JOURNAL OF BACTERIOLOGY, Aug. 1986, p. 480–486
0021-9193/86/080480-07$02.00/0

Vol. 167, No. 2

Transcriptional Regulation of Nitrogen Fixation by Molybdenum in *Azotobacter vinelandii*†

MARTY R. JACOBSON,‡ R. PREMAKUMAR, AND PAUL E. BISHOP*

Department of Microbiology, North Carolina State University, and U.S. Department of Agriculture, Agricultural Research Service, Raleigh, North Carolina 27695-7615

Received 13 January 1986/Accepted 29 April 1986

Multiple genomic regions homologous to *nifH* were found in the diazotroph *Azotobacter vinelandii*. The *nifHDK* gene cluster, located on a 12.8-kilobase (kb) *Xho*I fragment and two additional *Xho*I fragments (7.4 and 8.4 kb) hybridized to a *nifH*-specific DNA template but the 7.4- and 8.4-kb fragments did not hybridize to *nifD*- or *nifK*-specific DNA probes. In vivo transcription of the *nifHDK* gene cluster was ammonia-repressible and required the presence of at least 50 nM molybdenum in the derepression medium. Three mRNA species were transcribed from the *nifHDK* gene cluster, a 4.2-kb transcript homologous to *nifH*-, *nifD*-, and *nifK*-specific DNA templates, a 2.6-kb transcript homologous to *nifH*- and *nifD*-specific DNA templates, and a 1.2-kb transcript homologous only to the *nifH*-specific DNA template. In strain CA11, a *nifHDK* deletion mutant, the *nifHDK*-specific transcripts were not produced and the strain was unable to grow in N-free medium in the presence of Na_2MoO_4 at concentrations of 50 nM or higher. However, at concentrations of 25 nM Mo or less, growth occurred in N-free medium. Under these conditions two *nifH*-homologous (but not *nifD*- or *nifK*-homologous) transcripts were observed (1.2 and 1.8 kb). Presumably these were transcribed from the additional *nifH*-homologous sequences present in the genome. These results are consistent with the existence of two N_2 fixation systems in *A. vinelandii* which are regulated by molybdenum at the level of transcription.

Biological reduction of dinitrogen to ammonia is catalyzed by the enzyme complex nitrogenase, consisting of dinitrogenase, a molybdenum-iron protein, and dinitrogenase reductase, an iron protein. Dinitrogenase is a tetramer composed of two pairs of nonidentical subunits, the α- and β-subunits, which are encoded by the *nifD* and *nifK* genes, respectively. Active dinitrogenase contains four [4Fe-4S] clusters and two molecules of FeMo cofactor (5). Dinitrogenase reductase is a dimer composed of two identical subunits and is encoded by the *nifH* gene. A single [4Fe-4S] cluster is bridged between the two subunits of dinitrogenase reductase (5).

Biological N_2 fixation in all diazotrophic organisms is influenced by a number of environmental parameters (for a review, see references 26 and 27). As a general rule, both NH_4^+ and O_2 repress N_2 fixation. Since an Mo-containing dinitrogenase has been observed in all known diazotrophs, one might speculate that Mo also regulates nitrogenase expression. This has led to a number of studies on the effect of Mo deprivation on nitrogenase in a variety of organisms, revealing no uniform response. In the cyanobacteria *Plectonema boryanum* and *Anabaena cylindrica* (10, 20), Mo itself appears to have no regulatory properties. In *Klebsiella pneumoniae* the nitrogenase genes are expressed in both the presence and the absence of molybdate (8, 11, 13); however, molybdate is required for maximal expression (8). In *Clostridium pasteurianum*, Mo is an essential requirement for both the biosynthesis and activity of nitrogenase (7). Nagatani and Brill (19) obtained evidence that molybdate is an inducer of dinitrogenase in *Azobacter vinelandii*; they found only dinitrogenase reductase activity in cells derepressed for nitrogen fixation under Mo deprivation conditions.

Bishop et al. (1) provided evidence for the existence of an alternative nitrogen fixation system in *A. vinelandii*. This was based on the observation that Mo deprivation caused Nif^- mutants to undergo phenotypic reversal to Nif^+. Since then, the existence of two dinitrogenase reductases under regulatory control by Mo in *A. vinelandii* has been reported (23).

Multiple copies of *nif* genes have been observed in a number of diazotrophs. In *Rhodopseudomonas capsulata*, sequences homologous to the structural genes for nitrogenase components are present in multiple copies (29). In *Rhizobium phaseoli* three identical *nifH* genes, located in three different regions on a 250-kilobase (kb) plasmid, have been identified (24). By using heterologous specific probes, *nifD* and *nifK* sequences have been identified downstream from two of these *nifH* regions. Multiple *nifH*-homologous regions have also been identified in *Anabaena* spp. (25), *Calothrix* spp. (14), and *Azobacter chroococcum* (12).

In this study we describe the existence of multiple genomic regions homologous to a cloned *nifH* fragment from *A. vinelandii*. We also present data which demonstrate regulation of the "conventional" *nifHDK* gene cluster and an "alternative" *nifH* gene(s) by molybdenum at the level of transcription.

MATERIALS AND METHODS

Bacterial strains and media. The strains used in this study were *A. vinelandii* CA (6) and mutants derived from this strain (see Fig. 1A). Cells were cultured in a modified Burk medium (32). When fixed nitrogen was included in the medium, ammonium acetate was added to a concentration of 400 μg of N per ml. Molybdenum-deficient medium was prepared as previously described (1) with ultrapure chemi-

* Corresponding author.

† Paper 10284 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, N.C.

‡ Present address: Department of Anaerobic Microbiology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061.

