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The Isolation and Characterization of a Sequence-Specific Endonuclease from *Anabaena subcylindrica*

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An endonuclease, *Asu*I, was isolated from extracts of *Anabaena subcylindrica* on the basis of gel-electrophoretic analysis of digests of bacteriophage-λ DNA with the partially purified extracts. The enzyme requires Mg^{2+}, but no other cofactors. Endonuclease *Asu*I recognizes the interrupted tetranucleotide sequence:

5′-G↓G-N-C-C-3′
-C-C-N-G↑G-

and breaks the phosphodiester bonds indicated by the arrows to leave single-stranded trinucleotide projections at the 5′-termini of the DNA fragments.

During the relatively short period that has elapsed since the discovery of a sequence-specific endonuclease in extracts from *Haemophilus influenzae* (Smith & Wilcox, 1970; Kelly & Smith, 1970) and the recognition that a number of restriction enzymes are also sequence-specific endonucleases (Hedgpeth *et al.*, 1972; Bigger *et al.*, 1973; Bron & Murray, 1975) these enzymes have found dramatic application in molecular biology and especially in the structural analysis of DNA. Enzymes exhibiting a wide range of differing nucleotide-sequence-specificities have subsequently been isolated from many species of bacteria, and the intense interest in the enzymes, both as reagents for the biochemical and genetic analysis of DNA (including genetic-manipulation experiments *in vitro*) and as systems for the study of specific interactions between proteins and DNA, has stimulated the quest for more enzymes with different sequence-specificities. Many are described in a review by Roberts (1976). Among the bacteria examined for the presence of these enzymes are a number of species of blue–green algae (or cyanobacteria), and the isolation and properties of two sequence-specific endonucleases (*Ava*I and *Ava*II) from *Anabaena variabilis* have already been reported (Murray *et al.*, 1976). In the present paper are described the purification and properties of a similar enzyme (*Asu*I) from another cyanobacterium, *Anabaena subcylindrica*. Sequence-specific endonucleases have also been isolated from *Anabaena cylindrica* (de Waard *et al.*, 1978) and *Anabaena oscillarioides* (de Waard *et al.*, 1979).

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Materials and Methods

Nomenclature

Restriction enzymes are described by the abbreviations suggested by Smith & Nathans (1973) with the prefix R omitted. Since all the oligonucleotides discussed in the present paper are oligodeoxynucleotides the prefix d has been omitted. Sequences are written in the sense 5′ to 3′, left to right.

Chemicals and enzymes

AnalaR or equivalent-grade chemicals from BDH, Poole, Dorset, U.K. were mainly used. Tris (Trizma base) was obtained from Sigma, and all buffer solutions and stock reagents for enzyme preparations, reactions or electrophoresis experiments were sterilized by autoclaving. [γ-^{32}P]ATP of high specific radioactivity was from The Radiochemical Centre, Amersham, Bucks., U.K. Ion-exchange papers Whatman DE81 and Whatman AE81 were supplied by W. and R. Balston, Maidstone, Kent, U.K., and ion-exchange materials for chromatographic fractionation of the enzymes were Whatman Chromedia DE52 and P11, and these, as well as bacterial alkaline phosphatase, were from Whatman, Maidstone, Kent, U.K. Agarose was obtained from Miles Laboratories, Slough, Bucks., U.K. Pancreatic deoxyribonuclease and snake-venom phosphodiesterase were purchased from Worthington Corp., Freehold, NJ, U.S.A. Polynucleotide kinase was prepared from *Escherichia coli* B infected with bacteriophage T4 N*am*122 as described by Richardson (1965). T4 DNA ligase was prepared from cultures of *E. coli* infected with a λ/T4 hybrid phage carrying the ligase gene (Murray *et al.*, 1979).

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Bacteriophage λ and its DNA

These preparations were made as described previously (Murray *et al.*, 1976).

Culture of the organism

The strain of *Anabaena subcylindrica* used was CCAP 1403/4b and was maintained on slants of agar (Allen & Arnon, 1955). It was grown in liquid culture at 20°C in Kratz & Meyers (1955) medium in 20-litre carboys illuminated by normal fluorescent tube lighting and aerated with air enriched with CO_2 to a level of 5%. The culture was harvested after 20 days, in an Alfa Laval LAB 102B continuous-flow centrifuge, and the cells were stored frozen at −20° C.

