Identification of a Positive Transcription Regulatory Element within the Coding Region of the *nifLA* Operon in *Azotobacter vinelandii*

Ranjana Mitra,* Hirendra K. Das, and Aparna Dixit

Gene Regulation Laboratory, Centre for Biotechnology, Jawaharlal Nehru University, New Delhi 110067, India

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Nitrogen fixation in *Azotobacter vinelandii* is regulated by the *nifLA* operon. NifA activates the transcription of *nif* genes, while NifL antagonizes the transcriptional activator NifA in response to fixed nitrogen and molecular oxygen levels. However, transcriptional regulation of the *nifLA* operon of *A. vinelandii* itself is not fully understood. Using the S1 nuclease assay, we mapped the transcription start site of the *nifLA* operon, showing it to be similar to the σ^{54} -dependent promoters. We also identified a positive *cis*-acting regulatory element (+134 to +790) of the *nifLA* operon within the coding region of the *nifL* gene of *A. vinelandii*. Deletion of this element results in complete loss of promoter activity. Several protein factors bind to this region, and the specific binding sites have been mapped by DNase I foot printing. Two of these sites, namely dR1 (+134 to +204) and dR2 (+745 to +765), are involved in regulating the *nifLA* promoter activity. The absence of NtrC-like binding sites in the upstream region of the *nifLA* operon in *A. vinelandii* makes the identification of these downstream elements a highly significant finding. The interaction of the promoter with the proteins binding to the dR2 region spanning +745 to +765 appears to be dependent on the face of the helix as introduction of 4 bases just before this region completely disrupts promoter activity. Thus, the positive regulatory element present within the BglII-BglII fragment may play, in part, an important role in *nifLA* regulation in *A. vinelandii*.

Azotobacter vinelandii is a gram-negative bacterium which fixes N_2 in the free-living state in the presence of air (6, 15, 16, 39). *Klebsiella pneumoniae* is one of the organisms in which *nifL* regulation is well understood. Though N_2 fixation is a well-studied phenomenon, the regulation of genes differs from one organism to another. Similar to *K. pneumoniae*, the N_2 fixation of *A. vinelandii* is under two types of regulation: global and *nif* specific (19, 26, 28, 43). The *nif*-specific regulation is by the *nifLA* operon and is very complex (18, 32, 45, 46). The activity of *nifA* is decreased following interaction with the *nifL* gene product. It has been shown in *K. pneumoniae* that NifL acts as a redox-sensitive regulatory protein, which modulates NifA activity and allows it to function only in the absence of oxygen and low levels of fixed N_2 . Schmitz et al. (45) showed that different species-specific mechanisms of signal transduction are involved in *nifL* regulation in *K. pneumoniae* and *A. vinelandii*. In *A. vinelandii* the NifL-NifA system responds directly to the physiological concentration of 2-oxoglutarate, resulting in release of NifA from NifL when fixed nitrogen is limited (27). NifA activates the transcription of all other *nif* operons, including *nifHDK* (24). The NifA binding domain is present in the upstream region of all *nif* promoters, except *nifM* and *nifL* (11). However, an NtrC binding site is present upstream of the *nifLA* operon in most organisms. NtrC binding to this site is regulated globally, which in turn positively regulates transcription from the *nifLA* promoter. All the *nif* promoters are σ^{54} dependent (10, 12, 34), and the positive regulatory elements are thought to help in promoter clearance (1).

In response to increased levels of fixed nitrogen or oxygen, the *nifL* gene product directly interacts with NifA (2, 4, 9, 20, 21) and disrupts the contact between NifA and σ^{54} -dependent promoters (5, 33, 35). No such negative regulatory protein has been reported in relation to NtrC.

Brewin et al. (9) have reported that increasing NifA-mediated transcription by either elimination of NifL or overexpression of NifA resulted in ammonia release, correlating with enhanced levels of NifH mRNA, nitrogenase and acetylene reducing activity, and increased concentration of intracellular ammonium.

The *nifL* gene from *A. vinelandii* has been cloned and characterized (7, 41). The upstream region of the *nifLA* operon of *A. vinelandii* does not show any NtrC-like binding site involved in positive regulation of the *nifLA* operon of other nitrogen-fixing organisms. Therefore, it is possible that alternative mechanisms of regulation of *nifLA* operon exist in *A. vinelandii*. The present study was undertaken to identify regulatory regions that could positively regulate the *nifLA* operon, as it does not have the NtrC binding site.

