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Regulation of Nitrogenase Gene Expression in Anaerobic Cultures of *Anabaena variabilis*

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Derepression of nitrogenase gene expression was studied at the mRNA and enzyme activity levels in anaerobic cultures of *Anabaena variabilis* 29413. Cells, previously grown with ammonium chloride, were incubated in the absence of fixed nitrogen compounds under an Ar atmosphere with dichlorophenyldimethylurea present to inhibit oxygen evolution. The appearance of nitrogenase mRNA (measured by dot blot hybridization analysis) and nitrogenase activity (measured as acetylene-reducing activity) was followed, and the cells were also observed by phase-contrast microscopy. Nitrogenase mRNA could be detected after 1.5 to 2.0 h of nitrogen starvation; enzyme activity appeared about 1 h later. Although enzyme activity increased for many hours, mRNA levels reached a steady state rapidly. Neither heterocysts nor proheterocysts formed under these conditions; however, the cells were observed to shrink and become chlorotic. When anaerobic, derepressed cultures were exposed to oxygen, nitrogenase mRNA levels decreased very rapidly.

The filamentous cyanobacterium *Anabaena variabilis* is capable of both oxygenic photosynthesis and nitrogen fixation. Under aerobic conditions, nitrogen fixation occurs in specialized cells known as heterocysts, which develop in response to nitrogen starvation (11, 28, 30). In contrast to vegetative cells, heterocysts have thick cell envelopes and high rates of respiration; they also lack photosystem II activity and, thus, do not evolve oxygen (5, 28). These differences between heterocysts and photosynthetic vegetative cells are significant because heterocysts provide an anaerobic microenvironment for nitrogen fixation in an aerobic organism. Nitrogen fixation requires low potential electrons and is catalyzed by nitrogenase, an extremely oxygen-sensitive enzyme. Heterocyst formation requires about 14 to 24 h, depending on the species and conditions, during which time the cells differentiate both morphologically and biochemically; the appearance of nitrogenase activity is a relatively late event in this process (2, 11). Under normal aerobic circumstances, heterocyst formation and nitrogenase synthesis are regulated coordinately, and both are triggered by nitrogen starvation. Aerobic vegetative cells do not have nitrogenase activity, and heterocysts form only when nitrogen fixation is required (23). Both nitrogenase synthesis and heterocyst development are repressed by fixed nitrogen compounds such as ammonia (11, 28).

Under anaerobic conditions, some heterocyst-forming cyanobacteria can fix nitrogen without forming mature heterocysts (25). *Anabaena* sp. strain 6310 cells had substantial nitrogenase activity when incubated under an argon atmosphere for 20 h with dichlorophenyldimethylurea (DCMU) present to inhibit oxygen evolution; stage II proheterocysts, but no heterocysts, were present in the cultures as observed by electron microscopy. *Anabaena* sp. strain 7118, a non-heterocyst-forming mutant of *A. variabilis* which does not fix nitrogen aerobically, had nitrogenase activity under anaerobic conditions, and there were no apparent signs of proheterocyst or heterocyst formation (25). *Anabaena* sp. strain 7120 also fixes nitrogen anaerobically without forming mature heterocysts, and nitrogenase mRNA is present only under conditions in which fixed nitrogen is not present (12, 24). Thus, expression of nitrogenase genes and heterocyst development genes can be dissociated under some conditions.

We have previously cloned and characterized the genes for nitrogenase in *A. variabilis* (14; R. Hirschberg and J. Miller, unpublished observations), and we would like to define how their expression responds to environmental stimuli. Although there have been numerous studies on environmental factors that influence nitrogenase synthesis in various *Anabaena* strains and other nitrogen-fixing cyanobacteria, almost all have monitored gene expression by measuring nitrogenase activity. This is a very indirect assessment of regulatory events at the transcription level since subsequent translation, modification, and assembly of the enzyme must occur before activity can be detected. Furthermore, protein turnover as well as inactivation of nitrogenase by oxygen can alter observed levels of activity. In the studies reported in this and the accompanying paper, we have investigated nitrogenase gene expression more directly, using cloned genes as probes for nitrogenase mRNA levels. In addition, we have conducted our studies under anaerobic conditions so that we could study nitrogenase gene regulation without the potentially complicating effects of concomitant heterocyst formation. In this paper we report studies on the time course of mRNA and enzyme synthesis during derepression under anaerobic conditions and the effect of oxygen of nitrogenase mRNA levels.

