Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 5812–5816, October 1983
Biochemistry

Promoter mutations that allow *nifA*-independent expression of the nitrogen fixation *nifHDKY* operon

(nitrogenase genes/*Klebsiella pneumoniae*/*nifH'–'lacZ* fusion/cruciform structure)

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Communicated by Michael Sela, May 24, 1983

ABSTRACT **The *nifHDKY* operon of *Klebsiella pneumoniae* encodes for structural polypeptides of nitrogenase and requires the *nifA* gene product for transcription. Mutations that allow transcription of the *nifHDKY* operon in absence of the *nifA* gene product were characterized in plasmids containing the regulatory region of *nifHDKY* and *nifH* fused in phase to *lacZ*. β-Galactosidase activity served as a measure for *nifH* expression. Most mutations were located in the *nif* regulatory region and included insertion sequence 2 (IS2) insertions, a sequence duplication, and a base substitution. In *Escherichia coli*, β-galactosidase activity expressed from the mutant plasmids in the absence of *nifA* was 6–30% of the *nifA*-activated, parental level. Expression from most mutant plasmids was further increased by *nifA*. In *K. pneumoniae*, IS2-containing plasmids expressed low levels of β-galactosidase and responded poorly, if at all, to activation by *nifA*, whereas expression from other mutant types was similar to that observed in *E. coli*. Nucleotide sequence analysis of two mutants indicated that sequences within 41 base pairs upstream to the *nifH* coding sequence were involved in *nif*-specific regulation. The results suggest that an inverted repeat element in this region, which could theoretically form a cruciform structure in the DNA, is involved in the transcriptional control of the *nifHDKY* operon.**

Nitrogen fixation functions in *Klebsiella pneumoniae* are encoded by at least 17 genes. The *nif* genes are organized in seven or eight transcriptional units that are contiguously arranged in the chromosome. Expression of nitrogen fixation functions is repressed by fixed nitrogen and by oxygen (for reviews, see refs. 1–3). The *nifLA* operon, which mediates this regulation, is transcriptionally controlled by elements of the general nitrogen metabolism system (4) and is expressed when the bacteria are depleted of fixed N (5–7). The operon encodes two regulatory proteins acting on other *nif* promoters. The *nifL* gene product is thought to be a transcriptional repressor effective in the presence of O_2 and fixed N (8–10), whereas *nifA* encodes an activator required for transcription (7, 11, 12). The mechanism by which *nifA* activates transcription from multiple *nif* promoters and the target sites for *nifA* action have not been elucidated yet. The isolation and characterization of *nif* promoter mutations that relieve the requirement for the *nifA* gene product could be useful in understanding the mechanism of *nif*-specific regulation.

In the present study plasmids were constructed containing the regulatory region of the *nifHDKY* operon and part of the *nifH* coding sequence fused in phase to a truncated *lacZ* gene. Expression of β-galactosidase from these plasmids requires the *nifA* gene product. Mutant plasmids expressing β-galactosidase constitutively were initially isolated in *Escherichia coli* and then studied in *K. pneumoniae*. Most mutations mapped within the regulatory region of the *nif* operon and included insertion sequence 2 (IS2) insertions, a sequence duplication, and a base substitution. Nucleotide sequence analysis of two mutants indicated that sequences included in the 41 base pairs (bp) upstream to the coding sequence of *nifH* function in regulation of the *nif* operon. Furthermore, the results suggest that an inverted repeat within this region, which theoretically could generate a cruciform structure, is involved in the transcriptional control by *nifA*.