MATERIALS AND METHODS

Reagents. Strains were grown on LB and Burk's nitrogen-free medium (42) supplemented with antibiotics as needed (μ g/ml): ampicillin, 100; kanamycin, 50; chloramphenicol, 200 for *A. vinelandii* and 30 for *Escherichia coli*; and tetracycline, 5 for *A. vinelandii* and 10 for *E. coli*. IPTG (isopropyl- β -D-thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) were used at final concentrations of 0.5 mM and 25 μ g/ml, respectively.

Bacterial strains. *E. coli* DH5 α X-L1 Blue (Stratagene, La Jolla, CA) and *A. vinelandii* UW (6) were used in the study.

Nucleotide number designations in the *nifL* gene. The nucleotide number designation in the current study is based on the transcriptional start site of the *nifLA* operon of *A. vinelandii*, as mentioned in the Results section. The base 1 corresponds to the C at position 274 in the sequence published by Raina et al. (41; GenBank accession no. X70993).

* Corresponding author. Present address: Division of Haematology and Oncology, Indiana University, Department of Medicine, 1044 West Walnut Street, R4-202, Indianapolis, IN 46202-5121. Phone: (317) 274-8498. Fax: (317) 274-0396. E-mail: ranmitra@iupui.edu or rini98@hotmail.com.

FIG. 1. Transcription start site determination by S1 nuclease analysis. The transcription start site (shown by arrow) corresponds to a C present at base no. 274 of the sequence. Lanes marked with A, C, G, and T represent Sanger's dideoxy sequencing reactions using M13 (–40) universal primer and M13mp18 as a template, used as a marker reference. P indicates the single-stranded end-labeled probe.

Plasmids and gene constructs. The multicopy plasmid vectors pBR322, pUC18, and pUC19 (Cm^r, chloramphenicol-resistant version of pUC19 developed by Gary Ditta) were used as intermediate cloning vehicles (13, 44). The plasmid pGD499 (Amp^r, ampicillin resistance; Tet^r, tetracycline resistance), a low-copy-number promoter-cloning vector derived from plasmid RK2 (17) with a neomycin phosphotransferase (Kan^r) promoter and *lacZ* as the reporter gene, was used for the promoter assay. The transcriptional fusion of the *nifLA* promoter and the β -galactosidase gene (plasmid pUK121.2) was constructed by replacing the BamHI-HindIII fragment of pGD499 with the BamHI-HindIII fragment of plasmid pUK121, thus replacing the kanamycin promoter with the *nifLA* promoter and rendering the recombinants ampicillin sensitive (41). A large number of deletion/substitution mutants in the *nifL* gene were generated using simple deletion or oligonucleotide insertion/replacement. The different constructs used in the present study are as follows.

(i) **pUK121 and pRMΔBB.** pUK121 was made with part of the *nifL* gene and the upstream region of the *nifLA* operon of *A. vinelandii*, cloned into pUC19 (41). pRMΔBB was derived from pUK121 by deleting a 591-bp BglII-BglII fragment.

(ii) **pRR410BB.** pRR410BB was derived by inserting a 591-bp BglII-BglII fragment from pUK121 into the cosmid vector pRR410. pRR410 (RSF1010 origin of replication) can coexist with pGD499 (RK2 origin).

(iii) **pRMBBU.2 and pRMBBI.2.** pRMBBU.2 was constructed by cloning the BglII-BglII fragment (591 bp) into the BamHI site of pRMΔBB.2. pRMBBI.2 is similar to pUK121.2, except that the 591-bp fragment is inserted in the opposite orientation.

(iv) **pRMA10, pRMB10, and pRMB4** In pRMA10 and pRMB10, 10 bases were inserted into pUK121 at the BglII sites, respectively, using two partially complementary oligonucleotides (5'-GATCCGGATG-3' and 5'-GATCCATCCG-3') with BamHI overhangs (underlined).

In pRMB4, 4 bases were added at the BglII(b) site by partially digesting with BglII, end filling, and religating it.

(v) **pRMBN1 and pRMBN2.** In pRMBN1, the BglII(b)-NotI fragment was deleted from pUK121, and in pRMBN2, it was replaced by two partially complementary oligonucleotides (5'-GATCAAGGATCAAGTTGC-3' and 5'-GGC CGCAACTTGATCCTTG-3'), producing BamHI and NotI overhangs (underlined).

