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Deletion of a 55-Kilobase-Pair DNA Element from the Chromosome during Heterocyst Differentiation of *Anabaena* sp. Strain PCC 7120

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The filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 produces terminally differentiated heterocysts in response to a lack of combined nitrogen. Heterocysts are found approximately every 10th cell along the filament and are morphologically and biochemically specialized for nitrogen fixation. At least two DNA rearrangements occur during heterocyst differentiation in *Anabaena* sp. strain PCC 7120, both the result of developmentally regulated site-specific recombination. The first is an 11-kilobase-pair (kb) deletion from within the 3′ end of the *nifD* gene. The second rearrangement occurs near the *nifS* gene but has not been completely characterized. The DNA sequences found at the recombination sites for each of the two rearrangements show no similarity to each other. To determine the topology of the rearrangement near the *nifS* gene, cosmid libraries of vegetative-cell genomic DNA were constructed and used to clone the region of the chromosome involved in the rearrangement. Cosmid clones which spanned the DNA separating the two recombination sites that define the ends of the element were obtained. The restriction map of this region of the chromosome showed that the rearrangement was the deletion of a 55-kb DNA element from the heterocyst chromosome. The excised DNA was neither degraded nor amplified, and its function, if any, is unknown. The 55-kb element was not detectably transcribed in either vegetative cells or heterocysts. The deletion resulted in placement of the *rbcLS* operon about 10 kb from the *nifS* gene on the chromosome. Although the *nifD* 11-kb and *nifS* 55-kb rearrangements both occurred under normal aerobic heterocyst-inducing conditions, only the 55-kb excision occurred in argon-bubbled cultures, indicating that the two DNA rearrangements can be regulated differently.

Heterocyst differentiation in the cyanobacterium *Anabaena* sp. strain PCC 7120 is accompanied by two developmentally regulated genome rearrangements (8, 9, 14). Unlike most DNA rearrangements in procaryotic organisms, such as the movement of transposable elements and phase variation inversions which occur at very low frequencies (33), rearrangement of the heterocyst genome is tightly regulated by an environmentally induced developmental sequence.

Heterocysts are highly specialized, terminally differentiated cells which reduce atmospheric dinitrogen to ammonia and then export the fixed nitrogen to neighboring vegetative cells in the form of glutamine (reviewed in references 12 and 37). Differentiation of a vegetative cell into a heterocyst is repressed either by the presence of an external source of combined nitrogen such as ammonia or by the close proximity of neighboring heterocysts in the filament. In the absence of a combined nitrogen source, approximately 10% of the cells along a filament form heterocysts at a relatively constant interval, producing a simple one-dimensional pattern of development.

Global changes in gene expression occur during heterocyst differentiation (5, 22). Most notably, nitrogen fixation (*nif*) genes are induced and several genes necessary for carbon fixation and the oxygen-evolving reactions of photosynthesis are repressed (13). A 40-kilobase-pair (kb) region of the vegetative-cell chromosome comprising four adjacent *Eco*RI fragments that contain at least five nitrogen fixation genes has been cloned and mapped (31). The *Anabaena* sp. strain PCC 7120 nitrogen fixation genes *nifH*, *nifD*, and *nifK* have been sequenced (20, 24, 26), as have the *nifS* and *nifU* genes (M. Mulligan, manuscript in preparation). The two heterocyst DNA rearrangements involve this region of the chromosome (14).

One of the DNA rearrangements is the excision of an 11-kb element from within the open reading frame of the *nifD* gene (9). The element is bordered by 11-base-pair direct repeats. Site-specific recombination within these direct repeats results in deletion of the element from the chromosome. The excised 11-kb element is found as a stable circular molecule in heterocysts. The 11-kb rearrangement has two significant effects on *nif* gene expression. First, it results in formation of a complete *nifD* open reading frame; second, it places the *nifK* gene under the transcriptional control of the *nifH* promoter (14). Excision of the 11-kb element is tightly coupled to heterocyst differentiation; excision is not detected in vegetative cells, and the element is completely excised in heterocysts (9). Physical mapping of the nitrogen fixation genes in other cyanobacterial strains suggests that a rearrangement similar to the 11-kb excision found in *Anabaena* sp. strain PCC 7120 may occur in most, but not all, filamentous heterocystous species (6, 7, 15, 17, 25, 32).

The second genome rearrangement has one DNA recombination site near the *nifS* gene and is referred to here as the *nifS* rearrangement (8, 9). Like the *nifD* rearrangement, the *nifS* rearrangement is the result of a site-specific recombination between repeated DNA sequences, but the DNA sequences involved in recombination differ for the two rearrangements (8). Both the *nifD* 11-kb excision and the second rearrangement near the *nifS* gene occur late during hetero-

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cyst differentiation, at about the same time that *nif* gene expression can be detected and active nitrogenase can be assayed (9; J. Golden, unpublished data). Also, both rearrangements affect *nif* gene transcription; heterocyst-specific transcripts pass through the *nifD* and *nifS* chromosomal recombination sites after rearrangement (8, 14).

In this paper, we show that the genome rearrangement near *nifS* is a large, 55-kb deletion from the chromosome. The deleted DNA persists in heterocysts and is not detectably degraded or amplified. In the vegetative-cell chromosome, the 55-kb element is bordered by the short, directly repeated DNA sequences that are involved in site-specific recombination during heterocyst differentiation (8). The 55-kb element is positioned on the chromosome between the genes encoding the large and small subunits of ribulose-bisphosphate carboxylase (*rbcL* and *rbcS*) (3, 27) and the *nifS* gene. We also show that under microaerobic heterocyst-inducing conditions, the 55-kb element is excised from the chromosome but the 11-kb element is not.

