Isolation and Characterization of Two Sequence-Specific Endonucleases from *Anabaena variabilis*

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Two endonucleases, *AvaI* and *AvaII*, were isolated from *Anabaena variabilis* on the basis of their ability to make a limited number of breaks at specific points in bacteriophage λ DNA. Neither enzyme has cofactor requirements beyond Mg^{2+} . Endonuclease *AvaI* makes eight breaks in the phage λ chromosome at which the 5'-terminal sequence is pPy-C-G-N. *AvaII* endonuclease cuts phage λ DNA more extensively, yielding fragments with the 5'-terminal sequence G-T-C-N or G-A-C-N. Neither enzyme generates cohesive ends.

Site-specific endonucleases, some of which have been identified as restriction enzymes (Arber & Linn, 1969), have been isolated from several prokaryotes including species of *Haemophilus*, *Bacillus* and enterobacteriaceae (for reviews see Nathans & Smith, 1975; Roberts, 1976). The enzymes that have been studied in detail recognize short nucleotide sequences (four to six base pairs) containing twofold axes of symmetry (Kelly & Smith, 1970; Murray & Old, 1974). These enzymes have already found wide application in studies of the structure of DNA, chromosome mapping by biochemical methods, the characterization of DNA preparations and the biochemical manipulation of genomes and construction of simple hybrid chromosomes (Murray, 1977). We report here the isolation and characterization of two-sequence-specific endonucleases from the blue-green bacterium *Anabaena variabilis*, which are of interest both as additions to the library of sequence-specific endonucleases and as an indication of the existence of restriction and modification systems within what is regarded as a primitive group of prokaryotes.

Materials and Methods

Nomenclature

The abbreviated description for restriction enzymes suggested by Smith & Nathans (1973) has been used for the enzymes, but the prefix R. has been omitted because we do not know whether the enzymes from *Anabaena* are associated with a restriction and modification system or not. All the oligonucleotides discussed in this paper are deoxyribonucleotides, but for convenience the prefix d has been omitted throughout.

Chemicals

All chemicals used were of AnalaR or equivalent grade and supplied by BDH Chemicals, Poole,

Dorset, U.K., or Koch-Light, Colnbrook, Bucks., U.K. [γ - ^{32}P]ATP and $^{32}P_i$ were from The Radiochemical Centre, Amersham, Bucks., U.K. Ion-exchange papers Whatman AE81 and Whatman DE81 were from R. W. Balston, Maidstone, Kent, U.K. Pancreatic DNAase (deoxyribonuclease) and snake-venom phosphodiesterase were from Worthington Corp., Freehold, NJ, U.S.A., and bacterial alkaline phosphatase was from Whatman Biochemicals, Maidstone, Kent, U.K.

Culture of the organism

The strain of *Anabaena variabilis* (Kutzing) used in these experiments was supplied by Dr. N. G. Carr and came originally from the laboratory of Professor Myers (Department of Zoology, University of Texas, Austin, TX, U.S.A.). The strain was maintained on slopes of solid medium (Allen & Arnon, 1955). It was grown autotrophically in either 20-litre carboys or a New Brunswick 10-litre Microferm fermenter on either medium C (Kratz & Myers, 1955) or the medium of Allen & Arnon (1955). Air+CO₂ (95:5) was bubbled through the cultures which were illuminated by two 14in normal fluorescent lighting tubes. Cells were harvested in an Alfa Laval 102B continuous-flow centrifuge when growth had ceased, and the cell pellet was stored at -20°C.

Bacteriophage and DNA

Bacteriophage λ was isolated from lysates of *Escherichia coli* K, lysogenic for phage λ C1857 *Sam7* (Goldberg & Howe, 1969), by two cycles of equilibrium centrifugation in CsCl gradients (Kaiser & Hogness, 1960). DNA was isolated from phage by extraction with freshly distilled phenol (equilibrated with 0.5 M-Tris/HCl, pH8.5, immediately before use)

followed by extensive dialysis against 0.01 M-Tris/HCl (pH 8.0)/1 mM-EDTA. ^{32}P -labelled DNA was isolated from phages propagated in low-phosphate medium (Grossman, 1967) containing $0.5\ \mu\text{Ci}$ of $^{32}\text{P}_i/\text{ml}$.

