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In Situ Detection of Transcripts for Ribulose-1,5-Bisphosphate Carboxylase in Cyanobacterial Heterocysts

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Heterocysts of free-living cyanobacteria lack ribulose-1,5-bisphosphate carboxylase activity. Nevertheless, using in situ hybridizations, we demonstrate that transcripts for the *rbcL* and *rbcS* genes are present in both heterocysts and vegetative cells of *Anabaena* spp. in association with, or isolated from, the *Azolla-Anabaena* symbiosis. In contrast, *rbcLS* transcripts were detected only in vegetative cells of the free-living cyanobacterium *Anabaena* strain 7120. Under anaerobic growth conditions that inhibited heterocyst differentiation, transcripts for nitrogenase were present in all cells composing *Anabaena* strain 7120 filaments, whereas *rbcL* and *rbcS* transcripts were not detected. Thus, transcriptional regulation of genes related to photosynthesis and nitrogen fixation is under environmental, as well as developmental, control in *Anabaena* spp. In addition, these results suggest either the possible retention of regulatory patterns in symbiotically derived cyanobacterial isolates or differences in expression of *rbcLS* genes in different free-living cyanobacteria.

The *Azolla-Anabaena* symbiosis is an association between the eukaryotic water fern *Azolla* sp., nitrogen-fixing cyanobacteria (tentatively identified as *Anabaena* spp.), and eubacteria (1). The association is of agricultural importance due to its ability to serve as a biofertilizer, fodder, and weed suppressor (13). In this association, symbionts reside within specialized structures called leaf cavities, where exchange of nutrients takes place between the partners. The fern supplies a carbon source to the cyanobionts, which fix enough atmospheric nitrogen to satisfy the nitrogen requirements of both symbiotic partners. Nitrogen fixation in symbiotic and free-living *Anabaena* spp. occurs in specialized cells called heterocysts (17, 18, 28). Reduced carbon compounds are produced in vegetative cells by photosynthesis and transferred to heterocysts. Ribulose-1,5-bisphosphate carboxylase (RuBisCO) catalyzes the incorporation of CO_2 into carbohydrate in plant and algal chloroplasts as well as in photosynthetic prokaryotes. In the symbiosis, CO_2 fixed via the Calvin cycle by both *Azolla* and *Anabaena* vegetative cells is transferred to the heterocysts (21). The mutual dependence of heterocysts and vegetative cells is well documented (7).

In earlier studies, cloned genes from the free-living cyanobacterium *Anabaena* strain 7120 have been used to determine transcript levels of corresponding genes in freshly isolated cyanobionts of *Azolla caroliniana* (15). RuBisCO transcripts were determined to be fivefold less abundant in the cyanobionts (15) than in the free-living *Anabaena* sp. The endosymbiont also had reduced CO_2-fixing activity (16). To further study the regulation of RuBisCO expression in symbiotic *Anabaena* spp., we developed a method of in situ hybridization to localize transcripts within cyanobacterial cells. We believe that this is the first report of in situ detection of natural populations of mRNA within cyanobacterial or other prokaryotic cells. Moreover, the in situ hybridization technique described below has the potential to facilitate a more complete understanding of the molecular mechanisms of heterocyst differentiation.

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In situ hybridization is a powerful technique that has been used for years in animal biology for the localization of specific DNA and RNA sequences within cells or tissue sections. Only recently was this method adapted for plant (12) and bacterial (5) systems. In the present study, we have extended the use of this technique by adapting an in situ hybridization procedure for plant tissues (12) to localize transcripts in cyanobacterial cells. The sensitivity of this technique has allowed for the detection of transcripts in individual cells within cyanobacterial filaments.

MATERIALS AND METHODS

Organisms. Cyanobionts from *Azolla pinnata* R. Brown (IRRI Collection no. 2) and *A. caroliniana* Willd. (IRRI Collection no. 3001) were used in the present study. Free-living *Anabaena* species used in this study were *Anabaena* sp. Newton's strain (14), and *Anabaena* sp. strain PCC 7120 (4).

Growth conditions. *A. pinnata* no. 2 and *A. caroliniana* no. 3001 were grown at 27°C under a 17:7 (light-dark) photoperiod with light from fluorescent and incandescent lamps at a photon fluence rate of 50 to 70 $\mu mol \cdot m^{-2} s^{-1}$. Free-living cultures, *Anabaena* strain 7120 and *Anabaena* sp. Newton's isolate, were grown in 200 ml of BG-11 (nitrogen-deficient) medium (24) in 500-ml flasks plugged with cotton at 27 to 30°C with shaking at 200 rpm under incandescent and fluorescent lamps at a photon fluence rate of 50 to 60 $\mu mol \cdot m^{-2} s^{-1}$. For anaerobic induction experiments, *Anabaena* strain 7120 was first grown in BG-11 (nitrogen-rich) medium and then transferred to BG-11 (nitrogen-deficient) medium containing 3,3,4-dichlorophenyl dimethyl urea. Anaerobiosis was established by bubbling argon through the culture, and the cells were harvested after 10 h (23).

