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Characterization of an Insertion Sequence (IS*891*) of Novel Structure from the Cyanobacterium *Anabaena* sp. Strain M-131

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When recombinant plasmids that were transferred to the cyanobacterium *Anabaena* sp. strain M-131 were transferred back to *Escherichia coli*, some of the transformants contained inserts. One of the insertion sequences (ISs) was characterized by sequencing. This 1,351-base-pair IS contained an open reading frame that was capable of encoding a peptide of 310 amino acids and had terminal sequences with distinctive structures, but it lacked terminal inverted repeats and did not duplicate target DNA upon insertion. The element bore no significant sequence homology to any sequence stored in the GenBank data base. Restriction analysis of the genomes of *Anabaena* sp. strain M-131 and *Anabaena* sp. strain PCC 7120 showed those strains to be closely related. Sequences homologous to the IS element were also present in the DNA of *Anabaena* strain PCC 7120, but the copy numbers and chromosomal locations of such sequences differed in the two strains. The largest visualized plasmid was 425 kilobases (kb) in M-131 and 410 kb in PCC 7120; at least the former plasmid contained multiple copies of the element, as did a 115-kb plasmid in M-131.

Cyanobacteria are procaryotes that are capable of oxygenic photosynthesis. Under conditions of deprivation for combined nitrogen, strains of some filamentous genera, such as *Anabaena*, form differentiated cells called heterocysts at semiregular intervals along the filaments. The heterocysts are thought to provide the microaerobic environment necessary for protracted fixation of atmospheric dinitrogen by nitrogenase. Such strains are therefore valuable for the study of oxygenic photosynthesis, nitrogen fixation, and differentiation. A particularly successful way of isolating the genes involved in such processes starts by screening for the in vivo insertion of a mobile genetic element within those genes. The genes can then be recognized by the presence of the inserted element and cloned.

Numerous mobile genetic elements are known in bacteria. When such an element lacks a selectable marker, it is referred to as an insertion sequence (IS). Nearly all ISs identified to date have terminal inverted repeats of a few 10s of nucleotides, generate short (2- to 12-base-pair [bp]) duplications of target DNA sequences upon insertion, contain one or more open reading frames (ORFs), and are typically 0.8 to 2.6 kilobases (kb) in size (4). A typical 1.4-kb IS, IS*701*, is present and mobile in the DNA of the cyanobacterium *Calothrix* sp. strain PCC 7601 (D. Mazel, A.-M. Castets, J. Houmard, and N. Tandeau de Marsac, Abstr. VI Int. Symp. Photosynthetic Prokaryotes, p. 227, 1988). A family of putative ISs with a typical structure has also been found in the DNA of *Anabaena* sp. strain PCC 7120, but their mobilities have not been demonstrated (J. Alam and S. E. Curtis, Abstr. First Int. Congr. Plant Molecular Biology, OR-22-07, p. 49, 1985). To date, no derivative of an exogenous IS has been shown to be mobile in *Anabaena* sp. We report here the characterization of an IS from *Anabaena* sp. strain M-131.

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MATERIALS AND METHODS

Strains, plasmids, and culture conditions. *Anabaena* sp. strain M-131 was grown in an eightfold dilution of the medium of Allen and Arnon (1), which was supplemented with 2.5 mM $NaNO_3$–2.5 mM KNO_3, at 30°C under illumination of 60 to 70 microeinsteins m^{-2} s^{-1}. *Escherichia coli* HB101 was grown on L agar or in LB medium by standard methods (11). The plasmids used for conjugal transfer into *Anabaena* sp. strain M-131 were based on pIB0505 and pIB0623 (I. Bancroft, Ph.D. thesis, University of Lancaster, United Kingdom, 1986). Triparental matings were performed between *Anabaena* sp. strain M-131, *E. coli* J-53 (bearing RP-4), and *E. coli* HB101 (bearing pDS4101, pRL449, and the plasmid to be transferred [8, 17]).