Endonuclease assays

These were based upon digestion of bacteriophage-λ DNA as described in Fig. 1, and electrophoresis in 1% agarose gels in 0.04M-Tris acetate buffer, pH8.0 (Thompson *et al.*, 1974); this is now a routine laboratory method. The gels were stained with ethidium bromide solution (about 0.5μg/ml) and photographed on Ilford FP4 film under u.v. light (4x red filters).

5'-Terminal labelling of DNA fragments and determination of 5'-terminal oligonucleotide sequences

The procedures followed in these experiments were as described previously (Murray *et al.*, 1976).

Results and Discussion

Purification of the enzymes

Frozen packed cells (20g) were allowed to thaw in 20ml of 10mM-Tris/HCl buffer, pH7.5 at 4°C. The cell suspension was refrozen and thawed three times before being disrupted by passage through a Manton–Gaulin extrusion press at 5620kPa. Rinsing of the press with buffer brought the volume of lysate up to 90ml. All subsequent operations were carried out at 4°C. The resulting lysate was centrifuged at 12000rev./min (MSE High Speed 18, 6×250ml rotor) for 30min. The supernatant was then subjected to fractional precipitation with $(NH_4)_2SO_4$. Each fraction was redissolved in 20ml of extraction buffer (20mM-Tris/HCl, pH7.5, 2mM-EDTA, 10mM-2-mercaptoethanol) and dialysed extensively against the same buffer. Each fraction was assayed for its ability to degrade bacteriophage-λ DNA, so as to give a discrete set of fragments as described in the Materials and Methods section. Such activity, observed against a background of non-specific cleavage, was found in the fractions precipitated between 0 and 40% saturation and 40 and 50% saturation. Detectable but weak activity was found in the fraction precipitated between 50 and 70% saturation, but this activity was not investigated further.

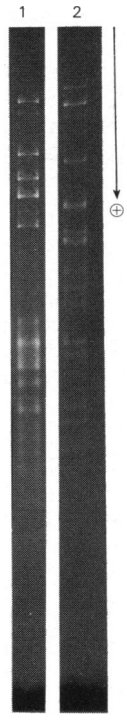

Fig. 1. *Digestion of bacteriophage-λ DNA by endonuclease AsuI*

Bacteriophage-λ DNA (1μg) was digested with 20μl of purified endonuclease *Asu*I (track 1) and 5μl of endonuclease *Ava*II (track 2) for 2h in the presence of 20mM-Tris/HCl (pH7.5)/10mM-$MgCl_2$/100mM-NaCl, and the products were analysed by zone electrophoresis on a 1% agarose gel as described by Thompson *et al.* (1974). The gel was stained in ethidium bromide and photographed by u.v. fluorescence.

The two active fractions were separately fractionated further by chromatography on DEAE-cellulose. Each fraction was adsorbed on a column (6cm× 3.5cm diameter) of DEAE-cellulose previously equilibrated with extraction buffer, and eluted with a 1-litre gradient (total volume) of from 0 to 0.5M-NaCl in extraction buffer. Fractions were assayed as above, and activity from both $(NH_4)_2SO_4$ fractions was found in those fractions eluted at about 0.2M-NaCl. Active fractions were pooled and dialysed overnight (against extraction buffer made 20%, w/v, with respect to glycerol) and further purified by chromatography on phosphocellulose P11. Each pool was adsorbed on a column (3cm×2cm diameter) of phosphocellulose and eluted with a 300ml

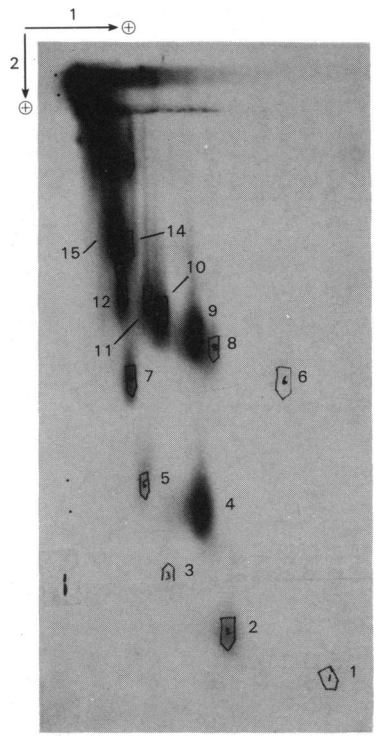

Fig. 2. *Separation of oligonucleotides derived from 5'-terminally labelled DNA fragments*