FIG. 2. Maps of different *nifLA* constructs. The parent plasmid used for all these constructs is pGD499. Names of the plasmids are shown on the left side. The sites shown in smaller font are the ones lost during cloning. Amp^r, β -lactamase gene; PnifLA, *nifLA* promoter; E, EcoRI; B, BamHI; Bg, BglII; H, HindIII. "Kan^r promoter" represents the neomycin phosphotransferase promoter. The effect of the different alterations is shown in Table 1.

(vi) **pRMMB.** In pRMMB, the MluI-BglII(b) fragment was deleted from pUK121 using two partially complementary oligonucleotides (5'-CGCGTGGC AAGGG-3' and 5'-GATCCCCCTTGCCA-3') with BamHI and MluI overhangs (underlined).

All the above mentioned variants of the promoters were cloned in vector pGD499 in the same manner and were designated as *.2 (where * is the original clone in pUC19).

β -Galactosidase assay. The specific activity of β -galactosidase was determined in lysates of exponential-phase culture grown at 30°C by the method of Miller (31). The cells were diluted in *lacZ* buffer (60 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, and 58 mM β -mercaptoethanol) and lysed with sodium dodecyl sulfate (0.01% [wt/vol]) and chloroform (20% [vol/vol]). The reaction was initiated by the addition of ONPG (*O*-nitrophenyl- β -D-galactopyranoside) as a substrate (0.53 mg/ml) and terminated by the addition of Na₂CO₃ (0.25 M) after the appearance of a yellow color. The activity is expressed as Miller units.

S1 nuclease mapping analysis. Total RNA from log-phase cultures of *A. vinelandii* was isolated by the method of Ausubel et al. (3). The S1 nuclease assay was performed with total RNA and one end-labeled single-stranded probe (SmaI-BglII* fragment; labeled at the BglII end). Approximately 1.5 pmol of ³²P-labeled probe was hybridized with 40 μ g of total RNA and digested with S1 nuclease (3). The protected fragments were resolved on a 5% polyacrylamide–7

TABLE 1. Effect of deletion and altered placement of the BglII(a)-BglII(b) fragment on the activity of the *nifLA* promoter in *Azotobacter vinelandii* UW

Construct	Placement of the BglII(a)-BglII(b) fragment	β -Galactosidase activity (Miller units) ^a	
		+NH ₄ ⁺	-NH ₄ ⁺
pUK121.2	As in wild type	1,500 $\pm$ 178	820 $\pm$ 23
pRM Δ BB.2	Deleted	7 $\pm$ 3	6 $\pm$ 1
pRM Δ BB.2 + pRR410BB.2	At BamHI site of pRR410 (different vector)	5 $\pm$ 0.1	6 $\pm$ 0.3
pRMBBU.2	At BamHI site of pRM Δ BB.2	41 $\pm$ 17	66 $\pm$ 20
pRMBBI.2	In inverted orientation in same position as in pUK121.2	6 $\pm$ 0.1	7 $\pm$ 1

^a Values represent the mean $\pm$ standard error of the mean of four experiments.

M urea gel and detected by autoradiography. A standard sequencing reaction (M13mp18 using a ~40-mer universal primer) was included in order to determine the exact base position to the end point of S1 nuclease action, thus determining the start site.

Cell extract preparation. Total cell extract of UW was prepared from mid-log-phase cultures of *A. vinelandii* grown in the presence of ammonia. Cells were lysed in lysis buffer (50 mM Tris-Cl, pH 7.5, 2.5 mM EDTA, 50 mM NaCl, 10% sucrose, 2 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 0.2% lysozyme [wt/vol], and 0.6% Brij-58 [wt/vol]) by repeated freeze-thaw cycles and ultrasonication. Protein content was estimated by the method of Bradford (8).

EMSA. For the electrophoretic mobility shift assay (EMSA), the fragment of interest was end labeled with [α -³²P]dCTP by a filling-in reaction with the Klenow fragment of DNA polymerase I. Total cell extract (~50 μ g protein) was incubated with ³²P-labeled DNA fragment (~1 ng, 2,000 cpm) in binding buffer (50 mM Tris-Cl, pH 7.4, 1 mM dithiothreitol, 2 mM EDTA, 50% [vol/vol] glycerol, 30 μ g/ml bovine serum albumin, 50 μ g/ml fragmented calf thymus DNA) for 0 to 15 min at 37°C. Reaction products were resolved on a 6% nondenaturing pre-electrophoresed acrylamide (29:1) gel in Tris-glycine buffer (0.192 M glycine and 25 mM Tris-HCl, pH 8.3) and visualized by autoradiography.

