Activation of the *Klebsiella pneumoniae nifU* promoter: identification of multiple and overlapping upstream NifA binding sites

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Received January 31, 1990; Revised and Accepted March 14, 1990

ABSTRACT

The *Klebsiella pneumoniae nifU* promoter is positively controlled by the NifA protein and requires a form of RNA polymerase holoenzyme containing the *rpoN* encoded sigma factor, σ^{54} . Occupancy of the *K. pneumoniae nifU* promoter by NifA was examined using *in vivo* dimethyl sulphate footprinting. Three binding sites for NifA (Upstream Activator Sequences, UASs 1,2 and 3) located at -125, -116 and -72 were identified which conform to the UAS consensus sequence TGT-N₁₀-ACA. An additional NifA binding site was identified at position -90. The UASs located at -125 (UAS1) and -116 (UAS2) overlap and do not appear to bind NifA as independent sites. They may represent a NifA binding site interacting with two NifA dimers. UAS3 is located at -72, and abuts a binding site for integration host factor (IHF) and is not normally highly occupied by NifA. In the absence of IHF UAS3 showed increased occupancy by NifA. Mutational and footprinting analysis of the three UASs indicates (1) IHF and NifA can compete for binding and that this competition influences the level of expression from the *nifU* promoter (2) that UAS2 is a principle sequence of the UAS 1,2 region required for activation and (3) that none of the NifA binding sites interacts with NifA independently. *In vivo* KMnO₄ footprinting demonstrated that NifA catalyses open complex formation at the *nifU* promoter. IHF was required for maximal expression from the *nifU* and *nifH* promoters in *Escherichia coli*, and for the establishment of a Nif⁺ phenotype in *E. coli* from the *nif* plasmid pRD1.

INTRODUCTION

The nitrogen fixation (*nif*) genes of *Klebsiella pneumoniae* are among a class of genes present in prokaryotes which employ a form of RNA polymerase that contains the *rpoN*-encoded sigma factor (σ^{54}) for expression (1). The σ^{54} -containing form of RNA polymerase holoenzyme recognises the nucleotide sequence 5'-YTGGCACRR-N₃-TTGCA located 11 to 12 bp from the

start of transcription (2, 3) of genes utilising this form of holoenzyme for expression. The binding of RNA polymerase- σ^{54} to this downstream promoter element (DPE) to form a closed complex in the absence of a positive control protein is non-productive with respect to the transcription of *nif* and other σ^{54} -dependent genes. This conclusion is based on the observation that the promoter DNA is not denatured when RNA polymerase- σ^{54} is bound (3, 4, 5). Activation of transcription has been best studied from the σ^{54} -dependent *glnAp2*, *nifLA* and *nifH* promoters and it can be concluded that the positive control proteins required for expression from these promoters catalyse the rate of open complex formation (3-6). The *glnAp2* and *nifLA* promoters are positively controlled by the general nitrogen regulatory protein NtrC, the *nifH* and other *nif* promoters are controlled by the NifA protein (7, 8). Both NtrC and NifA are DNA binding proteins which specifically recognise upstream sequences (called upstream activator sequences [UASs] or enhancers) of the promoters which they regulate. Their DNA binding domains reside in their carboxy termini and are likely to be helix-turn-helix structures (9, 10, 11). Activators functioning with σ^{54} -containing holoenzyme have a conserved central domain (1, 9). This domain contains a putative ATP binding pocket, consistent with the ATP requirement for the positive control function of NtrC (1, 5). In the case of NifA the central domain is sufficient to activate transcription when a closed RNA polymerase- σ^{54} promoter complex forms in the absence of upstream bound activator and may thus be the catalytic domain (11-13). In the absence of preformation a closed promoter complex, positive control of *nif* promoters requires that NifA is bound upstream at the UAS (13, 14). The UAS is usually located >100bp upstream of the start of transcription, and upstream bound NifA is believed to make productive contacts with RNAPolymerase- σ^{54} at the downstream promoter element through the formation of a DNA loop (15, 16). The UAS may therefore serve to topologically constrain NifA in the region of the RNA polymerase- σ^{54} recognition sequence and to increase its local concentration.

The UASs of *nif* promoters are characterised by a TGT-N₁₀-ACA motif, and the binding of NifA to UASs of the *nifH* and *nifJ* promoters has been demonstrated using *in vivo*

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Figure 1. Organisation of the *K. pneumoniae nifU* promoter. The three putative NifA binding sites, the upstream activator sequences (UASs) are shown together with the downstream promoter element (DPE) which is the RNA polymerase- σ^{54} recognition sequence. The conserved TGT and ACA motifs of the UASs are underlined, and the invariant dinucleotides GG and GC of the DPE indicated.

