Transcriptional Analysis of Promoter Mutations in the *Klebsiella pneumoniae* nifHDKY Operon

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Previously isolated promoter mutations that allow expression of the *Klebsiella pneumoniae* nifHDKY operon in the absence of nifA (R. Bitoun, J. Berman, A. Zilberstein, D. Holland, J. B. Cohen, D. Givol, and A. Zamir, Proc. Natl. Acad. Sci. USA, 80:5812–5816, 1983) were further characterized. pRB1 and pRB5, containing, respectively, point and duplication mutations in the nifHDKY regulatory region, were transformed into *Escherichia coli* and *K. pneumoniae* hosts with different nifA and ntrA backgrounds. nif transcription start sites were determined by nuclease S1 mapping. The results indicated that nifA-independent expression from both mutants did not require ntrA. Transcription from pRB5 started 3 base pairs (bp) upstream of the start site of nif-regulated transcription and could stem from a canonical promoter sequence generated at the junction between the two copies of the duplicated sequence. In the presence of nifA-ntrA, transcription from pRB5 started predominantly at the site characteristic of the nif-regulated promoter. The site of constitutive transcription initiation in pRB1 was located 33 bp upstream of the point mutation and 40 bp upstream of the start of nifA-ntrA-activated transcription. Low-level transcription from the upstream site was also evident, in the absence of nifA or nifA or both, with the plasmid containing the wild-type nifHDKY regulatory region. However, when nifA and ntrA were present to activate transcription from the major nif promoter, no activity was evident from the upstream site in either pRB1 or the parental plasmid. Thus, the mutation enhanced the activity of a pre-existing constitutive promoter, the activity of which was repressed on nifA-ntrA activation of the major nif promoter.

In *Klebsiella pneumoniae*, the genetically best studied diazotroph, the nitrogen fixation function is encoded in 17 contiguous nif genes, arranged in seven to eight operons. nif gene expression is repressed in the presence of ammonia, or certain amino acids, and in the presence of oxygen; derepression occurs under anaerobic, nitrogen-limiting conditions (for reviews, see references 7 and 14). Nitrogen availability to the cell is sensed by the ntr system which regulates, in addition to nif, several other pathways of nitrogen assimilation (10). Under nitrogen starvation conditions, products of ntrA and ntrC jointly activate transcription from the nifLA promoter. The nifA product, together with the ntrA product, then activate all the remaining nif promoters. The function of the nifL product, on the other hand, is to repress nif transcription in response to rising concentrations of fixed nitrogen or oxygen (7).

The mechanisms underlying nif-specific regulation are only partially understood. Mapping of transcription start sites in different nif operons revealed a common structural feature in the putative promoter regions: two regions of conserved sequence located around positions −12 and −24 with respect to the transcription start sites (3). These nif-specific sequences show no homology to the sequences at positions −10 and −35 characteristic of most *Escherichia coli* promoters (8, 15).

We have previously isolated and characterized nif promoter mutations that allow expression in absence of the nifA product (4). The mutations were detected by selection of Lac+ derivatives of a Lac− ntrA+ *E. coli* strain (that normally does not contain nifA), transformed with plasmids containing the nifHDKY promoter followed by a nifH'−lacZ hybrid gene. One of the mutants, pRB1, had a C to A transversion at position −7 with respect to the wild-type transcription start site. In another mutant, pRB5, an 89-base-pair (bp) duplication of the sequence between positions −12 and +77 occurred. The level of β-galactosidase expressed from the mutant plasmids was lower than that observed from the parental, wild-type plasmids in the presence of nifA (constitutively expressed from a cotransformed plasmid). However, when the nifA product was provided to each of the mutants, expression was elevated to nearly the level of the wild type, indicating that the mutations did not abolish nifA action.

In this study, two additional criteria were used to compare expression from wild-type and mutant plasmids: the requirement for activation by ntrA and the sites of transcription initiation. The results show that while one of the mutations generates an adventitious promoter sequence, another mutation enhances the activity of a pre-existing, upstream promoter. The activity of this promoter, in both mutant and parental plasmids, is repressed when nifA and ntrA are present to activate the major downstream promoter. Thus, the mutations generate, or activate, promoters other than the positively regulated major nif promoter.

**MATERIALS AND METHODS**

Bacterial strains, plasmids, and growth conditions. *E. coli* and *K. pneumoniae* strains and the plasmids used in this study are listed in Table 1. Bacterial cultures were grown aerobically in LB medium containing, as necessary, antibiotics for the selection of transformants: 200 μg of penicillin G per ml, 10 μg of tetracycline per ml, 25 μg of chloramphenicol per ml. *E. coli* was grown at 37°C, and *K. pneumoniae* was grown at 30°C.

