Differences in mRNA levels in *Anabaena* living freely or in symbiotic association with *Azolla*

Sandra A. Nierzwicki-Bauer¹ and Robert Haselkorn

Department of Molecular Genetics and Cell Biology, The University of Chicago, 920 E 58th Street, Chicago, IL 60637, USA

¹Present address: Biology Department, Rensselaer Polytechnical Institute, Troy, NY 12181, USA

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***Azolla* is a small water fern in whose leaf cavities the filamentous nitrogen-fixing cyanobacterium *Anabaena azollae* is symbiotically associated. Using cloned genes from *Anabaena* 7120 for glutamine synthetase (GS), ribulose-1,5-bisphosphate (RuBP) carboxylase, nitrogenase and the 32-kd protein of photosystem II, mRNA levels of the corresponding genes in the *Anabaena* endosymbiont were studied by Northern hybridization. In RNA isolated from the endosymbiont there is a 10-fold reduction of GS transcript levels, a >5-fold increase in 32-kd transcript levels and a >5-fold decrease in RuBP carboxylase transcript levels, compared with levels in the free-living *Anabaena azollae*. In the endosymbiont and in heterocysts of the free-living *Anabaena azollae* the *nif* H, *nif* D, and *nif* K genes are transcribed from a single *nif* HDK operon.**

Key words: glutamine synthetase/RuBP carboxylase/32-kd protein/nitrogen fixation genes

Introduction

The symbiotic association of the eukaryotic water fern *Azolla* with its prokaryotic cyanobacterial (blue-green algal) endosymbiont *Anabaena azollae* has received considerable attention because of its ability to fix N₂, allowing it to serve as a nitrogen source in agriculture, especially in rice paddies (Moore, 1969). The usefulness of this association as a nitrogen fertilizer has been widely documented (Talley *et al.*, 1977; Watanabe *et al.*, 1977; Talley and Rains, 1980). In the *Azolla*-*Anabaena* symbiosis, specific functions are performed by each of the partners. It is believed that the fern provides the endosymbiont with a carbon source, probably sucrose (Ray *et al.*, 1979). The *Anabaena* endosymbiont contributes by fixing enough atmospheric N₂ to satisfy both its own requirement for combined nitrogen and that of its host (Peters and Mayne, 1974a, 1974b; Ray *et al.*, 1978; Peters *et al.*, 1980a, 1980b). Both partners in the *Azolla*-*Anabaena* association carry out photosynthesis of the higher plant type (Peters, 1976, 1977), enabling them to fix atmospheric CO₂ via the Calvin cycle (Ray *et al.*, 1979).

In the *Azolla*-*Anabaena* association the endosymbiont is contained within specialized leaf cavities of the fern. Throughout formation and maturation of the leaf cavities the endosymbiont is associated with specialized multicellular hairs, produced by the fern (Moore, 1969; Calvert and Peters, 1981). These hairs have elaborate cell wall ingrowths and numerous organelles which are characteristic of transfer cells (Duckett *et al.*, 1975). Peters *et al.* (1978) have suggested that the hairs in mature and immature cavities may be involved in nitrogen assimilation and the release

of fixed nitrogen, respectively.

Nitrogen fixation by the endosymbiont is carried out in specialized cells called heterocysts. Under certain environmental conditions heterocysts are formed by many of the filamentous cyanobacteria. Most of our current knowledge of heterocysts has come from studies involving the genus *Anabaena*. When cultured in the presence of combined nitrogen, *Anabaena* grows as long filaments of undifferentiated and morphologically uniform vegetative cells. In the absence of combined nitrogen, however, heterocysts differentiate from vegetative cells at regularly spaced intervals along the filaments (Haselkorn, 1978; Adams and Carr, 1981). It is now known that these heterocysts function as the exclusive sites of nitrogen fixation in aerobically grown cultures (Fay *et al.*, 1968; Wolk, 1973; Haselkorn, 1978).