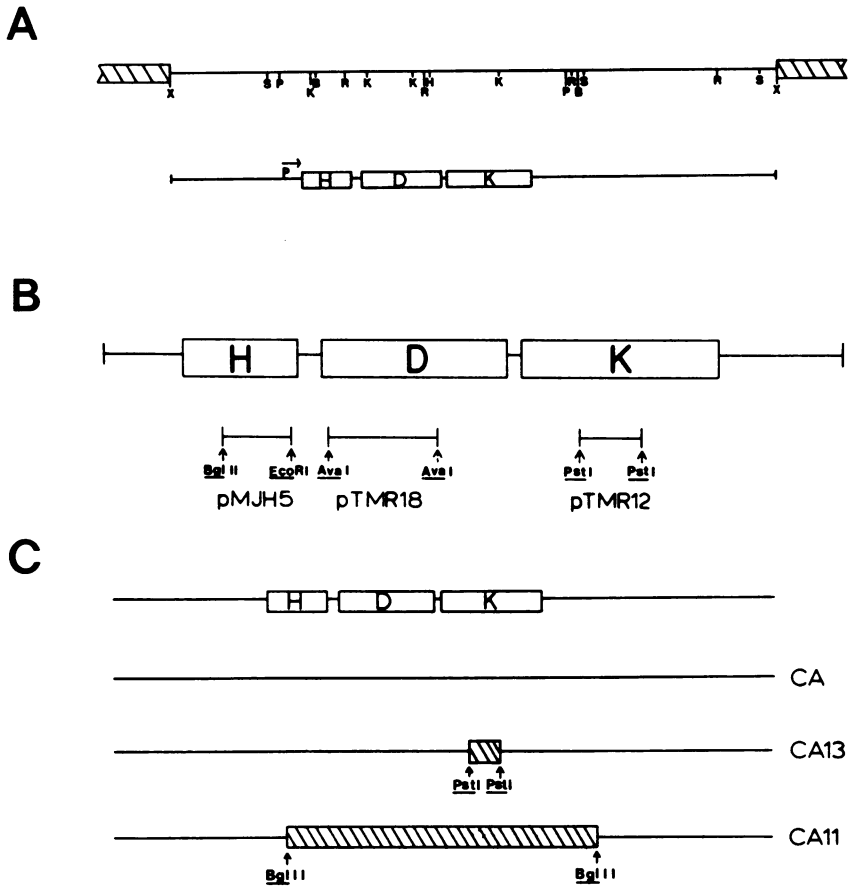

FIG. 1. *nifHDK* gene cluster, *nif*-specific probes, and strains of *A. vinelandii* used in this study. (A) Genetic and restriction map of the *nifHDK* gene cluster in *A. vinelandii*. The plasmid pDB2 (which consists of a 12.8-kb *Xho*I fragment containing the *A. vinelandii nifHDK* region cloned into the plasmid pKT230) and the location of the restriction sites were obtained from D. Dean. (B) Location of the *nif*-specific DNA templates used to probe both Southern and Northern blots. The plasmids pMJH5, pTMR18, and pTMR12 were used to isolate these *nif*-specific templates. (C) *A. vinelandii* strains used in this study. The hatched bars indicate the extent of the deletions in strains CA13 and CA11. Abbreviations: X, *Xho*I; S, *Sma*I; P, *Pvu*II; K, *Kpn*I; B, *Bgl*II; R, *Eco*RI; H, *Hin*dIII.

cals. Precautions were taken to minimize contamination by Mo. All glassware was base- and acid-washed.

Derepression of nitrogenase. Cultures grown in modified Burk medium containing fixed nitrogen were harvested at a density of 80 Klett units (2×10^6 CFU/Klett unit) by centrifugation at 10,000 × *g* for 10 min. The cells were suspended in nitrogen-free medium and shaken vigorously for 60 min at 28°C. Molybdenum was added as indicated prior to derepression.

Purification of DNA templates. Plasmid DNA was isolated by the procedure of Norgard (21). DNA fragments to be used as probes for hybridization were prepared as described by Dretzen et al. (9).

Southern blot analysis. Total genomic DNA was isolated from *A. vinelandii* and digested with the appropriate restriction enzyme. The digested DNA was then fractionated by agarose gel electrophoresis. Electrophoretic transfer to GeneScreen (New England Nuclear Corp., Boston, Mass.) hybridization transfer membranes was carried out as suggested by the manufacturer. Hybridizations were performed as described by the suppliers of GeneScreen in the presence of 50% formamide at 42°C. The DNAs to be used as probes were labeled with [α-^{32}P]dCTP with the nick translation procedure of Maniatis et al. (17).

Northern blot analysis. Total RNA was isolated as described by Krol et al. (16). Approximate 10-μg portions of RNA samples were glyoxylated and electrophoresed on a 1.1% agarose gel as described by McMaster et al. (18). Electrophoretic transfer of RNA onto GeneScreen hybridization transfer membranes was carried out as suggested by the suppliers. Hybridizations were performed in the presence of 50% formamide at 42°C as described by the suppliers of GeneScreen. The DNAs to be used as probes were labeled with ^{32}P as previously described.

