A NEW SEQUENCE-SPECIFIC ENDONUCLEASE FROM *ANABAENA CYLINDRICA**

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1. Introduction

The isolation of sequence-specific endodeoxyribonucleases from the cyanophytes *Anabaena variabilis* (ATCC 27892), *Anabaena subcylindrica* (CCAP 1403/4b) and *Anabaena catenula* (CCAP 1403/1) has been reported [1,2]. We have found a new endonuclease from *Anabaena cylindrica* (CCAP 1403/2a) and describe here the procedure of its isolation as well as the elucidation of the nucleotide sequences:

5′ G Pu ↓ C G Py C 3′

3′ C Py G C ↑ Pu G 5′

recognized by the purified enzyme *Acy*I.

2. Materials and methods

The culture of *A. cylindrica* was a gift from A. G. de Wit, of the Laboratory of Biophysics at Leiden University, and originated from culture 1403/2a from the Culture Centre of Algae and Protozoa at Cambridge. It was grown at 27°C in three 1 l cylindrical flasks with sterile air/CO_2 (5%) bubbling from below, and with illumination from 1 or 2 55 cm fluorescent lighting tubes placed at 40 cm distance. The growth medium consisted of (mg/l): $MgSO_4 \cdot 7\ H_2O$, 250; $CaCl_2$, 50; NaCl, 230; K_2HPO_4, 350; Fe (as EDTA complex) 5 (a 1:2.5 mixture of $FeSO_4 \cdot 7\ H_2O$ and EDTA aerated by shaking overnight); H_3BO_3, 3; $MnCl_2 \cdot 4\ H_2O$, 2; traces (0.2–0.5 mg/l) of Zn^{2+}, Cu^{2+}, Ti^{4+}, Cr^{3+}, Wo_4^{2-}; and MoO_4^{2-}; at pH ~7.5. No nitrogen source other than N_2 present in the air was provided. From an inoculum of 100 ml, 3 weeks were required to collect (from 10 l medium) 100 g wet cells, which were harvested and then stored at −20°C.

2.1. *Materials*

T4 polynucleotide kinase and DNA from phage λ and from SV40 virus were prepared as in [5,6]. Octathymidylate (8) obtained from New England Biolabs, Beverly, MA was converted to [5′-^{32}P]T8 with polynucleotide kinase and [γ-^{32}P]ATP [5] (diluted to 150 Ci/mmol) and purified by passage through a 20 × 1 cm column of Sephadex G-25 (Pharmacia) in 50 mM triethyl ammonium bicarbonate, pH 8.0. Pancreatic DNase and snake venom exonuclease were purchased from Boehringer, Mannheim. Restriction endonucleases were either purchased from New England Bioloabs (*Hae*II, *Hha*I) or prepared in an analogous fashion to established procedures [9,13]. One unit of enzyme activity was the amount which cleaves 1 μg λ DNA to completion in 1 h at 37°C. DEAE-cellulose thin-layer plates (Polygram Cel 300 DEAE/HR-2/15) were obtained from Macherey-Nagel, Dueren. Ethidium bromide was from British Drug Houses, polyethylene imine (Polymin P) from BASF Aktiengesellschaft, 6700 Ludwigshafen. The Radiochemical Centre, Amersham, supplied [γ-^{32}P]ATP at a spec. act 3000 Ci/mmol.

* The authors dedicate this communication to Professor E. Havinga on the occasion of his retirement from Leyden University to which he has given so much. They also recognize his contributions to the independent development of biochemistry at this University

2.2. *Assay of* Acy *endonuclease activity*

Acy enzyme (column fraction) 15 μl, and 1 μg λ DNA were added to incubation mixtures (30 μl) containing 20 mM Tris–HCl (pH 7.4) and 10 mM $MgCl_2$. After incubation for 45 min the reactions were stopped by adding 5 μl 12.5% Ficoll/0.05% bromophenol blue/0.05 M EDTA and subjected to slab gel electrophoresis as in [3].