TABLE 1. Bacterial Strains and Plasmids

		Reference/source
<i>E. coli</i> ET8894	$\Delta(rha\ glnA\ ntrB\ ntrC)1703\ rbs$	(38)
	<i>gyrA hutC⁺ lacZ::IS1 Mucts62</i>	
<i>E. coli</i> S90C	$\Delta(lac\ pro)rpsL$	(39)
<i>E. coli</i> DPB101	$\Delta(lac\ pro)rpsL\ himD451::mini-tet$	"
<i>E. coli</i> DPB102	$\Delta(lac\ pro)rpsL\ himA452::mini-tet$	"
<i>E. coli</i> DPB316	$\Delta(lac\ pro)rpsL\ himA453::mini-tet$	"
<i>E. coli</i> DPB223	$\Delta(lac\ pro)rpsL\ himA452::mini-tet\ topA66$	(28)
<i>E. coli</i> JC5466	<i>trp recA56 rpsE his</i>	(R. Dixon)
<i>K. pneumoniae</i> UNF932	$\Delta lac2001\ recA56\ hsdR1sbl300::Tn10$	(40)
Plasmids	Relevant character	
pMD1106	<i>nifU-lacZ</i> translation fusion, Cb ^R	(14)
pWVC1106	<i>nifU</i> promoter cloned in pACYC184, CA ^R	(This paper)
pWVC88072	as pWVC1106 but G-T at -125 in UAS ₁ , CA ^R	(" ")
pWVC88073	as pWVC1106 but G-T at -72 in UAS ₃ , CA ^R	(" ")
pWVC88079	As pWVC1106 but C-A at -103 in UAS ₂ , CA ^R	(" ")
pWVC11061	<i>nifU-lacZ'</i> translation fusion in pNM482, Cb ^R	(This paper)
pWVC880721	as PWVC11061 but G-T at -125 in USA ₁ , Cb ^R	(" ")
pWVC880731	as pWVC11061 but G-T at -72 in UAS ₃ , Cb ^R	(" ")
pWVC880791	as pWVC11061 but C-A at -103 in UAS ₂ , Cb ^R	(" ")
pMJ160	NifA expressed from <i>lac</i> promoter in pEMBL8 ⁺ , Cb ^R	(13)
pMJ220	NifA expressed from <i>lac</i> promoter in pMD220, CA ^R	(13)
pMB880131	Mutant NifA (arg513@lys513) expressed from <i>lac</i> promoter in pMD220, CA ^R	(11)
pMC71A	NifA expressed from <i>tet</i> promoter in pACYC184, CA ^R	(41)
pRD1	<i>his⁺ nif⁺ Cb^R, Tc^R, Km^R</i>	(21)
pMS160A	<i>nifL-lacZ</i> translational fusion, Cb ^R	(42)
pMB12461	<i>Rhizobium meliloti nifH-lacZ'</i> translational fusion, lacking UAS, Cb ^R	(16)

dimethylsulphate protection experiments (13). In the *nifU* promoter three potential UASs are present (17, Figure 1). This is quite atypical of other *K. pneumoniae nif* promoters where single UASs conforming to the TGT-N₁₀-ACA consensus have been identified (14, 17). The two most upstream *nifU* UASs (UAS1, UAS2) overlap each other and deletion of these diminishes activation of the *nifU* promoter dramatically (11, 14). The binding of NifA at the UAS 1 and 2 region is itself of interest since these UAS sites overlap each other. Thus they could represent two discrete sites or one atypical NifA binding site. The UAS2 shows the greatest homology to the UAS of the *nifH* promoter, particularly in the retention of one half-site: 5'-TGTGGG at positions 102–107 (bottom strand, Fig. 1) which

includes four guanine residues which in the *nifH* UAS are changed in their reactivity to dimethylsulphate in the presence of NifA (13). We have now examined the occupancy of the *nifU* promoter by NifA using *in vivo* dimethylsulphate footprinting to identify functional NifA binding sites and selectively mutated each of its three UASs.

MATERIALS AND METHODS

Bacterial Strains and Plasmids

These are listed in Table 1. Plasmids carrying the *nifU* promoter were constructed by cloning a 355bp *Bam*HI-*Sal*I fragment (17) from pMD1106 (14) into *Bam*HI-*Sal*I restricted pACYC184 (18)

or pNM482 (19), the latter to generate *nifU-lacZ'* translational fusions. Such fusions carry 46 amino acids of *nifU* before the fusion junction. For mutagenesis of the UASs of the *nifU* promoter the same *Bam*HI-*Sal*I fragment was cloned into M13mp19 and oligonucleotide directed mutagenesis carried out as described previously (20). Subsequently the mutagenised *nifU* promoter fragment was cloned into pACYC184 or pNM482. pRD1 (21) was transferred from *E. coli* JC5466 to *E. coli* S90C, DPB101, 102, 316 and 223 by selecting for growth on minimal media supplemented with L-proline (15 µg/ml), carbenicillin (100 µg/ml) and kanamycin (15 µg/ml). The presence of pRD1 was confirmed by back transfer to *E. coli* JC5466 and subsequently measuring whole cell acetylene reduction in the recipient cells. Carbenicillin (100 µg/ml) was used to maintain pRD1 during growth and derepression.