MATERIALS AND METHODS

Bacteria and Plasmids. Bacterial strains and plasmids are listed in Table 1. Plasmids pBZ147 and pBZ3230, containing translational *nifH'–'lacZ* fusions (primes denote the deleted parts of the respective genes), were constructed by cloning fragments of *nif* DNA from pSA30 [containing the *nifHDKY* operon (15)] in pMC1403 (13). The latter plasmid is a derivative of pBR322 containing the *lac* operon lacking the regulatory region and the first eight codons of *lacZ*. The absence of a promoter and a translation initiation site prevents the expression of β-galactosidase from pMC1403. To construct pBZ147, a 903-bp fragment of pSA30, containing 291 bp upstream to *nifH* and 204 codons of *nifH* (13, 16), was prepared by digesting pSA30 with *Kpn* I, removing the single-stranded extensions with nuclease S1 and then digesting with *Eco*RI. To generate an in-phase *nifH'–'lacZ* fusion, as defined by DNA sequence analyses of the two genes (13, 16, 17), the fragment was ligated to pMC1403, which was first digested with *Bam*HI, treated with nuclease S1 and then treated with *Eco*RI. Plasmid pBZ3230 was constructed from a fragment generated by *Eco*RI and partial *Sau*3A digestion of the *nif* DNA from pSA30, which was cloned into pMC1403 digested with *Eco*RI and *Bam*HI. Ligation of the *nif* DNA cleaved by *Sau*3A in the site overlapping codons 119 and 120 of *nifH*, with the *Bam*HI-generated end of *lacZ*, theoretically forms another in-phase *nifH'–'lacZ* fusion. Plasmid pNR300 (identical to pMC71A; ref. 12) contains a *Sal* I fragment from pGR112 (18), including part of *nifL* and the entire *nifA* gene. The plasmid directs the constitutive expression of *nifA*, presumably from the *tet* promoter in the cloning vector (12).

Growth Conditions. *E. coli* cells were grown aerobically in LB or M9 minimal medium supplemented with 0.4% lactose and the appropriate amino acids. *K. pneumoniae* cells were grown aerobically in LB medium. Depending on the strains and transforming plasmids, antibiotics were added as follows: penicillin G (200 μg/ml), streptomycin (100 μg/ml), chloramphenicol (25 μg/ml), and tetracycline (10 μg/ml).

Assays of β-Galactosidase Activity. *E. coli* or *K. pneumoniae* cultures growing exponentially in LB medium at 30°C were assayed as described (19).

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Abbreviations: IS2, insertion sequence 2; bp, base pair(s); kb, kilobase(s); kDa, kilodalton(s).

Table 1. Bacterial strains and plasmids

Strain	Relevant genotype or phenotype	Source or ref.
E. coli		
MC1061	*hsd*R Δ*lac*X74	13
pp2010	*cya rec*A Δ*lac*X74	14
K. pneumoniae		
KP5611	His⁻ *nifA*⁻	F. Ausubel
Plasmids		
pNR300	*nifA* Amp^R	12
pBZ147	*nifH'–'lacZ*, Amp^R	This work
pBZ3230	*nifH'–'lacZ*, Amp^R	This work
pRB1	Lac$^+$ derivative of pBZ147	This work
pRB5	Lac$^+$ derivative of pBZ3230	This work
pRBI9	Lac$^+$ derivative of pBZ3230 (contains IS2)	This work
pRBI10	Lac$^+$ derivative of pBZ147 (contains IS2)	This work
pRBI20	Lac$^+$ derivative of pBZ3230 (contains IS2)	This work
pRBI61	Lac$^+$ derivative of pBZ147 (contains IS2)	This work

Blot Immunoassays of Cell Extracts. *E. coli* cells growing exponentially in LB medium were extracted essentially as described (20). Aliquots containing 400 μg of protein were electrophoresed on a 5% polyacrylamide gel in the presence of $NaDodSO_4$. The gel was blotted electrophoretically onto a nitrocellulose sheet that was then treated with rabbit antibodies against nitrogenase reductase (*Kp2–nifH* gene product), followed by fluorescein-labeled, goat anti-rabbit immunoglobulin antibodies (Miles-Yeda, Rehovot, Israel), essentially as described (21).

DNA Sequence Analysis. Nucleotide sequence analysis was done by using the procedure of Maxam and Gilbert (22) with some modifications (23).