2.3. *Assays for contaminating exonucleases*

Exonucleotic activity was detected in two ways:

(i) By inspecting the width and sharpness of the bands obtained as above after extensive incubation of λ DNA with *Acy*I enzyme;

(ii) By detection of the breakdown of terminally-labeled [^{32}P]T8 on DEAE thin-layer plates [4].

2.4. *Enzyme purification*

Wet cells, 100 g, were put through 3 freezing–thawing cycles [1] before being disrupted by 2 passages in a French pressure cell at 3000 kg/cm^2 at 0°C. The paste was mixed with an equal volume of buffer A (10 mM Tris–HCl, pH 7.4, 0.1 mM EDTA, 0.2 mM $MgCl_2$, 2 mM mercaptoethanol) and centrifuged at 30 000 × *g* for 30 min at 2°C. The deep-green supernatant was carefully poured off the thick sediment and diluted further with 1 vol. buffer A. NaCl was dissolved to 0.1 M (*Acy* supernatant). 1/10 Polymin P, solution 10%, 1/10 the vol., was stirred in for 10 min and the mixture centrifuged as before. The supernatant (not containing any detectable enzyme activity) was decanted and the precipitate extracted 3 times with 25 ml 0.6 M NaCl in buffer A for 100 ml *Acy* supernatant. Extraction was done by grinding the precipitate for 1 min with a ground glass Potter homogenizer in a 45 ml Sorvall centrifuge tube at 0°C. The extract was collected after centrifugation at 20 000 × *g* for 10 min at 2°C. The deep blue extract was fractionated with ammonium sulphate to give 2 fractions, 0–40% and 40–70% saturated. Only the latter contained enzyme activity. The 40–70% $(NH_4)_2SO_4$ precipitate was dissolved in 20 ml buffer B (20 mM KPO_4, pH 7.4, 0.1 mM EDTA, 0.2 mM $MgCl_2$, 2 mM mercaptoethanol) and dialyzed overnight (one change) against 2 l buffer Bg (buffer B with 10% glycerol). The enzyme solution was chromatographed on a 20 × 2.5 cm column of Whatman P11 phosphocellulose equilibrated against Bg buffer, using a linear 800 ml 0–0.5 M NaCl gradient in Bg buffer. Fractions, 80, 10 ml, were collected. An activity appearing 1/2-way through the gradient (*Acy*II) was not further investigated. A more pronounced peak of activity (*Acy*I) observed at 2/3-way through the gradient was pooled and dialyzed against 2 l of buffer Ag (buffer A with 10% glycerol). Subsequent chromatography on a 15 × 2 cm DEAE–Sephadex A-50 (Pharmacia) column (equilibrated against Ag buffer) with a 400 ml linear gradient from 0.05–0.40 M NaCl in Ag buffer yielded active fractions appearing 1/2-way through the gradient. (The column shrank considerably during the operation.) The enzyme was further purified on a 6 × 1 cm DE52 (Whatman) column after dilution with 2.5 vol. buffer Ag containing autoclaved gelatin at 25 μg/ml; a linear 150 ml 0.05–0.40 M NaCl gradient (Ag buffer) was used. The fractions of highest quality were concentrated by adsorption onto a 15 × 4 mm DE52 column and elution with 0.20 M NaCl in buffer Ag. These fractions of 1.5 ml were stored in an ice-salt bath at –8°C with 10^{-4} M NaN_3 for preservation.

2.5. *Determination of enzyme cleavage specificity*

Two approaches were used:

1. The DNA of phage λ was incubated with purified *Acy*I enzyme under conditions optimal for extensive cleavage. 5′-^{32}P-label was introduced using [γ-^{32}P]-ATP and polynucleotide kinase [5]. The labeled DNA was then treated with DNase, at 130 μg/ml at 37°C and 3 samples were withdrawn at 45 min intervals in order to isolate a suitable mixture of di-, tri- and oligonucleotides which were separated by two-dimensional homochromatography [4] and autoradiographed. An aliquot of the sample treated longest with DNase was also treated with snake venom exonuclease to yield the 5′-^{32}P-labeled mononucleotide present at the termini generated by *Acy*I enzyme. Its identity was established by paper electrophoresis in pyridine acetate, pH 3.5.
2. A DNA fragment of known sequence (*Hpa*I-E from adenovirus 5 DNA), 1574 nucleotides long [5] was cleft at 4 points with *Acy*I. These sites were localized with respect to known positions of various restriction enzyme sites [6] at approx. nucleotides 800, 1300 (2 ×) and 1450 (fig.2). The 5 resulting *Acy*I × *Hpa*I-E fragments were labeled at their 5′-termini with ^{32}P and cut once using

either *Taq*I or *Hin*fI endonuclease R [5]. These fragments were then isolated on 5% polyacrylamide gel and subjected to nucleotide sequence analysis as in [7]. The sequences were aligned with the complete *Hpa*I-E sequence, thus directly demonstrating the sequences at the cleavage sites. The plasmid pBR322 [8,14] was treated in an identical manner.

3. Results and discussion

The purified endodeoxyribonuclease from *A.cylindrica* (*Acy*I) made as in section 2 appears to be essentially free of exonuclease activity as revealed by prolonged incubation with ^{32}P-labeled pT8 or unlabeled λ DNA (no band widening). The 5′-termini of fragments produced by *Acy*I can be labeled with ^{32}P by polynucleotide kinase and [γ-^{32}P]ATP, after treatment with phosphomonoesterase. The presence of Mg^{2+} is sufficient for activity. The enzyme does not seem to cut SV40 DNA but cleaves λ DNA and adenovirus 2 DNA at a minimum of 14 sites (not determined exactly).

The first approach (see section 2) to establish the structure of the *Acy*I cleavage sites in λ DNA revealed that the 5′-terminal nucleotide is pC. In addition the following oligonucleotides were identified by their position on the chromatogram (fig.1): pCpG, pCpGpC, pCpGpT, pCpGpCpC and pCpGp(TpC) (spot 6). The latter was shown to be pCpGpTpC by partial digestion with snake venom exonuclease. The recognition site suggested by these data is the symmetrical hexanucleotide:

5′ G Pu ↓ C G Py C 3′

3′ C Py G C ↑ Pu G 5′

Firm proof for this tentative conclusion was obtained with the second approach using the known DNA sequence of the *Hpa*I-E fragment from adenovirus 5 (Ad5) DNA, schematically presented in fig.2. The double-stranded DNA sequences at the 4 *Acy*I cleavage sites are of two types, those at sites 1 and 2 (5′GGCGCC 3′) and those at sites 3 and 4 (5′ GACGCC 3′ = 5′ GGCGTC 3′).

It may also be noted that the *Hpa*I-E fragment contains, in addition to these 4 sites, 7 hexanucleotide sequences of the general form 5′ GNCGNC 3′. There was no evidence for cleavage at any of these 7 sites.