MATERIALS AND METHODS

Cyanobacterial and bacterial strains and culture conditions. Stocks of *Anabaena* sp. strain PCC 7120 were grown in 100-ml BG-11 liquid cultures and on plates of BG-11 solidified with 1.5% agar (1). Large-scale cultures of 1 to 15 liters were grown in Kratz and Myers medium C (9) or AA/8 medium (16) containing 0.5 mM potassium phosphate (dibasic) and 5 mM MOPS (morpholinepropanesulfonic acid) buffer, pH 8.0. Cultures were bubbled with 1% CO_2 in air. Heterocyst differentiation was suppressed by the addition of 2.5 mM ammonium sulfate. Cultures were synchronously induced to form heterocysts by transfer of vegetative cells from medium containing ammonia to medium lacking a source of combined nitrogen. The microaerobic induction shown in Fig. 5 (lanes c) was done by bubbling the culture with argon.

Escherichia coli strains were grown in LB liquid or agar-solidified medium with appropriate antibiotics according to standard procedures (23, 34). Plasmids were maintained in *E. coli* DH5α (Bethesda Research Laboratories, Inc.), and cosmids were maintained in strain ED8767 (23).

DNA manipulations. High-molecular-weight genomic DNA from *Anabaena* sp. strain PCC 7120, used for the construction of cosmid recombinant libraries, was prepared as follows. *Anabaena* sp. strain PCC 7120 filaments from a growing 3-liter culture were collected by centrifugation, washed once with 50 mM Tris (pH 8.0)–100 mM EDTA (pH 8.0), suspended in 100 ml of the same buffer containing lysozyme (2 mg/ml), and incubated at 37°C for 1 h. Proteinase K and sodium sarcosyl were added to 100 μg/ml and 0.5%, respectively. The mixture was incubated at 50°C with gentle shaking for 90 min. The sample of lysed cells was adjusted to 100 mM NaCl and then gently extracted sequentially with phenol, phenol-chloroform (1:1), and chloroform according to standard procedures (23). Aqueous DNA solutions were transferred between containers by pouring or by pipetting with the wide end of a pipette. The DNA was precipitated with 2 volumes of 95% ethanol, hooked with a sealed Pasteur pipette, transferred to 50 ml of Tris (50 mM)–EDTA (100 mM), pH 8.0, and dissolved overnight at 37°C. Heat-treated RNase (23) was added to 20 μg/ml, and the solution was incubated at 37°C for 30 min. Proteinase K was added to 100 μg/ml, and the solution was incubated for 60 min at 50°C. Sodium perchlorate was added to 1 M, and the solution was extracted twice with phenol-chloroform (1:1) and once with chloroform. The DNA was precipitated as described above, rinsed with 70% ethanol, and dissolved in 25 ml of Tris (10 mM)–EDTA (1 mM), pH 8.0. The resulting solution contained 0.2 mg of DNA per ml (total of 5 mg) with an average size greater than 150 kb as determined by pulsed-field gel electrophoresis (35).

Vegetative-cell DNA and heterocyst DNA (isolated from purified heterocysts) used in the experiments shown in Fig. 3 and 5 were prepared as previously described (9).

Enzymes were obtained from various suppliers and used according to the recommendations of the manufacturers. Recombinant DNA procedures were performed by standard methods (23) with the following exceptions. DNA was transferred from agarose gels to GeneScreen Plus (Dupont, NEN Research Products) with 0.4 N NaOH (30). DNA probes for the experiment shown in Fig. 5 were prepared by the mixed-oligonucleotide primer procedure (4). Southern hybridizations were performed in 5× SSPE–1% sodium dodecyl sulfate at 65°C (SSPE is described in reference 23). Filters were washed in 0.5× SSPE–0.2% sodium dodecyl sulfate at 65°C. DNA probes were denatured in hybridization solution by autoclaving for 5 min, and probes were reused by the same procedure.

Construction of cosmid libraries. Two different cosmid libraries were constructed in the cosmid vector pTBE (11). The S library consisted of *Cpf*I (an isoschizomer of *Sau*3AI with identical cleavage sites) partials cloned into the *Bam*HI site of the vector, and the T library consisted of *Taq*I partials cloned into the *Cla*I site of the vector. Both libraries were constructed as follows. High-molecular-weight genomic DNA was isolated as described above, partially digested with either *Cpf*I (the S library) or *Taq*I (the T library), and dephosphorylated. The insert DNA was then ligated to gel-purified vector arms. The vector arms for the S library were the 4.2-kb *Bam*HI-*Hpa*I fragment and the 2.4-kb *Cla*I-*Bam*HI fragment in which the *Hpa*I and *Cla*I ends were dephosphorylated. The vector arms for the T library were the 4.5-kb *Cla*I-*Pvu*II fragment and the 2.4-kb *Bam*HI-*Cla*I fragment in which the *Pvu*II and *Bam*HI ends were dephosphorylated. For each library, the ligations were packaged in vitro, using extracts prepared as described previously (23); the resulting bacteriophage particles were used to infect *E. coli* ED8767.

A library of individual cosmid clones from primary recombinants was established in the wells of 12 96-well plates, giving a total of 1,152 clones. Clones were named by the library, the number of the 96-well plate, the row letter, and the column number. The libraries were replica plated with a 96-pin replicator to new 96-well plates for storage and onto nitrocellulose filters for hybridization screening. Colony screening was done according to standard procedures, using nick-translated DNA probes (23).