**Recombinant DNA procedures.** Manipulations of DNA and transformations were carried out as described previously (12).

**DNA sequencing.** Nucleotide sequence analysis was per-
TABLE 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains and plasmids</th>
<th>Relevant Genotype or Phenotype</th>
<th>Source or reference</th>
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</thead>
<tbody>
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<tr>
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<td>K. pneumoniae</td>
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</tr>
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<td>F. Ausubel</td>
</tr>
<tr>
<td>KG6237</td>
<td>ntrA</td>
<td>9</td>
</tr>
<tr>
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<tr>
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<td>nifA Cm'</td>
<td>4</td>
</tr>
<tr>
<td>pBZ147</td>
<td>nifH'-lacZ Ap'</td>
<td>4</td>
</tr>
<tr>
<td>pBZ3230</td>
<td>nifH'-lacZ Ap'</td>
<td>4</td>
</tr>
<tr>
<td>pRB1</td>
<td>Lac+ derivative of PBZ147</td>
<td>4</td>
</tr>
<tr>
<td>pRB5</td>
<td>Lac+ derivative of pBZ3230</td>
<td>4</td>
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formed by the procedure of Maxam and Gilbert, with some modifications (18).

**RNA preparation.** RNA from *E. coli* and *K. pneumoniae* was isolated essentially as described previously (19) and purified by centrifugation in a CsCl gradient (12).

**Nuclease S1 mapping.** The procedure used was essentially that described previously (12) with some modifications. DNA probes (10 to 50 ng, 5 × 10^6 to 10^7 cpn) were hybridized to 20 μg of DNA at 52°C for 12 h. Digestion with 300 U of nuclease S1 (Boehringer Mannheim) was carried out at 37°C for 45 min. The protected DNA fragments were analyzed by electrophoresis on 6% polyacrylamide gels.

**RESULTS**

The general structure of pBZ147 and pBZ3230, the parental plasmids containing the wild-type *nifHDKY* promoter followed by a nifH'-lacZ hybrid gene, is shown in Fig. 1. pBZ147 and the mutant derivatives pRB1 (Fig. 2C) and pRB5 (Fig. 3C) were transformed into *E. coli* and *K. pneumoniae* hosts with different nifA and ntrA backgrounds. (It should be noted that the ntrA+ and ntrA strains of *K. pneumoniae* compared in this study were nonisogenic.) Results of β-galactosidase assays (data not shown) indicated that, in the absence of nifA, the levels of activity expressed from pRB1 and pRB5 in a ntrA+ *K. pneumoniae* host were similar to the levels previously observed in a ntrA strain (4). Introduction of nifA into the ntrA strain had no effect on the levels of expression in the ntrA+ host (4).

To further characterize nif expression from the mutant plasmids, nif transcripts from the different transformants were subjected to nuclease S1 mapping.

**Mapping of pRB1 transcripts.** The DNA probe used for S1 mapping was prepared as follows. The 730-bp EcoRI-BglII fragment from pRB1 (Fig. 1) was digested with Hinfl; the resulting fragments were 5' 32P-phosphorylated with T4 polynucleotide kinase and digested with NcoI; and the largest of the labeled fragments was isolated. Products of nuclease S1 reactions were electrophoresed alongside with products of sequencing reactions of the probe fragment.

The analysis (Fig. 2A) indicates that nifA-independent transcription from pRB1 (lane c) initiates upstream to the major start site of nifA-ntrA-activated transcription (lane d). As already suggested by results of β-galactosidase assays, transcription from the upstream site in pRB1 does not require ntrA, both in *E. coli* (lanes c and h) and in *K. pneumoniae* (lanes e and f). Transcripts initiated at this upstream site are not unique to pRB1, but they are also found in much lower amounts in nifA *E. coli* transformants with the wild-type pBZ147 (seen faintly in lane b). In the presence of nifA (and ntrA) (Fig. 2B), transcription from both pBZ147 (lane c) and pRB1 (lane b) occurs predominantly from the major nif-regulated promoter, and transcription from the upstream promoter is repressed.

The start site of nifA-ntrA-independent transcription in pRB1 (Fig. 2C) is located 33 bp upstream of the point mutation and 40 bp upstream of the start of nifA-ntrA-activated transcription. Examination of the sequence upstream of the start site reveals some homologies to the canonical promoter sequences at positions −10 and −35.