RESULTS

Isolation and Characterization of Mutants. Two different translational *nifH'–'lacZ* fusions were constructed starting from pSA30 and pMC1403. Both constructs contain 291 bp of *nif* DNA upstream to the *nifH* coding sequence and 204 codons (pBZ147), or 119 codons (pBZ 3230), of *nifH* fused to the eighth codon of *lacZ* (Fig. 1). The in-phase fusions were designed on the basis of the published nucleotide sequences of *nifH* (16, 17) and *lacZ* (13). None of the recombinant plasmids expressed β-galactosidase in *E. coli* MC1061, in keeping with previous observations (12). To select for Lac$^+$ spontaneous mutants, the transformed *E. coli* cells were plated on minimal medium containing lactose. Lac$^+$ colonies appeared at a frequency of approximately 10^{-8}. Plasmid DNA isolated from mutant clones was used to transform *E. coli* pp2010. All the transformants selected for penicillin resistance also displayed a Lac$^+$ phenotype on McConkey indicator plates (19) and grew on lactose minimal medium. These results showed that the Lac$^+$ phenotype was due to mutations in the plasmid and not in the host chromosome.

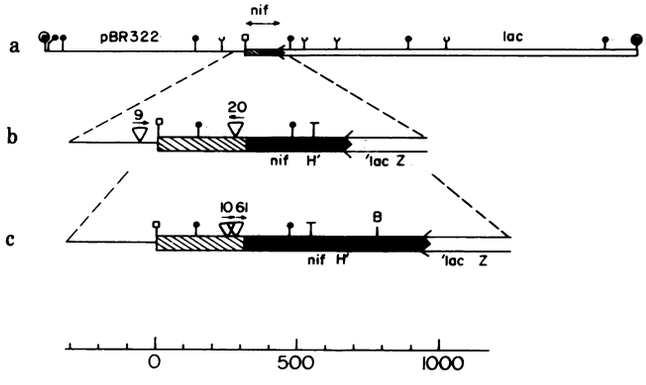

FIG. 1. Structure of recombinant plasmids containing *nifH'–'lacZ* fusions. General structure of pBZ3230 and pBZ147 linearized with *Sal* I (*a*), showing some of the restriction sites present. An expanded map of the *nif* DNA and adjacent vector sequences is shown for pBZ3230 (*b*) and for pBZ147 (*c*). The maps show restriction sites used in the analysis of the parental plasmids as well as the derived mutants. ♀, *Sal* I; ♖, *Eco*RI; ♦, *Bgl* I; ♀, *Hin*cII; ⊤, *Taq* I; ♦, *Bgl* II. The scale describes the map coordinates in bp for *b* and *c*. ▽, sites of IS2 insertion in the pRBI plasmids of the numbers designated. An arrow above the triangle pointing rightward describes the antipolar (II) orientation of the element. ▨, *nif* sequence upstream to the *nifH* coding sequence; ■, *nifH* coding sequence.

Mutant plasmids were first characterized by restriction analysis (data not shown). Most of the plasmid isolates contained an insertion of ≈1.3 kilobases (kb). With one exception, all insertions were mapped to the larger of the two *Eco*RI–*Sal* I fragments containing the *nif* and *lac* sequences (Fig. 1). In one plasmid, pRBI9, the insertion was located in the pBR322 part of the recombinant plasmid. To characterize the 1.3-kb insertion, several plasmids were further analyzed by restriction with *Hin*dIII, *Bgl* I, *Hin*dII, and *Eco*RI. The results indicated that the restriction map of the inserted DNA fully corresponded to the map of IS2 (24) and that the element in these plasmids had inserted within the *nif* DNA upstream to the *nifH* coding sequence. In most plasmids analyzed the element was inserted in its antipolar (II) orientation (24, 25) with respect to *nifH*. In pRBI20, IS2 was inserted in the polar (I) orientation. The sites of insertion and the polarity of IS2 in four mutant plasmids, pRBI9, pRBI10, pRBI20, and pRBI61, are shown in Fig. 1. Of the mutants without IS2 insertions, the restriction map of pRB1 was indistinguishable from that of the Lac$^-$ parent plasmid and another plasmid, pRB5, had an ≈90-bp insertion in the *nif* DNA.