Restriction fragment mapping. Restriction fragments were initially ordered by alignment of the cosmid clones. This order was refined with single and double restriction enzyme digests of cosmid clones and subcloned fragments isolated from the cosmid clones. Additional data were obtained from Southern blots of genomic DNA digested with the appropriate restriction enzymes and probed with labeled cosmid clones (see Fig. 3). This last procedure was used to identify the restriction fragments in which the cosmid insert terminated. All restriction fragments greater than 1 kb were mapped; a few restriction fragments smaller than 1 kb were sometimes faintly visible in digests of cosmid clones, but these fragments were not subcloned or mapped.

RNA-probed DNA slot blot analysis. DNA slot blots were

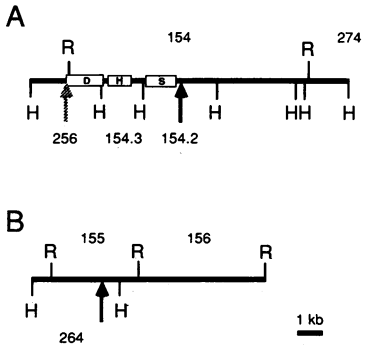

FIG. 1. Restriction maps of the *nifS* rearrangement recombination sites in the vegetative-cell genome. (A) Map of the *nifS*-proximal recombination site. (B) Map of the *nifS*-distal recombination site. Restriction sites are labeled R (*Eco*RI) and H (*Hin*dIII). Restriction fragments discussed in the text are labeled (the "An" prefix is omitted in the figure). Locations of the *nifS* rearrangement recombination sites are indicated with solid arrows, and the location of the 11-kb rearrangement *nifD*-proximal recombination site is indicated with a striped arrow. The genes *nifD*, *nifH*, and *nifS* are labeled D, H, and S, respectively.

prepared with *Eco*RI plasmid clones of the 55-kb element and flanking regions of the chromosome. The *Eco*RI fragments from the 55-kb element were subcloned from cosmid clones into the *Eco*RI site of pUC18. Plasmids containing flanking DNA were pAn207, pAn154, and pAn274 (31) and pAn600 (3). Plasmid pAn621 (28), containing the 23S rRNA gene from *Anabaena* sp. strain PCC 7120, was included as a control for the RNA probe specific activity. A 5-μl sample of plasmid DNA containing 0.1 μg of insert DNA per 1 kb of insert was transferred to a GeneScreen Plus membrane by using a 24-slot manifold (Bethesda Research Laboratories). The DNA was denatured and bound to the membrane as recommended by the supplier.

End-labeled RNA probes were prepared from total RNA isolated from *Anabaena* sp. strain PCC 7120 vegetative cells and purified heterocysts (isolated 36 h after aerobic induction in nitrogen-free medium) as described elsewhere (9, 10, 28) except that the cultures were grown in AA/8 medium. A 10-μg sample of total RNA was end labeled by the procedure of Williams and Lloyd (36). The RNA was fragmented with 0.1 M NaOH on ice for 30 min, followed by neutralization and ethanol precipitation before end labeling. The DNA slot blots were hybridized overnight at 65°C with the RNA probe in 5× SSPE–1% sodium dodecyl sulfate–tRNA (100 μg/ml) and then washed with 0.5× SSPE–0.2% sodium dodecyl sulfate at 70°C.

RESULTS

Chromosome walking. The region of the vegetative-cell chromosome surrounding the two DNA recombination sites involved in the heterocyst genome rearrangement near the *nifS* gene is shown in Fig. 1 (8). The *nifS*-proximal recombination site was located within the 3.3-kb *Hin*dIII fragment An154.2 and the 10.4-kb *Eco*RI fragment An154 (Fig. 1A). Approximately 15 kb of the chromosome to the right of the *nifS* gene has been cloned and mapped (31). The 8.1-kb *Eco*RI fragment An274 was adjacent to fragment An154 (Fig. 1A). The *nifS*-distal recombination site was located within the 3.6-kb *Hin*dIII fragment An264 and the 3.6-kb *Eco*RI fragment An155 (Fig. 1B). In our earlier experiments that identified this region, we isolated approximately 20 kb of the chromosome flanking the distal recombination site on two overlapping lambda clones (8). Since the cloned regions of the chromosome containing each of the two recombination sites shown in Fig. 1 did not overlap, the distance between the sites and their orientation relative to one another were not known.

The first attempts at linking the *nifS* rearrangement DNA recombination sites were chromosome-walking experiments using the same lambda library that was used to obtain the *nifS*-distal DNA recombination site (8). The library consisted of *Anabaena* vegetative-cell DNA partially digested with *Sau*3AI cloned into the *Bam*HI site of the lambda vector L47.1 (21). Chromosome walks were initiated with a probe from An274 (Fig. 1A) followed by a second step which extended the cloned region of the chromosome adjacent to An274 approximately 12 kb (data not shown). Two fragments, 274WW#1 and 274WW.78H3, failed to identify additional clones from the lambda L47.1 library that would extend the cloned region.

After each step in these chromosome-walking experiments, linkage between the two DNA recombination sites was tested by Southern analysis. The two overlapping L47.1 clones containing the distal DNA recombination site within An155 were used to probe Southern blots of genomic DNA and clones linked to the proximal DNA recombination site. These experiments never showed linkage between the two DNA recombination sites, but fortuitously resulted in linking of the genes for ribulose-bisphosphate carboxylase (*rbcL* and *rbcS*) to An155, which contained the *nifS*-distal recombination site. The *rbcLS* operon, which has been cloned and sequenced (3, 27), was located on a 17-kb *Eco*RI fragment (An600) and was approximately 10 kb from the recombination site within An155 (Fig. 2).