DNase I footprinting assay. The labeled DNA fragment (~20,000 cpm) was incubated with 25 or 50 μ g of total cell extract protein in binding buffer for 10 min. The reaction was supplemented with 1 mM CaCl₂, 5 mM MgCl₂, and 50 μ g of fragmented calf thymus DNA prior to digestion with DNase I (0.20 U/ml) at 37°C for 90 seconds. The reaction mixtures were analyzed by electrophoresis on a DNA sequencing gel. A standard M13mp18 sequencing reaction with a ~40-mer universal primer was used as a reference.

RESULTS

Mapping of the transcriptional start site of the *nifLA* operon of *A. vinelandii*. The transcription start site mapped using the S1 nuclease assay (Fig. 1) corresponds to the C at position 274 in the sequence reported by Raina et al. (41) and is placed exactly 12 bases downstream of the σ^{54} -like promoter. The transcription start site of the *nifL* gene earlier reported by Blanco et al. (7) is not correctly placed relative to the consensus σ^{54} binding site, which is an absolute requirement for proper promoter activation.

Effect of deletion and altered placement of the BglII(a)-BglII(b) fragment on the activity of the σ^{54} -dependent *nifLA* promoter. Deletion of the 591-bp BglII-BglII fragment present in the coding region of the *nifL* gene from pUK121.2 (construct pRM Δ BB.2) resulted in complete loss of promoter activity (Fig. 2, Table 1). This suggests that the BglII-BglII fragment plays a regulatory role in transcription from the *nifLA* promoter. In order to determine whether this element is able to exert its positive effect in *trans*, the 591-bp fragment was cloned in a different plasmid, pRR410 (compatible with pRM Δ BB.2). Transformation of *A. vinelandii* UW harboring pRM Δ BB.2 with pRR410BB did not increase the activity of the *nifLA* promoter (Fig. 2, Table 1).

To determine if the element can act as an enhancer, it was cloned upstream of the promoter (pRMBBU.2) and in an inverted orientation (pRMBBI.2) and assayed for promoter activity. The promoter activity of these plasmids was found to be negligible in comparison to that of the control plasmid, pUK121.2 (Fig. 2, Table 1).

The BglII-BglII fragment directly affects transcription from the *nifLA* promoter. To establish if deletion of the BglII-BglII fragment blocks transcription initiation from the σ^{54} -dependent promoter, the S1 nuclease assay was performed with total RNA isolated from *A. vinelandii* cells transformed with plasmid pUK121.2 and pRM Δ BB.2. As evident from Fig. 3, the loss in the β -galactosidase activity is due to a significant loss of

FIG. 3. Effect of BglII(a)-BglII(b) deletion on transcription from *nifLA* promoter: Total RNA (30 μ g each) isolated from *A. vinelandii* cells transformed with pUK121.2 (lane 2) and pRM Δ BB.2 [591-bp BglII(a)-BglII(b) fragment deleted] (lane 1) was hybridized with a SmaI-BglII fragment followed by S1 nuclease digestion. P represents the end-labeled probe. An arrow points to the protected fragment.

FIG. 4. Potential hairpin loop regions in the BglII(a)-BglII(b) fragment of the *nifL* gene. Shown is the potential hairpin loop formation of sequences present between positions (A) 309 to 324, (B) 322 to 335, and (C) 543 to 558 in the BglII(a)-BglII(b) fragment.

transcription from *nifLA* promoter in pRMΔBB.2. No signal was obtained when total RNA from the parental UW was subjected to S1 mapping (data not shown). Therefore, the decrease in the intensity of protected RNA isolated from cells transformed with the deletion construct is solely an effect of deletion on promoter activity in the fusion construct (Fig. 3). It has earlier been reported that the fusion construct with *lacZ* probably stabilizes *nifLA* RNA (7). This further ruled out the possibility of an *nifL* protein coding region present upstream of the β-galactosidase gene adversely affecting the enzyme activity and confirmed that the observed effects are due to the direct effect of the BglII-BglII fragment deletion on *nifLA* promoter activity.