Preparation and manipulation of DNA. DNA was isolated from *Anabaena* sp. strain M-131 by a method involving the preparation of spheroplasts by treatment with lysozyme, lysis by sodium dodecyl sulfate, and phenol extraction (15). DNA for pulsed-field gel electrophoresis was prepared from *Anabaena* sp. strain M-131 and from *Anabaena* sp. strain PCC 7120; the strains had been embedded in agarose microbeads (3). Yeast (*Saccharomyces cerevisiae*) chromosomes and oligomers of the DNA of coliphage lambda were prepared and embedded in agarose as described previously (2). Restriction endonuclease digests of *Anabaena* DNA in agarose microbeads were performed, pulsed homogeneous orthogonal field electrophoresis gels were run, and genetic sequences were mapped by hybridization as described previously (3). Southern transfers onto nitrocellulose membranes were performed by standard techniques (11). Ethidium bromide-stained gels were exposed to shortwave UV light to induce strand cleavage before denaturation and transfer.

Cloning of DNA in *E. coli* and most preparations of DNA from *E. coli* were performed by standard techniques (11). RNA-free plasmid DNA for *Bal* 31 deletion and for subsequent cloning into M13 for sequencing was prepared by a method involving RPC-5 analog chromatography (Bancroft, Ph.D. thesis). DNA fragments to be used as probes were

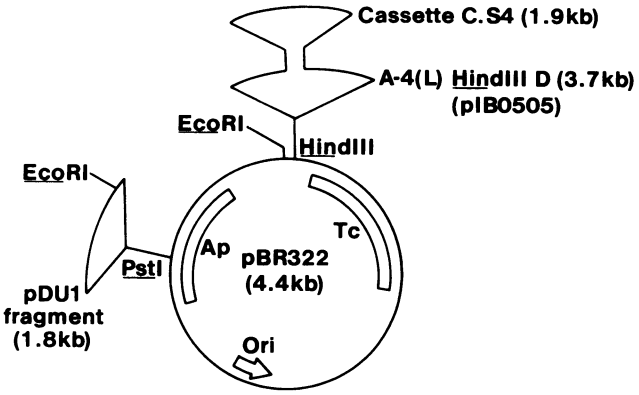

FIG. 1. Plasmids for investigation of transduction by cyanophage A-4(L). Construct 1, Cassette C.S4 (see text), which conferred resistance to streptomycin and spectinomycin, was inserted into *Hin*dIII fragment D of A-4(L), a distance of ca. 1.0 kb from the *Eco*RI site of pBR322, in place of ca. 0.8 kb of DNA deleted between two *Alu*I sites; the orientation of the pDU1 fragment was as shown. Construct 2, Cassette C.S4 was inserted into an *Alu*I site ca. 1.0 kb from *Eco*RI, with no deletion of DNA; the pDU1 fragment was in the orientation opposite to the orientation shown. Construct 3, Cassette C.S4 was inserted into an *Alu*I site ca. 0.7 kb from *Eco*RI, ca. 1.2 kb of DNA was deleted, and the pDU1 fragment was in the orientation shown. See the text for sources of components.

recovered from agarose gels (SeaKem GTG; FMC BioProducts, Rockland, Maine) by elution from NA45 membranes (7). DNA probes were labeled with [α-^{32}P]dATP by extension from random primers (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). Hybridization procedures were standard (11) and included three washes with 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate at 65°C. Sequencing reactions incorporated [^{35}S]thio-dATP by using a kilobase sequencing kit (Bethesda Research Laboratories).