Since linkage between the two DNA recombination sites was not achieved with the lambda library, two cosmid libraries of vegetative-cell DNA were constructed for use in additional chromosome-walking experiments. Both libraries were composed of 1,152 primary clones. The libraries were screened with DNA probes representing the two DNA recombination sites followed by new probes obtained from the cosmids identified in the previous screening (chromosome walking). Each new probe hybridized to the clone from which it was derived, to clones identified in previous hybridizations, and to new clones which extended the chromosome walk. Since the libraries consisted of identifiable isolated clones, new clones could be used as probes for the next step in the walk.

Chromosomal walks were initiated in the S library by using probes from each end of the cloned region surrounding the two DNA recombination sites involved in the *nifS* rearrangement, for a total of four different probes. For the *nifS*-proximal DNA recombination site, the probes were *Hin*dIII fragment An319 (S. Robinson, unpublished data; H. Böhme and R. Haselkorn, Mol. Gen. Genet., in press), to the left of both the *nif* region and the recombination site, and *Hin*dIII fragment 274WW.78H3 (not shown), isolated from a lambda clone extending to the right of An274. For the *nifS*-distal DNA recombination site, the probes were *Eco*RI fragment An155, which contained the recombination site, and *Eco*RI fragment An600, which contained the *rbcLS* operon (shown to be linked to An155 as described above). A total of 18 cosmid clones were identified in these screens, but analysis of these clones showed that they did not establish linkage between the two *nifS* rearrangement DNA recombination sites. Chromosome walking was continued with two additional steps, using probes isolated from cosmid clone

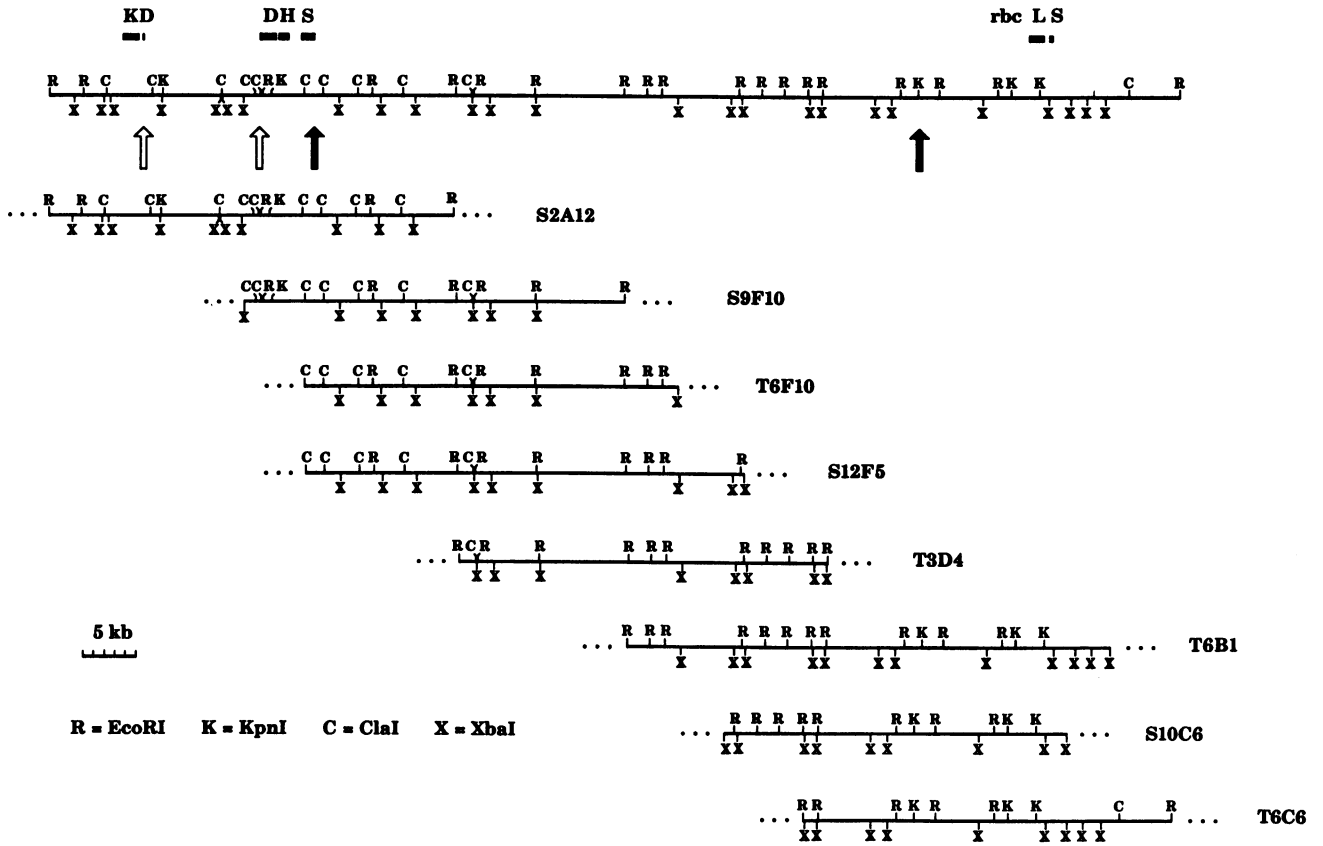

FIG. 2. Map of the *Anabaena* sp. strain PCC 7120 vegetative-cell chromosome and cosmid clones covering the region involved in the *nifD* and *nifS* genome rearrangements. The sites for the restriction enzymes *Eco*RI (R), *Xba*I (X), *Cla*I (C), and *Kpn*I (K) are shown. The location of the nitrogen fixation genes *nifK* (K), *nifD* (D), *nifH* (H), and *nifS* (S) and the ribulose-bisphosphate carboxylase genes *rbcL* and *rbcS* are shown (27, 31). The *nif* genes are transcribed from right to left, and the *rbc* genes are transcribed from left to right. The *rbcLS* genes are approximately 68 kb from the *nifS* gene. The open arrows indicate the *nifD* rearrangement recombination sites and are separated by 11 kb. The solid arrows indicate the *nifS* rearrangement recombination sites and are separated by 55 kb. The restriction maps of eight cosmid clones covering this region of the vegetative cell chromosome are shown.