In free-living *Anabaena* the heterocyst frequency varies from <1% in cultures containing combined nitrogen to ~10% in cultures growing on N₂. In contrast, when *Anabaena azollae* is symbiotically associated with the fern its tendency to form the nitrogen-fixing heterocysts varies according to the age of the leaf. Heterocyst frequencies range from only a few percent in young leaves to 30% or more in the oldest leaves (Hill, 1975).

Studies of the program of protein synthesis during heterocyst differentiation have shown that large numbers of genes in free-living *Anabaena* must be regulated in order to convert a vegetative cell into a mature heterocyst (Fleming and Haselkorn, 1973, 1974). All of the results obtained to date concerning the molecular mechanisms that regulate gene activation and gene repression during heterocyst differentiation in cyanobacteria indicate that regulation is effected at the level of transcription (Tumer *et al.*, 1983; Haselkorn *et al.*, 1985).

In the work reported here, cloned genes from *Anabaena* 7120 for glutamine synthetase (GS), ribulose-1,5-bisphosphate (RuBP) carboxylase, nitrogenase, and the 32-kd protein of photosystem II were used to measure mRNA levels of the corresponding genes in endosymbiotic *Anabaena azollae* by Northern hybridization. These genes code for proteins that are regulated differentially during heterocyst development (for review, see Haselkorn *et al.*, 1983, 1985). Transcript levels within the endosymbiont were compared with transcript levels within free-living *Anabaena*, grown on a combined nitrogen source (N+) or fixing atmospheric nitrogen (N-).

Results

The free-living *Anabaena* strain used for these experiments was reported to have been isolated from *Azolla caroliniana* (Newton and Herman, 1979). This strain has the advantage over *Anabaena* 7120, from which all of the specific gene probes were cloned, of growth in the dark using fructose as the carbon source. In the course of this work, we found that the free-living *Anabaena azollae* is not identical with the major endosymbiont of *Azolla*, based on the sizes of DNA fragments containing particular genes following digestion with the restriction enzyme *Hind*III (Figure 1). Nevertheless, the genes of both symbiotic and free-living *Anabaena* were found to be sufficiently homologous that the cloned

Fig. 1. Southern hybridization of *Anabaena* 7120 gene-specific probes to total DNA from free-living *Anabaena azollae* (FL) or freshly isolated endosymbiont (S) from *Azolla caroliniana*. The DNA in each case was digested with *Hind*III. Sizes of λ DNA fragments run as standards are shown in kbp at the left. Probes are described in Materials and methods. Although the free-living and symbiotic *Anabaenas* are clearly different organisms, the heterologous *Anabaena* 7120 probes recognize their cognate genes with equal efficiency.

probes could be used to analyze transcription equally well in both organisms.

In the experiments that follow, mRNA levels for various genes were determined by Northern dot blots, comparing RNA preparations from the symbiont and free-living *Anabaena* grown on nitrate or N_2 . The RNA preparations were normalized on the basis of hybridization with ribosomal DNA as described in Materials and methods.

Glutamine synthetase

Glutamine synthetase, which is encoded by the *glnA* gene, is a key enzyme for nitrogen assimilation in all organisms. In *Anabaena* all of the nitrogen reduced to ammonia by nitrogenase is normally added to glutamate by this enzyme (Thomas *et al.*, 1977). The glutamine is then transported from the heterocyst to neighboring vegetative cells, where it is used in the biosynthesis of purines, pyrimidines, and amino acids. All *Anabaena* cells require GS regardless of whether they are grown on a combined nitrogen source or fixing atmospheric nitrogen. Thus, it is not surprising that GS is present in both vegetative cells and heterocysts. Different promoters are used, however, to transcribe the *glnA* gene in each of these cell types in *Anabaena* 7120 (Tumer *et al.*, 1983).

While a vast amount of information on the regulation of GS activity in a number of different systems is now available (for reviews, see Stewart, 1977, 1980), relatively little has been agreed on regarding the regulation of GS synthesis within the endosymbiont. It was reported that GS protein levels in the *Anabaena* symbiont are only 5–10% of those present in the free-living form (Orr and Haselkorn, 1982). On the other hand, Peters *et al.* (1980) suggested that regulation of GS in the association is at the level of function rather than synthesis.