In vivo Dimethylsulphate footprinting

The accessibility of promoter DNA to dimethylsulphate (DMS) in the presence or absence of NifA was assayed as described previously (13). Primer extension was carried out at 52°C and the products analysed on 12%, 9% or 6% polyacrylamide gels as appropriate. The methylation of guanine residues on the bottom strand of the promoter was examined using the oligonucleotide 5'-TGCTCGCTTCGCTACTTGG complementary to sequence in pACYC184 44bp upstream of the *Bam*HI site and on the top strand using the oligonucleotide 5'-AGCGGTGGCGATAACGAAC which primes at position -11 in the *nifU* promoter. Autoradiograms were densitometrically scanned, and the data expressed as the logarithm of the quotient of the peak area without NifA to that with NifA.

In vivo footprinting of single stranded DNA using KMnO₄

The reactivity of single stranded pyrimidine residues to KMnO₄ was assessed as described previously (3, 4). Briefly cells with or without NifA were grown to an A₆₀₀ of 0.6, rifampicin (200 µg/ml) was added for 7 minutes, followed by the addition of KMnO₄ for 2.5 minutes. Plasmid DNA was isolated, and DNA adducts on the bottom strand of the promoter DNA resulting from reaction with KMnO₄ visualised by primer extension using the oligonucleotide 5'-GCCCCAAAAACATCGCGAC which primes synthesis 13bp downstream of UAS2. Appropriate controls generated from the reaction of single stranded M13mp19:*nifU* DNA with KMnO₄ *in vitro* were also run on gels to identify reactive residues.

In vitro integration host factor binding assays

Purified *E. coli* integration host factor protein was a gift from Prof. Howard Nash. Prior to use this was diluted down to 15 µg/ml in 50 mM tris, pH 7.2, 500 mM KCl, 2.5 mg/ml BSA, 10% glycerol. Binding assays contained in 40 µl *ca.* 0.1 pmoles of the *Bam*HI-*Sal*I *nifU* promoter DNA fragment (isolated from pWVC11061 and labelled with α-dGTP at the *Bam*HI site, bottom strand) and 30 ng of IHF in 45 mM tris, pH 7.8, 100 mM KCl, 10 mM MgCl₂, 2 mM dithiothreitol, 77.5 µg/ml BSA, 1% glycerol. Following incubation at 25°C for 15 minutes, 1 ng of freshly diluted DNase I was added for 2 minutes and the reaction then terminated by phenol extraction. DNA recovered by ethanol precipitation was analysed on sequencing gels.

Assays of promoter activity

Activation of the *nifU* promoter by NifA was measured in *E. coli* ET8894 with the wild-type NifA provided by pMC71A or

pJM220, or with a mutant form of NifA in which arginine 513 was replaced with lysine (pMB880131, 11). The ability of the *nifU* promoter, when present on a multicopy plasmid, to titrate NifA (multicopy inhibition) was assayed in the *nif*⁺ *K. pneumoniae* strain UNF932 by measuring nitrogenase levels as an indication of chromosomal *nif* expression (22). Conditions for cell growth prior to assays of activation or multicopy inhibition were as described previously (20).

Sequencing of the *Enterobacter agglomerans nifU* promoter

For sequencing, a 435bp *Pst*I-*Sau*3a fragment of the *Enterobacter agglomerans nifU-lacZ'* fusion plasmid pMK182P8 (23) was cloned into pUC18 (24). Both strands were sequenced on double stranded template DNA using dideoxynucleotide termination sequencing methods. The sequence was aligned with the corresponding *K. pneumoniae* region.

RESULTS

Interaction of NifA with the *nifU* promoter

The reactivity of guanine residues in the *nifU* promoter towards DMS *in vivo* was assayed as described in Materials and Methods. In these experiments plasmid pMJ160 provided *nifA* and the *nifU* promoter was carried on pWVC1106. Representative autoradiograms from the primer extension analysis are shown in Figure 2, and a densitometric analysis in Figure 3. It is quite clear that in the presence of NifA (lanes b,d and f) the reactivity of guanine residues in UASs 1 and 2 (guanines present in base pairs -125 to -103) is markedly influenced by the presence of NifA. In contrast guanines in the UAS3 region (base pairs -72 to -59) show relatively little change in reactivity when NifA is present. Therefore NifA appears to interact with the binding site constituted by UASs 1 and 2, but only weakly with UAS3. Somewhat unexpectedly guanine residues in the -90 region showed strong NifA-dependent changes in reactivity towards DMS. Guanine of the G:C base pair -90 falls within a ACA motif and may therefore form part of an imperfect UAS to account for an interaction of NifA with this sequence. This is labelled as binding site 4 (BS4) in Figure 7. NifA would appear to interact significantly more strongly with the BS4 sequence than UAS3.