Endonuclease assays

The substrate DNA (1–2 μg) was incubated with samples (1–10 μl) of enzyme in 20 mM-Tris/HCl, pH 7.4, and 10 mM-MgCl₂ in a final volume of 30 μl at 37°C for 30 min, and analysed by electrophoresis on 1% agarose slab gels (Sharp *et al.*, 1973; Thompson *et al.*, 1974). Such gels separate DNA fragments on the basis of size. Fragments of radioactively labelled DNA were located by radioautography of the dried gel (Fairbanks *et al.*, 1965) and unlabelled fragments were detected by their fluorescence with ethidium bromide (Sharp *et al.*, 1973). The appearance of a number of sharp bands on the gel (see Plate 1), representing discrete fragments of the substrate DNA, was taken as evidence for the presence of a site-specific endonuclease in the assayed fraction.

5'-Terminal labelling of DNA fragments

Bacteriophage λ DNA (20–50 μg) was digested with the endonuclease preparations in 0.02 M-Tris/HCl (pH 7.4)/0.01 M-MgCl₂ (total volume 0.5–1.0 ml) at 37°C for 30 min, and the reaction stopped by extraction with freshly distilled phenol that had been equilibrated with 0.5 M-Tris/HCl, pH 8.5. After extensive dialysis against 0.01 M-Tris/HCl (pH 8.0)/1 mM-EDTA to remove phenol, the solution was treated with bacterial alkaline phosphatase (1 mg/ml of 0.1 M-NH₄HCO₃; 25 μl /ml of reaction mixture) at 37°C for 2 h and again extracted with phenol (twice) and dialysed against 0.02 M-Tris/HCl, pH 7.4. The samples were then incubated at 37°C for 60 min, with polynucleotide kinase (Richardson, 1965) and [γ - ^{32}P]ATP in 0.02 M-Tris/HCl (pH 7.4)/0.01 M-MgCl₂/0.01 M-2-mercaptoethanol, extracted with phenol, and dialysed (36 h) against 0.3 M-NaCl to remove ATP (four changes) and 0.1 mM-NaCl. They were then filtered through columns (30 cm $\times$ 0.7 cm) of Sephadex G-100 in 0.1 M-NaCl, and the column fractions were monitored by counting the Čerenkov radiation (Clausen, 1967). The labelled DNA fragments were clearly separated from residual ATP, and appropriate fractions were combined, concentrated by evaporation in a vacuum desiccator and digested with pancreatic DNAase [25 μg /ml of 0.01 M-Tris/HCl (pH 7.4)/5 mM-MgCl₂ at 37°C for 2 h].

Fractionation of oligonucleotides and sequence determination

The pancreatic DNAase digests of 5'-terminally labelled DNA fragments were fractionated by iono-

phoresis on Whatman DE81 paper (pH 2.0) or Whatman AE81 paper (pH 3.5) or by two-dimensional ionophoresis on both of these papers (Murray, 1973). Some digests were fractionated by ionophoresis on cellulose acetate, pH 3.5, and t.l.c. on polyethyleneimine-cellulose (Southern & Mitchell, 1971; Southern, 1974). Radioactive oligonucleotides were located radioautographically, eluted from the papers with 30% triethylammonium bicarbonate and analysed by digestion with snake-venom phosphodiesterase. The 5'-terminal nucleotides were readily obtained from complete digests and the sequence of the oligonucleotides was deduced from M values (Sanger *et al.*, 1965) obtained for the products of partial digests on Whatman DE81 paper at pH 2.0 and Whatman AE81 paper at pH 3.5 (Murray, 1970, 1973). The conditions used for partial digestion were 0.01 mg of venom phosphodiesterase/ml in 0.02 M-Tris/HCl, pH 8.5, at 23°C for 20 and 60 min. Deduction of sequences from such results has been described by Murray & Old (1974).

Electron microscopy

Samples (30 μl , containing 1 μg of DNA) of bacteriophage λ DNA digested with endonuclease *Ava*I or *Ava*II were made up to 0.1 M-ammonium acetate in a final volume of 60 μl , and left at 0°C for 4 days (Mertz & Davis, 1972). They were then mounted for electron microscopy by the method of Lang & Mitani (1970) in a cold-room (4°C), care being taken to avoid warming of grids and samples by proximity to the bodies of the experimenters.