Isolation of cyanobionts. Cyanobionts were isolated by crushing whole *Azolla* fronds and filtering plant debris through cheesecloth (15). Cyanobacteria were pelleted, after which a 40- to 50-μl pellet was resuspended in 40 μl of phosphate-buffered saline (PBS; pH 7.4). Coverslips were dipped into a 0.1-mg/ml solution of polylysine hydrobromide (Sigma Chemical Co., St. Louis, Mo.) and allowed to air dry. Approximately 10 μl of cells was spread on each coverslip and allowed to air dry.

In situ hybridization. Cyanobacterial cells were fixed with 4% paraformaldehyde in PBS for 0, 5, 10, 15, and 25 min. Following two rinses with PBS, the coverslips were stored (for up to 10 days) in 70% ethanol at 4°C. Each in situ hybridization was performed in duplicate. Pretreatments were performed to increase the accessibility of the fixed mRNA within cells to the probes. Solutions (approximately 20 ml) were added to petri dishes; incubations were conducted without agitation. The initial treatment (four to five coverslips per dish) was in 0.2 M HCl at room temperature for 5 min. Following two rinses with sterile distilled water, the coverslips were placed in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]) (at 50°C) for 15 min. Cells attached to coverslips were then incubated in a solution containing 1 mg of proteinase K (Sigma) per ml in Tris-EDTA (pH 7.5) for 10 min at room temperature, rinsed in sterile distilled water, treated with 2× SSPE for 5 min, and rinsed twice with sterile distilled water. Dehydrations were in 70% ethanol for 5 min followed by 95% ethanol for 5 min. The cells were then air dried (5 to 15 min). Probes were nick translated with [α-^{35}S]dATP (Amersham Corp., Arlington Heights, Ill.) to specific activities of approximately 10^8 cpm/μg. Approximately 10^6 cpm of labeled probe (ethanol precipitated with 1 mg of salmon sperm DNA per ml [final concentration]) was used in hybridization reactions for each coverslip. Dried probes were resuspended in 20 μl of hybridization solution (70°C for 5 to 10 min), denatured for 3 min at 95°C, and chilled on ice until used. Coverslips were placed cell side down on the probe-hybridization solution, which had been spotted onto Parafilm. Hybridization was performed in a humid atmosphere at room temperature for 20 to 22 h. Coverslips were rinsed twice in the following solution: 50% deionized formamide, 600 mM NaCl, 10 mM Tris (pH 7.5), and 1 mM EDTA. Subsequent washes were in 2× SSPE at room temperature for 2 to 3 h (five to seven changes). Cells were dehydrated (in 70% ethanol–0.3% ammonium hydroxide) for 5 min and air dried. Coverslips were mounted on glass slides (cell side up) with permount (Fisher Scientific, Rochester, N.Y.), coated with Kodak NTB-2 nuclear track emulsion, and incubated in the dark at 4°C for 12 to 25 days. Slides developed with D-19 developer were mounted with coverslips, examined with an Olympus BH light microscope, and photographed with Tech Pan film (Eastman Kodak Co., Rochester, N.Y.). For control experiments demonstrating that the probes specifically label RNA, isolated cyanobionts were treated with DNase-free RNase (pancreatic, from Sigma Chemical Co.) at a concentration of 50 μg/ml for 10 min at room temperature, prior to the fixation of cells with paraformaldehyde, as described above.

Probes. The probes used were obtained from recombinant plasmids containing cloned fragments of *Anabaena* strain 7120 DNA. The *nifK* probe was pAn207.8, containing the 0.7-kb *Hin*dIII fragment (22), the *nifH* probe was a 1.8-kb *Hin*dIII fragment of the plasmid pAn154.3 (11), the *rbcL* probe was pAn602, and the *rbcS* probe was pAn606, as described previously (8).