The primer extension strategy used permitted detection of methylation of the C:G base pair at position -12 in the RNA polymerase-σ⁵⁴ recognition sequence. No changes in reactivity of the guanine of this base pair were detected when NifA was present (data not shown). However NifA-dependent protection of the guanine of the base pair at -6 was detected (see below—Open Promoter Complex Formation).

The *nifU*, UAS1, UAS2-NifA interaction

Results obtained with the *nifH* and *nifJ* UASs using the *in vivo* DMS protection assay (13) have shown that the guanine of the UAS TGT motif is protected by NifA and that the guanine residues in positions 4, 5, 6 of the half-site 5'-TGT⁴G⁵G⁶ are hypermethylated (position 4) and protected (positions 5 and 6). In the upstream region of the *nifU* promoter guanine residues corresponding to UAS TGT motifs are at base pairs -125 and -112 (for UAS1) and base pairs -116 and -103 (for UAS2). Results of DMS protection experiments with the TGT sequences of the *nifU* UASs are complex (see Figure 3). NifA-dependent protection of G at base pair -125 is relatively strong and that at -112 relatively weak, indicating the half-sites of UAS1 do

Figure 2. Autoradiograph of primer extension analysis of cleaved DNA following exposure of *E. coli* ET8894 cells harbouring the *nifU* plasmid pWVC1106 to DMS in the presence (lanes b, d, f) of NifA (provided by pMJ160) or its absence (lanes a, c, e). Open circles indicate regions showing protection, closed circles enhanced methylation. For positions of reactive guanine residues see Figures 3 and 7. Extensions were carried out with deaza GTP (lanes c, d) or the usual triphosphates (other lanes). Densitometric analysis of the autoradiographs is presented in Figure 3.

not interact equivalently with NifA. The situation of non-equivalent interaction of half-sites appears more complex for UAS2. Here G of base pair -103 is clearly protected, but G-116 of the other half site TGT motif is hyper-methylated rather than being protected in the presence of NifA. The predicted hypermethylation of the G in the base pair at position -105 in the half-site sequence TGTGGG of UAS2 is observed, but hypermethylation rather than protection in positions 106 and 107 is also observed. In contrast to the result with base pair G-105, G-114 was protected rather than showing the anticipated hypermethylation. The changes in reactivity towards DMS observed, which contrast those predicted to separate UASs (11,13), may reflect that UASs 1 and 2 overlap and thus constitute a single atypical NifA binding site.

The influence of mutations in UASs 1, 2 and 3 upon NifA binding

To examine the influence of UAS1 upon the interaction of NifA at UAS2 and vice versa mutations in the UAS TGT and ACA motifs at positions -125 (UAS1) and -103 (UAS2) were constructed. To further examine any role for UAS3 in *nifU* promoter function a mutation at -72 was also constructed. Each mutation alters the sequence of the TGT and ACA motif to TTT or AAA, which in the *nifH* promoter results in a diminished binding of NifA at the UAS (13) and a significant reduction in activation of the *nifH* promoter (11, 25) and *nifF* promoter (26). Results of the *in vivo* footprinting are shown in Figure 3. In the G to T at -125 mutant the G of the TGT motif at -116 in UAS2 showed protection, indicating that its unexpected increased reactivity to DMS in the wild-type *nifU* promoter (see above) is probably a consequence of the binding of NifA to UAS 1. UAS3 occupancy was also increased slightly in the -125 mutant. Mutation at position -103 increased occupancy of UAS1 and resulted in the expected protection of the UAS1 bottom strand TGT motif at -112, a result anticipated if UAS2 were not binding NifA and therefore not interfering with UAS1 binding. Mutation at -72 in UAS3 decreased the binding of NifA at UAS1 slightly, but increased UAS2 and BS4 occupancies.

Activation of the *nifU* promoter by NifA

Examination of activation of the mutant *nifU* promoters by NifA revealed that the integrity of the UAS2 is required for the most efficient activation (Table 2). This was demonstrated by the promoter down phenotype of a mutation in UAS2 and increased activation when UAS1 was mutated, changes which diminished and increased the occupancy of UAS2 respectively. The phenotype of the UAS2 mutation was more exaggerated in assays employing a mutant form of NifA partially defective in binding

Table 2. Activation of mutant and wild-type *nifU* promoters by NifA

Plasmid	Relevant character	β -gal activity (U)			Multicopy
		(None)	(Activator present) (NifA)	(NifA lys513)	Inhibition
pWVC11061	wild-type	60	18,000	4,300	0.1%
pWVC880721	UAS1 mutant	50	30,000	2,900	0.1%
pWVC880731	UAS3 mutant	60	29,000	3,300	0.1%
pWVC880791	UAS2 mutant	110	12,800	1,500	2.5%

Activation assays were conducted as described previously (20) in *E. coli* ET8894 with either pMC71A or pMB880131 providing NifA or the lys513 NifA mutant respectively. The multicopy inhibition assays were conducted in *K. pneumoniae* UNF932 as described previously (20). Percentages given are acetylene reduction activities expressed relative to UNF932 with the fusion vector plasmid pMN482, the control value of 100%.

to UASs, supporting the idea that occupancy of UAS2 is important for activation. Interestingly in the presence of wild-type NifA the UAS3 mutation increased activation, but not with the mutant NifA. We argue (see below) that the wild-type NifA at UAS3 competes with the binding of an additional factor, IHF, to the *nifU* promoter which is required for maximal expression of the *nifU* promoter. The UAS3 mutation prevents this competition and the mutant NifA, due to its reduced affinity for a UAS, does not compete effectively regardless of whether UAS3 is wild-type or mutant.