RESULTS

Visualization of the IS. As part of an experiment in which transduction of DNA by the cyanophage A-4(L) was unsuccessfully sought, plasmids (Fig. 1) were constructed in which a cassette, C.S4, which confers resistance to streptomycin and spectinomycin in both *E. coli* and *Anabaena* sp. was inserted into pIB0505. Cassette C.S4 was constructed from sequenced fragments as follows. First, the promoter sequence (Prbc) of the ribulose bisphosphate carboxylase gene of *Anacystis nidulans* (14) from *Sst*I at bp −420 to *Nla*III at bp −240 was inserted between the *Sst*I and *Sph*I sites in a pUC18 (18) polylinker. Next, the Sm^r Sp^r gene that was excised from Tn*7* (9) with *Ava*I (the extension was trimmed with S1 nuclease) and *Eco*RI was inserted into a second pUC18 polylinker between *Sph*I (blunted with S1 nuclease) and *Eco*RI. The *Hin*dIII sites from the two constructs were then joined to form the 1,882-bp cassette C.S4 (*Eco*RI *Sst*I Prbc *Hin*dIII Sm^r Sp^r *Eco*RI). This cassette is present at the center of polylinker L.HEH1 in plasmid pRL244 (S.A1 [7]). Plasmid pIB0505 (Bancroft, Ph.D. thesis) is a clone of a *Hin*dIII fragment of the DNA of cyanophage A-4(L) in pBR322. The ability to replicate in *Anabaena* sp. strain M-131 was conferred by a 1.8-kb fragment of *Nostoc* sp. strain PCC 7524 plasmid pDU1 (13). The plasmids that were constructed were transferred to *Anabaena* sp. strain M-131 by conjugation, and plasmid DNA was prepared from Sm^r Sp^r clones. This DNA, which was transformed into *E. coli* HB101, was restriction mapped to check the identity of the plasmids. Plasmids were recovered in this manner from exconjugant isolates of *Anabaena* sp. strain M-131 to which three derivatives of pIB0505 were transferred. Both of two independently transformed derivatives (one designated pRL707) of one of the pIB0505-based plasmids (construct 2; see Fig. 1) showed insertions of ca. 1.4 kb. Of three independently transformed copies of another of the pIB0505-based plasmids (construct 1), one was recovered in the original form (pRL705) and two showed a ca. 1.4-kb insertion in the same position (one was designated pRL706).

The insertion in pRL706 was found to be within a viral DNA sequence, whereas that in pRL707 was found to be within the drug resistance cassette (although it did not interfere with the expression of resistance). The DNA fragment from pRL707, consisting of the drug resistance cassette plus the IS, was used as a probe to investigate the source of the IS. This fragment hybridized to the IS in pRL706 (and to the drug resistance cassette) but to nowhere else in either plasmid (data not shown). Furthermore, both ISs were found to contain restriction sites for *Eco*RV, *Hin*dIII, and *Dra*I in similar locations. We conclude that the two independent insertions are of either identical or very similar elements.

Structure of the element. We chose to sequence the copy of the element that was found within the drug resistance cassette because the sequence at the site of insertion corresponded to that between the *Stu*I site and the *Eco*RI site 3′ from the *aadA* (Sm^r Sp^r) gene from Tn*7* (9). In contrast, the sequence of cyanophage A-4(L) DNA, in which the other insertions were found, is not known. The *Stu*I-*Eco*RI fragment containing the element plus approximately 150 bp of flanking DNA was subcloned into pUC18, which was cut with *Sma*I and *Eco*RI. Sets of *Bal* 31 deletions were generated (from the *Pst*I and *Eco*RI sites), and both strands were sequenced in M13mp18 and M13mp19. The sequence of the element, along with the sequence of the region into which it is inserted, is shown in Fig. 2 (GenBank accession no. M24855). Some restriction endonuclease cleavage sites and other features of the sequence are indicated.

Genomic location of cross-hybridizing elements in *Anabaena* sp. strains M-131 and PCC 7120. Genomic DNAs of *Anabaena* sp. strains M-131, PCC 7120, and ATCC 29413 were prepared in liquid and were cleaved with *Eco*RI (which did not cut within the IS), resolved by electrophoresis through 0.4% agarose, transferred to nitrocellulose, and probed with DNA of the whole IS. There were many hybridization signals with the DNA of strain M-131, several with the DNA of strain PCC 7120, but none with the DNA of strain ATCC 29413 (Fig. 3). Restricted DNA from *E. coli* HB101 also showed no hybridization signals (data not shown). These results indicate that there are numerous copies (or, at least,

FIG. 2. Sequence of IS*891*. Two sets (one for the sequencing of each strand) of overlapping *Bal* 31 deletions of IS*891* DNA plus ca. 150 bp of target DNA were sequenced in M13mp18 and M13mp19. The sequence of the target DNA that we read is given in parentheses. Boxed broken arrow, Imperfect terminal repeats; boxed solid arrow, perfect repeats; *, translational termination codon. An ORF of 930 nucleotides extended from the putative initiation codon to the tandem termination codons.