Anaerobic inductions. For anaerobic induction experiments, *Anabaena* strain 7120 was initially grown in BG-11 (nitrogen-rich) (24). After the absence of heterocysts was ascertained via microscopic observation, cells were pelleted by centrifugation and inoculated into 500 ml of BG-11 (nitrogen-deficient) medium (24). The culture was gassed with air-CO_2 (99%:1%) and incubated for 24 h in the light on a shaker. 3,3,4-Dichlorophenyl dimethyl urea was added to a final concentration of 10^{-5} M, after which the flask was flushed thoroughly with argon-CO_2 (99%:1%). Anaerobiosis was maintained by capping the flask tightly, and the cells were harvested after 10 h (23). Subsamples were checked for nitrogenase activity by measuring acetylene reduction (18). Once measurable acetylene reduction was observed, filaments were subjected to RNA isolation or in situ hybridization.

Heterocyst isolations and Northern blot hybridizations. The method of Jensen et al. (9) was used for heterocyst isolations. RNA isolated from heterocysts of *A. pinnata* cyanobionts, *Anabaena* strain 7120 grown on a medium deficient in combined nitrogen, and *Anabaena* strain 7120 filaments anaerobically induced for nitrogenase was used for Northern (RNA) blot analysis essentially as described previously (15). However, the RNA was transferred to Nytran and baked in a vacuum oven at 65°C for 30 min. Hybridization was carried out using probes that were multi-prime labeled with [α-^{32}P]dCTP according to the manufacturer's specifications (Amersham Corp.). A NICK column (Pharmacia LKB Biotechnology, Piscataway, N.J.) was used to remove the unincorporated label.

RESULTS

The in situ hybridization procedure requires attachment of cells to polylysine-coated coverslips, fixation of cellular mRNA, permeabilization of the cells to allow entrance of ^{35}S-labeled probes, and autoradiography to detect specific mRNAs. The most important parameter influencing the detection of mRNA within individual cyanobacterial cells was the length of fixation with 4% paraformaldehyde. A 5-min change in fixation time drastically altered detection of mRNA within cyanobacterial filaments (Fig. 1; see Fig. 3). Initially, transcripts for the *rbcL* and *rbcS* genes, encoding the large and small subunits of RuBisCO, were localized in cyanobionts freshly isolated from *A. pinnata* (Fig. 1). No label was detected within cyanobacterial cells in control hybridizations, using pBR322 without an insert as the probe (Fig. 1A). In addition, no label was detected with cyanobacterial cells treated with RNase, as described in Materials and Methods (data not shown). Fixation times of 0 min (Fig. 1B) or 5 min (Fig. 1C) are presumed to be insufficient to preserve transcripts and resulted in failure to detect them with an *rbcL* probe. Cells fixed for 25 min or longer also contained no label (data not shown). With an optimal fixation time of 10 or 15 min (Fig. 1D and E, respectively), RuBisCO-specific transcripts were clearly detected in both vegetative cells and heterocysts. Similar results were obtained with an *rbcS* specific probe (Fig. 1F to I). At times it was difficult to distinguish heterocysts from vegetative cells when filaments were examined by light microscopy because of the exposed silver grains around and within the labeled cells. To conclusively distinguish heterocysts from vegetative cells, in some instances, a series of photographs of the same filament was taken with the focus changing from the silver grains to the filaments. An example of such a through-focus series, examining an in situ hybridization of *rbcS* transcripts in an *Anabaena* strain 7120 filament, is shown in Fig. 2. The labeling results for *Anabaena* strain 7120 are discussed in detail below.

Additional hybridizations were performed with a free-living cyanobacterial isolate originally obtained from *A. caroliniana* leaf cavities (14). A 5-min fixation time was optimal for detection of transcripts in this organism, which is smaller than the *A. pinnata* cyanobiont. *rbcLS* transcripts were not detected in unfixed filaments (Fig. 3A and D) or in filaments fixed for 10 min (Fig. 3C and F) or longer. However, when fixed for 5 min, *rbcL* and *rbcS* transcripts were again detected within both heterocysts and vegetative cells (Fig. 3B and E, respectively).

To determine whether the expression of *rbcLS* transcripts in heterocysts was unique to cyanobacteria in or isolated from the