MATERIALS AND METHODS

Media and growth conditions. *A. variabilis* ATCC 29413 was used for all studies. Cultures were routinely grown in BG-11 medium (27), supplemented with 2 mM NH_4Cl and 10 mM fructose, at 29 to 30°C. Continuous illumination was provided by cool white fluorescent lamps (approximately 2,100 lx), and the cultures were shaken at 150 rpm on gyratory shakers.

Anaerobic derepression experiments. Cultures (500 ml) were grown to an optical density at 600 nm of 0.5 to 0.7 and

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harvested by centrifugation at 7,500 × *g* for 5 min at 10°C. The cells were washed twice and suspended at 1/10 their original volume in nitrogen-free medium (AA/8 [1]) containing 20 μM DCMU, 0.5% fructose, and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer, pH 7.0. Samples of the cells were put into serum vials, which were then purged with oxygen-free argon gas for 20 min. Each vial contained the cells from 40 to 100 ml of the original culture. The sealed vials were then incubated in the growth conditions described above. For the time course experiments, separate vials were set up for each time at which nitrogenase activity was to be measured and for each time RNA would be prepared.

Nitrogenase activity measurements. Enzyme activity in whole cells was measured by the acetylene reduction technique. At desired times, vials were injected with a 10% headspace volume of acetylene, and incubation was continued for 60 min; 1.0-ml gas samples were removed, and ethylene was determined with a Varian 1740 gas chromatograph, using a Poropak N column. Results are expressed as micromoles of C_2H_2 per milligram of protein per hour or micromoles of C_2H_2 per milligram of chlorophyll *a* per hour.

RNA preparation. At desired times, serum vials were opened, 1/10 volume of 10× RNA preparation buffer (10× = 0.5 M Tris chloride buffer, pH 7.9, 0.5 M NaCl, 0.5 M sodium EDTA) was added, the samples were immediately transferred to a chilled French pressure cell, and the cells were disrupted at about 8,000 lb/in^2. The lysate was ejected into an equal volume of phenol equilibrated with DNA dialysis buffer (DDB; 0.01 M Tris chloride, pH 7.9, 0.01 M NaCl, 1 mM EDTA). After a 15-min extraction, the aqueous supernatant was collected, and total cell nucleic acids were ethanol precipitated. The nucleic acids were redissolved in DDB and fractionated by pelleting the RNA through a 5.7 M CsCl cushion (5.7 M CsCl in 0.01 M EDTA, pH 7.5), using the procedure of Chirgwin et al. (6). Finally, the RNA pellets were dissolved in DDB, reprecipitated with ethanol, and redissolved in DDB at a concentration of about 1 mg/ml, based on A_{260}. RNA yields were 50 to 200 μg per sample. Agarose gel electrophoresis of the RNA showed distinct rRNA bands, suggesting that the RNA is relatively undegraded.

Dot blot hybridizations. Purified RNA was spotted onto nitrocellulose filters which had been soaked in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (19) and then air dried (29) or on untreated dry Biodyne filters. The filters were then air dried and baked for either 2 h at 65°C in a vacuum oven (nitrocellulose) or 1 h at 80°C (Biodyne). The filters were then hybridized to a 2.8-kilobase *Hin*dIII fragment which contains the entire *nifH* and *nifD* genes of *A. variabilis* (14), using 1 × 10^6 cpm of nick-translated probe per ml in a 5-ml total volume. Preparation of DNA probe fragments, nick translation, and other recombinant DNA procedures were performed as previously described (14, 19).