Bacteriophage-λ DNA (10μg) was incubated with 25μl of endonuclease *Asu*I in the presence of 100mM-NaCl/2mM-Tris/HCl (pH7.5)/10mM-$MgCl_2$ in a volume of 500μl for 2h at 37°C. Samples were analysed by zone electrophoresis to check that the DNA was restricted. The reaction was stopped by heating at 75°C for 5min, and then bacterial alkaline phosphatase (1mg/ml in 12.5μl) was added. Incubation was continued for 2h at 37°C and then the dephosphorylated DNA was extracted twice with freshly redistilled phenol and dialysed exhaustively against 20mM-Tris/HCl, pH7.5. Polynucleotide kinase was then used to label the 5'-ends of the DNA fragments. The reaction mixture contained 10μl of polynucleotide kinase, 1μM-ATP {containing [γ-^{32}P]-ATP (150Ci/mol)}, 20mM-Tris/HCl, pH7.5, 10mM-$MgCl_2$, 10mM-2-mercaptoethanol and the dialysed DNA. Incubation at 37°C was continued for 3h and followed by heating at 70°C for 5min; the solution was then dialysed against 0.3M-NaCl to remove the bulk of the unchanged ATP. The DNA was further purified by passage through a column (2cm×70cm) of Sephadex G-100 in 0.3mM-NaCl. Radioactive fractions eluted in the exclusion volume were collected, pooled and concentrated by evaporation to a volume of 100μl (this contained 180000c.p.m. of radioactivity). Half of this material was digested with pancreatic deoxyribonuclease and the resulting oligonucleotides were separated by two-dimensional electrophoresis on AE81 paper at pH3.5 in the first dimension and on DE81 paper at pH2.0 in the second. The electrophoretogram was dried and radioautographed. The Figure shows a photograph of the radioautograph with the various oligonucleotides marked and numbered. Table 1 gives the identification of these oligonucleotides.

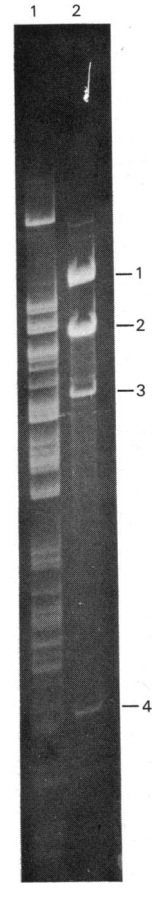

Fig. 3. *Digestion products of bacteriophage-ϕX174 DNA with endonuclease AsuI*

Bacteriophage-ϕX174 RFII DNA (0.5μg) was digested with 2μl of endonuclease *Asu*I under standard conditions and the digest analysed by zone electrophoresis on a 1% agarose gel (track 2) alongside a mixture of DNA fragments of known size derived by digesting bacteriophage-λ DNA with endonucleases *Eco*RI and *Hin*dIII singly and together (track 1). Band 1 is the circular form of phage-ϕX174 RFII DNA, band 2 the linear form generated by cleavage of one of the endonuclease-*Asu*I sites, and bands 3 and 4 the two fragments derived by cleavage of both endonuclease-*Asu*I sites in phage-ϕX174 DNA. Bands 3 and 4 have sizes of 0.41 kilobases and 0.13 kilobases.

Table 1. *5′-Terminal oligonucleotides from DNA fragments released by endonuclease AsuI*

Radioactive areas indicated in Fig. 2 were cut from the paper and the oligonucleotides eluted with 30% triethylamine carbonate solution. Each oligonucleotide was partially digested with venom phosphodiesterase and analysed by electrophoresis on Whatman AE81 paper at pH3.5 and on Whatman DE81 paper at pH2.0. Complete digests with venom phosphodiesterase showed that the 5′-terminus of each oligonucleotide was guanosine. The identities of the oligonucleotides were deduced from the mobilities of their partial-digestion products in the two electrophoretic systems and from the mobility shifts (*M* values) between immediate pairs in each homologous series of nucleotides generated by the partial digestion (Murray, 1973).