Fig. 2. Northern blot analysis of *glnA* gene expression in symbiotic and free-living *Anabaena azollae*. **Lane 1:** total RNA from free-living *Anabaena azollae* grown in BG-11 medium supplemented with nitrate. **Lane 2:** free-living *Anabaena azollae* grown in BG-11 medium lacking combined nitrogen. **Lane 3:** symbiotic *Anabaena azollae*. Fifteen micrograms of RNA were denatured, fractionated on an agarose gel, and transferred to a nitrocellulose filter. The blot was hybridized with 32 P-labeled *glnA* specific DNA of *Anabaena* 7120. A *Hae*III digest of Φ X174 replicative form DNA was run as a standard. Sizes of these DNA fragments are indicated.

Transcript levels of the *glnA* gene in symbiotic and free-living *Anabaena* cells were determined using as a probe the 1.4-kb *Eco*RI fragment from pAn503. This fragment contains a portion of the *Anabaena* 7120 *glnA* gene from amino acids 161–474 and 3' flanking nucleotides (Fisher *et al.*, 1981; Tumer *et al.*, 1983). Northern blot hybridizations show the presence of *glnA* message in RNA isolated from both nitrate-grown (N+) and nitrogen fixing (N-) cells of free-living *Anabaena azollae* (Figure 2). In both cases, the predominant band corresponds to a message size of ~1.8 kb. No *glnA* message is detected in the RNA isolated from the endosymbiont.

Northern dot blot hybridizations show that transcript levels in the N- RNA are slightly lower than those of the N+ RNA. Equal intensity hybridization to 1 μ g of endosymbiont RNA and 0.1 μ g of N+ RNA is observed (Figure 3). This indicates at least a 10-fold reduction of GS transcript levels in RNA isolated from the endosymbiont compared with RNA isolated from free-living *Anabaena azollae*.

32-kd protein

The 32-kd protein, which is encoded by the *psbA* gene, is required for PSII quinone-mediated electron transfer. This protein has been characterized by abundant light-induced synthesis and rapid turnover in chloroplasts (Eaglesham and Ellis, 1974; Reisfeld *et al.*, 1982) and in unicellular cyanobacteria. In plant chloroplast DNA the *psbA* gene is single-copy, whereas in *Ana-*

Fig. 3. Quantitative analysis of *glnA* specific transcripts using RNA dot blot hybridization. (A) Normalization of RNA concentrations for N+, N- and S (endosymbiont) samples. Total RNA from cells was isolated and spotted for dot blot hybridization as described in Materials and methods. To ascertain that equivalent amounts of RNA had been applied to each lane, the filter was initially hybridized with ^{32}P -labeled 23S rDNA from *Anabaena* 7120. Equivalent RNA concentrations of all samples (ranging from 1 to 0.01 $\mu\text{g}/\text{dot}$) are indicated by equal hybridization intensities. (B) The dot blot, used for normalization in A, was washed for 3 min in boiling water to remove the 23S rRNA probe. This blot was rehybridized with ^{32}P -labeled *glnA* DNA. Equal intensity hybridizations to 1 μg of endosymbiont (S) RNA and 0.1 μg N+ RNA is observed.

baena 7120 (Curtis and Haselkorn, 1984) and *Anabaena azollae* (Figure 1) there are multiple copies of this gene. It is not known how many copies are actually expressed in these *Anabaena* species. While it is known that the 32-kd protein is expressed in vegetative cells, the status of this protein in heterocysts remains unknown. Because PSII reaction centers are absent from heterocysts (Tel-Or and Stewart, 1977) it seemed likely that the expression of PSII components would be regulated down during heterocyst differentiation.

Transcript levels of the gene encoding the 32-kd protein in symbiotic and free-living *Anabaena* cells were determined. Northern blot hybridizations were probed using a 779-bp *Sau96/XbaI* fragment from the clone pAn625 (Curtis and Haselkorn, 1984). This fragment is internal to the *psbA* gene of *Anabaena* 7120. 32-kd specific message is present in N+, N- and endosymbiont RNA samples (Figure 4). In the N+ RNA there are two predominant transcripts, 1.4 and 1.0 kb. The relative intensities of these two bands is the same. The endosymbiont RNA contains unequal amounts of these transcripts, with the predominant species being the 1.4-kb message, while the N- RNA contains only the 1.4-kb transcript. These data indicate that when a combined nitrogen source is not readily available, the 1.0-kb 32-kd specific transcript is present in reduced amounts or completely absent.