Hybridizations were performed at 42°C in 5× SSD (19) containing 50% formamide, 0.05 M phosphate buffer, pH 7.0, 250 μg of denatured salmon sperm DNA per ml, and 10% dextran sulfate for nitrocellulose or in 5× SSD containing 50% formamide, 0.05 M phosphate buffer, pH 6.5, and 25 μg of denatured salmon sperm DNA per ml for Biodyne (as recommended by the manufacturer). After hybridization was complete, the filters were washed twice for 5 min in 2× SSC at 25°C and then twice for 15 min in 0.1× SSC at 50°C, and autoradiograms were prepared. Under our conditions, nitrocellulose and Biodyne membranes gave similar results.

Analytical methods. Protein was determined by the Bio-Rad protein assay as described by the manufacturer. For chlorophyll *a* determinations, cultures were filtered onto Whatman GF/C filters and then extracted with methanol. The A_{665} of the extracted material was determined, and chlorophyll *a* content was calculated from a standard curve.

RESULTS

Derepression in anaerobic cultures. Several *Anabaena* strains are known to fix nitrogen under anaerobic conditions (25), but this had not been confirmed in *A. variabilis* 29413. In addition, the time course of nitrogenase gene transcription and the appearance of nitrogenase activity had not been examined together in the same study with any organism. Our first experiments were designed to demonstrate anaerobic nitrogen fixation in *A. variabilis* and to follow the timing of derepression during nitrogen starvation at both the mRNA and enzyme activity levels. A culture which had been grown with 10 mM NH_4Cl to repress heterocyst formation was washed and transferred to nitrogen-free medium in an argon atmosphere. DCMU (5 × 10^{-5} M) was present to inhibit oxygen evolution, and fructose (0.5%) was provided to serve as a source of reductant and energy. The cells were then incubated in conditions otherwise suitable for growth. At 1- to 2-h intervals nitrogenase activity was determined. RNA preparations were made at 0, 3.5, 5.5, 10, 14, and 18 h; periodically, cells were also observed by phase-contract microscopy.

The time course of appearance of acetylene-reducing activity during derepression in an argon atmosphere is shown in Fig. 1. There was no nitrogenase activity until 3 h, when low but significant activity was observed. The level of activity increased throughout the remainder of the experiment, reaching a maximum of about 700 nmol min^{-1} mg of

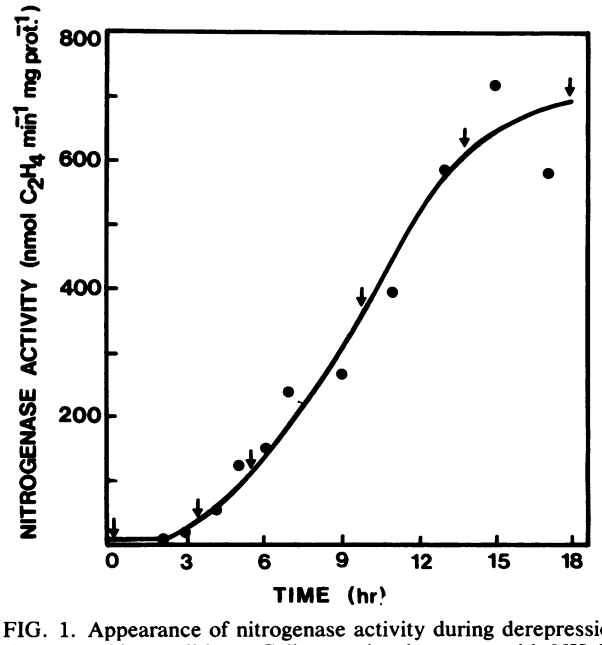

FIG. 1. Appearance of nitrogenase activity during derepression under anaerobic conditions. Cells, previously grown with NH_4Cl, were transferred to nitrogen-free medium and incubated under an argon atmosphere. At the times indicated by the closed circles, one vial was injected with acetylene; 60 min later, acetylene-reducing activity was determined. At the times indicated by the arrows, one vial was used to prepare RNA. Details of the procedures are described in Materials and Methods.

chloraphyll a^{-1}. At 15 h, activity was beginning to plateau. At later times (data not shown), activity decreased 20 to 25%. This probably reflects the poor physiological condition of the cells at the end of these experiments. Since nitrogen fixation cannot occur with an argon atmosphere, the cells continue to be starved for nitrogen even though nitrogenase activity is high.