S9F10 (linked to the *nifS*-proximal recombination site; Fig. 2) and cosmid clone S8A7 (linked to the *nifS*-distal recombination site; not shown). Both of these probes failed to hybridize to any new clones. Therefore, chromosome walking was continued in the T cosmid library.

The T library was screened with probes obtained from cosmid S8A7 and with two probes linked to the *nifS*-proximal DNA recombination site: a *Hin*dIII fragment, 274WW#1, isolated from the right end of a lambda clone that extended from An274 to the right; and An154.3 (Fig. 1A). A total of 22 clones were identified with these probes. A probe from the cosmid clone T3D4, which was identified with the 274WW#1 probe, was then prepared and used to rescreen the T library to continue the walk. The T3D4 probe hybridized with clones that, in previous screens, had been linked to the *nifS*-proximal DNA recombination site and also to clones that were linked to the *nifS*-distal DNA recombination site. Linkage was therefore established between the two DNA recombination sites on the vegetative-cell chromosome.

Physical map. Figure 2 shows a restriction map of eight cosmid clones that cover the region of the chromosome involved in the two known heterocyst DNA rearrangements. Several additional cosmid clones that contained DNA from this region were identified but not extensively mapped. The map shows approximately 106 kb of the vegetative-cell chromosome. The recombination sites involved in the *nifS* rearrangement are separated by approximately 55 kb on the vegetative-cell chromosome.

The restriction map established by the cosmid clones indicates that the repeated DNA sequences (8) present at the ends of the 55-kb element are directly repeated on the chromosome. Site-specific recombination between the directly repeated sequences would result in a deletion of the intervening DNA from the chromosome as a 55-kb circle. Comparison of the vegetative-cell chromosome map with the map of rearranged heterocyst DNA (8, 9) confirms that the *nifS* rearrangement is the deletion of the 55-kb region from the chromosome. The deletion results in placement of the *rbcLS* operon approximately 10 kb away from the *nifS* gene on the heterocyst chromosome. Although the ends of the 55-kb element are ligated as a result of the recombination event and are the only recombination products identified by Southern analysis (8, 9; Fig. 3), the predicted 55-kb circle has not been detected in uncut DNA isolated from heterocysts (data not shown).

Rearrangement of the 55-kb element. Three cosmid clones, S9F10, T3D4, and T6B1 (Fig. 2), which together spanned the 55-kb element (including several kilobases of flanking DNA), were used as probes against Southern blots of vegetative-cell and heterocyst DNA digested with *Eco*RI or *Xba*I (Fig. 3). The restriction fragments identified by each of the cosmid probes were as predicted from the vegetative-cell chromo-

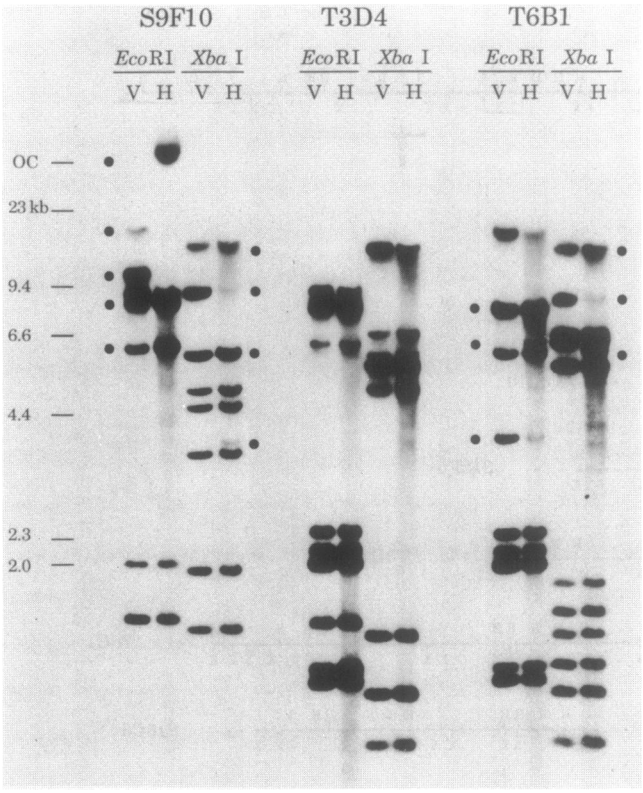

FIG. 3. Southern analysis of vegetative-cell and heterocyst DNA involved in the *nifS* rearrangement. The cosmid clones S9F10, T3D4, and T6B1 (Fig. 2) were used as probes against Southern blots of vegetative-cell (V) and heterocyst (H) DNA digested with *Eco*RI or *Xba*I. The positions of lambda *Hin*dIII fragments used as size markers are shown. The position of the slowly migrating open circular form of the excised 11-kb element is labeled OC. The S9F10 cosmid probe identifies both the *nifD* and *nifS* rearrangements. The T3D4 cosmid probe identifies fragments internal to the 55-kb element and does not contain a DNA recombination site. The T6B1 cosmid probe identifies the *nifS*-distal DNA recombination site near the *rbcLS* operon. Restriction fragments that contain a DNA recombination site and are mentioned in the text are indicated with a dot.

some map (Fig. 2) and the two known heterocyst DNA rearrangements. These results confirm the map of the vegetative-cell chromosome generated from the cosmid clones and rule out the possibility of artifacts created during cloning.