In the unicellular cyanobacterium *Anacystis*, two transcripts of sizes 1.2 and 0.9 kb are produced by the same *psbA* gene; the shorter RNA has been shown to lack the N-terminal part of the 32-kd open reading frame (S.S.Golden, personal communication).

Northern dot blot hybridizations show that 32-kd specific transcripts are ~7–10 times more abundant in the endosymbiont RNA than in the N+ or N- RNA, respectively (Figure 5). The fact that the *psbA* gene(s) is so highly expressed in the endosymbiont cannot be explained by variations in the cell

Fig. 4. Northern blot analysis of *psbA* gene expression in symbiotic and free-living *Anabaena azollae*. RNA preparations and hybridization procedures were the same as in Figure 2, except that the probe was from the *Anabaena* 7120 *psbA* gene.

Fig. 5. Quantitative analysis of *psbA* specific transcripts using RNA dot blot hybridization. RNA preparations and procedures were the same as those shown in Figure 3, except that the probe was from the *psbA* gene. *PsbA* transcript levels are 5–10 times more abundant in the endosymbiont RNA (lane 3) than in the N+ (lane 1) or the N- (lane 2) RNA.

populations (i.e., percentages of heterocysts and vegetative cells). While the only reported function for the 32-kd protein is its role in PSII quinone-mediated electron transfer (Mattoo *et al.*, 1981; Arntzen *et al.*, 1982), it seems likely that this protein has additional functions in the endosymbiont.

Ribulose 1,5 biphosphate (RuBP) carboxylase

RuBP carboxylase, which is encoded by the *rbcL* and *rbcS* genes, is the first enzyme required in the Calvin pathway for the fixation of atmospheric CO_2 . Like that in higher plants, the enzyme from *Anabaena* consists of eight large subunits and eight small

Fig. 6. Quantitative analysis of *rbcS* specific transcripts using RNA dot blot hybridization. RNA preparations and procedures were the same as those shown in Figures 3 and 5, except that the probe in (B) was from the *rbcS* gene encoding the small subunit of RuBP carboxylase.

subunits. From *Anabaena* 7120 the *rbcL* (Curtis and Haselkorn, 1983) and *rbcS* (Nierzwicki-Bauer *et al.*, 1984) genes have been isolated and sequenced. *Anabaena* 7120 (Nierzwicki-Bauer *et al.*, 1984) and *Anabaena azollae* (Figure 1) contain only one copy each of the large- and small-subunit genes.

Transcript levels of the RuBP carboxylase genes in symbiotic and free-living *Anabaena* cells were determined. Northern blot hybridizations using either a *rbcL* or *rbcS* specific probe from *Anabaena* 7120 identified a preponderant message species of ~3.2 kb in all RNA samples (data not shown). Northern dot blots show that RuBP carboxylase specific transcripts are ~5–7 times less abundant in the endosymbiont RNA than in the N+ or N- RNA (Figure 6). The very low amount of carboxylase message in the endosymbiont RNA is consistent with the reduced CO₂ fixation in the endosymbiont observed by Peters *et al.* (1980). RuBP carboxylase transcripts are 2–5 times more abundant in the N- RNA than in the N+ RNA.

Nitrogenase

The biological fixation of nitrogen is catalyzed by the nitrogenase enzyme complex. The general properties of this complex have been highly conserved within a variety of microorganisms (Mortenson and Thorneley, 1979). The genes coding for components of the nitrogenase complex in *Klebsiella* (*nif* HDK) are contiguous and co-transcribed (Ausubel and Cannon, 1980). In *Anabaena* vegetative cells the *nif* D coding sequence is interrupted by 11 kb of DNA which is precisely excised during heterocyst differentiation, restoring the *nif* HDK operon structure (Golden *et al.*, 1985).