The DNA mounted on carbon grids was shadowed with platinum from an angle of 7° and screened for circular DNA molecules in a Siemens Emiscop 1A at $\times 18000$ magnification.

Results

Preliminary enzyme isolation and identification

Disruption and extraction of cells. Frozen packed cells (90 g) were thawed and mixed into an equal volume of extraction buffer [10 mM-Tris/HCl (pH 7.4)/0.1 mM-EDTA/0.2 mM-MgCl₂/2 mM-2-mercaptoethanol]. The suspension was frozen and thawed four times before disruption at 27.5 MPa (4000 lb/in²) in a Manton-Gaulin homogenizer, with recycling, during which the temperature was not allowed to exceed 25°C. Although repeated freezing and thawing was not required for successful cell disruption, it appeared necessary for release of the endonucleases. The crude homogenate was centrifuged (10000 g for 20 min) and the pellet discarded.

(NH₄)₂SO₄ fractionation. The supernatant (170 ml) of the homogenate was fractionated by the addition of solid (NH₄)₂SO₄. This resolved the two endonucleases, *Ava*I appearing in the fraction precipitated

between 20 and 40% saturation (a dark blue-green colour) and *AvaII* appearing in the fraction precipitated between 50 and 70% saturation (a royal-blue colour). The precipitates were redissolved in the extraction buffer and dialysed extensively against extraction buffer.

Further purification

Endonuclease *AvaI*. The 20–40%-satd.-(NH₄)₂SO₄ fraction (in a volume of 40 ml) was adsorbed to a column (20 cm × 3 cm diam.) of DEAE-cellulose (Whatman DE-52). The column was eluted with a 500 ml (total volume) linear gradient of 0.1–0.4 M-NaCl in extraction buffer containing 10% (v/v) glycerol, and 10 ml fractions were collected. The fractions were assayed for *AvaI* endonuclease activity, which was found in fractions 18–28 (from 0.18 to 0.21 M-NaCl).

Active fractions were pooled, dialysed (overnight) against extraction buffer, diluted to 180 ml and adsorbed to a column (10 cm × 3 cm diam.) containing freshly equilibrated phosphocellulose (Whatman P11). The column was eluted with a 400 ml (total volume) linear gradient of 0–0.4 M-NaCl in extraction buffer containing 10% (v/v) glycerol, and 8 ml fractions were collected. Dilute *AvaI* endonuclease activity was found in fractions 24a–32 (from 0.18 to 0.23 M-NaCl). These fractions were pooled, dialysed against buffer and concentrated on a small (2 ml) column of DEAE-cellulose, from which the enzyme was eluted in a small volume (3 ml) by step elution with 0.4 M-NaCl in extraction buffer.

Endonuclease *AvaII*. The 50–70%-satd.-(NH₄)₂SO₄ fraction in a volume of 20 ml was adsorbed to a column (10 cm × 3 cm diam.) of DEAE-cellulose. The column was eluted with a 250 ml (total volume) linear gradient of 0–0.4 M-NaCl in extraction buffer containing 10% (v/v) glycerol. Assays showed that *AvaII* endonuclease activity was present in fractions eluted between 0.05 and 0.15 M-NaCl. These fractions were pooled (volume 50 ml), dialysed (15 h) against extraction buffer and adsorbed on a column (10 cm × 2 cm diam.) of phosphocellulose (P11). The column was eluted with a 200 ml (total volume) linear gradient of 0–0.4 M-NaCl in extraction buffer containing 10% (v/v) glycerol. *AvaII* endonuclease activity was found between 0.17 and 0.19 M-NaCl.

Stability of endonucleases *AvaI* and *AvaII*. Endonuclease *AvaI* has been stored at –20°C in 50% glycerol and remained stable for about 1 year. Preparations of endonuclease *AvaII* have usually been less stable.

Fragmentation of bacteriophage λ DNA by the endonucleases

Analysis by gel electrophoresis. Both endonucleases made a limited number of cuts at specific points in

bacteriophage λ DNA to yield characteristic patterns of bands when the digested DNA was analysed by gel electrophoresis (Plate 1). The fragmentation patterns provided a specific assay for the enzymes, for clear sharp bands were obtained on gels at the (NH₄)₂SO₄ fractionation stage of purification.