The known DNA recombination sites for both the *nifD* 11-kb deletion and the *nifS* 55-kb deletion were evident in these experiments, and no additional rearrangements or DNA recombination sites were detected with any of the three cosmid probes. The cosmid S9F10 contained the *nifD*-proximal DNA recombination site of the 11-kb element and the *nifS*-proximal DNA recombination site of the 55-kb element (Fig. 2). These DNA recombination sites are seen in the *Eco*RI-digested genomic DNA shown in Fig. 3 (S9F10 probe). The *nifD* rearrangement is seen as the disappearance of the 17-kb fragment from the vegetative-cell chromosome and the appearance of both an 11-kb element and a 6-kb fragment in the heterocyst chromosome. The *nifS* rearrangement is evidenced by the disappearance of a 10.5-kb fragment from the vegetative-cell chromosome and the appearance in heterocyst DNA from fragments of 6.2 kb (on the chromosome) and 7.9 kb (on the 55-kb element). In the *Xba*I-digested DNA, an 8.8-kb fragment in vegetative-cell DNA, containing both the *nifD*- and *nifS*-proximal DNA recombination sites, was replaced in heterocyst DNA by fragments of 3.3 kb (very faint band on the 11-kb element), 5.5 kb (seen as a doublet on the 55-kb element), and 13.8 kb (faint doublet on the chromosome).

The cosmid T3D4 insert was completely within the borders of the 55-kb element and did not contain a DNA recombination site (Fig. 2). No differences between vegetative-cell and heterocyst DNA were detected (Fig. 3, T3D4 probe).

Cosmid T6B1 contained the *nifS*-distal DNA recombination site of the 55-kb element (Fig. 2). In *Eco*RI-digested DNA, the recombination site was seen as the replacement of a 3.6-kb fragment on the vegetative-cell chromosome with fragments in the heterocyst genome of 6.2 kb (on the chromosome) and 7.9 kb (on the 55-kb element). The corresponding *Xba*I-generated fragments were an 8.6-kb fragment on the vegetative-cell chromosome and fragments in the heterocyst genome of 13.8 kb (faint doublet on the chromosome) and 5.5 kb (on the 55-kb element).

The *Anabaena* sp. strain PCC 7120 genome contains a relatively high level of N^6-methyladenine and 5-methylcytosine and shows *dcm*- and *dam*-like methylation patterns (29). Because *Xba*I and *Cla*I restriction endonucleases are sensitive to *dam* methylation (18), only those sites that are unmethylated in *Anabaena* sp. strain PCC 7120 and in *E. coli* DH5α would be detected by our mapping studies. There are, in fact, *Xba*I and *Cla*I restriction sites in the *nifS* gene that do not cut and are not shown in our restriction map but have been identified in sequenced DNA (Mulligan, unpublished data).

The sequences deleted from the chromosome in heterocysts retain their stoichiometry with sequences on the chromosome of vegetative cells, showing that the excised 55 kb of DNA persists in heterocysts and is not rapidly degraded or amplified after excision from the chromosome. The experiment also shows that the 55-kb element is single copy in the chromosome and does not contain repetitive DNA sequences.

Transcription of the 55-kb element. Plasmid clones of *Eco*RI fragments that covered the 55-kb element and flanking regions of the vegetative-cell chromosome were immobilized onto nylon filters and hybridized with end-labeled RNA isolated from vegetative cells or purified heterocysts to determine the extent of transcription from this region in the two cell types (Fig. 4). A clone containing the *Anabaena* sp. strain PCC 7120 23S ribosomal RNA gene (28) was used as a control for labeling efficiency and showed strong hybridization with both vegetative-cell and heterocyst RNA (Fig. 4, slot 18). *Eco*RI fragment clones that flanked the 55-kb element and contained *nif* genes (An207 and An154) hybridized strongly with RNA isolated from heterocysts but not with vegetative-cell RNA (slots 1 and 2). The converse was true for the *rbcLS* operon (An600): it hybridized strongly with vegetative-cell RNA but not with heterocyst RNA (slot 17). *Eco*RI fragment An155, which contained the *nifS*-distal recombination site, hybridized very weakly with heterocyst RNA (observed only after longer exposures than that shown in Fig. 4). This is expected, because after excision of the 55-kb element, a transcript passes through the recombination site on the heterocyst chromosome (8). *Eco*RI fragment clone An156 showed significant hybridization to heterocyst RNA. The *nifB* gene has been shown to reside on the An155 and An156 fragments (Mulligan, unpublished data).