RESULTS

Presence of multiple *nifH*-homologous regions in *A. vinelandii*. Ruvkun and Ausubel (28) observed five *Eco*RI fragments in digests of genomic DNA from *A. vinelandii* UW10 which hybridized to the A fragment of plasmid pSA30 (which contains the *nifHDK* gene cluster of *K. pneumoniae*). This observation would indicate either that there are more restriction fragments present than would be expected if the *A. vinelandii nifHDK* genes were contiguous or that there are multiple regions homologous to the *nif* structural genes in the genome of *A. vinelandii*. Bishop et al. (3) cloned two of these five *Eco*RI fragments. They determined that these fragments were contiguous and that the 1.4-kb insert of plasmid pLB3 contained *nifD* homology while the 2.6-kb

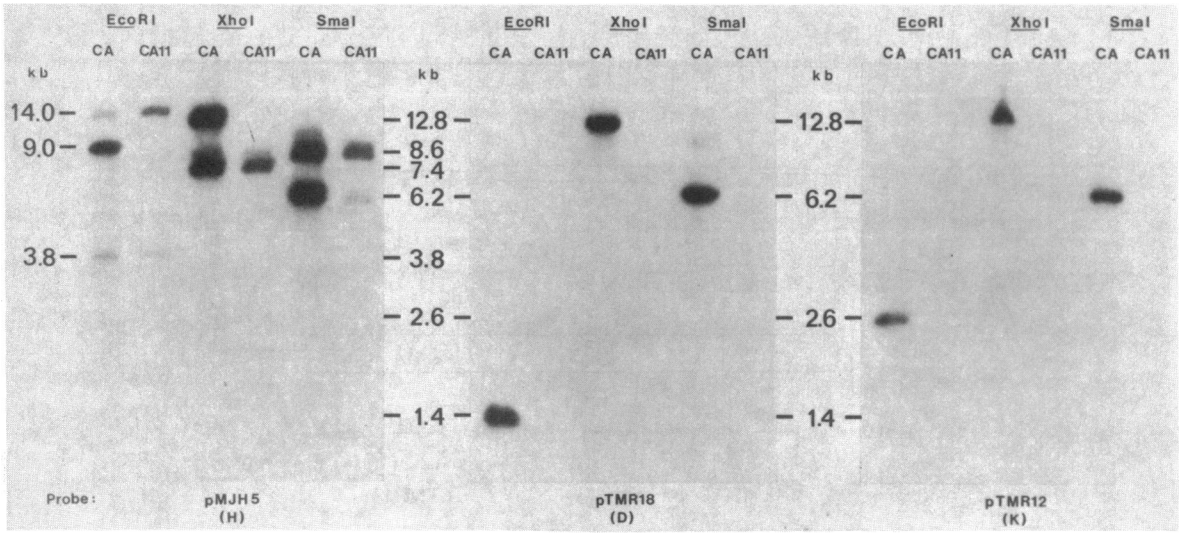

FIG. 2. Southern hybridization analysis of *A. vinelandii* genomic DNA probed with intragenic *nif*-specific DNA templates.

insert of pLB1 contained *nifK* homology. They also identified the 4.1-kb *Eco*RI fragment as a partial digestion product which contained the 1.4- and 2.6-kb *Eco*RI fragments. Brigle et al. (4) have shown that a *nifHDK* gene cluster is present in *A. vinelandii* (Fig. 1A) similar to that previously observed in *K. pneumoniae*. They confirmed that the cloned *Eco*RI restriction fragments of pLB1 and pLB3 are contiguous and also demonstrated that these are immediately preceded by a *nifH* gene. This leaves two *Eco*RI fragments (9.2 and 15.1 kb) unaccounted for.

To further characterize these restriction fragments, hybridization experiments were conducted with *nifH*-, *nifD*-, and *nifK*-specific DNA probes (Fig. 1B) on genomic DNA digests of the wild type and a *nifHDK* deletion strain, CA11 (2) (Fig. 1C). Hybridization of the 0.5-kb *Bgl*II-*Eco*RI insert of pMJH5 (Fig. 1B), which contains internal *nifH* sequences from *A. vinelandiii*, to digests of *A. vinelandii* genomic DNA revealed multiple hybridizing bands in each digest (Fig. 2). Three bands were observed in an *Xho*I digest of the wild-type strain CA; an intense band of 12.8 kb, which corresponds to the cloned *Xho*I fragment of pDB2 (5) (Fig. 1A), and two additional bands of lesser intensity with sizes of 8.4 and 7.4 kb. Only two bands (8.4 and 7.4 kb) were observed in *Xho*I digests of genomic DNA from the *nifHDK* deletion strain CA11. Three bands (14.0, 9.0, and 3.8 kb) were also observed in *Eco*RI digests of genomic DNA from strain CA. The 9.0-kb band must correspond to the *nifH*-containing *Eco*RI fragment associated with the *nifHDK* gene cluster, since only the 14.0- and 3.8-kb bands were observed in *Eco*RI-digested DNA isolated from strain CA11. *Sma*I digests of DNA isolated from both strains CA and CA11 contained two bands (8.6 and 6.2 kb) which hybridized to the *nifH*-specific DNA template. However, the intensity of the 6.2-kb *Sma*I band in DNA from strain CA11 was significantly reduced. Based on the restriction map of the *nifHDK* region (4) (Fig. 1A) and the *Eco*RI and *Xho*I hybridization data, it appears that the 6.2-kb *nifHDK*-containing *Sma*I fragment comigrates with another *nifH*-homologous *Sma*I fragment which can only be observed in DNA from a strain carrying a deletion in the *nifHDK* region.

Hybridization of either the intragenic *nifD*- or the intragenic *nifK*-specific DNA template (Fig. 1A) to digests of genomic DNA from the wild-type strain CA (Fig. 2) revealed only those bands which would be expected based on the restriction map of the *nifHDK* region shown in Fig. 1A. No hybridization was observed with these probes on DNA isolated from strain CA11 (Fig. 1C). These data demonstrate the presence of multiple regions containing *nifH* homology in the *A. vinelandii* genome. However, under our hybridization conditions, multiple regions homologous to *nifD* or *nifK* do not appear to be present in the genome of *A. vinelandii*.