In vitro recombination of restriction fragments from the parent and mutant plasmids localized most mutations in the *Eco*RI–*Sal* I fragment containing the *nif* and *lac* sequences (Fig. 1). The mutation in pRBI9 was located in the *Eco*RI–*Sal* I fragment containing the pBR322 sequence.

The following experiments compare the efficiency of expression from the mutant and parental plasmids in *E. coli* and in a *nifA*⁻ strain of *K. pneumoniae*, in the presence or absence of the *nifA* gene product. [Although the *K. pneumoniae* strain used contains *nifL*, this gene is not expressed in cells grown in a medium rich in fixed N (1).] To test the effect of *nifA*, the cells were cotransformed with pNR300, a plasmid identical to pMC71A constructed by Buchanan-Wollaston *et al.* (12). The plasmid expresses *nifA* constitutively and is compatible with the plasmids containing *nifH'–'lacZ*. The results (Table 2) show that both *E. coli* and *K. pneumoniae* transformed with the parental plasmid pBZ147 or pBZ3230 do not express detectable levels of β-galactosidase, unless the cells are cotransformed with pNR300. In *E. coli*, different mutant plasmids expressed constitutively 6–30% of the β-galactosidase activity observed with the parental plasmids in the presence of *nifA*. In four of the plasmids tested the mutations did not abolish the response to the *nifA* gene product because cotransformation with pNR300 raised the expression to 76–98% of the fully activated parental level. One IS2-containing plasmid, pRBI20, did not respond to *nifA*.

Comparison of β-galactosidase levels in *E. coli* and in *K. pneumoniae* revealed a pronounced difference between pRB1 and pRB5 as compared to the IS2-containing plasmids. The rel-

Table 2. β-Galactosidase activity expressed from different plasmids in *E. coli* and *K. pneumoniae*

Plasmid	Type of mutation	*E. coli nifA* −	*E. coli nifA* +	*K. pneumoniae nifA* −	*K. pneumoniae nifA* +
pBZ147	Parent	<0.1	100	<0.2	100
pBZ3230	Parent	<0.1	100	<0.2	100
pRB1	Point	7	98	7	96
pRB5	Duplication	27	95	18	82
pRBI9	IS2	28	ND	0.7	3.5
pRBI10	IS2	30	83	1.2	1.5
pRBI20	IS2	6	6	<0.1	<0.1
pRBI61	IS2	14	76	<0.1	3.5

The bacterial strains used were *E. coli* pp2010 and *K. pneumoniae* 5611. *nifA* was introduced by cotransformation with pNR300. Growth of bacteria and β-galactosidase assay conditions were as described in the text. Enzymatic activity specified by mutant plasmids is expressed relative to the respective parental plasmids. Activity of 100% ranged in different experiments between 6,000 and 7,000 units (as defined in ref. 19) in *E. coli*, and it ranged between 4,000 and 5,000 units in *K. pneumoniae*. ND, not determined.

ative levels of expression from pRB1 and pRB5 were very similar in the two organisms both in the presence and absence of *nifA*, whereas expression from the IS2-containing plasmids was strikingly different in *K. pneumoniae* and in *E. coli*. The constitutive level of expression from the IS2-containing plasmids, pRBI9 and pRBI10, was very low in *K. pneumoniae* compared with *E. coli*. The expression from two other plasmids, pRBI20 and pRBI61, was below the level detectable in the assay. Low levels of activation by *nifA* were observed with pRBI9 and pRBI61 but not with pRBI10 or pRBI20.