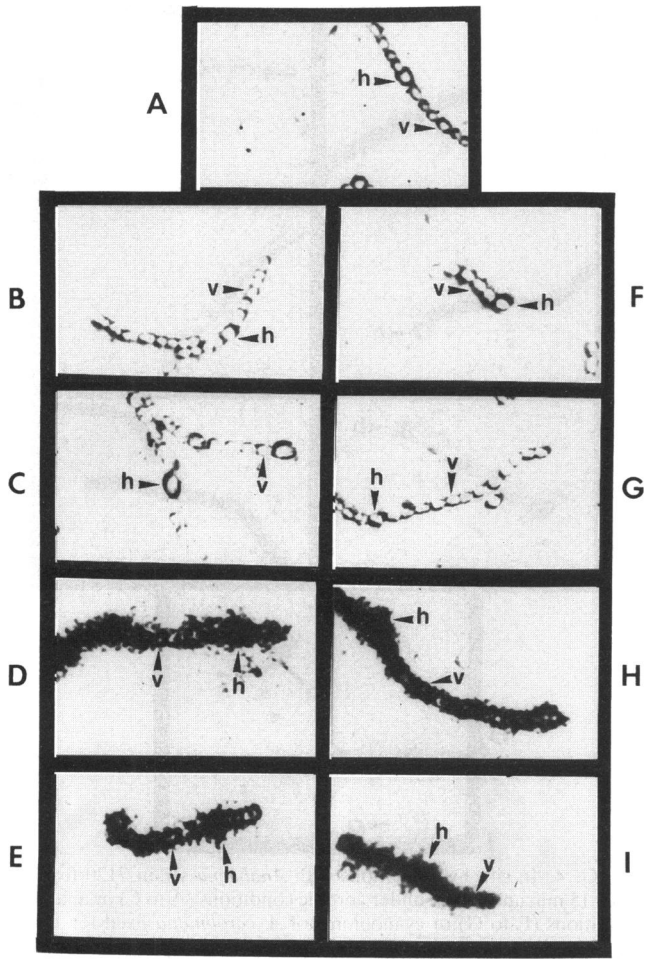

FIG. 1. In situ hybridizations with the cyanobionts of *A. pinnata*. Cells were attached to polylysine-coated coverslips, fixed with 4% paraformaldehyde for 0 to 15 min, and hybridized with the designated probe. (A) Cells fixed for 15 min and hybridized with a ^{35}S-labeled pBR322 control probe contained no detectable label. (B to E) Cells were fixed for 0, 5, 10, and 15 min (respectively) and hybridized with a ^{35}S-labeled *rbcL* probe. In filaments fixed for 10 and 15 min, *rbcL* transcripts were detected in both vegetative cells (v) and heterocysts (h). (F to I) Cells were fixed for 0, 5, 10, and 15 min (respectively) and hybridized with a ^{35}S-labeled *rbcS* probe. Vegetative cells and heterocysts within filaments fixed for 10 and 15 min contained *rbcS* transcripts.

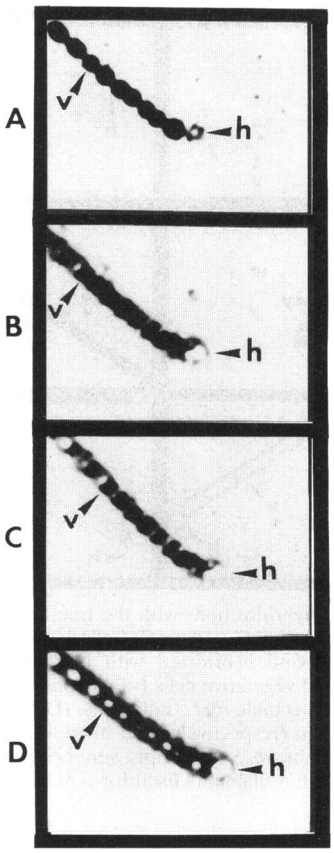

FIG. 2. A through-focus series of photographs examining an in situ hybridization of *rbcS* transcripts in an *Anabaena* strain 7120 filament. When the point of focus is changed from the exposed silver grain around the filament to individual cells in the filament, it is possible to clearly demonstrate the morphological difference between vegetative cells (v), which were labeled, and heterocysts (h), which were not labeled.

symbiotic association, in situ hybridizations were performed using the free-living cyanobacterium *Anabaena* strain 7120. When cultured aerobically in a medium lacking combined nitrogen, *Anabaena* strain 7120 filaments contained vegetative cells and heterocysts. Hybridizations with either the *rbcL* or *rbcS* probe detected transcripts only within the vegetative cells of these filaments (Fig. 4A and B, respectively). As expected, control hybridizations using a *nifK* gene probe detected dinitrogenase reductase transcripts within heterocysts, but not vegetative cells, of *Anabaena* strain 7120 (Fig. 4C) fixed for 10 to 15 min. An additional control was carried out to ensure that labeling of heterocysts was not due to nonspecific sticking of the probe to these cells due to their extracellular envelope. The cyanobionts of *A. caroliniana* that differentiate heterocysts and akinetes, which both possess an envelope of similar chemical composition, were used for this work. In situ hybridization using the *nifK* probe detected transcripts only within heterocysts (Fig. 4D), thereby demonstrating an absence of nonspecific labeling of the probe due to the envelope of the akinetes.