Transcriptional regulation of *nif* genes by molybdenum. The presence of a second dinitrogenase reductase (23) which functions in the alternative nitrogen fixation system suggests that these additional *nifH*-homologous regions might serve as coding sequences for the alternative dinitrogenase reductase. It was therefore of interest to determine whether these sequences are transcribed and, if so, under what conditions. Total RNA was isolated from *A. vinelandii* and analyzed as described in Materials and Methods. Three *nifH*-homologous mRNA species were observed in strains CA and CA13 (3) (Fig. 3; see also Fig. 1C) derepressed for N_2 fixation in the presence of 1 μM Na_2MoO_4. In RNA isolated from strain CA, a 4.2-kb transcript homologous to *nifH*-, *nifD*-, and *nifK*-specific DNA templates, a 2.6-kb transcript homologous to *nifH*- and *nifD*-specific DNA templates, and a 1.2-kb transcript homologous to only the *nifH*-specific DNA template were observed. All three of the transcripts were present in RNA isolated from strain CA13; however, the large transcript (4.2 kb) was reduced in size by approximately 0.5 kb, which corresponds to the size of the deletion in the *nifK* gene in strain CA13 (3). No *nifH*-homologous transcripts were detected in RNA isolated from the *nifHDK* deletion strain CA11 when this strain was derepressed for N_2 fixation in the presence of 1 μM Na_2MoO_4. Also, no *nif*-homologous transcripts were detected in RNA isolated from any of these strains when they were derepressed in the presence of 29 mM ammonia acetate (data not shown).

A similar analysis of RNA isolated from strains CA, CA13, and CA11 derepressed for N_2 fixation under Mo deprivation conditions revealed two transcripts (1.8 and 1.2 kb) homologous to the *nifH*-specific DNA template (Fig. 4) which were also ammonia-repressible (data not shown). In addition, no transcripts homologous to *nifD*- or *nifK*-specific DNA templates could be detected in RNA isolated from

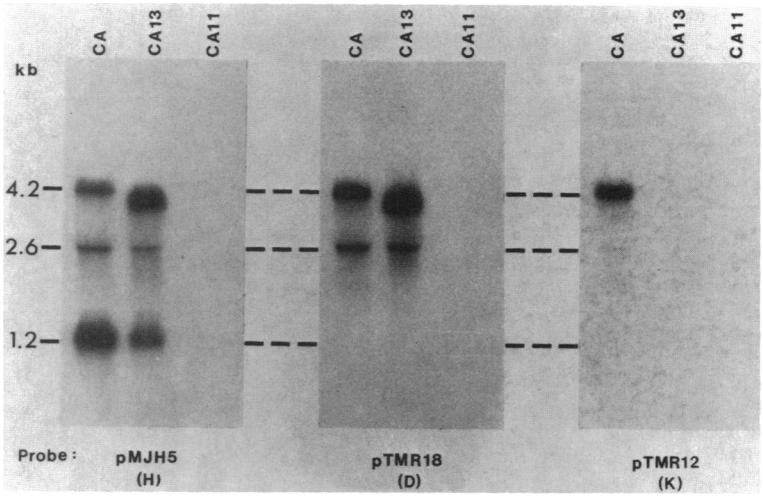

FIG. 3. Identification of *nif*-homologous transcripts from strains CA, CA13, and CA11 derepressed for nitrogen fixation in the presence of 1 μM Na_2MoO_4.

these strains under these conditions (data not shown). Presumably these *nifH*-homologous transcripts (1.8 and 1.2 kb) result from transcription of the additional *nifH*-homologous sequences present outside the *nifHDK* gene cluster.

Effect of molybdenum concentration on transcription of *nif*-homologous sequences. The data shown in Fig. 4 demonstrate that the presence or absence of Na_2MoO_4 regulates the expression of the *nifHDK* gene cluster and some other *nifH*-homologous region(s). We therefore examined the effect of different concentrations of molybdate on the expression of these two transcript patterns. The results of one such experiment are shown in Fig. 5. When no Na_2MoO_4 was added to the derepression medium, the two *nifH*-homologous transcripts (1.2 and 1.8 kb) were observed in RNA isolated from both strains CA and CA11. As the initial

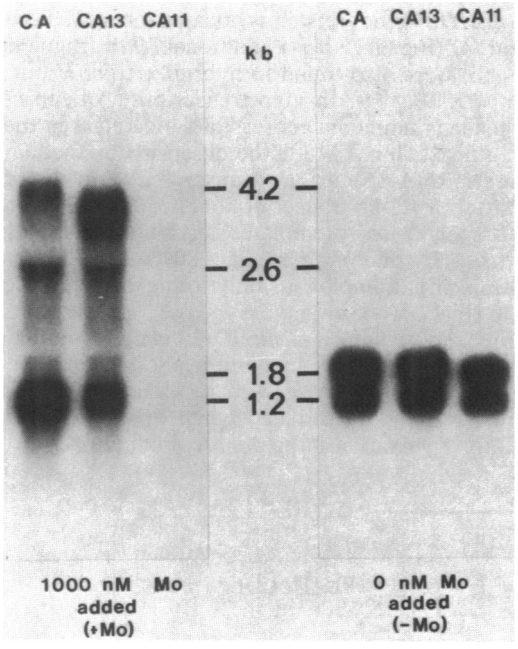

FIG. 4. Comparison of *nif*-homologous transcripts from cells derepressed for nitrogen fixation in either the presence (1 μM Na_2MoO_4) or absence of molybdenum.

concentration of Na_2MoO_4 was increased to between 25 and 50 nM, a change in the *nifH*-homologous transcript pattern was observed. In strain CA, repression of the two-transcript pattern occurred with Mo concentrations above 25 nM, while derepression of the three-transcript pattern (1.2, 2.6, and 4.2 kb) was observed. For strain CA11, repression of the two-transcript pattern was observed with Na_2MoO_4 concentrations above 50 nM.