Immunological Characterization of the Hybrid Proteins. Blot immunoassays were performed to confirm that the bacteria expressing β-galactosidase contained proteins bearing antigenic determinants of the *nifH* gene product with molecular weights expected of the *nifH'–'lacZ* fusions. Extracts of *E. coli* cells transformed with mutant plasmids, or cotransformed with the parental plasmids and pNR300, were separated by gel electrophoresis and electroeluted onto nitrocellulose sheets. Proteins containing *nifH* antigenic determinants were detected by using anti-Kp2 antibodies (Fig. 2). Each of the extracts showed one strongly immunoreactive band and several faint bands. The faint bands were also visible in extracts of untransformed cells (not shown) and were not *nif* specific. The mutant plasmids pRB1 and pRB5 directed the constitutive synthesis of proteins of ≈150 and ≈145 kilodaltons (kDa), respectively. These proteins comigrated with the major immunoreactive proteins synthesized in the presence of *nifA* from pBZ147 (not shown) and pBZ3230, respectively. The molecular weights of the Kp2 crossreacting proteins are in good agreement with the values expected from the *nifH'–'lacZ* fusions constructed. Similar proteins were also observed in extracts of cells transformed with the IS2-containing plasmids pRBI9, pRBI10, pRBI20, and pRBI61. Thus, β-galactosidase activity indeed reflects the expression of *nifH* and the mutations do not affect the nature of the hybrid proteins synthesized.

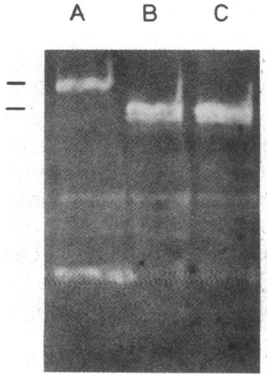

FIG. 2. Immunological characterization of the *nifH'–'lacZ* gene product. Blot immunoassays were performed as described and the analyses included extracts of *E. coli* pp2010 transformed with pRB1 (lane A); pRB5 (lane B); and pBZ3230 and pNR300 (lane C). The lines indicate the bands corresponding to proteins of ≈150 and ≈145 kDa.

Nucleotide Sequence Analysis of Mutants. The nucleotide sequence in the region of the mutated sites was determined in plasmids pRB1, pRB5, as well as in pRBI9 and pRBI20. In all cases sequence analysis was performed from the *Eco*RI site toward the *nifH* coding sequence. In pRBI9, the sequence was also determined from the *Eco*RI site into the pBR322 sequence. The nucleotide sequences of the parental pBZ147 and mutants pRB1 and pRB5 are shown in Fig. 3. Our sequence analysis of the wild-type *nif* sequence upstream to *nifH* generally confirmed the previously reported sequence (16), except for an additional A·C dinucleotide at positions −189 and −190 (the A of the start codon of *nifH* is defined as +1). The addition of the A·C sequence generates a *Bgl* I recognition site between positions −193 and −183. The presence of this site was confirmed experimentally.

The sequence of pRB1 was found to differ from that of pBZ147 in a single position. In pRB1 an A residue replaces a C at position −37. This mutation introduces a mismatch into a perfect inverted repeat found between positions −41 to −35 and −11 to −5 (Figs. 3 and 4). The sequence of pRB5 is identical to that of the wild-type sequence between the *Eco*RI site and position +48, the last nucleotide of codon 16 of *nifH*. Following this position is an 89-bp-long duplication of the sequence between −41 and +48, after which the pBZ147 sequence resumes with the first nucleotide of codon 17. The sequence of pRB5 thus contains an interrupted and a complete *nifH* coding sequence. Interestingly, position −41 is also the start of the 7-bp inverted-repeat sequence distal to *nifH*.