Plate 1(a) shows that an endonuclease *AvaI* digest of bacteriophage λ DNA contains nine size classes of fragments represented by nine bands on the gel. However, band 1a represents a fragment formed by the annealing of fragment 2 and a fragment from band 4 (which contains two fragments from different parts of the molecule) via the cohesive 5'-ends of bacteriophage λ DNA; this was determined during the mapping of the cleavage sites for endonuclease *AvaI* in the phage λ chromosome (S. G. Hughes, unpublished work). We conclude that endonuclease *AvaI* makes eight cuts in the phage λ chromosome to give nine fragments; we cannot, however, exclude the possibility that there are extra sites at which endonuclease *AvaI* cuts the DNA to produce fragments that are too small to be resolved and detected by the gel system.

Endonuclease *AvaII* gives a more complex fragmentation pattern than does endonuclease *AvaI*, and contains at least 18 bands. More detailed analysis of this pattern shows that an endonuclease-*AvaII* digest of bacteriophage λ DNA contains at least 25 discrete fragments, since several of the bands in the gel pattern contain more than one fragment.

Electron microscopy. In a random sample of 200 DNA fragments, no circular molecules were observed in DNA digested with endonuclease *AvaI* or *AvaII*. This finding was consistent with sequence data and makes it unlikely that *AvaI* and *AvaII* endonucleases cut DNA to leave single-strand projections at the break point which allow circularization of fragments. This procedure, derived from that of Mertz & Davis (1972), gave circularization of bacteriophage λ DNA fragments generated by R.EcoRII (Bigger *et al.*, 1973).

Determination of pH optimum and Mg²⁺ requirements

The pH optimum for endonuclease *AvaI* was determined by carrying out assays with constant amounts of DNA and enzyme over a range of pH from 6.5 to 9 in steps of 0.5 pH unit. The relative extents of degradation of the DNA were assessed by examination of the gel after electrophoresis. The pH values giving the most effective digestion were from pH 7.5 to 8.8.

Similar experiments showed that Mg²⁺ was required for activity and the optimum concentration was between 5 and 25 mM. Ca²⁺ could not restore activity in the absence of Mg²⁺. Similarly, the pH optimum of endonuclease *AvaII* was shown to be between pH 7.5 and 9.

Specificity of the enzymes

Position of breakage. In a preliminary 5'-labelling experiment bacteriophage λ DNA (20 μ g) was digested with endonuclease *Ava*I. The digest was split into two parts, one of which was treated with alkaline phosphatase and the other remained untreated. The two samples were then labelled in parallel with polynucleotide kinase and [γ - 32 P]ATP, and the labelled DNA was separated from residual [32 P]ATP as described in the Materials and Methods section. The dephosphorylated DNA contained 948 c.p.m./ μ g compared with 354 c.p.m./ μ g in the sample that had not been incubated with alkaline phosphatase. DNA samples were digested with pancreatic DNAase and snake venom phosphodiesterase and analysed electrophoretically on Whatman AE81 paper at pH 3.5 (Plate 2). This showed that the radioactivity incorporated into dephosphorylated DNA was predominantly in pC and pT, whereas most of the radioactivity in the non-dephosphorylated DNA remained at the origin during electrophoresis (presumably ATP), very little radioactivity moving with 5'-mononucleotides. We conclude that *Ava*I cuts DNA to leave a 5'-phosphate. We did not deter-

mine whether endonuclease *Ava*II leaves a 5'-phosphate or hydroxyl group and for this reason do not include -p or -OH in writing the 5'-terminal sequences produced by this enzyme.

Nucleotide sequences at the 5'-ends of DNA fragments. The oligonucleotides in pancreatic DNAase digests of 5'-terminally labelled DNA fragments from endonuclease-*Ava*I and -*Ava*II digests of phage λ DNA were fractionated in various experiments by one-dimensional ionophoresis on Whatman AE81 and DE81 ion-exchange papers, by two-dimensional ionophoresis on these two papers, and two-dimensionally by ionophoresis on cellulose acetate followed by chromatography on thin layers of polyethyleneimine-cellulose. Examples of the two-dimensional separations are shown in Plates 3 and 4. Smaller oligonucleotides (not indicated in Plates 3 and 4) were isolated in high yield by the one-dimensional ionophoretic separations. Results of the analyses of oligonucleotides eluted from these papers and thin layers are given in Tables 1 and 2. These show that two families of sequences were obtained from the DNA fragments produced by each enzyme. Digestion of DNA with endonuclease *Ava*I gave