The two-transcript pattern which has homology to the *nifH*-specific DNA template and is expressed at Mo concentrations below 50 nM is indicative of the alternative N_2 fixation system since it is expressed in both the *nifHDK* deletion strain CA11 and the wild-type strain CA. The three-transcript pattern which has transcripts homologous to *nifH*, *nifD*, and *nifK* is indicative of the conventional N_2 fixation system and is expressed at Mo concentrations above 50 nM.

Identification of sequences homologous to an Fd-like gene in *A. vinelandii*. R. Robson (personal communication) has identified an open reading frame which appears to be within the same transcriptional unit as a second copy of *nifH* (*nifH**) in *A. chroococcum* (Fig. 6). The primary amino acid sequence predicted from the nucleic acid sequence of this gene is very similar to that of known ferredoxin (Fd) genes (R. Robson, personal communication). A comparison of Southern hybridizations of digests of genomic DNA isolated from strains CA and CA11 probed with the *nifH*-specific DNA probe (Fig. 1B) and a DNA template which encodes this Fd-like gene (Fig. 6) is presented in Fig. 7.

A 7.4-kb *Xho*I fragment and an 8.6-kb *Sma*I fragment were found to be homologous to the Fd probe in DNA isolated from both strains CA and CA11. These bands comigrated with *nifH*-homologous fragments found in DNA isolated from these strains. A 2.6-kb *Eco*RI fragment also hybridized to the Fd probe. This *Eco*RI fragment did not hybridize to the *nifH*-specific DNA template.

These results suggest that the Fd-like gene comigrates with one of the additional ("alternative") *nifH*-homologous regions on *Xho*I and *Sma*I restriction fragments. These results also indicate that there is an *Eco*RI restriction site between this Fd-like and the *nifH*-homologous regions, similar to that observed in *A. chroococcum* (R. Robson, personal communication; see Fig. 6). Restriction analysis of the cloned 8.6-kb *nifH*-homologous *Sma*I fragment (Fig. 2)

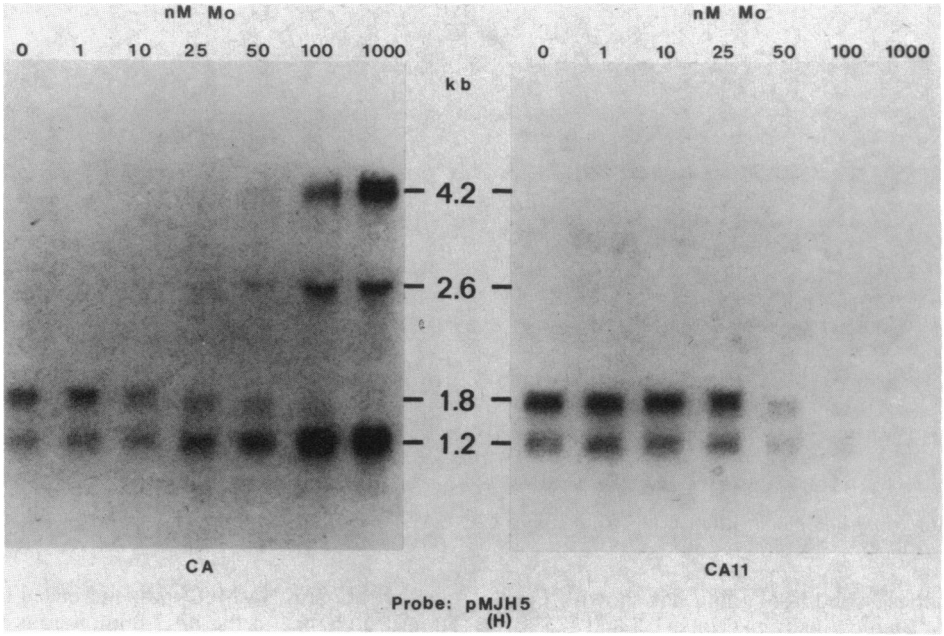

FIG. 5. Effect of Na_2MoO_4 concentration on the transcription of *nifH*-homologous sequences.

confirmed the existence of this *Eco*RI site between the *nifH*-homologous region and the Fd-like sequences in *A. vinelandii* (data not shown).

Transcriptional regulation of Fd-like sequences by molybdenum in *A. vinelandii*. Since this Fd-like gene appeared to be linked to a *nifH*-homologous gene which is transcribed under conditions of Mo deprivation, we examined transcription of the Fd gene in the presence and absence of molybdenum. Hybridization of the *nifH*-specific DNA template or the Fd probe to RNAs isolated from cultures derepressed for N_2 fixation in either the presence or absence of 1 μM Na_2MoO_4 is shown in Fig. 8. No hybridization of the Fd probe to RNA isolated from either strain CA or strain CA11 was observed when these strains were grown in medium containing ammonia (data not shown) or derepressed for N_2 fixation in the presence of 1 μM Na_2MoO_4. However, the FD probe hybridized to a 1.8-kb band in RNA isolated from these strains when they were derepressed for N_2 fixation under Mo deprivation conditions. This Fd-homologous transcript comigrated with the 1.8-kb *nifH*-homologous transcript which is expressed under conditions of Mo deprivation. These data suggest that the *A. vinelandii* Fd-like gene is cotranscribed with one copy of the alternative *nifH* gene(s) and that its transcription is regulated by molybdenum.

DISCUSSION

In this study we have demonstrated the presence of multiple genomic regions in the diazotroph *A. vinelandii* which are homologous to *nifH* and that transcription of these sequences is regulated by molybdenum. This regulation of the two N_2 fixation systems in *A. vinelandii* by Mo at the level of transcription is different from the situation observed in *K. pneumoniae*, in which transcription of the *nifHDK* gene cluster occurs in both the presence and absence of Mo (8, 11, 13, 15). This role of Mo in the regulation of *nif* transcripts also appears to be in contrast to the regulatory role that has been assigned to other metals which act as cofactors (Mn, Fe, Mg, Ca, etc.). These metals act by derepressing very efficient scavenging systems in response to nutritional limitation (30, 31).