A 1.8-kb *Hind*III fragment from pAn154.3, containing the entire *nif* H gene and some 5' flanking sequence from *Anabaena* 7120, was used as the transcriptional probe for nitrogenase. The *nif* H gene, which encodes dinitrogenase reductase, was previously isolated and sequenced (Mevarech *et al.*, 1980). Northern blots hybridized with this probe show the presence of nitrogenase-specific message in the N- and endosymbiont RNA. No *nif* H

Fig. 7. The *nif* H gene of the endosymbiont is transcribed from a fused *nif* HDK operon. Fifteen micrograms of endosymbiont RNA was denatured, fractionated on an agarose gel and transferred to a nitrocellulose filter. The blot was hybridized with ³²P-labeled *nif* H DNA. Three *nif* H transcripts 4.9, 3.0 and 1.2 kb in size are identified. The 4.9 kb transcript corresponds to the complete *nif* HDK operon.

message is present in the N+ RNA.

The N- and endosymbiont RNA samples contain three *nif* H specific transcripts, 4.9, 3.0 and 1.2 kb in size (Figure 7). The largest of these transcripts could only have been transcribed from a *nif* HDK operon (Golden *et al.*, 1985). The sizes of these *nif* H transcripts are almost identical to the *nif* H messages transcribed from rearranged DNA of *Anabaena* 7120 (Golden *et al.*, 1985); they correspond to messages containing genes HDK, genes H and D, and gene H alone, respectively.

Discussion

The quantitative results of mRNA abundance determinations for free-living and endosymbiotic *Anabaena azollae* are summarized in Table I. Some of these results are consistent with the expectations based on determinations of enzyme activity in the association, while others are sufficiently unexpected to require further explanation.

Glutamine synthetase has been shown by Orr and Haselkorn (1982) to be barely detectable in the association, either by enzyme activity or by radioimmune assay. We find the GS mRNA abundance to be 10-fold lower in the association than in free-living *Anabaena*. This result suggests that the host fern regulates transcription of the *Anabaena glnA* gene. This, of course, is a sensible thing to do because the fern is prepared to utilize the ammonia given up by *Anabaena* unable to convert it to glutamine. It will be of considerable interest to determine how the fern regulates *Anabaena glnA* gene transcription. In enteric bacteria, *glnA* transcription is regulated by the products of two genes, *ntrA*, and *ntrC*, that respond to the ratio of glutamine to α -ketoglutarate, a high ratio leading to repression. However, it would be futile

Table I. Transcript levels in free-living and symbiotic *Anabaena*

Gene	Product	mRNA level		
		Free-living		Symbiont
		N+	N-	
<i>glnA</i>	GS	100	90	10
<i>rbcL-rbcS</i>	RuBP carboxylase	50	100	10
<i>psbA</i>	32 kd	15	10	100
<i>nifH</i>	Nitrogenase reductase	0	(100)	100

mRNA levels are expressed relative to the highest concentration for a given probe set at 100. Comparisons cannot be made between genes because the probes were nick-translated to different specific activities. *Nif* mRNA from free-living *A. azollae* was degraded to varying extent in different RNA preparations, as judged by Northern gels. These samples were not used for dot blots. The level of *nif* message was only estimated from the Northern gels, so the figure in parentheses has a large possible error.

for the fern to pump glutamine into the endosymbiont to shut down *glnA*, because the glutamine would simply be metabolized (Orr and Haselkron, 1982). The possibility we favor is that the fern makes a non-metabolizable analog of glutamine that acts as co-repressor of the prokaryotic *glnA* gene but has no effect on the eukaryotic gene.