Total cellular RNA was prepared at the times indicated by arrows in Fig. 1. The presence of nitrogenase mRNA at these times was analyzed by DNA-RNA dot blot hybridization, using a probe which contains the *nifH* and *nifD* genes of *A. variabilis*. Significant levels of nitrogenase RNA were observed at 3.5 h (the first time sampled), and the level did not appear to change substantially during the remainder of the experiment (Fig. 2). The apparent decrease seen at 5.5 h was not observed in other derepression experiments and is not considered significant.

In another experiment, the appearances of nitrogenase mRNA and nitrogenase activity were followed more closely during the first 3 h of nitrogen starvation. Nitrogenase mRNA was first detected at 1.5 h (Fig. 3); in this experiment, acetylene-reducing activity was first detected at 2.0 h (see legend to Fig. 3). Thus, derepression of nitrogenase gene expression in anaerobic conditions is very rapid. The initial appearance of enzyme activity is 5 to 6 h earlier under these conditions than with aerobic cultures which must form heterocysts before nitrogen fixation can occur (J. A. Myers, J. E. Miller, L. R. Yarbrough, and R. Hirschberg, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, p. 136).

In these and other experiments, nitrogenase mRNA was detected 0.5 to 1.0 h before the appearance of enzyme activity. Although nitrogenase activity levels increased for many hours after the first appearance of activity, mRNA reached a steady-state level soon after the initial appearance of hybridizable material, and the level did not change significantly during the rest of the experiment. By comparing the extent of hybridization with the different amounts of total RNA in the dots, the relative amount of nitrogenase mRNA in the various samples can be estimated. For instance, the 0-, 0.5-, and 1.0-h samples had no detectable nitrogenase mRNA even in the samples that have 1 μg of total RNA; by comparison, the 1.5-, 2.0-, and 2.5-h samples showed detectable hybridization to the dots with only 0.01 μg of total RNA. Thus, we can say that the amount of nitrogenase mRNA increased at least 100-fold during derepression. A similar relative increase was observed in other experiments.

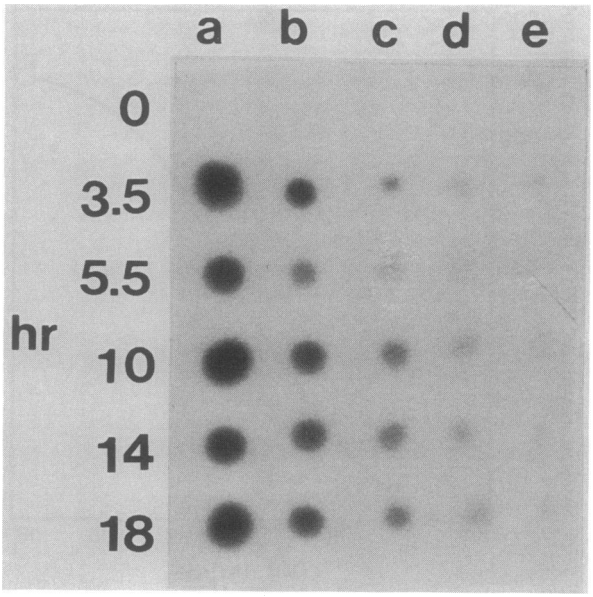

FIG. 2. Appearance of nitrogenase mRNA during derepression under anaerobic conditions. RNA was prepared at the times indicated by arrows in Fig. 1. Various dilutions were spotted onto nitrocellulose such that the dots contain 1.0 (a), 0.33 (b), 0.11 (c), 0.033 (d), and 0.011 (e) μg of total cell RNA. The filter was hybridized to a probe which contains the *nifH* and *nifD* genes, and an autoradiogram was prepared. Details of the procedures are given in Materials and Methods.