Identification of specific protein binding site(s) on the 591-bp BglII-BglII DNA fragment of the *nifL* gene. The 591-bp fragment of *nifL* is GC rich and could form hairpin-like structures (Fig. 4) that can be recognized by DNA-binding proteins. EMSA reactions with the total cell extract revealed that while the mobility of MluI-BglII(b) subfragments was unaltered, the mobility of the other fragment, BglII(a)-MluI, was retarded

FIG. 5. Partial restriction map of construct pUK121 and electrophoretic mobility shift assay using the downstream region of the *nifL* gene [BglII(a)-BglII(b) fragment] and *A. vinelandii* cell extract. (A) The base positions are shown with respect to the transcription start site. For restriction enzymes which are present in this region more than once (*), only the sites used in this study are shown. The thick bar represents the 591-bp BglII-BglII fragment of the *nifLA* operon. The sites are shown as follows: E, EcoRI; Sm, SmaI; B, BamHI; S, SalI; X, XhoI; Bg, BglII; M, MluI; Hi, HincII; A, AvaII; N, NotI; Af, AflIII; and H, HindIII. (B) Gel binding reaction was carried out with 50 μg of total cell extract and 1 ng radiolabeled fragments (~2,000 cpm) in the presence of 1 μg of nonspecific calf thymus DNA both in the presence (+) and in the absence (–) of 10 mM MgCl₂ for 0 or 2 min. BglII(a)-MluI and MluI-BglII(b) are the two fragments derived from a BglII(a)-BglII(b) fragment by digestion with MluI. Lanes 1, 6, and 11 represent respective labeled free fragments.

TABLE 2. Effect of insertion of 10 bases and 4 bases at the BglII(a) and the BglII(b) restriction endonuclease sites on the activity of the *nifLA* promoter^a

Construct	Oligonucleotides used	Site of insertion	No. of bases inserted	β -Galactosidase activity (Miller units) ^b	
				+NH ₄ ⁺	-NH ₄ ⁺
pRM A10.2	5'-GATCCGGATG-3' 3'-GCCTACCTAG-5'	BglII(a)	10	390 $\pm$ 26	350 $\pm$ 21
pRM B10.2	5'-GATCCGGATG-3' 3'-GCCTACCTAG-5'	BglII(b)	10	1,400 $\pm$ 290	940 $\pm$ 29
pRM B4.2	5' Overhangs filled up	BglII(b)	4	45 $\pm$ 1.0	0.0

^a Ten bases represents one full turn of the DNA helix, and 4 bases represents about half a turn.

^b Values represent the mean $\pm$ standard error of the mean of four experiments. Lysates prepared from cells transformed with control plasmid pUK121.2 exhibited 1521 $\pm$ 178 and 818 $\pm$ 23 Miller units of β -galactosidase in the presence and absence of NH₄⁺, respectively.

2). Thus, the protein binding to the BglII(a) site is relevant to regulation of transcription from the *nifLA* promoter.

Role of the DNA sequences immediately downstream of the BglII(b) restriction site in transcriptional regulation of the *nifLA* operon. Unlike disruption of the BglII(a) site, disruption of the BglII(b) site, by introducing 10 bases (construct pRMB10; Fig. 7), did not affect the promoter activity significantly (pRMB10.2, Table 2). However, when only 4 bases were introduced at the BglII(b) site (construct pRMB4.2), it disrupted the face of the DNA helix, leading to a drastic reduction in activity (Table 2). Thus, though the DNA sequence at the BglII(b) site itself is not involved in protein binding and regulation of transcription, sequences downstream of the BglII(b) site are involved in transcriptional regulation. The interaction of the promoter with downstream sequences also appears to be dependent on the face of the DNA helix.

Interaction of sequences immediately downstream of the BglII(b) restriction site with protein factor(s) present in the cell extract. A NotI site is present 12 bp downstream of the BglII(b) site. Addition of these 12 bp to MluI-BglII(b) results in a distinct retarded band (Fig. 8). This is consistent with the involvement of the DNA region just downstream of the BglII(b) site in transcriptional regulation. The protein-binding sequences in this region were identified by DNase I footprint-

ing of the HincII-AflIII fragment labeled at the AflIII end (Fig. 9). Three protected regions, +701 to +720, +745 to +765, and +776 to +790, can be visualized. The second protected region (dR2, +745 to +765) overlaps with the NotI site.