(TACTTAC)GAGCCGTGAAGCTAAAGCCCCGTATTTTTAA 31
TCGGGGGATATAAGCGAATGACCGAATTTATTCGTCGTAACATGGTATAATTACGTCAGAGAG 94
Met Leu Val Phe Glu Thr Lys Leu Glu Gly Thr Asn Glu
TTTGACTTAAAAATG CTA GTA TTT GAG ACA AAA CTT GAA GGA ACA AAC GAG 145
Gln Tyr Gln Leu Leu Met Arg Arg Leu Lys Leu Leu Val Leu Ser Asn
CAG TAT CAA TTG CTG ATG AGG CGA TTA AAA CTG CTC GTT TTG TCG AAT 193
Ala Cys Leu Arg Thr Trp Ile Gly Gln Pro Asn Ile Gly Arg Tyr Asp
GCT TGC CTC CGT ACT TGG ATT GGA CAA CCA AAC ATC GGC AGG TAT GAT 241
Leu Ser Ala Tyr Cys Ala Val Leu Leu Pro Met Lys Thr Phe Arg Ser
TTG AGT GCT TAT TGC GCT GTC CTG CTG CCA ATG AAA ACT TTC CGT TCG 289
HindIII
Leu Pro Asn Ser Thr Leu Trp Leu Asp Lys Leu Leu Leu Lys Glu Arg
TTG CCA AAC TCA ACT CTA TGG CTC GAC AAG CTT CTG CTG AAA GAG CGT 337
Gly Val Gln Leu Leu Gly Phe Leu Thr Ile Ala Ser Lys Thr Lys Pro
GGA GTG CAA TTG CTC GGT TTT TTG ACA ATT GCA AGC AAA ACA AAA CCG 385
DraI
Gly Arg Lys Val Ile His Ala Leu Lys Lys Asn Arg Arg Met Gly Val
GGA AGA AAG GTT ATC CAC GCT TTA AAA AAG AAC AGA CGC ATG GGA GTG 433
HindIII
Leu Ser Ile Lys Leu Ala Ala Gly Ser Leu Val Val Thr Val Ala Tyr
TTG AGT ATA AAA CTA GCG GCT GGA AGC TTA GTA GTG ACC GTC GCT TAT 481
DraI
Val Thr Phe Ser Asp Gly Phe Lys Ala Gly Thr Phe Lys Leu Trp Gly
GTC ACT TTT AGC GAC GGA TTT AAA GCA GGA ACT TTC AAA CTC TGG GGA 529
Thr Arg Asp Leu His Phe Tyr Gln Leu Lys Gln Phe Lys Arg Val Arg
ACT CGT GAC TTG CAT TTC TAC CAG TTG AAA CAG TTC AAG AGG GTG CGG 577
Val Val Arg Arg Ala Asp Gly Tyr Tyr Ala Gln Phe Cys Ile Asp Gln