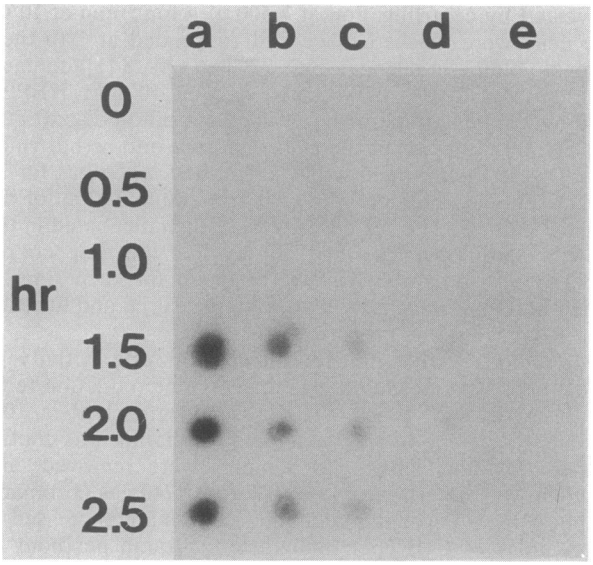

FIG. 3. Appearance of nitrogenase mRNA during the early phase of derepression under anaerobic conditions. At the times indicated RNA was prepared, and dot blot hybridization analysis was performed with a Biodyne filter. Dots a to e contained the same amounts of RNA as those in Fig. 2. Experimental details are given in Materials and Methods. Acetylene-reducing activity at the corresponding times follows: 0 h, <0.02; 0.5 h, <0.02; 1.0 h, <0.02; 1.5 h, <0.02; 2.0 h, 0.13; 2.5 h, 0.78; and 3.5 h, 3.30 μmol of C_2H_4 mg of chlorophyll a^{-1}.

During the course of derepression experiments, the cells shrank in size and eventually appeared very small and depleted compared with the ammonia-grown cells at the beginning of the experiment, reflecting the effects of nitrogen starvation (Fig. 4). However, neither heterocysts nor proheterocysts were observed in significant numbers. After 15 h the culture had <1% proheterocysts and essentially no mature heterocysts (Fig. 4B). Thus, nitrogen fixation gene expression has been disassociated from heterocyst development gene expression.

Effect of oxygen on nitrogenase mRNA levels. In *Klebsiella pneumoniae*, oxygen prevents *nif* gene expression (4, 7, 15). In aerobic cultures, *Anabaena* nitrogen fixation occurs predominately in heterocysts where the microenvironment is anaerobic. The effect of oxygen on *nif* gene transcription in derepressed anaerobic cells was examined in the following experiment. A culture was incubated under derepressing anaerobic conditions for 6 h, a time at which nitrogenase mRNA levels would be at a maximum and stable. The cells were then exposed to oxygen by removing the stoppers from the flasks and allowing the cultures to shake in air, and at 0, 5, 10, 20, and 30 min RNA was prepared. RNA dot blot analysis was then performed to determine the effect of