Table 1. Analysis of the oligonucleotides obtained by digestion with pancreatic DNAase of 5'-terminally labelled fragments of phage λ DNA formed by the action of endonuclease *Ava*I

The oligonucleotides other than 9, 17 and 18 were separated by ionophoresis on Whatman AE81 paper at pH 3.5 or Whatman DE81 paper at pH 2, or two-dimensionally by ionophoresis on cellulose acetate, pH 3.5, followed by chromatography on thin layers of polyethyleneimine-cellulose (Plate 3), or both; their mobilities with respect to the blue dye Xylene Cyanol FF were determined on the two ion-exchange papers and the base sequence of each oligonucleotide was determined from a combination of these values and M values (Sanger *et al.*, 1965) of the products of partial digestion with venom phosphodiesterase after ionophoretic separation on both AE81 and DE81 papers (Murray, 1973). Oligonucleotides 9, 17 and 18 were not obtained in sufficient yield for the sequences to be confirmed in this way. The results given for these oligonucleotides are based on their 5'-termini, limited information from partial digests with venom phosphodiesterase and their behaviour on the two-dimensional separation system (Morrison & Murray, 1974); they are probably correct, but should be regarded as tentative.

Oligonucleotide no.	Mobility on ion-exchange papers			Partial digestion products			Identity
	AE81 (pH 3.5)	DE81 (pH 2.0)	5'-Terminus				
1	0.93	2.6	pC				pC-C
2	0.30	1.8	pC	pC-C			pC-C-G
3	0.20	1.5	pC	pC-C	pC-C-G		pC-C-G-C
4	0.15	1.4	pC	pC-C	pC-C-G		pC-C-G-A
5	0.08	0.69	pC	pC-C	pC-C-G		pC-C-G-G
6	0.026	0.42	pC	pC-C	pC-C-G	pC-C-G-A	pC-C-G-A-G
7	0.04	0.48	pC	pC-C	pC-C-G	pC-C-G-T	pC-C-G-T-A
8	0.017	0.42	pC	pC-C	pC-C-G	pC-C-G-G	pC-C-G-G-A
9			pC	pC-C	pC-C-G		pC-C-G-G-T
10	0.021	0.21	pC	pC-C	pC-C-G	pC-C-G-G	pC-C-G-G-G
11	0.89	2.12	pT				pT-C
12	0.23	0.80	pT	pT-C			pT-C-G
13	0.09	0.69	pT	pT-C	pT-C-G		pT-C-G-C
14	0.11	0.50	pT	pT-C	pT-C-G		pT-C-G-A
15	0.065	0.24	pT	pT-C	pT-C-G		pT-C-G-G
16	0.04	0.48	pT	pT-C	pT-C-G	pT-C-G-C	pT-C-G-C-A
17			pT	pT-C	pT-C-G		pT-C-G-G-T
18			pT	pT-C	pT-C-G		pT-C-G-G-G

EXPLANATION OF PLATE I

*Agarose-gel electrophoresis of samples of bacteriophage λ DNA digested with endonucleases *AvaI* and *AvaII**

The DNA ($2\mu\text{g}$) was treated with (a) endonuclease *AvaI* ($2\mu\text{l}$ of the concentrated phosphocellulose fraction), and (b) endonuclease *AvaII* ($10\mu\text{l}$ of the DEAE-cellulose fraction) in 50 mm-Tris/HCl (pH 7.5)/ 10 mm-MgSO_4 ($20\mu\text{l}$) at 37°C for 30 min. The digests were analysed by electrophoresis in 1% agarose gels containing ethidium bromide ($5\mu\text{g/l}$). Gels were photographed with u.v. light in a Chromatovue short-wavelength u.v. illuminator on Ilford FP4 film ($4\times$ red filter).

EXPLANATION OF PLATE 2

*Effect of phosphatase on the 5'-termini of DNA fragments generated by the action of *Ava*I endonuclease*

Fragments were incubated with [γ - 32 P]ATP and polynucleotide kinase before (a) and after (b) treatment with bacterial alkaline phosphatase. The fragments were then digested with pancreatic DNAase and snake-venom phosphodiesterase and the 5'-mononucleotides separated by ionophoresis on Whatman AE81 paper at pH 3.5.