GTT GTG CGT CGT GCC GAT GGG TAC TAC GCG CAG TTT TGC ATT GAC CAA 625
Glu Arg Val Glu Arg Arg Glu Pro Thr Leu Lys Thr Ile Gly Leu Asp
GAG CGA GTA GAA AGG CGA GAA CCA ACG CTT AAA ACT ATT GGG CTG GAT 673
Val Gly Leu Asn His Phe Leu Thr Asp Ser Glu Gly Asn Thr Val Glu
GTG GGA TTG AAC CAT TTC TTG ACC GAT AGC GAA GGC AAT ACA GTT GAG 721
Asn Pro Arg His Leu Arg Lys Ser Glu Lys Ser Leu Lys Arg Leu Gln
AAC CCT AGA CAC TTG CGT AAA AGC GAA AAG TCT CTC AAG AGA TTG CAA 769
Arg Arg Leu Ser Lys Thr Lys Lys Gly Ser Asn Asn Arg Val Lys Ala
CGC AGA TTG TCT AAA ACC AAG AAG GGT TCT AAC AAC AGA GTC AAG GCA 817
Arg Asn Arg Leu Ser Arg Lys His Leu Lys Val Ser Arg Gln Arg Lys
AGA AAT CGC TTG AGT AGA AAA CAC CTT AAA GTA AGT AGG CAG CGT AAA 865
Asp Phe Ala Val Lys Leu Ala Arg Cys Val Val Gln Ser Ser Asp Leu
GAC TTC GCC GTA AAG TTG GCG AGG TGC GTA GTC CAG TCT AGC GAC TTG 913
Val Ala Tyr Glu Asp Leu Gln Val Arg Asn Met Val Arg Asn Arg His
GTA GCC TAT GAG GAT TTG CAG GTG CGG AAC ATG GTC AGG AAT AGA CAT 961
Leu Ala Lys Ser Ile Ser Asp Ala Arg Gly Arg Ser Phe Gly Asn Gly
CTT GCC AAG TCG ATT AGT GAT GCA CGT GGA CGC AGT TTC GGC AAT GGG 1009
Leu Ser Ile Ser Ala Lys Cys Leu Val * *
TTG AGT ATT TCG GCA AAG TGT TTG GTG TAG TGACTGTTGCAGTCCCACCCCAT 1062
CACACTTCGCAGAATTGTTCCAACTGTGGCGAAGTAGTGAAAAAGTCGCTGAGTACAAGAACT 1125
CATGCTTGCCCTCACTGTGGACATATTCAAGACAGGGATTGGAACGCTGCACGGAACATACTT 1188
SnaBI EcoRV
GAACTAGGACTACGTACTGTGGGACACCACGGATCTCAAGTCTCTGGAGATATCGACCTCTGT 1251
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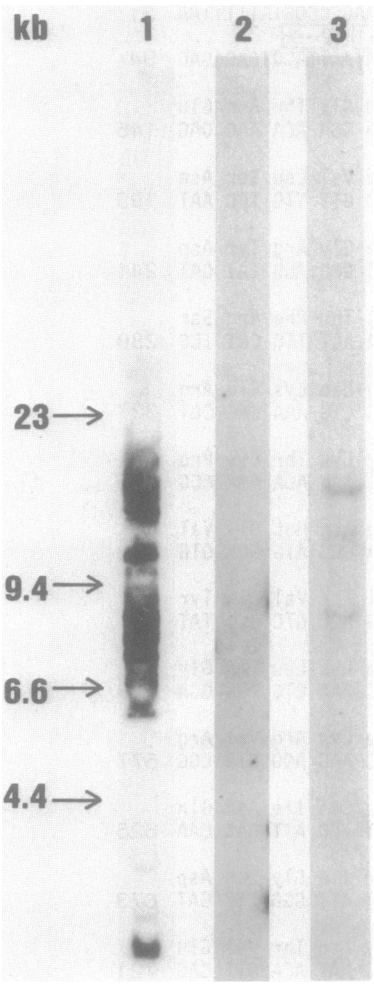