Our results for the relative abundance of *glnA* mRNA in free-living *Anabaena* grown with or without nitrate are slightly at variance with those of Tumer *et al.* (1983). In the latter study, *glnA* mRNA was about twice as abundant in N- RNA as in N+ RNA. However, Tumer *et al.* prepared their N- RNA by transferring ammonia-grown cells to N- medium and then adding DCMU to prevent O₂ evolution and purging the culture with argon. Under these conditions the cells cannot grow but they derepress the *nif* genes abundantly. Since the *glnA* gene also contains a *nif* promoter, that gene is also induced considerably. In the experiments reported here, we prepared RNA from cells growing aerobically in log phase, in all probability using the vegetative cell promoter(s) in vegetative cells and the *nif* promoter in heterocysts. Apparently, in the steady state, there is less overall transcription of the *glnA* gene in differentiated filaments than in vegetative cells growing on nitrate. In this respect the regulation of *glnA* transcription in *Anabaena* differs from that in enteric bacteria, in which *glnA* transcription is greater in low ammonia media than in high ammonia media.

The results for *glnA* mRNA are relatively straightforward compared with those for *psbA*. The latter are complicated by the fact that *Anabaena* contains more than one gene encoding the 32-kd protein. In the case of free-living *Anabaena* 7120 growing on ammonia, S1 nuclease protection, primer extension and Northern blot experiments indicate that only one *psbA* gene is transcribed (Curtis and Haselkorn, 1984). We have not determined the relationship between the two different-sized *psbA* transcripts from *Anabaena*. However, the unicellular cyanobacterium *Anacystis* also produces two *psbA* transcripts (1.2 kb and 0.9 kb); in that case it has been determined that the shorter transcript begins within the coding region of the 32-kd protein, i.e., it is missing the 5' end of the *psbA* mRNA, and both transcripts can be derived from a single gene (S.S. Golden, personal communication). If the shorter transcript is produced by processing the longer, we would infer that processing is reduced in free-living *Anabaena* fixing nitrogen and in the endosymbiont.

The 32-kd protein is part of the photosystem II reaction center apparatus. That reaction center is absent from heterocysts (Tel-Or and Stewart, 1977). Why then should the *psbA* gene be expressed so abundantly in the association? It has been reported

that the 32-kd protein is the target, in addition to the herbicides diuron and atrazine, of the inhibitor hydroquinoline-N-oxide (HQNO). But HQNO inhibits electron flow at three different locations in heterocysts (Houchins, 1985). Therefore it seems possible that the 32-kd protein functions in new capacities in the heterocyst, in addition to the role played by that protein in photosystem II.

Our results for RuBP carboxylase mRNA abundance in free-living *Anabaena* also appear to be at variance with the earlier observation that this mRNA disappears during nitrogen starvation (Haselkorn *et al.*, 1983). Again, the differences are due to the comparison of the early stages of differentiation of heterocysts, in the earlier work, with steady-state growth under nitrogen-fixing conditions in this work. When an ammonia-grown culture is transferred to nitrogen-free medium, RuBP carboxylase activity and antigen disappear completely. Only after differentiation is complete and nitrogen fixation has begun in the heterocysts does RuBP carboxylase activity reappear in vegetative cells. The products of CO₂ fixation are needed, in steady-state growth on N₂, for the generation of ATP and reductant in heterocysts, in addition to the usual requirements for energy and structural components in vegetative cells. Thus, our results are consistent with a model of transcriptional control of the *rbcL-rbcS* operon in which the operon is repressed during nitrogen starvation and the early stages of heterocyst differentiation but derepressed, compared with expression in cells growing on nitrate or ammonia, in the vegetative cells of differentiated filaments growing on N₂.

Why then is the level of RuBP carboxylase mRNA so low in the symbiont? The answer is likely to be that the host fern controls transcription of the *Anabaena* gene. It is known that sugars are transported from the host into the symbiont, presumably to provide substrate for production of ATP and reductant for nitrogen fixation (Ray *et al.*, 1979). *Anabaena azollae* will use fructose as a carbon source in the dark. In some strains of *Nostoc*, sugars have been shown to reduce severely CO₂ fixation. Thus, it seems possible that the host fern shuts down unnecessary CO₂ fixation activity in endosymbiotic *Anabaena* by repressing transcription of the *rbcL-rbcS* operon, using a diffusible carbohydrate as part of the regulatory apparatus.