The sequence of pRBI20 (data not shown) is identical to the

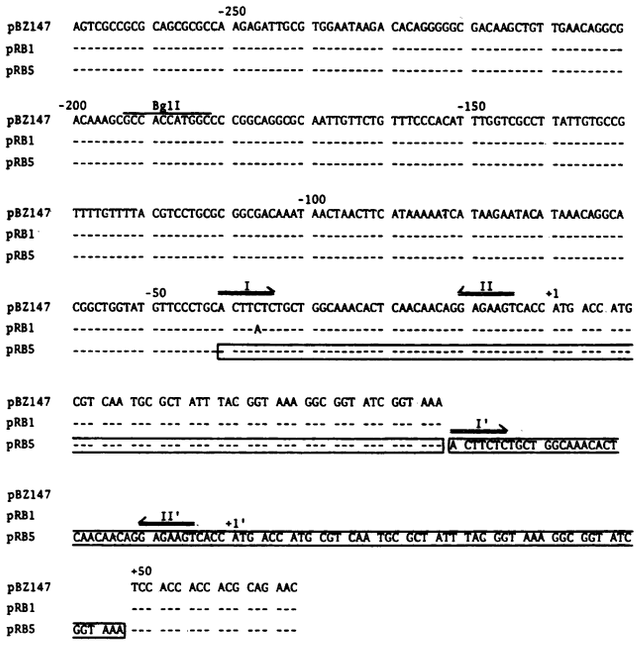

FIG. 3. Nucleotide sequence of parental and mutant plasmids. The sequence analysis method and strategy used are described in the text. The sequence analysis of pRB1 (containing a point mutation) was run in parallel to pBZ147 to facilitate the identification of the mutated site. Inverted repeats are overlined and marked I and II. The sequence duplicated in pRB5 is boxed. The inverted repeats in the duplicated sequence are marked I' and II'.

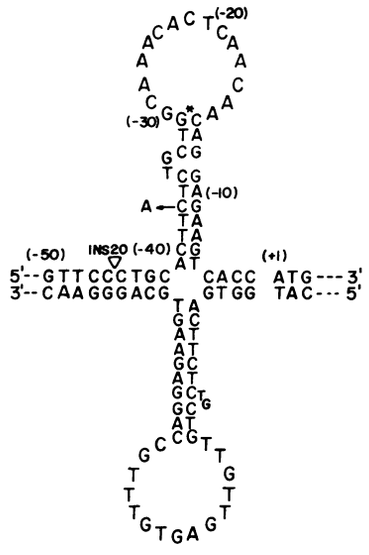

FIG. 4. Proposed cruciform structure in the regulatory region of the *nifHDKY* operon. The mutations in pRB1 and pRBI20 are indicated only in one of the strands. The IS2 element in pRBI20 is designated as INS20. The asterisk denotes the site of transcription initiation (26).

sequence of pBZ147 to position −45. From this point on, the sequence is identical to that of IS2 inserted in its polar (I) orientation (24, 25) with respect to *nifH* (Fig. 1). Sequence analysis of pRBI9 (data not shown) indicated that IS2 was inserted in its antipolar (II) orientation in the pBR322 sequence, between positions 4,314 and 4,315 (coordinates as in ref. 27). The site of insertion is 337 nucleotides upstream to the coding sequence of *nifH*.

DISCUSSION

The expression in *K. pneumoniae* as well as in *E. coli* of the *nifH'–'lacZ* fusions constructed in this study requires the *nifA* gene product. Similar results were reported previously for a different *nifH'–'lacZ* construction (12). This indicates that the hybrid gene in the plasmids studied is under *nif* specific control and hence that the regulatory region of the *nifHDKY* operon, containing the target for *nifA* action, is included within the 291 bp of *nif* DNA upstream to the coding region of *nifH*. In this report we describe several mutations that partially relieve the expression of the *nif* operon from the requirement for *nifA*. All of the mutations allowing partially constitutive expression of the *nifH'–'lacZ* fusions map in the plasmids containing the hybrid genes and not in the bacterial hosts. Polypeptides of similar size bearing nitrogenase reductase antigenic determinants were observed in extracts of *E. coli* transformed with mutant plasmids or cotransformed with the parent plasmids and the *nifA*-containing plasmid pNR300. These observations, together with the analysis of individual mutants, indicate that the mutations studied affected in *cis* the regulation of the *nifHDKY* operon.

In the following discussion we assume that the different plasmids do not differ in their copy number and hence that the levels of β-galactosidase activity provide an indication for expression efficiency. Also, because *lacZ* is fused to the promoter proximal *nifH* gene, effects on the levels of expression due to polarity within the operon are avoided.