FIG. 3. Cross-hybridization of IS*891* DNA to *Eco*RI-digested genomic DNA of *Anabaena* strains M-131, PCC 7120, and ATCC 29413. Lane 1, *Eco*RI-digested DNA of strain M-131; lane 2, *Eco*RI-digested DNA of strain ATCC 29413; lane 3, *Eco*RI-digested DNA of strain PCC 7120. Electrophoresis was performed through 0.4% agarose, followed by Southern transfer of DNA to nitrocellulose. IS*891* DNA was labeled with ^{32}P and used as a probe.

portions) of related elements in the genomic DNAs of *Anabaena* sp. strains M-131 and PCC 7120.

The degree of relatedness of *Anabaena* sp. strains M-131 and PCC 7120 was investigated next. DNA with a high molecular mass from both strains was prepared from cells that were embedded in agarose microbeads. This DNA was cleaved with *Avr*II, *Sal*I, and *Pst*I and resolved by the pulsed homogeneous orthogonal field gel electrophoresis system of pulsed-field electrophoresis (2). The ethidium bromide-stained electrohporetograms (Fig. 4a and c) show that the two strains are very closely related. The only conspicuous difference (most easily seen by comparing the patterns of bands in *Sal*I digests in Fig. 4a and c) is that the bands corresponding to the 410-kb, linearized plasmid α in the DNA of *Anabaena* sp. strain PCC 7120 (3) were increased in size by ca. 15 kb in *Anabaena* sp. strain M-131. The DNAs from the gels shown in Fig. 4a and c were transferred to nitrocellulose and were probed with IS DNA. The results (Fig. 4b and d) indicate that there are many more copies of DNA to which the IS hybridizes in strain M-131 than there are in strain PCC 7120. The signals from strain M-131 plasmids that corresponded to strain PCC 7120 plasmids α and γ were particularly strong, indicating that these plasmids contain multiple copies of the element. Plasmid α of PCC 7120 also showed a strong hybridization signal, indicating the presence of cross-hybridizing elements, but plasmid γ did not. The locations of the chromosomal copies of the cross-hybridizing elements in both strains were mapped (3) with respect to the chromosomal sites of cleavage of *Avr*II, *Sal*I, and *Pst*I in strain PCC 7120. In PCC 7120, they mapped between 4.455 and 4.670 Mb and between 4.890 and 5.300 Mb. In M-131 they mapped to eight locations, between 0.035 and 0.255 Mb, 0.345 and 0.825 Mb, 0.850 and 1.075 Mb, 1.245 and 1.420 Mb, 2.680 and 2.925 Mb, 3.040 and 3.305 Mb, 3.995 and 4.270 Mb, and 4.890 and 5.300 Mb. There were also hybridization signals to a DNA species of ca. 205 kb from strain M-131, although no band of that size was visible on gels that were stained with ethidium bromide. We suggest that this band may correspond to a plasmid (which we call δ) which contains the IS but no cleavage sites for *Avr*II, *Sal*I, or *Pst*I. We interpret the signals that we saw as being the result of occasional linearization of the plasmid, resulting in a small quantity of DNA entering the gel. Similarly, plasmid γ from M-131, when it was treated with *Sal*I or *Pst*I, gave a weak hybridization signal with the IS probe, although it contained no genuine sites for either restriction endonuclease.

DISCUSSION

We identified an IS, IS*891*, with a novel structure from the cyanobacterium *Anabaena* sp. strain M-131. This mobile element was identified in two of only three pIB0505-based plasmids that we investigated and may, therefore, insert into plasmids of exogenous origin at a relatively high frequency, although no such phenomenon was noted previously (17). Specific targeting of exogenous DNA for insertion would be a novel form of defense mechanism.

The size of IS*891*, 1,351 bp, is typical of ISs. It contains an ORF that can encode a peptide of 310 amino acids and has tandem translational stop codons. However, the nucleotide sequence of this ORF shows no homology to the sequences of known transposases or to other sequences in the GenBank data base (5). The highly basic amino acid composition (20.4% Arg + Lys; only 7.4% Asp + Glu) of its translation product (Fig. 2) is consistent with the idea that it encodes a DNA-binding protein. Although other mobile genetic elements are known that insert without the duplication of at least a few base pairs of target DNA, namely, IS*91*, IS*H1.8*, Tn*554*, Tn*916*, and Tn*1545* (4), we know of no other instance of terminal structures resembling those of IS*891*. IS*891* appears, therefore, to represent a previously unknown class of IS.

A comparison of restriction digests of DNA from *Anabaena* sp. strains M-131 and PCC 7120, which were resolved by pulsed homogeneous orthogonal field gel electrophoresis, revealed that these two strains are very closely related. This result was not wholly surprising, because the two strains both came from the midwestern United States (6, 10, 12) and both were initially identified as *Nostoc muscorum*; and the origin of PCC 7120, which was first used two decades after M-131 was, is unknown. Strain M-131 has lost its original ability to form heterocysts and to fix N_2. (Our attempts to convert strain M-131 to the *het*$^+$ *nif*$^+$ phenotype by complementation with DNA from strain PCC 7120 failed.) Our results illustrate the power of pulsed-field gel electrophoresis to show the relatedness of two procaryotic strains. We