EXPLANATION OF PLATE 3

*Two-dimensional separation of oligonucleotides in a pancreatic DNAase digest of 5'-terminally labelled fragments of bacteriophage DNA formed by the action of endonuclease *Ava*I*

After electrophoresis on cellulose acetate, pH3.5, 7M-urea, the oligonucleotides were transferred to a thin layer of polyethyleneimine-cellulose (Southern, 1974) and the chromatogram was developed with 1.6M-formic acid adjusted to pH3.5 with pyridine. The numbered oligonucleotides are identified in Table 1.

EXPLANATION OF PLATE 4

*Two-dimensional separation of oligonucleotides in a pancreatic DNAase digest of 5'-terminally labelled fragments of bacteriophage λ DNA formed by the action of endonuclease *Ava*II*

The first ionophoresis was on Whatman AE81 paper, pH 3.5; the strip of paper was then stitched to Whatman DE81 paper for ionophoresis in the second dimension at pH 2. The numbered oligonucleotides are identified in Table 2.

Table 2. Analysis of the oligonucleotides obtained by digestion with pancreatic DNAase of 5'-terminally labelled fragments of phage λ DNA formed by the action of endonuclease *AvaII*

Some of the oligonucleotides were separated by ionophoresis on Whatman AE81 paper at pH 3.5 or Whatman DE81 paper at pH 2, and the mobility of each oligonucleotide on the two ionophoretic systems was measured with respect to the blue dye Xylene Cyanol FF. Oligonucleotides in partial digests with venom phosphodiesterase were identified from their mobilities and M values on both AE81 and DE81 papers (Murray, 1973).

Oligo-nucleotide no.	Mobility on ion-exchange papers		5' Terminus	Partial digestion products	Identity
	AE81, pH 3.5	DE81, pH 2.0			
21	0.23	1.24	G	pG-A	pG-A-C
22	0.14	1.07	G	pG-A, pG-A-C	pG-A-C-C
23	0.08	0.80	G	pG-A, pG-A-C	pG-A-C-A
24	0.05	0.75	G	pG-A, pG-A-C, pG-A-C-A	pG-A-C-A-C
25	0.09	0.52	G	pG-A, pG-A-C	pG-A-C-T
26	0.04	0.46	G	pG-A, pG-A-C	pG-A-C-G
27	0.02	0.17	G	pG-A, pG-A-C, pG-A-C-T, pG-A-C-T-C	pG-A-C-T-C-T
28	0.01	0.16	G	pG-A, pG-A-C, pG-A-C-T, pG-A-C-T-C, pG-A-C-T-C-T	pG-A-C-T-C-T-C
31	0.24	0.72	G	pG-T	pG-T-C
32	0.13	0.65	G	pG-T, pG-T-C	pG-T-C-C
33	0.06	0.60	G	pG-T, pG-T-C, pG-T-C-C	pG-T-C-C-C
34	0.08	0.54	G	pG-T, pG-T-C	pG-T-C-A
35	0.08	0.28	G	pG-T, pG-T-C	pG-T-C-T
36	0.05	0.25	G	pG-T, pG-T-C	pG-T-C-G
37	0.03	0.23	G	pG-T, pG-T-C, pG-T-C-G	pG-T-C-G-C
38	0.03	0.17	G	pG-T, pG-T-C, pG-T-C-T	pG-T-C-T-A
39	0.02, 0.01	0.08	G	pG-T, pG-T-C, pG-T-C-T, pG-T-C-T-C	pG-T-C-T-C-T, pG-T-C-T-C-T-C
40	0.04	0.08	G	pG-T, pG-T-C, pG-T-C-T	pG-T-C-T-T

fragments terminating in pC-C-G-N- and/or pT-C-G-N-, and with endonuclease *AvaII* the 5'-terminal sequences were G-A-C-N- and G-T-C-N-.

Discussion

The results illustrate the purification and some of the general properties of two endonucleases from *A. variabilis*. Gel-electrophoretic analysis of the fragments generated from the DNA of phage λ by these endonucleases show that both enzymes are site-specific. This is confirmed by the determination of the nucleotide sequences at the ends of DNA fragments formed by the endonucleases.