The *nifHDK* gene cluster is located on a 12.8-kb *Xho*I fragment (4) (Fig. 1A). Two additional *Xho*I fragments (7.4 and 8.4 kb) were also found to hybridize to *nifH* but not to *nifD* or *nifK* (Fig. 4). In vivo transcription of the *nifHDK* gene cluster is ammonia repressible and requires the presence of at least 50 nM Mo in the derepression medium (Fig. 5). Three mRNA species are transcribed from the *nifHDK* gene cluster, a 4.2-kb transcript homologous to *nifH*-, *nifD*-, and *nifK*-specific DNA templates, a 2.6-kb transcript homologous to *nifH*- and *nifD*-specific DNA templates, and a 1.2-kb transcript homologous only to the *nifH*-specific DNA template (Fig. 3).

Strain CA11, which is a *nifHDK* deletion mutant (2), is unable to grow in N-free medium in the presence of Mo at concentrations of 50 nM or greater. The *nifHDK*-specific transcripts were not produced when this strain was derepressed for N_2 fixation in medium containing 50 nM Mo or

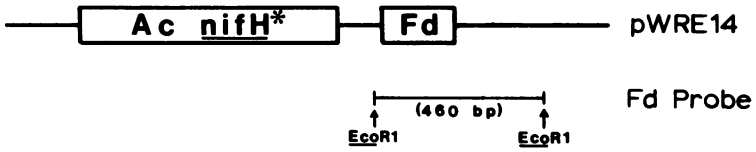

FIG. 6. Genetic map of the *A. chroococcum* (Ac) *nifH**-Fd region. The plasmid pWRE14, which contains a second copy of *nifH* (*nifH**) linked to an Fd-like gene, was obtained from R. Robson. The 460-base-pair (bp) Fd-containing *Eco*RI fragment was isolated and used as a probe in both Southern and Northern blot hybridization analysis.

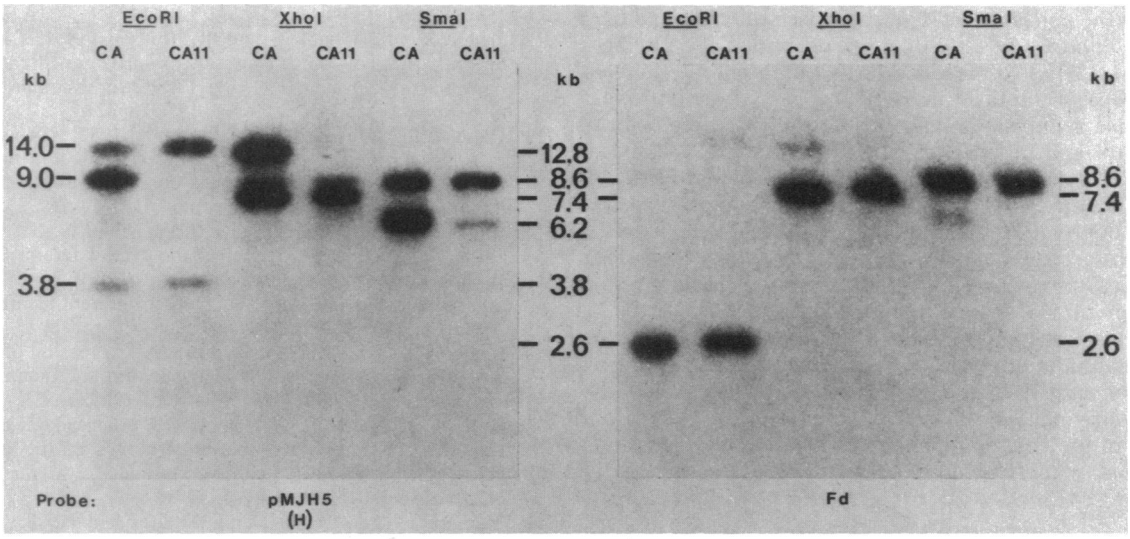

FIG. 7. Identification of sequences to an Fd-like gene in *A. vinelandii*.

greater (Fig. 5). However, at concentrations of 25 nM or less, strain CA11 was capable of diazotrophic growth, and two ammonia-repressible *nifH*-homologous (but not *nifD*- or *nifK*-homologous) transcripts were observed (1.2 and 1.8-kb). Thus, these transcripts probably result from transcription of the additional *nifH*-homologous sequences present in the genome of *A. vinelandii*.

The absence of *nifD*- and *nifK*-homologous DNA in the deletion strain CA11 suggests that the alternative dinitrogenase (*nifDK* homolog) may not be evolutionarily related to the conventional dinitrogenase. This suggestion is strengthened by our observations that crude extracts of Nif$^-$ mutant strains of *A. vinelandii*, derepressed for nitrogenase under conditions of Mo deprivation, do not contain cross-reacting material to antisera raised against the *A. vinelandii* conventional dinitrogenase (unpublished results). However, cross-reacting material to antisera raised against the *A. vinelandii* conventional dinitrogenase reductase was observed in these extracts.

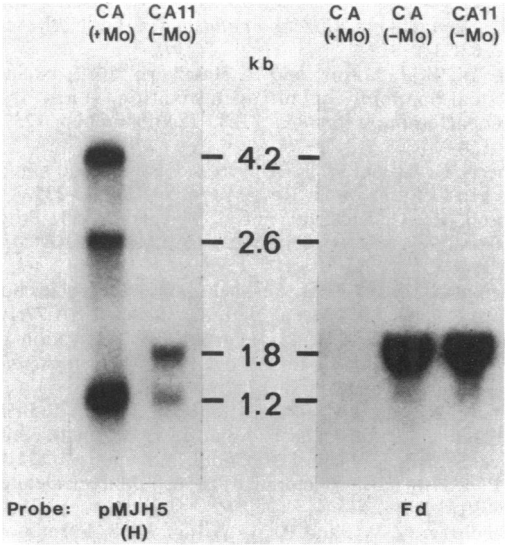

FIG. 8. Transcriptional regulation of sequences homologous to an Fd-like gene in *A. vinelandii* by molybdenum.