**Mapping of pRB5 transcripts.** The DNA probe used for nuclease S1 mapping was similar to the one used in the analysis of pRB1 but, in the absence of a BglII site, NarI was used together with EcoRI to generate the initial fragment. The S1 analysis (Fig. 3A) showed that, in the absence of nifA, transcription in both *E. coli* and *K. pneumoniae* transformants with pRB5 initiated 3 bp upstream of the major, wild-type start site, within the downstream copy of the 89-bp duplicated sequence. Examination of the sequence upstream of this start site (Fig. 3C) reveals a homology to a −10 element spanning the junction of the two repeated sequences, and a homology to a −35 element within the upstream nifH coding sequence.

Transcription from pRB5 was also studied in the presence of nifA-ntrA. The results (Fig. 3B, lane b) indicated that transcription started predominantly upstream to the duplicated sequence at the typical site of the major nif promoter (Fig. 3C). Transcription from the site used in constitutive expression from pRB5 was drastically decreased but still evident under these conditions.

**Basal transcription from the wild-type promoter.** As shown in Fig. 1, the RNA transcript of the wild-type promoter begins at a sequence 3' of the transcription start site. The RNA transcript is terminated at the lac operator site (34). The lac operator is located at a distance from the transcription start site that is consistent with the length of the RNA transcript.

FIG. 1. Structure of the parental plasmids pBZ147 and pBZ3230. The pBR322 sequence is indicated with a line; the nifHDKY regulatory region (17) is indicated with a filled box; the nifH coding region (612 bp in pBZ147, 357 bp in pBZ3230) is indicated with a dotted box; the truncated lacZ and the remainder of the lac operon are indicated with an open box. Sites of cleavage by restriction endonucleases are abbreviated as follows: S, Sall; R, EcoRI; B, BglII; H, HindIII; N, NcoI; Nr, NarI. The line over the expanded map of nif DNA denotes the span of the fragment used as probe in nuclease S1 mapping, with the radioactively labeled end marked with an asterisk.
above (Fig. 2A), some transcription from the parental plasmid pBZ147 occurs in E. coli in the absence of nifA. The transcripts are initiated at the major downstream site, as well as at the upstream site, typically used in pRB1. To further examine these activities, transcription from pBZ147 was studied in *K. pneumoniae* with different ntrA and nifA backgrounds (Fig. 4). The results show weak transcription activity from the major wild-type promoter in a nifA<sup>+</sup> ntrA strain (lane c), but not in a nifA ntrA<sup>+</sup> strain (lane b). Transcription from the upstream site does not require nifA and ntrA and, moreover, appears to be completely repressed when the major promoter is fully activated by the products of these genes (lane d). However, when the major promoter is only weakly active, as in the nifA<sup>+</sup> ntrA strain, transcription activity from the upstream promoter is still evident (lane c).

**DISCUSSION**

The major question posed in this study was whether the mutations relieving the nifA requirement for transcription from the nifHDKY promoter directly affected sites involved in the nif-specific control. Transcription from the mutant plasmids was therefore examined according to two criteria of nif-specific regulation: the requirement for ntrA and the
FIG. 3. Nuclease S1 mapping of nifH transcription start site in pRB5. (A) Nuclease S1 mapping procedure was as described in the text with the following RNAs: lane a, K. pneumoniae KP5611(pRB5) (nifA ntrA+); lane b, E. coli MC1061(pRB5) (nifA ntrA+); C, C + T, G + A, and G are products of sequencing reactions of the probe fragment. Abbreviations: m, transcription start site in pRB5; wt, start site of nifA-ntrA-activated transcription. The vertical line spans the upstream copy of the duplicated sequence. (B) Effect of NifA-NtrA. Lane a, E. coli MC1061(pRB5) (nifA ntrA+); lane b, E. coli MC1061(pRB5 pNR300) (nifA+ ntrA+); lane c, E. coli MC1061(pBZ147 pNR300) (nifA+ ntrA+). Abbreviations are as described above for panel A. (C) Nucleotide sequence of the region upstream to nifH in pRB5. The duplicated sequence is boxed; the nifH coding sequence is shaded; arrows indicate inverted repeats; asterisks indicate wild-type transcription start sites; filled circles indicate start sites of transcription in pRB5 (the major start site is indicated by the largest circle); potential promoter sequences in pRB5 at positions −10 and −35 and homologies to the canonical sequences.
characteristic site of transcription initiation. By these two criteria, constitutive expression from pRB1 and pRB5 does not result from a modification in the nif-specific control but by alternative mechanisms.