In accordance with the DMS protection data and activation assays, we observed that mutation of UAS2 partially relieved the inhibition of *nif* expression shown by multicopy *nif* promoters bearing NifA binding sites. Mutations in UAS1 or 3 had little effect upon this property of the promoter (see Table 2). Therefore UAS2 appears to be the most significant NifA binding site required for activity of the *nifU* promoter.

Open promoter complex formation

The role of NifA in positive control of the *nifH* promoter has been shown to be that of isomerising a closed promoter complex to the open form (3). Experimentally this was determined by measuring the reactivity of pyrimidine residues towards KMnO_4 in order to detect local NifA-dependent denaturation of DNA around the transcription start site. Using this methodology we were able to demonstrate that NifA is required for open complex formation at the *nifU* promoter also. Figure 4 shows the increased reactivity of the AT base pair at -2 to KMnO_4 in the presence of NifA. Thus the -2 region of the *nifU* promoter is denatured in the presence of NifA. We also note that the reactivity of the AT base pair at -41 is diminished in the presence of NifA (the lowest band of lanes b,c, Figure 4). This base pair lies within the IHF binding site of the *nifU* promoter (see below). Formation of an open promoter complex at the *nifH* promoter affords protection to guanine residues in the -12 , -24 region in the *in vivo* DMS assay (3). The base pair at -7 in the *nifH* promoter is protected in the open complex (3). At the *nifU* promoter we observed NifA dependent protection of the guanine of the base pair at -6 (data not shown). Since this base pair is not within a sequence likely to bind NifA, we attribute its protection (value of $+0.23$ in densitometric scans) to NifA-dependent open promoter complex formation, and therefore protection of the base pair at -6 by the RNA polymerase- σ^{54} complex.

The binding of integration host factor to the *nifU* promoter

The region immediately downstream of UAS3 binds a factor present in crude extracts of *K. pneumoniae* and *E. coli* (2). Recent work (27 and S. Kustu, personal communication) has demonstrated that the AT rich sequence of the *K. pneumoniae* *nifH* promoter located from -36 to -71 binds *E. coli* IHF. This prompted us to consider whether the *nifU* promoter might bind IHF and whether this could interfere with the interaction of NifA with UAS3. Gel retardation assays demonstrated that the 355bp *Bam*HI-*Sal*I fragment carrying the *nifU* promoter bound IHF (data not shown) and DNaseI footprints (Fig. 5) demonstrated that the binding covered a *ca.* 40bp sequence upstream from position -27 , thus partially overlapping UAS3. Some IHF-dependent protection of the guanine of base pair -57 from methylation by DMS *in vitro* was also observed (data not shown). The weaker DNaseI protection afforded by IHF outside the -27 to -67 region may reflect the expected bend in DNA structure resulting from IHF binding (37).

To examine the possible occupancy of UAS3 by NifA in the absence of IHF, DMS footprints of the *nifU* promoter were obtained in *E. coli* cells with (*E. coli* S90C) or without (*E. coli* DPB101) IHF in the presence and absence of NifA. Results obtained (Figure 6) clearly showed increased NifA-dependent methylation protection of UAS3 in the absence of IHF, confirming that IHF competes with NifA binding at UAS3. The absence of IHF also significantly influenced the relative occupancy of UAS1 and UAS2 by NifA, IHF appeared to be required for maximal UAS1 and UAS2 occupancy. It is possible that NifA bound at UAS3 in the absence of IHF destabilises the binding of NifA at UAS1, or IHF is in some other way influences NifA binding. We estimate a *ca.* 4-fold increase in UAS3 occupancy relative to UAS1 in the IHF mutant compared to its parent based on the reactivities of base pairs at -72 and -125 . We also observed that in the absence of NifA protection the guanine of base pair -57 towards DMS was IHF dependent *in vivo* (data not shown). This finding is consistent with the protection of base pair -57 by IHF *in vitro*, indicating an interaction of IHF with this base pair. Furthermore the presence of NifA diminished the apparent protection afforded by IHF *in vivo* to this sequence (Figure 6), indicating that the binding of IHF and NifA binding to UAS3 cannot be readily accommodated simultaneously. The base pair at -59 showed different NifA-dependent changes in reactivity towards DMS in the UAS mutants (Figure 3) and in the IHF mutant (Figure 6), compared to the wild type situation. This is likely to reflect direct interactions of NifA and IHF at this sequence.