Constitutive expression from plasmids with IS2 insertions was much more efficient in *E. coli* than in *K. pneumoniae*. The IS2 element in pRBI9, PRBI10, and pRBI61 has been inserted in the antipolar (II) orientation with respect to *nifH'–'lacZ*. The simplest interpretation of the constitutive expression from these plasmids in *E. coli* assumes that the IS2 element provides an alternative promoter function. Evidence for transcription starting within IS2 and proceeding into the adjacent DNA has been described (25). The site of IS2 insertion in pRBI9 is in the pBR322 sequence and is separated from the *nifH'–'lacZ* fusion by 337 bp of *nif* DNA (Fig. 1). Scott *et al.* (16) pointed out that the sequence between positions −149 and −122 can be arranged in a structure characteristic of a transcription termination signal and proposed it to be the site of transcription termination of the *nifJ* operon, located upstream to the *nifHDKY* operon. However, this signal cannot be effective in pRBI9 if transcription starts within the IS2 element.

In contrast to the other IS2-containing plasmids, pRBI20 contains an IS2 element inserted in the polar (I) orientation with respect to *nifH'–'lacZ*. Activation of downstream genes by IS2 inserted in the polar orientation has been reported (25). In some cases, activation was correlated with structural alterations within the IS2 element (25). We cannot exclude the possibility that the IS2 present in pRBI20 has an altered structure. Alternatively, the IS2 element in pRBI20 may not provide a promoter but rather may perturb the structure of the DNA adjacent to the site of insertion and thus activate the *nifHDKY* promoter.

The observation that expression from pRBI20 is not activated by *nifA* suggests that the sequence around position −45 (or position −50, because IS2 insertion generates a 5-bp duplication of the target DNA) might be important for *nifA* recognition or binding. This conclusion is compatible with recent studies with *K. pneumoniae*. Multicopy plasmids carrying the *nifHDKY* promoter were found to inhibit nitrogenase expression in wild-type *K. pneumoniae*. This effect was attributed to competition between the multicopy *nif* promoter and other *nif* promoters for the *nifA* gene product (10). Mutations relieving the inhibition of nitrogenase expression by a cloned *nifHDKY* promoter were mapped to positions −42 and −44 (F. M. Ausubel, personal communication) (Figs. 3 and 4). These mutations presumably reduce the affinity of the target site for the *nifA* gene product. Homologies were found between this region and corresponding regions in the *nifLA* operon from *K. pneumoniae* (28) as well as in *nifH* from *Rhizobium meliloti* (26). Recent studies showed these two operons could also be activated by the *nifA* gene product (26, 28).

The IS2-containing plasmids expressed in *K. pneumoniae* low or undetectable levels of β-galactosidase and the *nifA* gene product had little or no activating effect. The low activity was not due to plasmid loss as indicated by recovery of plasmid DNA from the transformed cells. Thus it appears that the presumed IS2 promoter functional in *E. coli* has little or no activity in *K. pneumoniae*. Furthermore, in *K. pneumoniae* IS2 appears to hinder the activation by *nifA* of transcription from the adjacent *nifHDKY* promoter. The cause for this effect is unknown.

In contrast to the IS2-containing plasmids, expression efficiencies from pRB1 and pRB5 are similar in *E. coli* and *K. pneumoniae*. In the presence of *nifA*, expression from both plasmids is enhanced to a level close to that of the fully activated, parent plasmids. A plausible interpretation of these results is that the mutations in pRB1 and pRB5 partially relieve the constraints on the *nifHDKY* promoter, which prevent its expression in the absence of *nifA* while still conserving the response to *nifA*.

As previously pointed out (16), a 10-bp imperfect inverted repeat (including a 7-bp perfect repeat) is found 5′ to the *nifH* coding sequence (Figs. 3 and 4). A putative ribosomal binding site is encoded by the repeat closer to the coding sequence. On this basis it was proposed that a stem and loop structure in the mRNA transcribed from this region could function as a translational regulatory element (16). However, recent studies have identified position −30 as the start of the *nifHDKY* transcript