In pRB1, the point mutation appears to have activated a pre-existing, low-efficiency promoter sequence. While homologies to canonical promoter elements at positions -10 and -35 are found in the sequence upstream to the transcription start site, there is still no evidence to indicate their role in promoter function. Although the mechanism responsible for the phenotypic expression of this promoter activity in the point mutant remains unclear, several possibilities can be considered. The sequence downstream to the start site may play some direct, or indirect role in the binding or positioning of RNA polymerase. For example, evidence for the potential significance of sequences within the transcribed region in the selection of transcription initiation sites has recently been reported for the TOL plasmid-borne meta pathway operon (13). Alternatively, the mutation, rather than affecting transcription initiation, may enhance transcript stability or relieve premature transcription termination. As noted previously (4), the mutation in pRB1 alters the upstream of two 7-bp inverted repeats located at a distance of 23 bp from each other. We have previously postulated that intrastrand base pairing between these repeats in the DNA could be involved in nif-specific regulation, such that destabilization of the cruciform structure by the C to A transversion would relieve the requirement for nifA. The present findings, that transcription from pRB1 differs from nif-regulated transcription in its start site as well as ntrA requirement, do not support this model. However, it is conceivable that the inverted repeats, included in the region transcribed from the upstream promoter (but not from the downstream nifA-activated promoter) may be responsible for the normally low expression from this promoter. Pairing between the repeats to form a stem and loop structure may sequester the nifH Shine and Dalgarno sequence (17), making translation initiation inefficient and the transcript short-lived. Alternatively, the secondary structure assumed by the transcript could lead to premature transcription termination. The mutation, by reducing the stability of this structure, may counteract both these effects.

The mutation in pRB1 does not interfere with nifA-ntrA activation of the major downstream promoter. Under these conditions, transcription from the upstream site is reduced to an undetectable level, possibly due to a competitive advantage of the major site in transcription initiation. Alternatively, NifA, NtrA, or their complexed forms may directly block the activity of the upstream promoter. Somewhat analogous observations were reported for the E. coli gal and lac operons (2, 5, 11), in which the activity of an upstream promoter was found to be repressed on activation of a positively regulated promoter downstream.

The physiological significance of the upstream promoter is unclear. In the wild type, transcription activity from the upstream promoter was too low to be detected in a previous analysis (19) or to provide for sufficient synthesis of $\beta$-galactosidase from the $nifH''$-'lacZ fusion to allow growth on lactose. However, it cannot be excluded that some low, constitutive expression of nifHDKY, and perhaps other nif operons, may become important at some specific instance during nif derepression or under particular physiological conditions. No evidence has been provided so far to indicate similar promoter arrangements in other nif operons in K. pneumoniae or in other nitrogen-fixing organisms. However, the presence of dual nifH promoters was suggested in transcript analyses of Rhizobium japonicum (1) and Rhizobium sp. strain IRC78 (20).

Results of analysis of transcripts from pRB5 suggest that a typical promoter structure has been generated as a result of the sequence duplication. A possible region of this promoter at position -10 spans the junction between the two sequence repeats, and a potential -35 region is provided by a sequence that was originally part of the nifH coding sequence. Each of these presumed promoter elements shares a 4-bp homology with the corresponding consensus sequence. In pRB5, an intact nifHDKY promoter is present upstream to the start site of constitutive transcription. The strong reduction in the activity of the constitutive promoter, evident on nifA-ntrA activation of the nif promoter, could at least partly be due to the transcription proceeding from the nif promoter into the presumed pRB5 constitutive promoter.

**ACKNOWLEDGMENTS**

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FIG. 4. Nuclease S1 mapping of nifH transcription start site in pBZ147. Nuclease S1 mapping procedure was as described in the text with the following RNAs; lane a, K. pneumoniae KG6237(pBZ147) (nifA ntrA); lane b, K. pneumoniae KP-5611(pBZ147) (nifA ntrA*); lane c, K. pneumoniae KG6237(pBZ147 pNR300) (nifA ntrA); lane d, K. pneumoniae KP5611(pBZ147 pNR300) (nifA ntrA*); G, G + A, C + T, and C are products of sequencing reactions of the probe fragment; 1, the start site of nifA-ntrA-activated transcription in pBZ147; 2, the major transcription start site in pRB1.
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LITERATURE CITED