In the absence of IHF, no NifA-dependent protection of the base pair at -6 was detected. This presumably reflects a lower rate of open complex formation at the *nifU* promoter in the IHF mutants (see below).

The influence of IHF upon activation of the *nifU* promoter

The binding of IHF to a sequence overlapping a NifA binding site could diminish activation of the *nifU* promoter by NifA or could be required for some aspect of *nifU* promoter function. We observed that activation of the *nifH* and *nifU* promoters by NifA was diminished in a mutant lacking IHF (Table 3). Some negative effect of NifA binding close to the DPE in the IHF mutant could be involved in the diminished activation of the *nifU* promoter, but not in the *nifH* promoter as no UAS is located near the DPE region.

Expression of *nif* in *E. coli* IHF mutants

The possible requirement for IHF to establish a Nif⁺ phenotype in *E. coli* was examined by transferring the plasmid pRD1 which carries the entire *K. pneumoniae* *nif* gene cluster into *E. coli* strains proficient or defective in IHF synthesis. Nitrogenase synthesis was then measured by acetylene reduction assays after derepression. In the absence of IHF no significant acetylene reduction was detected (Table 4). In addition, an IHF compensating *topA* mutation which allows pSC101 replication in the absence of IHF (28) did not permit establishment of a Nif⁺ phenotype in IHF backgrounds. Activation of the regulatory *nifLA* operon by NtrC was diminished in the absence of IHF (Table 3), probably reflecting the requirement that the *nifL* promoter must be negatively supercoiled to be activated (29, 30). This conclusion is based on the knowledge that (a) the level of DNA gyrase is significantly reduced in IHF mutants (31, 28) and (b) the *nifL* promoter does not bind IHF (R. Dixon, personal communication). However, constitutively produced NifA did not

Figure 3. Densitometric scans of the autoradiographs demonstrating changes in guanine reactivity towards DMS mediated by NifA. Both the wild-type promoter and the three UAS mutants were analysed. The scanned sequence from UAS1 to UAS3 is shown below. Top strand guanine residue reactivities are represented with open bars, bottom strand reactivities with solid bars, positions of mutations with arrows. Positive values indicate protection, negative values hypermethylation. The relevant *nifU* promoter is shown below the figure (3' to 5').

Figure 4. Detection of an open promoter complex at the *nifU* promoter. DNA was reacted with KMnO_4 *in vivo* and analysed as described in materials and methods. The arrow indicates a hyper-reactive residue (assigned to A:T base pair -2, lane b) present in the *nifU* promoter. Lanes labelled G, A, T, C are dideoxy sequencing reaction products corresponding to top strand G, A, T, C bases respectively, lane a represents single stranded *nifU* promoter DNA reacted *in vitro* with KMnO_4 and analysed by primer extension, lanes b and c are primer extension products derived from DNA reacted *in vivo* with KMnO_4 in the presence (lane b) or absence (lane c) of NifA. All primer extension products are from the same oligonucleotide (see materials and methods). Assays were conducted in *E. coli* ET8894 with pWVC11061 providing the *nifU* promoter sequence and pMJ220 NifA.

result in a Nif^+ phenotype from pRD1 in the absence of IHF (data not shown). Activation of the *Rhizobium meliloti nifH* promoter by NtrC was unaffected by the absence of IHF (Table 3), indicating that IHF does not influence σ^{54} -dependent promoters in some general way, for example through diminishing the level or activity of σ^{54} *per se*.

The *Enterobacter agglomerans nifU* promoter sequence

The *K. pneumoniae nifU* promoter is rather different from other *nif* promoters subject to activation by NifA in having three potential UASs and one further NifA binding site (see Introduction). To examine whether this could reflect some general specialisation of *nifU* promoter function the sequence of the *E. agglomerans nifU* promoter was determined and compared to that of *K. pneumoniae*. The *E. agglomerans nif* gene cluster is closely related to that of *K. pneumoniae* (23). The *nifU* promoter alignment (Figure 7) clearly shows that UAS1 is retained in *E. agglomerans* and that UAS2 is absent by virtue of a single base change in the TGT motif mutating it to TAT. This change is

Figure 5. DNase 1 footprinting of the *nifU* promoter in the presence of IHF. End labelled *nifU* promoter DNA was digested with DNase 1 in the absence (lane a) or presence (lane b) of bound *E. coli* IHF. Lane c is the product of a guanine cleavage reaction on the same end labelled *nifU* promoter DNA strand.

expected to diminish binding of NifA to this sequence (13). UAS3 is absent since both TGT and ACA motifs are lacking, but the fourth binding site is clearly present at the -90 region (BS4, Fig. 7). The RNA polymerase recognition sequence is highly conserved (the sequence marked Promoter in Figure 7) when the *K. pneumoniae* and *E. agglomerans* sequences are compared. A potential IHF binding site is present, and has been assigned on the basis of (a) its relative location to that of the *K. pneumoniae* *nifU* promoter IHF binding site and (b) the IHF binding site consensus A/TATCAANNNTTG/A AT A/T (32, 33). Based on these observations, we suggest the *E. agglomerans* *nifU* promoter may bind RNA polymerase- σ^{54} with similar affinity to the *K. pneumoniae* *nifU* promoter and require IHF for maximal expression but may differ in its response to increasing NifA concentrations by virtue of lacking UAS2 and 3. We suggest NifA binds to the *E. agglomerans* *nifU* promoter at its UAS1 and BS4 homologues and weakly to the UAS2-like sequence.