Oligonucleotide number (Fig. 2)	5′-Terminal nucleotide	Partial digestion products	*M* values on DE81	*M* values on AE81	*M* values relative to oligonucleotide	Identity
1	pG-C	pG-C	0.12	0.70	pG-C	pG-C-C
2	pG-C	pG-C, pG-C-C	0.12	0.76	1	pG-C-C-C
3	pG-C	pG-C, pG-C-C, pG-C-C-C	0.13	0.92	2	pG-C-C-C-C
4	pG-A	pG-A, pG-A-C	0.14	0.71	pG-A-C	pG-A-C-C
5	pG-C	pG-C-C, pG-C-C-C		1.5	2	pG-C-C-C-A
6	pG-T	pG-T	0.14	0.84	pG-T	pG-T-C
7	pG-A	pG-A, pG-A-C, pG-A-C-C	0.54	1.6	4	pG-A-C-C-A
8	pG-G	pG-G	0.11	0.63	pG-G	pG-G-C
9	pG-T	pG-T, pG-T-C	0.16	0.81	6	pG-T-C-C
10	pG-G	pG-G, pG-G-C	0.13	0.72	8	pG-G-C-C
11	pG-T	pG-T, pG-T-C, pG-T-C-C	0.14	0.78	9	pG-T-C-C-C
12	pG-C	pG-C, pG-C-C, pG-C-C-C	1.6	2.8	2	pG-C-C-C-G
14	pG-T	pG-T, pG-T-C, pG-T-C-C	0.45	1.8	9	pG-T-C-C-A
15	pG-G	pG-G-C, pG-G-C-C	0.45		10	pG-G-C-C-A

gradient (total volume) of from 0 to 0.5M-NaCl. Active fractions were pooled, dialysed as above and concentrated by adsorption on a small column (1cm×1cm diameter) of DEAE-cellulose followed by elution with 0.5M-NaCl. The concentrated preparation was made up to 40% glycerol and stored at −20°C. At this stage the enzymic activity from both $(NH_4)_2SO_4$ fractions was found to be identical by the criterion of the fragmentation of bacteriophage-λ DNA (Fig. 1), and the two preparations were used interchangeably and designated endonuclease *Asu*I. Bacteriophage-λ DNA is cut into more than 30 fragments by endonuclease *Asu*I.

This enzyme has also been purified by following the procedure developed by Smith & Wilcox (1970), in which gel filtrations on Bio-Gel A0.5m are used in place of $(NH_4)_2SO_4$ precipitation.

Identification of terminal nucleotides generated by endonuclease AsuI

Fig. 2 shows the separation of the radioactive nucleotides generated by pancreatic deoxyribonuclease digestion of bacteriophage-λ DNA labelled *in vitro* by using polynucleotide kinase and [γ-^{32}P]-ATP after fragmentation by endonuclease *Asu*I.

The nucleotides were eluted from the ion-exchange papers and analysed by partial digestion with venom phosphodiesterase and electrophoresis on Whatman AE81 and DE81 papers. The identification of nucleotides and their partial-digestion products was made on the basis of mobility shift values and the known mobility of dinucleotides in these systems. The results, which are presented in Table 1, show that endonuclease *Asu*I cleaves 5′-termini of the generalized structure G-N-C-C-N, from which it is inferred that endonuclease *Asu*I recognizes the rotationally symmetrical sequence:

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      ↓
G-G-N-C-C
C-C-N-G-G-
      ↑
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cutting as shown by the arrows. This interpretation is supported by the sizes of the endonuclease-*Asu*I digestion products of bacteriophage-φX174 RFII DNA. In phage-φX174 DNA (Sanger *et al.*, 1977) the sequence G-G-N-C-C occurs only twice, at nucleotide positions 980 and 5031, such that cleavage of the replicative form of phage-φX174 DNA by endonuclease *Asu*I should yield fragments of 4051 and 1324 base-pairs. Comparison of the mobilities of the endonuclease-*Asu*I digestion products of phage-φX174 RF DNA with those of endonucleases *Eco*RI and *Hin*dIII of phage-λ DNA (Fig. 3) in zone electrophoresis on agarose gels gives sizes for the two phage-φX174 DNA fragments of 0.41 and 0.13 kilobases, supporting the conclusion that endonuclease *Asu*I recognizes and cleaves the sequence G-G-N-C-C. An endonuclease with the same sequence-specificity has been isolated from *Staphylococcus aureus* by Sussenbach *et al.* (1978).

We thank Mrs. Sandra Bruce for excellent technical assistance and Dr. F. Sanger for directing us to the positions of the sequence G-G-N-C-C within the phage-φX174 DNA nucleotide sequence.

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