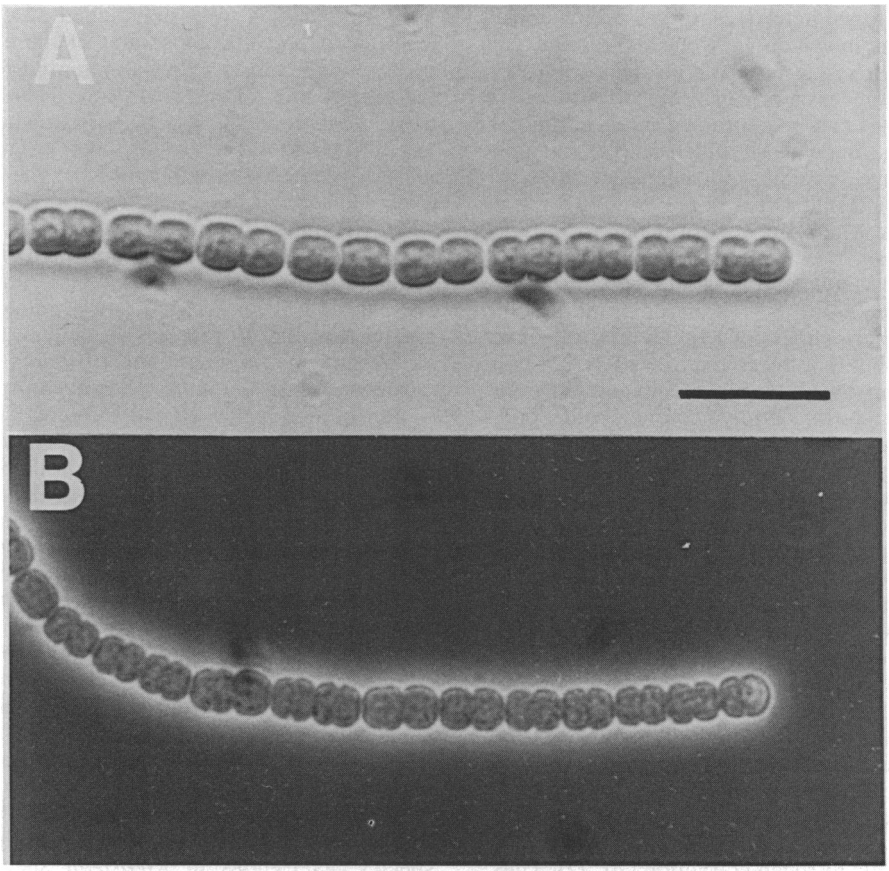

FIG. 4. Phase-contrast photomicrographs of anaerobic derepressed cells: cells at beginning of starvation (A) and after 15 h (B). Bar, 15 μm.

exposure to oxygen on nitrogenase mRNA levels. The level of hybridizing material dropped rapidly after oxygen was admitted to the cultures, and by 30 min very little remained (Fig. 5). Thus, oxygen has a very rapid effect on nitrogenase mRNA levels. The half-time of the decrease in mRNA was estimated to be in the range of 8 to 10 min by comparing the relative amount of hybridization at the various times. Whether this represents repression of transcription, an increase in the rate of nitrogenase mRNA degradation, or a combination of the two cannot be determined from these data.

DISCUSSION

We have studied *A. variabilis nif* gene expression under strictly anaerobic conditions. This species is particularly suitable for these experiments since it can use exogenous fructose as a source of reductant for nitrogen fixation under conditions in which CO_2 fixation is inhibited. Fructose may also be used to supply some of the energy requirements of the cells, although cyclic photophosphorylation is probably the primary energy source (5).

Under the conditions we have used, *nif* gene derepression is very rapid; significant levels of nitrogenase transcripts were detected after 1.5 to 2.0 h of nitrogen starvation; enzyme activity was detected after 2.0 to 3.0 h. The appearance of enzyme activity is 5 to 6 h earlier than observed in comparable aerobic experiments with the same strain (R. Hirschberg and J. A. Myers, unpublished data). One explanation for this difference is that it takes 8 to 9 h to synthesize