No significant hybridization to any of the *Eco*RI fragments

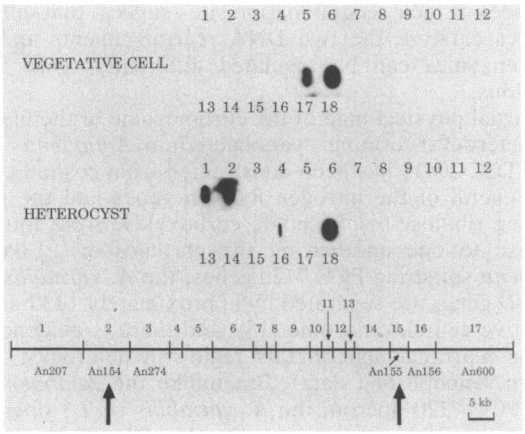

FIG. 4. Transcription of the 55-kb element in vegetative cells and heterocysts. DNA from plasmid clones of *Eco*RI fragments covering the 55-kb element and flanking regions of the vegetative-cell chromosome were immobilized onto a GeneScreen Plus membrane and hybridized with end-labeled RNA isolated from vegetative cells (upper panel) or purified heterocysts (lower panel). Slots 1 through 17 contain DNA from the *Eco*RI fragments shown and numbered on the restriction map. Restriction fragments that have been previously named are labeled below the map. The borders of the 55-kb element are within fragments 2 and 15; positions of the recombination sites are indicated with large arrows. Fragments 3 through 14 are contained entirely within the 55-kb element. Slot 18 contains a 23S rRNA gene clone, pAn621.

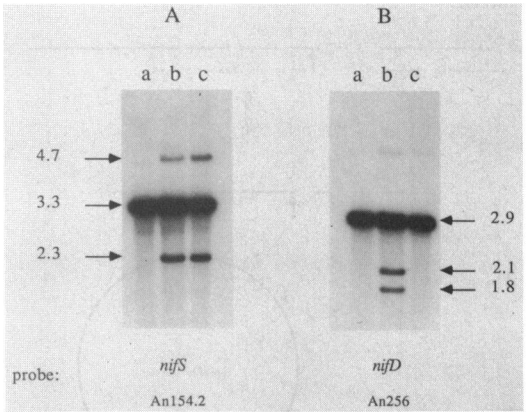

FIG. 5. Regulation of the *nifS* and *nifD* rearrangements. The *nifS* rearrangement can occur independently of the *nifD* rearrangement under microaerobic heterocyst-inducing conditions. DNA was isolated from cultures of vegetative cells grown in medium containing ammonia and bubbled with 1% CO_2 in air (lanes 1), filaments transferred to medium lacking ammonia and bubbled with 1% CO_2 in air (lanes b), and filaments transferred to medium lacking ammonia and bubbled with argon (lanes c). For each sample, 1.5 μg of DNA was digested with *Hin*dIII, electrophoresed on a 0.8% agarose gel, and blotted onto GeneScreen Plus with 0.4 N NaOH (30). Identical loadings were made on a single gel, and the membrane was cut in half before hybridization. Probes were prepared from *Hin*dIII fragments specific for the two rearrangements isolated from cloned DNA and labeled by using mixed-oligonucleotide-primed synthesis (4). (A) *nifS* rearrangement probe An154.2; (B) *nifD* rearrangement probe An256.

contained entirely within the 55-kb element could be detected with RNA isolated from vegetative cells or purified heterocysts. The fact that this experiment showed no detectable gene expression from the 55-kb element does not exclude the possibility that this region is expressed at low levels or is expressed transiently during heterocyst differentiation or under different growth or environmental conditions than those used in our experiment.

Regulation of the heterocyst genome rearrangements. Both the *nifD* 11-kb deletion and the *nifS* 55-kb deletion are tightly coupled to heterocyst differentiation. Neither rearrangement can be detected in vegetative-cell DNA, and DNA from purified heterocysts is completely rearranged at both sites (8, 9). The two rearrangements occur at approximately the same time of development after synchronous aerobic induction of heterocyst formation (9). They occur after morphological differentiation is apparent and at about the same time that *nif* gene expression is first detectable (Golden, unpublished data). However, under certain artificial conditions a striking difference in the regulation of the two rearrangements is seen. Figure 5 shows Southern blots of *Hin*dIII-digested DNA isolated from vegetative cells, from filaments induced aerobically in nitrogen-free medium, and from filaments induced microaerobically. DNA was isolated from whole filaments in which only about 10% of the cells were differentiating; therefore, the majority of the DNA was from nondifferentiating vegetative cells and was unrearranged. The blots were hybridized with DNA probes that identified the *nifS* or *nifD* rearrangement DNA recombination site. The *nifS* rearrangement probe, An154.2 (Fig. 1A), identified a 3.3-kb *Hin*dIII fragment in vegetative cells and the two rearrangement products of 2.3 and 4.7 kb after induction (8, 9). The *nifD* rearrangement probe, An256, identified a 2.9-kb *Hin*dIII fragment in vegetative cells and the two rearrangement products of 1.8 and 2.1 kb after induction (9). The experiment shows that the *nifS* 55-kb deletion occurs when cultures are induced to form heterocysts under both aerobic and microaerobic conditions (Fig. 5A). The *nifD* 11-kb deletion occurred normally under aerobic conditions but failed to occur under microaerobic conditions (Fig. 5B).

DISCUSSION

In the experiments presented here, cosmid clones covering over 100 kb of the *Anabaena* sp. strain PCC 7120 vegetative-cell chromosome were isolated and mapped. This region of the chromosome contains the genes encoding the structural polypeptides for both nitrogenase and ribulose-bisphosphate carboxylase. Two different genome rearrangements that occur during heterocyst differentiation are located within this region of the genome. Chromosome-walking and mapping experiments show that the genome rearrangement near the *nifS* gene is the deletion of a large, 55-kb element from the chromosome. The excision of the 55-kb element is the result of a site-specific recombination between short, directly repeated DNA sequences present at the ends of the element (8).

The heterocyst genome rearrangements are diagramed in Fig. 6. In heterocysts, the 11-kb element is excised from the chromosome and the ends of the element are joined to form an 11-kb circular molecule. Recombination occurs within an 11-base-pair directly repeated sequence present at the ends of the 11-kb element. The crossover leaves one copy of the 11-base-pair sequence on the chromosome and one copy on the excised 11-kb circle. The intact excised circle can be detected in DNA prepared from heterocysts as supercoiled and open circular forms, although a significant proportion is present as linear molecules (9).