Involvement of the BglII(b)-NotI region in regulation of transcription. To further confirm the role of the 12-bp BglII(b)-NotI region in transcriptional regulation, the BglII(b)-NotI region was deleted from pUK121.2 (Fig. 10) and the resulting construct, pRMBN1.2, was assayed for *nifLA* promoter activity. As shown in Table 3, this deletion resulted in a considerable decline in β -galactosidase activity. In plasmid pRMBN2.2, the 12-bp region was replaced by entirely different bases (Fig. 10), keeping the number of bases between the BglII(b)-NotI sites unaltered. This construct also had decreased promoter activity relative to the control (Table 3).

Interaction between the proteins binding to the downstream regulatory region dR1 (+134 to +204) and dR2 (+745 to +765). The results indicate that the two regulatory regions interact with the protein factors present in the cellular extract and the intervening region between the two sites, i.e., MluI-BglII(b), is not recognized by any protein (Fig. 5 and 9). However, deletion of this region resulted in complete loss of promoter activity (Fig. 10, Table 3). These data suggest that this region is also important for efficient transcription from the *nifLA* promoter and is possibly required for the interaction of the protein factors bound to dR1 and dR2.

FIG. 8. Electrophoretic mobility shift assay using end-labeled MluI-NotI fragment and *A. vinelandii* extract. This was performed to establish if the region downstream of the BglII(b) site forms a complex with cellular proteins. The reaction was performed with 50 µg of total cell extract protein, 1 ng radiolabeled MluI-NotI fragment, and 1 µg of calf thymus DNA both in the absence (– [lane 2]) and in the presence (+ [lane 3]) of 10 mM MgCl₂. An arrow points to the free labeled fragment in lane 1.

DISCUSSION

S1 nuclease mapping data suggest that like *K. pneumoniae*, the *nifLA* promoter of *A. vinelandii* is a σ^{54} -dependent promoter. σ^{54} -dependent promoters are characterized by the consensus sequence 5'-TGGCAC-(N)₅-TTGCA located 11 to 12 base pairs upstream of the transcription start site (19, 30). The σ^{54} -dependent polymerase binds stably and tightly to the promoter and needs activators only for transcription initiation, which requires hydrolysis of ATP (40). In most *nif* operons, the activator is NifA that recognizes its binding site upstream of the promoter region. Unlike other *nif* promoters, the *nifLA* promoter of *K. pneumoniae* needs NtrC as an activator, which is able to sense the fixed nitrogen and oxygen status of the cells through the *glnB* gene product (29). The *nifLA* promoter of *A. vinelandii* shows a single NtrC-like binding site (base no. 144 to 182). For NtrC to regulate the transcription, two NtrC binding sites have to be present as palindromes as seen in *K. pneumoniae*. Earlier studies have shown that NtrC mutant strains

FIG. 9. DNase I footprinting analysis using MluI-BglII(b) and HincII-AflIII fragments. The MluI-BglII(b) fragment (lanes 1 to 3) labeled at BglII(b) and the HincII-AflIII fragment (lanes 4 to 7) labeled at the AflIII end were used for footprinting analysis. Note that no protection is observed in the MluI-BglII(b) fragment (lanes 1 to 3). The three protected regions in the HincII-AflIII fragment (lanes 4 to 7) are indicated at the margin, and the sequences of the same regions are given in panel B. The underlined sequence in panel B represents the NotI site.

are Nif positive, and it may not be playing a role in regulating the *nifLA* promoter of *A. vinelandii* (7, 41).

In the absence of the NtrC regulation, the presence of other positive regulatory elements is not ruled out. Interestingly, we found that the 591-bp BglII-BglII fragment in the coding region of the *nifL* gene contains positive regulatory elements and protein-binding regions. Deletion or altered placement of these elements results in the loss of interaction and obliterates promoter activity. This loss of activity cannot be due to transcriptional polarity as the number of deleted bases is a multiple of 3, keeping the reading frame unchanged. Blanco et al. (7) reported no effect of the deletion of this fragment on the ability of the bacteria to fix nitrogen and excrete ammonium. However, Brewin et al. (9) found no evidence for NifL control of an ammonium release transport system. Based on their studies, ammonium release in *nifL* mutants of *A. vinelandii* could be due to the passive loss of the ammonium ions that accumulated to high intracellular concentrations as a result of prolonged and enhanced nitrogenase expression. This increase in the nitrogenase expression could result from loss of NifL function