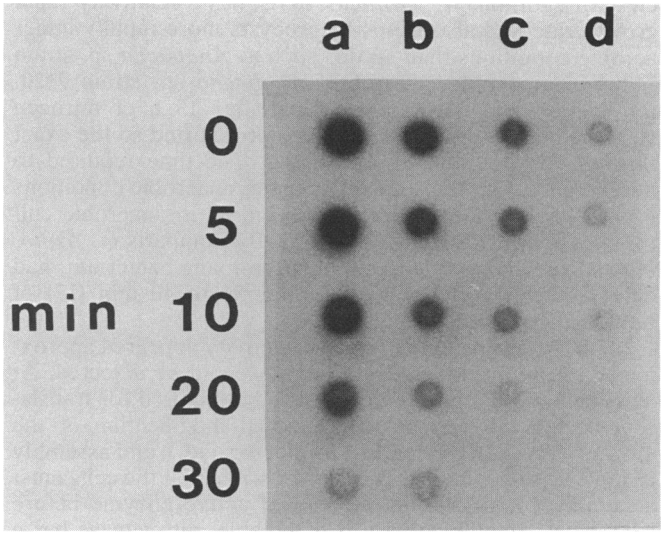

FIG. 5. Effect of oxygen on nitrogenase mRNA levels in cells derepressed under anaerobic conditions. A series of vials which contained cells that had been derepressed for 6 h was opened, and incubation was continued with shaking at 150 rpm. At 0, 5, 10, 20, and 30 min, RNA was prepared. RNA dot blot hybridization analysis was performed with a Biodyne membrane. Dots a to d contain 1.0, 0.33, 0.1, and 0.03 μg of total RNA, respectively.

a sufficiently well-developed heterocyst to provide an anaerobic microenvironment in which *nif* gene expression can occur; in other words, oxygen prevents *nif* gene transcription. This would not be necessary in anaerobic conditions, of course, so that the genes can be expressed much earlier. In support of this view is much work which shows that only mature heterocysts have significant nitrogenase activity or nitrogenase protein (20, 23) and that nonheterocystous mutants or cyanobacteria which do not form heterocysts fix nitrogen only under anaerobic or microaerophilic conditions (25, 28). An alternative explanation should be considered, however. It is possible that in both anaerobic and aerobic cells *nif* genes are transcribed within 1 to 2 h after the onset of nitrogen starvation, but that the enzyme or mRNA or both are rapidly degraded in the presence of oxygen. In other words, transcription and translation occur in both conditions, but RNA turnover or protein denaturation/degradation or both prevent the accumulation of significant enzyme activity if oxygen is present. Since we have not measured nitrogenase polypeptides, but only enzyme activity, we cannot rule out the possibility that protein was present earlier in the experiment. Interestingly, Fleming and Haselkorn (9) reported the appearance of nitrogenase proteins (but not enzyme activity) long before heterocysts had formed when *Anabaena* sp. strain 7120 cultures were starved for nitrogen and incubated without shaking (semiaerobic conditions).

Rippka and Stanier (25) first observed nitrogenase activity after 20 to 25 h of anaerobic nitrogen starvation in *Anabaena* sp. strain 6310 and after 4 to 5 h in *Anabaena* sp. strain 7118; nitrogenase proteins and mRNA were not studied. In *A. cylindrica* nitrogenase activity and proteins were detectable after 8 to 9 h of anaerobic nitrogen starvation (20, 22). Thus, the time required for derepression of nitrogenase varies considerably with the organism. *A. variabilis* is a facultative heterotroph which can use fructose to support anaerobic nitrogen fixation; strains which are strict phototrophs must mobilize endogenous reserves. This may, in part, explain the different time courses compared with other *Anabaena* species. In addition, *A. variabilis* 29413 has a relatively rapid growth rate and also forms heterocysts more rapidly under aerobic conditions than strains such as *Anabaena* sp. strain 7120. In anaerobic cultures of *Anabaena* sp. strain 7120, nitrogenase mRNA was abundant after 13 h of nitrogen starvation (24); earlier times were not reported so the exact time of derepression is not known. The time required to derepress *A. variabilis nif* genes under anaerobic conditions is only slightly longer than that required in anaerobic cultures of *K. pneumoniae* (4, 7, 15). By comparison, *Azotobacter vinelandii*, an aerobic nitrogen-fixing bacterium, had detectable nitrogenase mRNA after 35 to 40 min (17) of nitrogen starvation.

In our experiments, nitrogenase activity appeared approximately 1 h after nitrogenase mRNA could be detected. At least part of this time is assumed to be required for translation of the mRNA, posttranslational modification of the enzyme (addition of the Mo-Fe cofactor, etc.), and assembly of the enzyme complex. It is also possible that the cells must accumulate a substantial amount of active enzyme before acetylene-reducing activity is detectable; nitrogenase has a very slow turnover rate (3), and large quantities of enzyme protein are present in nitrogen-fixing cells (4, 23). A similar lag between appearance of mRNA and activity has been observed in *K. pneumoniae* (4, 15).