Anabaena strain 7120 was also grown anaerobically for nitrogenase induction (see Materials and Methods). Filaments grown under these conditions were devoid of heterocysts, and *rbcL* and *rbcS* transcripts were not detected within the vegetative cells (Fig. 4E and F, respectively). In contrast, a *nifK* probe detected *nif* transcripts in all of the vegetative cells (Fig. 4G). Similar results were obtained by Haselkorn et al. (8) using Northern blot analysis. Our work also demonstrates the presence of *nif* transcripts within every cell of the filament. Although this result would appear to be in conflict with those reported by Elhai and Wolk (3), differences in growth conditions used to obtain an anaerobic environment make direct comparisons inappropriate.

To confirm the in situ hybridization results, Northern blot analyses were conducted using RNA from heterocysts isolated from *A. pinnata* cyanobionts or free-living *Anabaena* strain 7120, as well as intact filaments of anaerobically induced *Anabaena* strain 7120 (Fig. 5). While the quality of RNA extracted from heterocysts was poorer than of RNA isolated from intact anaerobically growing filaments, transcripts were still detectable. Heterocyst RNA from cyanobionts of *A. pinnata* contained *nifH* transcripts approximately 4.9, 3.2, and

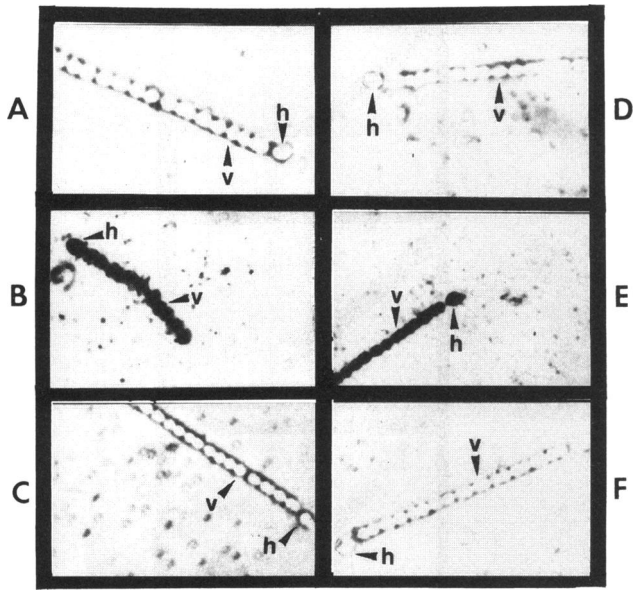

FIG. 3. In situ hybridizations with the free-living Newton's isolate obtained from *A. caroliniana*. (A to C) Cells were fixed for 0, 5, and 10 min (respectively) and hybridized with a ^{35}S-labeled *rbcL* probe. Heterocysts (h) and vegetative cells (v) composing filaments fixed for 5 min contained detectable *rbcL* transcripts. (D to F) Cells were fixed for 0, 5, and 10 min (respectively) and hybridized with a ^{35}S-labeled *rbcS* probe. Detectable *rbcS* transcripts were present in vegetative cells and heterocysts within filaments fixed for 5 min.