Like the 11-kb element, the 55-kb element is circularized during excision from the chromosome in heterocysts; the

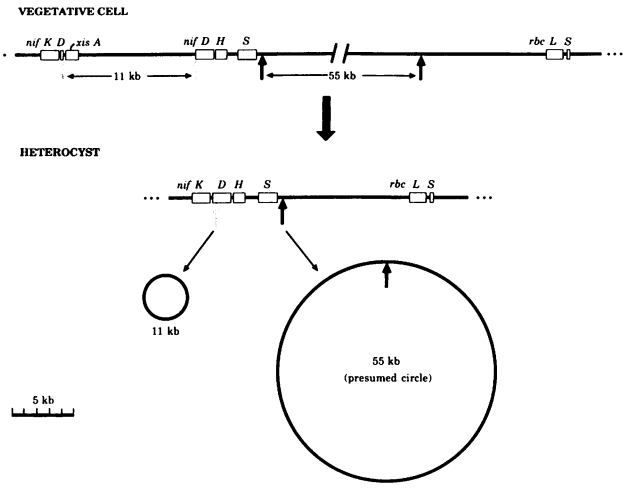

FIG. 6. Diagram of the *nifD* 11-kb and *nifS* 55-kb deletions from the *Anabaena* sp. strain PCC 7120 chromosome during heterocyst differentiation. Some of the nitrogen fixation (*nif*) genes and the ribulose-bisphosphate carboxylase (*rbc*) genes are shown. Also shown is the *xisA* gene, which is necessary for site-specific excision of the 11-kb *nifD* element in a rearrangement assay performed in *E. coli* (19). The positions of the recombination sites for the 11-kb *nifD* element (stippled arrows) and the 55-kb *nifS* element (solid arrows) are marked. The 11-kb element deleted from the chromosome is found as a circular molecule in heterocysts. The predicted 55-kb circle has not been found intact in DNA isolated from heterocysts.

ends of the element are joined together by recombination between directly repeated sequences that border the element (8). Unlike the 11-kb element, the intact 55-kb circle has not been detected in heterocyst DNA preparations (data not shown). The absence of an intact circle may be the result of random degradation or shearing which occurs during DNA isolation from heterocysts. It is unlikely that other secondary site-specific DNA rearrangements occur within the 55-kb excised circle. The Southern blots shown in Fig. 3 and other Southern blots used in mapping experiments (data not shown) detected only those changes expected from the two known rearrangements.

Both rearrangements are restricted to heterocysts and are undetectable in DNA isolated from vegetative cells grown in the presence of ammonia. DNA from purified heterocysts is nearly completely rearranged at both sites (8, 9). The small amount of unrearranged DNA is probably due to contaminating vegetative-cell DNA. During a normal synchronous aerobic induction of heterocyst differentiation, the two rearrangements follow the same time course (9): both are first detectable at 18 h after transfer to medium lacking ammonia and are completed by 30 h after induction.

Although the two genome rearrangements share many overt aspects of developmental regulation, the rearrangements can be uncoupled under certain conditions. Differences in the regulation of the two rearrangements were observed only when heterocyst differentiation was induced in cultures bubbled with argon. Under these artificial conditions, in which oxygen, nitrogen, and carbon dioxide were not supplied to the culture, only the 55-kb excision near *nifS* occurred; the 11-kb excision from *nifD* did not. Unlike a normal aerobic induction of heterocyst differentiation, the argon-bubbled cultures which failed to show excision of the *nifD* 11-kb element also lacked morphological differentiation and nitrogenase activity. The uncoupling of the two rearrangements under these conditions and the different DNA sequences at their recombination sites suggest that different enzymes catalyze the two DNA rearrangements and that these enzymes can be regulated differently under some conditions.

A partial physical map of the chromosome of the filamentous heterocyst-forming cyanobacterium *Anabaena variabilis* ATCC 29413 has been constructed from cosmid clones (15). Several of the nitrogen fixation genes and the genes encoding ribulose-bisphosphate carboxylase were found to be close to one another on the chromosome. Like the *Anabaena* sp. strain PCC 7120 genes, the *A. variabilis nifK* and *nifD* genes are separated by approximately 10 kb on the vegetative cell chromosome (15), and there is evidence for genome rearrangement in this region in heterocyst DNA (Golden, unpublished data). But unlike the *Anabaena* sp. strain PCC 7120 operon, the *A. variabilis rbcLS* operon is found within 10 kb of the *nif* genes (15). The 55-kb element found between the *nif* and *rbc* genes in *Anabaena* sp. strain PCC 7120 is absent in *A. variabilis* (Golden, unpublished data). Hence, the organization of the genes in this region of the *A. variabilis* vegetative-cell chromosome is similar to that found in the *Anabaena* sp. strain PCC 7120 heterocyst chromosome. This result suggests that the 55-kb element found in the *Anabaena* sp. strain PCC 7120 genome is dispensable.

In some ways, the heterocyst genome rearrangements may be modeled after the excision of a lysogenic phage (2). The elements might represent defective prophages whose excision from the chromosome has come under the control of heterocyst development to allow correct expression of the *nif* genes. Alternatively, rearrangement of the elements may provide a function in heterocyst differentiation that has not yet been discovered. The function, if any, of the elements in heterocyst differentiation will require genetic experiments which have only recently become possible through the development of methods for the conjugal transfer of shuttle vectors from *E. coli* to filamentous cyanobacteria (38, 39).

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