DISCUSSION

We have shown that the positive control protein NifA normally recognises a sequence in the upstream region of the *nifU* promoter which constitutes two UASs which overlap each other. A third UAS, which overlaps a binding site for IHF can also interact with NifA. The third UAS is not normally highly occupied by NifA unless IHF is absent. In addition to the UASs, sequences around -90 (the BS4 of Figure 7) are also recognised by NifA. BS4 is rationalised as being an imperfect UAS. We note that a BS4-like sequence 5'-GCGACA is present 3bp downstream of

Figure 6. Binding of NifA to the *nifU* promoter *in vivo* in the presence or absence of IHF. Primer extension analysis was conducted as for figures 2 and 3. *E. coli* S90C provided the control IHF positive background, *E. coli* DPB101 the background lacking IHF. pMJ160 provided *nifA*, pWVC1106 the *nifU* promoter target. Immunoblotting demonstrated that the level of NifA in DPB101 cells was 2–3 fold lower than in S90C cells (data not shown), probably reflecting reduced activity of the *lac* promoter on pMJ160 due to reduced DNA gyrase levels in DPB101 (28, 43).

the *nifJ* UAS and 41bp downstream of the *nifH* UAS. However neither of these sequences shows NifA-dependent methylation protection *in vivo* (data not shown). Thus, in contrast to the *nifH* promoter, functional multiple binding sites for NifA are present in the *nifU* promoter. Examination of the binding of NifA at the UAS1 and 2 region in the *nifU* promoter revealed that patterns of guanine methylation were not as predicted, and that the binding of NifA at UAS1 interfered with that at UAS2. The glutamine residue of the first helix of the helix-turn-helix structure of the DNA binding domain of NifA may hydrogen bond the phosphate of the first base of the DNA recognition sequence, by analogy with other DNA binding proteins (Anderson *et al.*, 1987). Thus the overlapping of UASs 1 and 2 may preclude these two sites acting as independent binding sites for steric reasons. The UAS1,2 region may therefore be an atypical NifA binding site, perhaps interacting with two dimers of NifA rather than one dimer as has been proposed for the UAS of the *nifH* promoter (13).

Increased binding of NifA to UAS2 (or the absence of NifA binding to UAS1) appeared to allow some NifA binding at UAS3. The occupancy of UAS3 when UAS1 is mutated could depend upon a cooperative interaction between NifA bound at UAS2. The binding of NifA to the BS4 sequence, which is not of the form TGT-N₁₀-ACA, is marked. The aggregative properties of NifA (35) could promote such binding if a conventional UAS is present nearby. It is possible that the multiple binding sites for NifA in the *nifU* upstream promoter region may have a role in increasing NifA concentration to facilitate open complex

Figure 7. Alignment of the *E. agglomerans* (Ea) *nifU* promoter with the *K. pneumoniae* (Kp) *nifU* promoter. The proposed UASs, DPE and IHF binding sites are indicated. BS4-fourth binding site for NifA identified in the *K. pneumoniae* *nifU* promoter.

Table 3. Expression of *nif* promoters in *E. coli* IHF mutants

Plasmid	Promoter	Assay Background	β -gal (U)		
	fusion		− NifA	+ NifA ^(a)	+ NifA ^(b)
pMB1	<i>Kp-nifH</i>	<i>E. coli</i> S90C, wt.	20	26,000	21,000
pMB1	<i>Kp-nifH</i>	<i>E. coli</i> DPB101 <i>himD</i> [−]	20	4,200	10,000
pMD1106	<i>Kp-nifU</i>	<i>E. coli</i> S90C, wt.	190	14,000	24,000
pMD1106	<i>Kp-nifU</i>	<i>E. coli</i> DPB101, <i>himD</i> [−]	210	1,300	4,500
			+ NH ₄	− NH ₄	
pMS160A	<i>Kp-nifL</i>	<i>E. coli</i> S90C wt.	30	5,300	
pMS106A	<i>Kp-nifL</i>	<i>E. coli</i> DPB101, <i>himD</i> [−]	30	1,200	
pMB12461	<i>Rm-nifH</i> -UAS	<i>E. coli</i> S90C wt.	80	1,600	
pMB12461	<i>Rm-nifH</i> -UAS	<i>E. coli</i> DPB101 <i>himD</i> [−]	150	1,500	