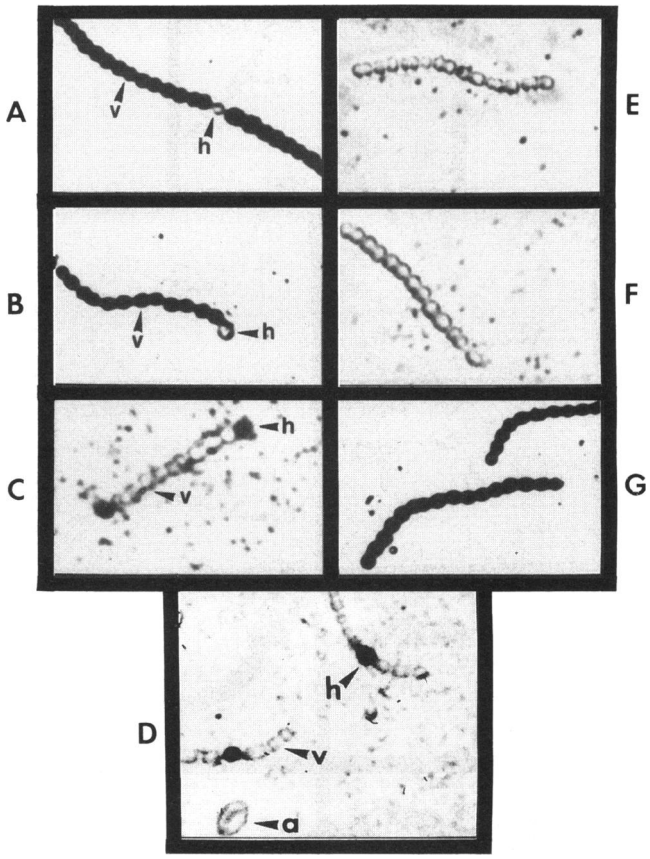

FIG. 4. In situ hybridizations with *Anabaena* strain 7120 fixed for 10 or 15 min and grown under aerobic conditions (A to C) or anaerobic conditions (E to G) or cyanobionts of *A. caroliniana* fixed for 15 min (D). (A) With a ^{35}S-labeled *rbcL* probe, transcripts were localized within vegetative cells (v) but absent from heterocysts (h). (B) Vegetative cells also contained *rbcS* transcripts which were absent from heterocysts. (C) With a ^{35}S-labeled *nifK* probe, *nif* transcripts were detected in heterocysts but absent from vegetative cells. (D) With a ^{35}S-labeled *nifK* probe, transcripts were detected in heterocysts but absent from akinetes (a), which possess an envelope composed of the same glycolipids as heterocysts. (E and F) Cells within filaments of anaerobically grown cultures were hybridized with *rbcL* and *rbcS* probes (respectively) but contained no detectable transcripts. (G) All cells within filaments grown under anaerobic conditions had detectable *nifK* transcripts.

2.1 kb in size (Fig. 5, lane A). These transcript sizes are comparable to those reported in an earlier study for the cyanobionts of *A. caroliniana* (15). In RNA from heterocysts of *Anabaena* strain 7120 (Fig. 5, lane D), *nifH* transcripts approximately 4.8 kb in size and smaller were detected, which is similar to observations of Golden et al. (6). In RNA isolated from intact filaments of anaerobically induced *Anabaena* strain 7120, *nifH* transcripts 3.8 and 2.7 kb in size were detected (Fig. 5, lane F), which corresponds to previous reports (8). Northern blot analyses using *rbcL* and *rbcS* probes identified transcripts of approximately 3.1 kb in size using RNA isolated from heterocysts of the cyanobionts. Transcripts for *rbcLS* were not detected in heterocyst RNA from *Anabaena* strain 7120 (Fig. 5, lane E) or in RNA prepared from anaerobically induced *Anabaena* strain 7120 filaments (Fig. 5, lane G). These results support those obtained by in situ hybridizations. Thus, the expression of *rbcL* and *rbcS* genes within heterocysts is different in the free-living cyanobacteria *Anabaena* strain 7120 versus symbiotic or symbiotically derived Newton's isolate. This suggests either the retention of certain regulatory patterns in symbiotically derived cyanobacteria or differences in expression of *rbcLS* genes in different free-living heterocystous cyanobacteria.

DISCUSSION

The in situ hybridization procedure that was developed allows for the detection of gene expression in single cells within cyanobacterial filaments. In addition, the method yielded both molecular and morphological data without disruption of filaments associated with fractionation procedures. Embedding and sectioning steps were unnecessary in conjunction with this procedure, and transcript localization was carried out within individual cyanobacterial cells composing filaments. It should be noted that while the in situ hybridization procedure allowed for the localization of transcripts in microscopically visible heterocysts, it did not allow for the distinction of heterocysts at different stages of development (i.e., proheterocysts or mature or old heterocysts).

In free-living cyanobacteria, it has been suggested that the level of RuBisCO is essentially invariant (26). In addition, it has been postulated that there is a lack of transcriptional control over enzymes of carbon metabolism in cyanobacteria. In the *Nostoc-Nephroma* symbiosis, cyanobionts contained RuBisCO protein although they lacked the ability to fix CO_2 in vivo (20). Also, cyanobionts associated with cycads displayed no in vivo CO_2 fixation (10), although measurements of in vitro RuBisCO activity were similar to those of free-living isolates. Thus, there appears to be inefficient control of RuBisCO expression in these cyanobionts, which synthesize RuBisCO that is not being used to fix CO_2. In contrast, there are