In anaerobic derepression experiments, nitrogenase mRNA levels increased very rapidly during a short period of 1 to 2 h and then were maintained at a relatively constant level throughout the remainder of the experiment. In contrast, enzyme activity levels increased for many hours and leveled off only after 15 to 20 h. This is what one would expect for a repressible gene system whose protein product has a long lifetime. In response to nitrogen starvation, mRNA synthesis is initiated; since the mRNA has a relatively short half-life (see below), the level reaches a steady state, when new synthesis is balanced by degradation, quite rapidly. By comparison, proteins, including nitrogenase, are comparatively stable. As long as translatable mRNA is present, and as long as the rate of nitrogenase synthesis is greater than that of proteins in general, the specific activity of the enzyme will continue to increase. The half-life of nitrogenase in *A. cylindrica* has been reported to be 6 to 25 h, depending on the oxygen tension (21). Eventually, new factors such as lack of energy or reductant will influence cell physiology, and activity will level off. Similar results have been observed in *K. pneumoniae* (15). All of our data are consistent with *nif* gene expression being controlled at the transcription level. Pulse-labeling experiments could help prove this. Unfortunately, these experiments are difficult to perform in cyanobacteria, but we are currently attempting to address this problem.

When oxygen was admitted into derepressed anaerobic cultures, nitrogenase mRNA levels decreased very rapidly. This could be the result of oxygen serving as an inhibitor of nitrogenase gene transcription; alternatively, oxygen might cause an increase in the rate of nitrogenase mRNA degradation. It is also possible that both factors contribute to the observed results. Probably the most likely explanation is that oxygen prevents *nif* gene transcription in *Anabaena* species, as it does in *Klebsiella* species, by preventing transcription initiation (4, 7) via the *nifL* protein. The half-time of nitrogenase mRNA disappearance we observed was about 8 to 10 min; this is similar to the half-life for *A. variabilis* total mRNA, which was reported to be 10 to 12 min (18). There is some evidence, however, that oxygen may also cause specific degradation of *nif* mRNAs in *K. pneumoniae* (8, 15), and this possibility cannot be rejected for *Anabaena* species without further information. It has also been reported that the *nifLA* promoter is sensitive to oxygen (16). We are currently investigating this issue. Haselkorn et al. (12, 13) have also reported that nitrogenase mRNA disappears rapidly in the presence of oxygen.

Proheterocysts were not observed in our anaerobic derepressed cultures. In *Anabaena* sp. strains 7120 and 6310, proheterocysts developed in similar conditions (24, 25). Differentiation is not essential in *Anabaena* species, however, since *Anabaena* sp. strain 7118 fixes nitrogen anaerobically and cannot form proheterocysts. Rogerson (26) reported the presence of heterocysts in *A. variabilis* 29413 grown with an atmosphere of N_2-CO_2, but it is likely that oxygen was produced by the cultures since DCMU was not used. In *A. cylindrica* mature heterocysts are formed under anaerobic conditions, and they appear to be the sole location of nitrogenase (20). Thus, our results may be a result of strain differences. Alternatively, the extent of differentiation in our experiments may have been too limited to be seen with phase-contrast microscopy.

Recently, Golden et al. reported that *nif* genes of *Anabaena* sp. strain 7120 undergo at least two DNA rearrangements during the course of heterocyst development (10). This rearrangement is apparently essential for nitrogenase gene expression, and it also occurs under anaerobic conditions. We have observed a large transcript of about 4.8

kilobases in RNA from anaerobic cultures that hybridizes to both *nifH* and *nifK* gene probes (unpublished observations). Since our previous mapping studies showed that the *nifK* gene is at least 4 kilobases away from the linked *nifH* and *nifD* genes (14), it is likely that a similar rearrangement occurs in *A. variabilis*.

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