Table 3. Bacteria were grown under nitrogen limiting conditions in the presence of 15 µg/ml L-proline for β-galactosidase assay as described previously (20). NifA was provided by pMJ220 (a) or pMC71A (b). Lower levels of activation in DPB101/pMJ220 compared to DPB101/pMC71A probably reflect lower levels of NifA, since the *lac* promoter from which NifA is expressed in pMJ220 is sensitive to supercoiling (43) and the level of DNA gyrase is lowered in IHF mutants (28). *Kp.* *K. pneumoniae*, *Rm.* *R. meliloti*.

formation not only at the *nifU* promoter but also at other downstream σ^{54} -dependent promoters, possibly the *nifZ* and *nifM* promoters which lack UASs (17, 36). Since the *E. agglomerans* *nifU* promoter shows some differences in the organisation of its UASs, as seen in the *K. pneumoniae* *nifU* promoter, it would be of interest to know the organisation of downstream promoters in *E. agglomerans*.

IHF appears to act positively, being required for maximal *nifA* dependent expression from the *nifU* and *nifH* promoters in *E. coli* *in vivo*, and possibly accounting, at least in part, for the failure of pRD1 to establish a Nif⁺ phenotype in *E. coli* IHF mutant backgrounds. One possible role for IHF in *nif* promoter activation may be in facilitating DNA loop formation (15) to allow upstream bound NifA to establish productive interactions with the downstream RNA polymerase- σ^{54} complex. This would be consistent with the ability of IHF to bend the DNA of *attP* upon binding (37). Alternatively, or in addition to a role in DNA loop formation, the proximity of the IHF binding site in the *nifU* promoter to the DPE suggests IHF could influence the RNA polymerase- σ^{54} promoter interaction. It is possible that the change in reactivity of the base pair at -6 to DMS observed in the IHF mutant background could be attributed in part to a weaker RNA polymerase- σ^{54} interaction as well as reduced open complex formation. IHF may also be required for maximal occupancy of the *nifU* UASs 1 and 2, by NifA. In *K. pneumoniae*

Table 4. Expression of *nif* genes from pRD1 in *E. coli* IHF mutants

Assay background	Nitrogenase activity (C ₂ H ₂ reduction)
<i>E. coli</i> S90C, wt.	100%
<i>E. coli</i> DPB101, <i>himD</i> ⁻	<0.5%
<i>E. coli</i> DPB102, <i>himA</i> ⁻	<0.5%
<i>E. coli</i> DPB316, <i>himA</i> ⁻	<0.5%
<i>E. coli</i> DPB223, <i>himA</i> ⁻ <i>topA66</i>	<0.5%

Table 4. Synthesis of nitrogenase was assayed by measuring whole cell acetylene reduction activity after 15 hours derepression. The value of 100% corresponds to ca. 5 nmoles of acetylene reduced per minute per A600 cells. This value was approximately 50% that obtained with *E. coli* JC5466/pRD1.

expression of the *nifH* promoter deleted from -27 to -76 (14) and therefore lacking the IHF binding site (2, 27) was significant, indicating the phenotype of the *E. coli* IHF mutants could be more severe than comparable *K. pneumoniae* mutants.

The binding of IHF to the *nifU* promoter may prevent NifA binding UAS3. Although IHF is thought to establish predominantly minor groove contacts in DNA (33), and NifA major groove contacts (13), some IHF mediated change in N⁷-Guanine reactivity does occur (32, 33) as also noted by us for the *nifU* promoter, indicating that IHF does approach the major groove of DNA. Therefore competitive binding of NifA

and IHF seems plausible. In the case of the *nifU* promoter it is possible that NifA can down-regulate expression by competing for the IHF binding site. This may reflect the increased activation observed when UAS3 is mutated. A more extreme example of this potential negative effect of NifA may be evident at the *nifE* promoter where a UAS is located (17) within a region which binds a factor, again probably IHF, present in crude extracts (2). Only an imperfect UAS is located upstream of this potential IHF binding site in *nifE* (14), and so the higher affinity NifA binding site within the *nifE* promoter region may be very close to the IHF site. Some negative regulation by NifA could limit expression of certain genes which have a catalytic role in nitrogenase biosynthesis, such as *nifE*, *U*, *V*, *M*, by virtue of adjacent IHF and NifA binding sites. Alternatively other features of *nif* promoters which suppress the requirement for upstream bound NifA (3,16) and IHF may be present in the *nifE* promoter to allow it to function efficiently.

ACKNOWLEDGEMENTS

We wish to thank A. Pühler for sequence information prior to publication, S. Kustu for communicating results of binding studies with the *nifH* promoter prior to publication, H. Nash for purified *E. coli* IHF and S. Cohen for *E. coli* IHF mutants. R. Kreutzer was supported by the Bundesministerium für Forschung und Technologie, E. Morett by the National University of Mexico. Ray Dixon, Mike Merrick and Barry Smith are thanked for their valuable comments upon the manuscript and Rosemary Foote for typing.

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