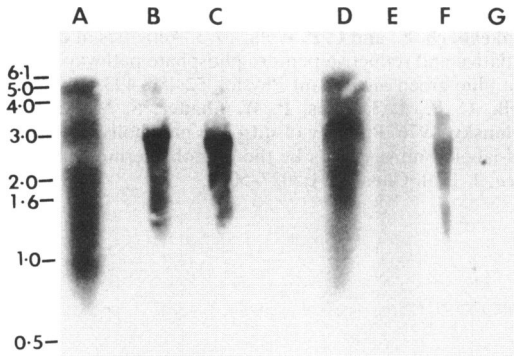

FIG. 5. Northern blot analyses using RNA prepared from heterocysts isolated from *A. pinnata* cyanobionts (lanes A to C) and free-living *Anabaena* strain 7120 (lanes D to E) and filaments of anaerobically induced *Anabaena* strain 7120 (lanes F to G). Transcripts for nitrogenase with a *nifH* probe were detected in each of the RNA samples (lanes A, D, and F). The *rbcL* transcripts were detected in heterocyst RNA from the cyanobionts (lane B) but were absent from heterocyst RNA from aerobically grown *Anabaena* strain 7120 (lane E) and RNA from anaerobically induced filaments (lane G). Heterocyst RNA from the cyanobionts also contained *rbcS* transcripts (lane C). Sizes are given on the left in kilobases.

precedents for regulation of RuBisCO expression at the translational and posttranslational levels in the symbiotic association between *Nostoc* and *Anthoceros* spp. (25). Previously, we have demonstrated transcriptional regulation of the *rbcLS* genes in the cyanobionts of *Azolla* spp. (15). Thus, inefficient transcriptional control of the *rbcLS* genes is unlikely to provide a complete explanation for the results obtained in the present study.

The presence of *rbcL* and *rbcS* transcripts in heterocysts of the cyanobionts of *A. pinnata*, *A. caroliniana*, and the symbiotically derived Newton's isolate was unexpected in light of previous studies reporting the absence of RuBisCO protein and activity in heterocysts of free-living and symbiotic cyanobacteria (2, 21, 27). While it is clear that transcripts are present in the heterocysts of these organisms, it is not yet known whether translation of these transcripts occurs. Immunolocalization of RuBisCO in symbiotic and symbiotically derived cyanobacteria will be conducted to determine this. If RuBisCO protein is detected within heterocysts, its function is unlikely to be CO_2 fixation. In a unicellular cyanobacterium, RuBisCO has been shown to be essential for both photoautotrophic and photoheterotrophic growth (19). In heterocysts of the cyanobionts, *rbcLS* expression may support an unknown dark function. However, the functional significance of RuBisCO in heterocysts remains to be elucidated.