Comparison of the results obtained for different genes reveals two paradoxes yet to be resolved. The first involves *glnA* and the *nif* genes. The work of Tumer *et al.* (1983) showed that the *glnA* gene has multiple promoters, one of which is used under nitrogen-fixing conditions. That promoter appears to have some similarities in sequence with the promoter for the *nifH* gene, so it has been thought that the *glnA* and *nif* genes are transcribed by the same machinery under nitrogen-fixing conditions. That cannot be true in the symbiont, which turns up the *nif* genes but turns off *glnA*. Thus, in spite of the apparent similarities of 5'-flanking sequences, the *nif* and *glnA* genes are likely to have different transcription requirements in *Anabaena*. The current model for transcriptional regulation of these genes in enteric bacteria can accommodate these results. The *glnA* gene, under nitrogen-fixing conditions, requires activation by the product of the *ntrC* gene. The *nif* genes require activation by the product of the *nifA* gene. The symbiont could shut down *glnA* by inactivation of *ntrC*. That might create a problem for the *nif* genes, because transcription of *nifA* usually requires *ntrC*. However, under certain conditions *Klebsiella nifA* can be shown to activate its own transcription (Ow and Ausubel, 1983; Drummond *et al.*, 1983), and *Azotobacter nif* gene expression does not require *ntrC* (A. Toukdarian and C. Kennedy, personal communication).

The second paradox involves *rbcL-rbcS* and *psbA*. These genes encode proteins involved in CO₂ fixation and O₂ evolution, respectively. Both functions are shut down in heterocysts. Yet the *psbA* gene(s) is abundantly transcribed in the symbiont. We have already mentioned the possibility that the *psbA* gene product may serve new roles in the heterocyst. What sort of promoters do these genes have and how might they be regulated differently? The *rbcL* promoter of *Anabaena* 7120 has been identified by S1 nuclease protection and primer extension, using RNA prepared from ammonia-grown cells (Nierzwicki-Bauer *et al.*, 1984). This promoter is not recognized by purified *Anabaena* vegetative cell RNA polymerase holoenzyme *in vitro* (G.Schneider, unpublished). It appears to require an extra factor for activation. The *psbA* gene promoter of *Anabaena* 7120 has been determined only in part, because the restriction fragment on which the gene is cloned ends just upstream of the -10 region (Curtis and Haselkorn, 1984). For this reason it is not possible to compare the two promoter regions directly or, as yet, to study transcription of the *psbA* gene *in vitro*. However, inactivation of a factor required for *rbcL* transcription could account for the low abundance of that mRNA in the symbiont.

Materials and methods

Strains and growth conditions

The fern *Azolla caroliniana* containing the symbiotic cyanobacterium *Anabaena azollae* was supplied by Jack Newton of the USDA Regional Laboratory, Peoria, IL (Newton and Herman, 1979). This organism was grown floating on 6 l of medium in air-filtered 15-l carboys. The medium itself was described by Peters and Mayne (1974a). The cyanobacterium *Anabaena azollae* strain 1a was also supplied by Jack Newton. Free-living *Anabaena azollae* was grown in BG-11 medium (Stainer *et al.*, 1971) either with (N+) or without (N-) nitrate. As described in the first section of Results, this organism does not correspond to the major component of the endosymbiont population.

Isolation of endosymbiont

The symbiotic cyanobacterium was isolated from the fern by a modification of the procedure of Peters and Mayne (1974a). All steps were carried out at 4°C. A single layer of *Azolla* fronds was placed in a large glass pyrex tray. 50 mM Tris/50 mM EDTA (~2 ml) was dispensed on the fronds and a marble rolling pin was used to gently squash them. Another 2 ml of the Tris/EDTA solution was used to wash the endosymbiont away from the crushed fronds. The solution, which now contained mostly cyanobacterial cells and cellular debris, was collected and filtered through four thicknesses of cheese cloth. The filtrate was centrifuged for 1 min at 4000 r.p.m. to pellet the cyanobacterial cells.