Sequences homologous to an Fd-like gene (cloned from *A. chroococcum*) have also been identified in *A. vinelandii* (Fig. 7). These Fd-like sequences appeared to comigrate on the same *Xho*I and *Sma*I restriction fragments with one of the additional *nifH*-homologous regions. The *A. vinelandii* Fd-like sequences are located on a 1.8-kb transcript, and transcription of these sequences is repressed by both ammonia and molybdenum. Since this 1.8-kb Fd-like transcript is the same size as one of the *nifH*-homologous transcripts which is expressed under the same conditions, these *nifH*- and Fd-homologous regions are probably in the same transcriptional unit.

The presence of multiple copies of *nif* structural genes has been observed in a number of diazotrophs (12, 14, 24, 25, 29). However, the function of these additional copies of *nifH* has not yet been identified. In contrast to this situation, we have demonstrated that in *A. vinelandii*, at least one of the additional *nifH* genes and an Fd-like gene are transcribed under conditions of Mo deprivation.

Dinitrogenase reductase (the *nifH* gene product) activity has been observed in whole cells and in extracts of *A. vinelandii* strains derepressed for nitrogen fixation under Mo deprivation conditions (19, 22, 23). This activity has recently been attributed to an alternative dinitrogenase reductase which is expressed only under Mo deprivation conditions (22, 23). The data presented here support these findings and demonstrate that under our conditions of derepression, transcription of this alternative *nifH* gene(s) can occur even in the presence of up to 25 nM Na_2MoO_4. Page and Collinson (22) observed that vanadium enhanced the acetylene reduction activity of cells derepressed in Mo-limited nitrogen-free medium. We examined the effect of vanadium on transcription of the *nif* genes and found no differences between transcripts from cells derepressed for nitrogen fixation under Mo deprivation conditions with and without added vanadium (unpublished results). These findings are in agreement with the biochemical observations of Premakumar et al. (23).

Recently, Chisnell and Bishop (Proceedings of the 6th International Symposium on Nitrogen Fixation, Corvallis, Oreg., 1985, p. 623) partially purified yet another dinitrogenase reductase activity which appears to be dif-

ferent from the conventional dinitrogenase reductase and the alternative dinitrogenase reductase described by Premakumar et al. (23). The presence of two different alternative dinitrogenase reductase activities and a conventional dinitrogenase reductase might explain the three regions of homology to *nifH* observed in *A. vinelandii* (Fig. 2 and 7). This might also explain the presence of two *nifH*-homologous transcripts (1.2 and 1.8 kb) which are expressed under Mo deprivation conditions (Fig. 4 and 8). However, the 1.2-kb transcript might also result either from attenuation or by processing of the 1.8-kb transcript (which contains a second copy of *nifH* linked to an Fd-like gene).

In summary, our findings suggest a role for molybdenum in the regulation of nitrogen fixation at the level of transcription in *A. vinelandii*. These findings are also consistent with the model originally proposed by Bishop et al. (1980) for regulation of the conventional and alternative nitrogen fixation systems by molybdenum.

ACKNOWLEDGMENTS

We thank R. Robson (A.F.R.C. Unit of Nitrogen Fixation, University of Sussex, Brighton, U.K.) for supplying us with the *A. chroococcum nifH** and Fd gene-containing plasmid pWRE14 prior to publication.

This project was supported by competitive grant 84-CRCR-1-1409 from the U.S. Department of Agriculture. These investigations were cooperative investigations of the Agricultural Research Service, the U.S. Department of Agriculture, and the North Carolina Agricultural Research Service, Raleigh.

