-specific residue (23). However, introduction of an A at position 4 of the UAS adjacent to the TGT motif (the C to T transition at -125) did not reduce NifA-dependent activation of the nifH promoter, but may have increased activation slightly. Presumably NifA still binds sufficiently well to this mutated UAS to activate transcription. An in vivo analysis of the interaction of proteins with the actively transcribed nifH promoter has shown the G of the C:G base pair at -126 to be protected from methylation with dimethylsulphate, presumably by NifA binding (E. Morrett, unpublished results). However a transition at position -126 does not reduce activation of the nifH promoter by NifA. As with the transition at -125, NifA must still be binding sufficiently well to the mutated UAS to activate transcription. This may reflect the contribution of the other non-mutated half-site of the UAS to the binding of NifA. Whether NifA utilises all or only some of the base specific contacts with the UAS which are predicted from analyses of other DNA-binding proteins (23) remains to be shown.

No single mutation within the UAS was found to reduce activation to a level as low as that observed with a UAS deletion, indicating that all the mutant UAS's examined interact with NifA to some extent. Presumably the ability of NifA to interact with altered forms of the UAS explains the observation that deletion of a variant of the nifF UAS with an 11bp spacing between the TGT and ACA triplets reduces NifA-dependent activation of the nifF promoter somewhat (18).

The silent nature of the mutations at -125 and -126, taken together with the naturally occurring sequences in comparable

positions in the UAS of other K. pneumoniae nif promoters (Figure 3), make it difficult to define a half-site UAS consensus sequence. This might suggest that NifA could bind any sequence of the form TGT-N_{10}-ACA. However, it appears that the nifH UAS (whether present singly or in tandem (ref. 9, and unpublished results) does not interact strongly with NifA in the absence of the downstream -12,-24 promoter element. Similarly, if the UAS is placed on the incorrect face of the DNA helix with respect to the downstream promoter element diminished titration of NifA is observed (11). Titration of NifA also appears to require ongoing transcription (14). In accord with the DNA looping model for transcriptional activation by NifA, the above findings suggest that the stable binding of NifA to the UAS requires the presence of downstream -12,-24 sequences and or proteins (RNA polymerase and NtrA) which interact with them. Therefore it can be argued that the binding of NifA upstream for activation is dependent upon a second specific sequence in the correct location, thus conferring considerable specificity to the stable binding of NifA upstream. Perhaps a contribution of downstream sequences or downstream-bound proteins to the binding of NifA upstream may explain, at least in part, the ability of NifA to activate the hybrid glnAp-nifH promoter which lacks a recognisable UAS motif. Unlike repressors which require a high affinity binding site on DNA to impeded RNA polymerase binding, activators such as NifA may need only to bind relatively weakly to DNA in order to function in activation. The differences in sequence between the half-sites of the K. pneumoniae nif UAS's may serve to modulate the affinity for these various sites for NifA, and so in turn contribute as a determinant of the relative activity of each nif promoter. Interestingly the nifU and nifH UAS's both have the half-sites: 5'-TGTGGG and are promoters which cause strong multicopy inhibition, perhaps indicating that G's in positions 4,5 and 6 of the UAS half-site favour NifA binding.

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