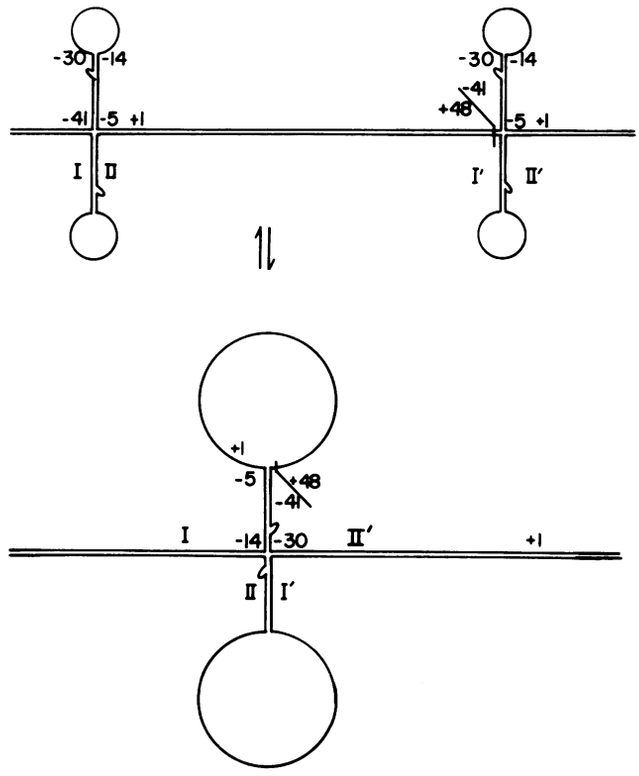

FIG. 5. Alternative intrastrand base pairing in the duplication mutant pRB5. A schematic representation of two possible structures. The inverted repeats are marked as in Fig. 3. The positions indicated mark the start and end of the intrastrand base-paired sequences. The inverted repeats are designated as in Fig. 3.

(26). Thus, transcription starts between the two inverted repeats and the stem and loop structure cannot be formed in the mRNA. Based on the mutations in pRB1 and pRB5 we propose that a cruciform structure generated by intrastrand base pairing between inverted repeats might be a critical element in the transcriptional control of the *nifHDKY* operon. Secondary structures involving intrastrand base pairing have been previously observed in supercoiled DNA (29) and proposed to play regulatory roles (e.g., ref. 30). The 7-bp region of perfect homology in the cruciform arm contains three noncontiguous C·G pairs. The elimination of the central pair by the C to A transversion should destabilize this structure. The duplication in pRB5 starts precisely at the branching point of the proposed cruciform structure and includes the inverted repeats. The duplication may thus result in the formation of two cruciform structures or may allow alternative intrastrand base pairing (Fig. 5). In the latter event, one pair of repeats may not form the secondary structure proposed.

According to this model, formation of the secondary structure in the DNA limits the access or hinders the activity of the transcription apparatus. One function of the *nifA* gene product would be to eliminate these constraints by destabilizing the secondary structure. Both the point and duplication mutations would be expected to affect the stability of the cruciform structure but not to eliminate it altogether. This is consistent with the observation that the mutants are only partially constitutive and still respond to *nifA*. The latter observation also indicates that the structural alterations in pRB1 and pRB5 do not abolish direct or indirect recognition by *nifA*, although perhaps the binding affinity is reduced. Activation of the *nifHDKY* operon by *nifA* may thus involve DNA sequences immediately flanking the start site of transcription. This is in contrast to operons activated by the cAMP receptor protein–cAMP complex (e.g., *lac, ara*), where target sites for positive regulation were located further than 30 bp upstream to the mRNA start site (31).

Activation by *nifA* is a feature common to most, if not all, *nif* promoters. Therefore, it will be of interest to search other *nif* promoter sequences for inverted repeat elements of the type proposed here as targets for the action of the *nifA* gene product.

We are grateful to R. R. Eady for anti-Kp2 antibodies and to F. M. Ausubel, A. Danchin, R. Dixon, and C. Elmerich for bacterial strains and for communicating results prior to publication. This work was supported by a grant from the U.S.–Israel Binational Science Foundation. RB was supported by Institut National de la Recherche Agronomique (France).

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