In producing the 5'-terminal sequences T-C-G-N and C-C-G-N, endonuclease *AvaI* could recognize some if not all of the family of three possible hexanucleotide sequences that result from rotation:

of the terminal trinucleotide sequence about a pro-

posed twofold axis of symmetry, and break the central phosphodiester bonds. An endonuclease (*Ball*) from *Brevibacterium albidum* gives seven DNA fragments with the unique 5'-terminal trinucleotide sequence C-C-G-, which arises from breakage of the central phosphodiester bonds of the hexanucleotide sequences -C-G-G-C-C-G- (R. S. Roberts, P. A. Myers, S. A. Bruce & K. Murray, unpublished work), which is one of the three possible sequences for endonuclease *AvaI*. Fragments of phage λ DNA formed by this enzyme, of which there are at least 14, are electrophoretically different from those formed on digestion of phage λ DNA with *AvaI* endonuclease, which suggests that in the recognition targets for this enzyme the central dinucleotide pairs may be asymmetrical. Additional evidence supporting the unique hexanucleotide -C-G-G-T-C-G- as the recognition target for endonuclease *AvaI* comes from digests of phage λ DNA made with endonuclease *AvaI* and the restriction enzyme *R.BsuR*, which, like the enzyme *R.HaeIII*, cleaves the central phosphodiester bonds of the tetranucleotide sequence -G-G-C-C- (Bron & Murray, 1975). Were the recognition sequence for endonuclease *AvaI* degenerate in its central dinucleotide, the enzyme would have some targets in common with the enzyme *R.BsuR*. In a digest of phage λ DNA with *R.BsuR*

enzyme some of the targets for endonuclease *AvaI* would then be lost and endonuclease *AvaI* would make fewer breaks in the products of the *R.BsuR* digest than in the undigested DNA. However, if the recognition sequence for endonuclease *AvaI* were the unique hexanucleotide, all the targets in phage λ DNA would be present within the fragments generated by the action of the *R.BsuR* enzyme. Gel-electrophoretic analysis of *R.BsuR* digests with and without the further action of endonuclease *AvaI* would then reveal changes in the pattern of DNA fragments corresponding to breakage at all eight of the targets for endonuclease *AvaI*. Such an experiment with phage λ DNA digested with the *R.BsuR* enzyme has provided evidence for seven of the eight possible breaks on treatment with endonuclease *AvaI* (S. G. Hughes & K. Murray, unpublished work), but because of the complexity of the *R.BsuR* digest these experiments are being continued with deletion strains of phage λ in which the DNA has fewer targets for endonuclease *AvaI* than has that of the wild-type. Confirmation of the unique recognition sequence for endonuclease *AvaI* requires the determination of sequences at individual targets, but will probably be obtained incidentally from other, more extensive, sequence results. Such information would be interesting in the context of reaction mechanisms between proteins and nucleotide sequences that are symmetrical at least in part.

Since there is no element of internal complementarity in the 5'-sequences determined, it is most unlikely that *AvaI* endonuclease cuts DNA to give a staggered break (unlike *R.EcoRI* and *R.EcoRII*), an argument that is supported by the experiments in which circularization of DNA fragments was screened by electron microscopy. None was found.

Similar arguments support the view that in generating G-T-C-N- or G-A-C-N-, endonuclease *AvaII* recognizes the degenerate hexanucleotide and cleaves it in the centre even though the two terminal sequences are complementary:

Since the two sequences become degenerate in the fourth and following nucleotides, base-pairing of these two complementary sequences would require a trinucleotide-recognition sequence, the frequency of which (1 in 64) is far from consistent with the number of cuts made by endonuclease *AvaII* in the DNA of phage λ , and again, examination of appropriately treated *AvaII* fragments of bacterio-

phage λ DNA by electron microscopy showed that no circular molecules were present.

Although these enzymes appear to be analogous to the restriction enzymes found in other prokaryotes, it is not known whether they are associated with a similar phenomenon in the Cyanophyceae. It will be interesting to compare the sensitivities of the DNA of *A. variabilis* and related organisms and their viruses with these enzymes *in vitro*. In a preliminary experiment, endonucleases with different sequence specificities have been identified in the related organism *Anabaena catenula* (S. G. Hughes & T. Bruce, unpublished work).

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