FIG. 10. Deletion/substitution constructs of the MluI-NotI region. The bases between the BglII(b) and NotI site have been deleted in pRMBN1 and have been replaced with other bases in the pRMBN2 construct. In the construct pRMBB, the MluI-BglII(b) region has been deleted. Different sites are shown as follows: E, EcoRI; B, BamHI; Bg, BglII; N, NotI; and H, HindIII. The effect of the above deletion and substitution on the promoter activity is shown in Table 3.

TABLE 3. Effect of deletion of the BglII(b)-NotI fragment or its replacement by an insert of the same size on the activity of the *nifLA* promoter

Construct	Fragment deleted or replaced	β -Galactosidase activity (Miller units) ^a	
		+NH ₄ ⁺	−NH ₄ ⁺
pRM BN1.2	<i>BglII(b)-NotI</i> fragment deleted	110 ± 25	120 ± 5
pRM BN2.2	<i>BglII(b)-NotI</i> fragment replaced	390 ± 26	330 ± 21
pRMMB.2	<i>BglII(a)-MluI</i> fragment deleted	100 ± 6	85 ± 2

^a Values represent the mean ± standard error of the mean of four experiments. Lysates prepared from cells transformed with control plasmid pUK121.2 exhibited 1,521 ± 178 and 818 ± 23 Miller units of β -galactosidase in the presence and absence of NH₄⁺, respectively.

or by changing the NifL/NifA ratio. In the present study, we have demonstrated the direct role of the 591-bp BglII-BglII fragment in regulating *nifLA* promoter activity.

The presence of regulatory elements in the coding region has been reported previously. A negative control element within a structural gene was reported in the *gal* operon of *Escherichia coli* (22) and in the *proU* gene (14). However, the presence of a positive regulatory element within the coding region of the gene is very rare. Positive regulatory elements within the coding region of the *flaN* gene of *Caulobacter crescentus* are known but have not been characterized completely (38).

The σ^{54} -dependent RNA polymerase is regarded as a defective enzyme and needs bacterial enhancer binding protein for transcription initiation (36). Kaufman and Nixon (23) have been able to isolate many genes coding for activators needed for initiation of transcription from σ^{54} -dependent promoter in diverse bacterial species. It appears that *A. vinelandii* also contains a family of NtrC-like activators which have binding sites downstream of the promoter within the coding region of the gene in the BglII-BglII fragment.

Two major protein binding sites recognized by DNase I footprinting are directly involved in promoter regulation. One of the sites overlaps the BglII(a) site, and the other overlaps a NotI site. The proteins binding to these sites are involved in regulating promoter activity. The interaction of these elements with promoter is dependent on the face of the helix as insertion of 4 bases decreased the activity.

The intervening sequence between dR1 and dR2, although not recognized by any protein(s), is also essential for promoter activity. It is likely that the factors interacting with dR1 and dR2 also interact with each other. The intervening sequences between the two sites may have a role in bringing the factors binding to these sites in contact with each other by creating the required bend in the DNA. Indeed two integration host factor-like binding sites are present within this region starting at 504 and 520. When integration host factor binds to its recognition site, it creates a bend in the DNA and facilitates the interaction between the proteins bound to either side of the bend. Knaus and Bujard (25) have also shown that interactions between distantly located protein-bound DNA elements are dependent on intervening distances and the proper bending angle. The interaction of the proteins bound to dR1 and dR2 could alter the topology of the DNA lying downstream of the promoter. This in turn might facilitate change in DNA conformation in

the promoter region and initiate transition from a closed complex to an open complex, which is the rate-limiting step of the σ^{54} -dependent promoters (37). In *Caulobacter crescentus* the *flaN* gene, which is dependent on a σ^{54} holoenzyme, requires two *flr* elements present downstream of the transcription start site for initiation. A similar hypothesis has been proposed in other bacteria in which regulatory elements downstream of the promoter have been identified (38).

Thus, the present study has resulted in the identification of a *cis*-acting positive regulatory element of the *nifL* gene located within the coding region. This is the first time that a positive regulatory element downstream of the σ^{54} -dependent promoter has been identified with reference to *nif* genes of *A. vinelandii*.

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