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REFERENCES

1. **Braun-Howland, E. B., and S. A. Nierzwicki-Bauer.** 1990. *Azolla-Anabaena* symbiosis: biochemistry, ultrastructure, and molecular biology, p. 65–117. *In* A. N. Rai (ed.), CRC handbook of symbiotic cyanobacteria. CRC Press, Inc., Boca Raton, Fla.
2. **Cossar, J. D., P. Rowell, A. J. Darling, S. Murray, G. A. Codd, and W. D. P. Stewart.** 1985. Localization of ribulose 1,5-bisphosphate carboxylase/oxygenase in the N_2-fixing cyanobacterium *Anabaena cylindrica*. FEMS Microbiol. Let. **28:**65–68.
3. **Elhai, J., and C. P. Wolk.** 1990. Developmental regulation and spatial pattern of expression of the structural genes for nitrogenase in the cyanobacterium *Anabaena*. EMBO J. **9:**3379–3388.
4. **Fleming, H., and R. Haselkorn.** 1974. The program of protein synthesis during heterocyst differentiation in nitrogen-fixing blue green algae. Cell **3:**159–170.
5. **Giovannoni, S. J., E. F. DeLong, G. J. Olsen, and N. R. Pace.** 1988. Phylogenetic group-specific oligodeoxynucleotide probes for identification of single microbial cells. J. Bacteriol. **170:**720–726.
6. **Golden, J. W., S. J. Robinson, and R. Haselkorn.** 1985. Rearrangement of nitrogen fixation genes during heterocyst differentiation in the cyanobacterium *Anabaena*. Nature (London) **314:**419–423.
7. **Haselkorn, R.** 1978. Heterocysts. Annu. Rev. Plant Physiol. **29:** 319–344.
8. **Haselkorn, R., S. E. Curtis, R. Fisher, B. J. Mazur, M. Mevarech, D. Rice, R. Nagaraja, S. J. Robinson, and R. Tuli.** 1983. Cloning and physical characterization of *Anabaena* genes that code for important functions in heterocyst differentiation: nitrogenase, glutamine synthetase, and RUBP carboxylase, p. 315–331. *In* G. Papageorgiou and L. Packer (ed.), Photosynthetic procaryotes: cell differentiation and function. Elsevier Science Publishing Co., Amsterdam.
9. **Jensen, B. B., R. P. Cox, and R. H. Burris.** 1986. Isolation of cyanobacterial heterocysts with high and sustained dinitrogen-fixation capacity supported by endogenous reductants. Arch. Microbiol. **145:**241–247.
10. **Lindblad, P., A. N. Rai, and B. Bergman.** 1987. The *Cycas revoluta-Nostoc* symbiosis: enzyme activities of nitrogen and carbon metabolism in the cyanobiont. J. Gen. Microbiol. **133:**1695–1699.
11. **Mevarech, M., D. Rice, and R. Haselkorn.** 1980. Nucleotide sequence of a cyanobacterial *nif*H gene coding for nitrogenase reductase. Proc. Natl. Acad. Sci. USA **77:**6476–6480.
12. **Meyerowitz, E. M.** 1987. *In situ* hybridization to RNA in plant tissue. Plant Mol. Biol. Rep. **5:**242–250.
13. **Moore, A. W.** 1969. *Azolla* biology and agronomic significance. Bot. Rev. **35:**17–34.
14. **Newton, J. W., and A. I. Herman.** 1979. Isolation of cyanobacteria from the aquatic fern *Azolla*. Arch. Microbiol. **120:**161–165.
15. **Nierzwicki-Bauer, S. A., and R. Haselkorn.** 1986. Differences in mRNA levels in *Anabaena* living freely or in symbiotic association with *Azolla*. EMBO J. **5:**29–35.
16. **Peters, G. A.** 1975. The *Azolla-Anabaena azollae* relationship. III. Studies on metabolic capabilities and a further characterization of the symbiont. Arch. Microbiol. **103:**113–122.
17. **Peters, G. A.** 1977. The *Azolla-Anabaena azollae* symbiosis, p. 231–258. *In* A. Hollaender (ed.), Genetic engineering in nitrogen fixation. Plenum Publishing Corp., New York.
18. **Peters, G. A., and B. C. Mayne.** 1974. The *Azolla*, *Anabaena azollae* relationship. II. Localization of nitrogenase activity as assayed by acetylene reduction. Plant Physiol. **53:**820–824.
19. **Pierce, J., T. J. Carlson, and J. G. K. Williams.** 1989. A cyanobacterial mutant requiring the expression of ribulose bisphosphate carboxylase from a photosynthetic anaerobe. Proc. Natl. Acad. Sci. USA **86:**5753–5757.
20. **Rai, A. N., M. Borthakur, S. Singh, and B. Bergman.** 1989. *Anthoceros-Nostoc* symbiosis: immunoelectron microscopic localization of nitrogenase, glutamine synthetase, phycoerythrin, and ribulose 1,5-bisphosphate carboxylase/oxygenase in the cyanobiont and the cultured (free-living) isolate *Nostoc* 7801. J. Gen. Microbiol. **135:**385–395.
21. **Ray, T. B., B. C. Mayne, R. E. Toia, Jr., and G. A. Peters.** 1979. *Azolla-Anabaena* relationship. VIII. Photosynthetic characterization of the association and individual partners. Plant Physiol. **64:**791–795.
22. **Rice, D., B. Mazur, and R. Haselkorn.** 1982. Isolation and physical mapping of nitrogen fixation genes from the cyanobacterium *Anabaena* 7120. J. Biol. Chem. **257:**13157–13163.
23. **Rippka, R., and J. B. Waterbury.** 1977. The synthesis of nitrogenase by non-heterocystous cyanobacteria. FEMS Microbiol. Lett. **2:**83–86.
24. **Stanier, R. Y., R. Kunisawa, M. Mandel, and G. Cohen-Bazire.**

1971. Purification and properties of unicellular blue-green algae (order *Chroococcales*). Bacteriol. Rev. **35:**171–205.

25. **Steinberg, N. A., and J. C. Meeks.** 1989. Photosynthetic CO_2 fixation and ribulose bisphosphate carboxylase/oxygenase activity of *Nostoc* sp. strain UCD 7801 in symbiotic association with *Anthoceros punctatus*. J. Bacteriol. **171:**6227–6233.
26. **Tabita, R. F.** 1987. Carbon dioxide fixation and its regulation in cyanobacteria, p. 95–117. *In* P. Fay and C. Van Baalen (ed.), The cyanobacteria. Elsevier Science Publishing Co., Amsterdam.
27. **Winkenbach, F., and C. P. Wolk.** 1973. Activities of enzymes of the oxidative and reductive pentose phosphate pathway in heterocysts of a blue-green alga. Plant Physiol. **52:**480–493.
28. **Wolk, C. P., J. Thomas, P. W. Shaffer, S. M. Austin, and A. Galonsky.** 1976. Pathway of nitrogen metabolism after fixation of ^{13}N-labeled nitrogen gas by the cyanobacterium *Anabaena cylindrica*. J. Biol. Chem. **251:**5027–5034.