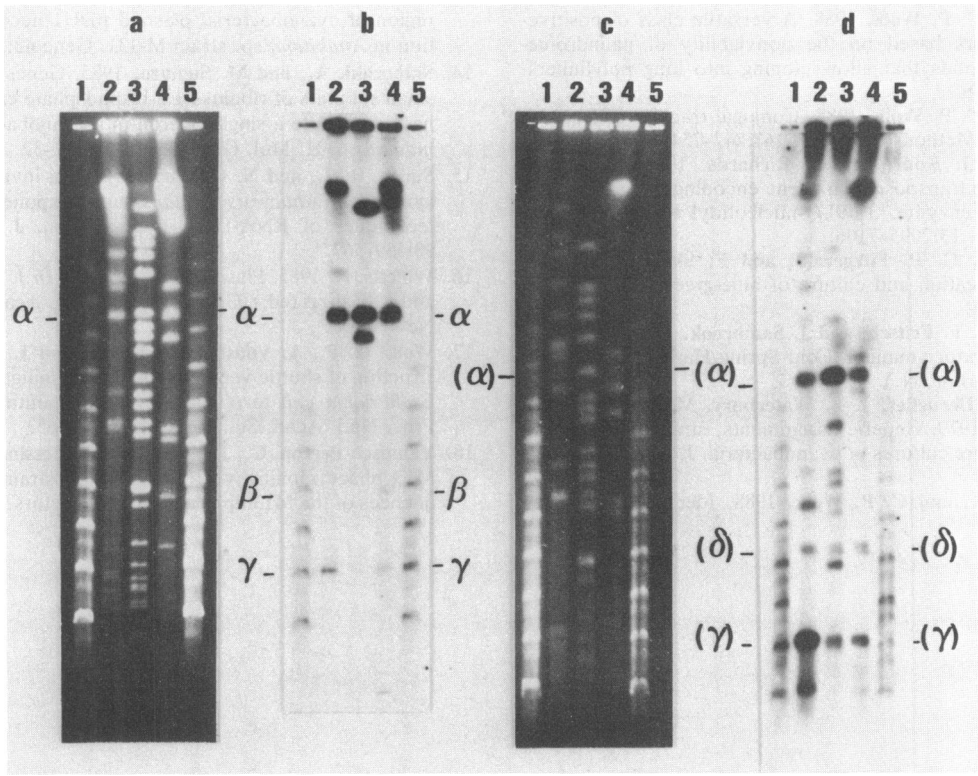

FIG. 4. Comparison of the positions of restriction endonuclease cleavage sites and IS locations in genomic DNAs of *Anabaena* strains M-131 and PCC 7120. (a) Lanes 1 and 5, Oligomers of lambda DNA (48.5 kb per monomeric unit); lanes 2 to 4, DNA from *Anabaena* strain PCC 7120 incubated with 0.5 U of *Avr*II (lane 2), 15 U of *Sal*I (lane 3), and 50 U of *Pst*I (lane 4). (b) Southern transfer of the gel shown in panel a probed with ^{32}P-labeled DNA from IS*891* and lambda. (c) As in panel a, but with DNA from *Anabaena* strain M-131. (d) As in panel b, but with the gel shown in panel c. See the text for protocols for restriction endonuclease digestion and electrophoresis. The positions of plasmids α, β, and γ are shown in panels a and b; because plasmids of strain M-131 were only presumptively identified, their positions in panels c and d are indicated by Greek letters within parentheses.

demonstrated that IS*891* originates from the DNA of *Anabaena* strain M-131. The very intense hybridization signal from the 425-kb plasmid (α) indicates the presence of numerous copies of cross-hybridizing sequences in that plasmid. There are also at least eight copies in the chromosome. *Anabaena* strain PCC 7120 contained fewer copies of the element but contained at least two copies in the chromosome and one copy in plasmid α. The most obvious difference between the restriction digests of the two strains is the ca. 15-kb relative increase in size of plasmid α in strain M-131. The intensities of hybridization signals were consistent with the interpretation that this increase in size is due to the presence of an extra ca. 11 copies of IS*891* in plasmid α of strain M-131. The facts that plasmid α contains the element in both strains and that the locations of the chromosomal copies differ between the two apparently very closely related strains suggest that the element may have been introduced into these strains of *Anabaena* borne on plasmid α. Many large plasmids are self-transmissible by conjugation (16). Conjugal transfer of DNA from *E. coli* to *Anabaena* sp. proceeds efficiently (17), but conjugation between cells of *Anabaena* sp. has yet to be demonstrated and should be sought with a marked form of plasmid α.

The fact that copies of IS*891* (or cross-hybridizing relatives) are present in multiple chromosomal locations in both strains of *Anabaena* that we studied suggests that it may have some mobility in these strains. IS*891* contains, 3′ from the ORF, restriction sites for *Eco*RV, *Xho*II, and *Sna*BI, into which a selectable marker might be inserted. It may also prove to be possible to construct a compound transposon consisting of a selectable marker flanked by copies of IS*891*, as is possible with other ISs. We are attempting to develop a system for in vivo transposon mutagenesis of *Anabaena* spp. based on this element.

ACKNOWLEDGMENTS

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