Preparation and analysis of DNA

Total DNA from free-living and symbiotic *Anabaena azollae* was prepared as described by Davis *et al.* (1980). Phage and *Escherichia coli* strains were grown and phage and plasmid DNAs were isolated as described (Mazur *et al.*, 1980; Rice *et al.*, 1982). Chromosomal and plasmid DNA were digested with restriction endonucleases, subjected to agarose gel electrophoresis and transferred to nitrocellulose filters as described (Southern, 1975; Mazur *et al.*, 1980; Rice *et al.*, 1982).

Identification, isolation and subcloning of 23S rRNA gene

A recombinant phage library of *EcoRI* fragments of total *Anabaena* 7120 DNA in λ gt7 was screened by plaque hybridization according to Benton and Davis (1977). Kinased *Anabaena azollae* 23S rRNA purified from an agarose gel was used as the probe. A lambda clone designated An78, previously identified by B.Mazur, contained a 4.0-kb *EcoRI* insert that showed homology with the *A. azollae* 23S rRNA. This 4.0-kb fragment of An78 was recloned into the plasmid vector pBR328 and designated pAn621. *Anabaena* 7120 DNA fragments were prepared from pAn621 by digestion with *EcoRI* and purified by agarose gel electrophoresis, nick-translated and hybridized to Northern dot blots for normalization of the different RNA samples.

Preparation and analysis of RNA

Total RNA was isolated from free-living and symbiotic *Anabaena* cells as previously described (Golden *et al.*, 1985). This procedure breaks proheterocysts and young heterocysts as well as vegetative cells. Aliquots containing 15 μ g of total RNA were denatured using dimethyl sulfoxide and glyoxal, fractionated on 1.2% agarose gels and transferred to nitrocellulose paper as previously described (Tumer *et al.*, 1983). Size markers, consisting of DNA fragments of a *HindIII* digest

of λ DNA or a *HaeIII* digest of Φ X174 replicative form, were similarly denatured and fractionated. For dot blots, RNA samples were denatured according to Thomas (1983). Serial dilutions of the RNA samples were made with concentrations ranging from 1 to 0.01 μ g, dotted onto nitrocellulose and probed with the cloned 23S rRNA gene of *Anabaena* 7120 (described above). Equivalent RNA concentrations (to 0.01 μ g RNA) were demonstrated by equal hybridization intensities. This normalization procedure assured that comparable amounts of total RNA were bound to the filters for each subsequent mRNA comparison. We assume that the amount of 23S rRNA per cell is the same in vegetative cells and in heterocysts and that there is no selective loss of either 23S RNA or individual mRNAs during the RNA isolation. For all of the hybridizations described, filters were pre-hybridized at 58°C for 3 h in 100 ml of 6 $\times$ SSPE buffer, containing 0.5% Denhardt's solution and then hybridized for 12 h at 58°C in 5 ml of 6 $\times$ SSPE buffer/0.5% NaDodSO₄, Denhardt's solution and $\sim 1 \times 10^6$ c.p.m. of ³²P-labeled probe. Filters were rinsed at 58°C as previously described (Mazur *et al.*, 1980; Rice *et al.*, 1982).

Anabaena 7120 gene-specific probes

GS: the 1.4-kb *EcoRI* fragment of pAn503, which contains most of the *Anabaena* 7120 *glnA* structural gene (Fisher *et al.*, 1981; Tumer *et al.*, 1983). 32-kd: the 1.4-kb *EcoHI-HindIII* fragment of pAn625, which contains the *Anabaena* 7120 *psbA* gene (Curtis and Haselkorn, 1984), was purified by agarose gel electrophoresis. The purified fragment was digested with both *Sau96* and *XbaI* to yield a 779-bp fragment entirely within the coding region. *rbc*: the 980-bp *HindIII-XbaI* fragment of pAn606, which contains the complete *Anabaena* 7120 *rbcS* gene (Nierzwicki-Bauer *et al.*, 1984). *nif H*: the 1.8-kb *HindIII* fragment of pAn154.3, which contains all of an *Anabaena* 7120 *nif H* gene and some 5' flanking sequence (Mevarech *et al.*, 1980). All fragments were purified by agarose gel electrophoresis and nick-translated.

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