LITERATURE CITED

1. **Bishop, P. E., D. M. L. Jarlenski, and D. R. Hetherington.** 1980. Evidence for an alternative nitrogen fixation system in *Azotobacter vinelandii*. Proc. Natl. Acad. Sci. USA **77**: 7342–7346.
2. **Bishop, P. E., R. Premakumar, D. R. Dean, M. R. Jacobson, J. R. Chisnell, T. M. Rizzo, and J. Kopczynski.** 1986. Nitrogen fixation by *Azotobacter vinelandii* strains having deletions in structural genes for nitrogenase. Science **232**:92–94.
3. **Bishop, P. E., T. M. Rizzo, and K. F. Bott.** 1985. Molecular cloning of *nif* DNA from *Azobacter vinelandii*. J. Bacteriol. **162**:21–28.
4. **Brigle, K. E., W. E. Newton, and D. R. Dean.** 1985. Complete nucleotide sequence of *Azotobacter vinelandii* nitrogenase structural gene cluster. Gene **37**:37–44.
5. **Burgess, B. K.** 1984. Structure and reactivity of nitrogenase—an overview, p. 103–114. *In* W. E. Newton and C. Veeger (ed.), Advances in nitrogen fixation research. Nijhoff/Junk, The Hague.
6. **Bush, J. A., and P. W. Wilson.** 1959. A non-gummy chromogenic strain of *Azotobacter vinelandii*. Nature (London) **184**:381.
7. **Cardenas, J., and L. E. Mortenson.** 1975. Role of molybdenum in dinitrogen fixation by *Clostridium pasteurianum*. J. Bacteriol. **123**:978–984.
8. **Dixon, R., R. R. Eady, G. Espin, S. Hill, M. Iaccarino, D. Kahn, and M. Merrick.** 1980. Analysis of regulation of *Klebsiella pneumoniae* nitrogen fixation (*nif*) gene cluster with gene fusions. Nature (London) **286**:128–132.
9. **Dretzen, G., M. Bellard, P. Sassone-Corsi, and P. Chambon.** 1981. A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. Anal. Biochem. **112**:295–298.
10. **Hallenbeck, P. C., and J. R. Benemann.** 1980. Effect of molybdenum starvation and tungsten on the synthesis and activity of nitrogenase in *Anabaena cylindrica*. FEMS Microbiol. Lett. **9**:121–124.
11. **Imperial, J., R. A. Ugalde, V. K. Shah, and W. J. Brill.** 1984. Role of the *nifQ* gene product in the incorporation of molybdenum into nitrogenase in *Klebsiella pneumoniae*. J. Bacteriol. **158**:187–194.
12. **Jones, R., P. Woodley, and R. Robson.** 1984. Cloning and organisation of some genes for nitrogen fixation from *Azotobacter chroococcum* and their expression in *Klebsiella pneumoniae*. Mol. Gen. Genet. **197**:318–327.
13. **Kahn, D., M. Hawkins, and R. R. Eady.** 1982. Nitrogen fixation in *Klebsiella pneumoniae*: nitrogenase levels and the effect of added molybdate on nitrogenase derepressed under molybdenum deprivation. J. Gen. Microbiol. **128**:779–787.
14. **Kallas, T., M.-C. Rebiere, R. Rippka, and N. T. de Marsac.** 1983. The structural *nif* genes of the cyanobacteria *Gloeothece* sp. and *Calothrix* sp. share homology with those of *Anabaena* sp., but the *Gloeothece* genes have a different arrangement. J. Bacteriol. **155**:427–431.
15. **Kennedy, C., and J. R. Postgate.** 1977. Expression of *Klebsiella pneumoniae* nitrogen fixation genes in nitrate reductase mutants of *Escherichia coli*. J. Gen. Microbiol. **98**:551–557.
16. **Krol, A. J. M., J. G. J. Hontelez, B. Roozendaal, and A. van Kammen.** 1982. On the operon structure of the nitrogenase genes of *Rhizobium leguminosarum* and *Azobacter vinelandii*. Nucleic Acids Res. **10**:4147–4157.
17. **Maniatis, T., A. Jeffrey, and D. G. Kleid.** 1975. Nucleotide sequence of the rightward operator of phage λ. Proc. Natl. Acad. Sci. USA **72**:1184–1188.
18. **McMaster, G. K., and G. G. Carmichael.** 1977. Analysis of single- and double-stranded nucleic acids on polyacrylamide and agarose gels by using glyoxal and acridine orange. Proc. Natl. Acad. Sci. USA **74**:4835–4838.
19. **Nagatani, H. H., and W. J. Brill.** 1974. The effect of Mo, W, and V on the synthesis of nitrogenase components in *Azotobacter vinelandii*. Biochim. Biophys. Acta **362**:160–166.
20. **Nagatani, H. H., and R. Haselkorn.** 1978. Molybdenum independence of nitrogenase component synthesis in the non-heterocystous cyanobacterium *Plectonema*. J. Bacteriol. **134**:597–605.
21. **Norgard, M. V.** 1981. Rapid and simple removal of contaminating RNA from plasmid DNA without the use of RNase. Anal. Biochem. **113**:34–42.
22. **Page, W. J., and S. K. Collinson.** 1982. Molybdenum enhancement of nitrogen fixation in a Mo-starved *Azotobacter vinelandii* Nif$^-$ mutant. Can. J. Microbiol. **28**:1173–1180.
23. **Premakumar, R., E. M. Lemos, and P. E. Bishop.** 1984. Evidence for two dinitrogenase reductases under regulatory control by molybdenum in *Azobacter vinelandii*. Biochim. Biophys. Acta **797**:64–70.
24. **Quinto, C., H. de la Vega, M. Flores, J. Leemans, M. A. Cevallos, M. A. Pardo, R. Azpiroz, M. D. L. Girard, E. Calva, and R. Palacois.** 1985. Nitrogenase reductase: a functional multigene family in *Rhizobium phaseoli*. Proc. Natl. Acad. Sci. USA **82**:1170–1174.
25. **Rice, D., B. J. Mazur, and R. Haselkorn.** 1982. Isolation and physical mapping of nitrogen fixation genes from the cyanobacterium *Anabaena* 7120. J. Biol. Chem. **257**:13157–13163.
26. **Roberts, G. P., and W. J. Brill.** 1981. Genetics and regulation of nitrogen fixation. Annu. Rev. Microbiol. **35**:207–235.
27. **Robson, R., C. Kennedy, and J. Postgate.** 1983. Progress in comparative genetics of nitrogen fixation. Can. J. Microbiol. **29**:954–967.
28. **Ruvkun, G. B., and F. M. Ausubel.** 1980. Interspecies homology of nitrogenase genes. Proc. Natl. Acad. Sci. USA **77**:191–195.
29. **Scolnik, P. A., and R. Haselkorn.** 1984. Activation of extra copies of genes coding for nitrogenase in *Rhodopseudomonas capsulata*. Nature (London) **307**:289–292.
30. **Shah, V. K., R. A. Ugalde, J. Imperial, and W. J. Brill.** 1984. Molybdenum in nitrogenase. Annu. Rev. Biochem. **53**:231–257.
31. **Silver, S.** 1978. Transport of cations and anions, p. 221–324. *In* B. P. Rosen (ed.), Bacterial transport. Marcel Dekker, Inc., New York.
32. **Strandberg, G. W., and P. W. Wilson.** 1968. Formation of the nitrogen-fixing enzyme system in *Azotobacter vinelandii*. Can. J. Microbiol. **14**:25–31.