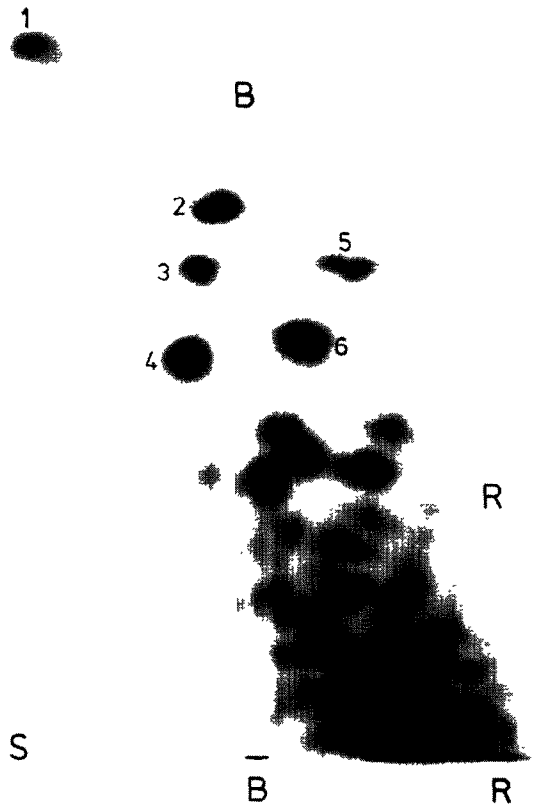

Fig.1. Autoradiograph of chromatogram [4] of ^{32}P-labeled oligonucleotides originating from the *Acy*I cleavage sequence: 1, pC, 2, pCpG; 3, pCpGpC; 4, pCpGpCpC; 5, pCpGpT, 6, pCpGpTpC. The figure suggests that the specificity is lost after the fourth nucleotide. Electrophoresis was from left to right.

Ad5 *Hpa*I-E does not contain the sequence 5′ GACGTC 3′. However, DNA of the plasmid pBR322 [8,14] contains this hexanucleotide sequence once, at nucleotides 4284–4290 (J. G. Sutcliffe, personal communication). We were able to show that the site is cleft by *Acy*I by sequencing to either side of the 5′ GACGTC 3′ site.

It is of interest to compare the recognition site of *Acy*I with those of other sequence-specific endonucleases. *Hae*II [9,10] from *Haemophilus aegyptius*

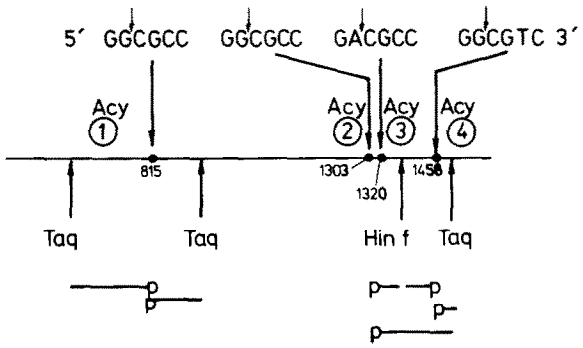

Fig.2. Elucidation of the structure of the *Acy*I recognition sites present on Ad5 *Hpa*I-E. After labeling of the 5′-termini generated by *Acy*I, DNA fragments (schematically displayed in the lower part of the figure) bearing only one labeled terminus were isolated following cleavage with *Taq*I endo R (in one instance *Hin*fI endo R) and gel electrophoretic separation. The sequences of these fragments were determined [7] and compared with that of the complete *Hpa*I-E fragment [5]. Cleavage site *Acy*I-2 was not approached experimentally in this way as its presence only 17 nucleotides away from site 3 was noticed at a late stage.

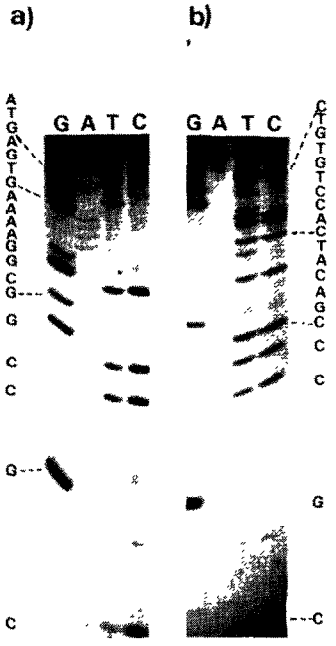

Fig.3. Sequences derived from cleavage of Ad5 *Hpa*I-E fragment [5] by *Acy*I. Fragments obtained as described in fig.2 were chemically cleaved according to [7]. The cleavage products were separated on a 25% polyacrylamide gel (24:1 acrylamide: *N,N′*-methylenediacrylamide, in 50 mM Tris–borate, pH 8.3/1 mM EDTA at 1000 V. The lanes are: G only, A > C, T + C and C only, respectively. (a) Sequence from site 1 reading from nucleotide 817 to nucleotide 798. (b) Sequence from site 3 reading from nucleotide 1321 to nucleotide 1340.

cleaves at a site similar to that of *Acy*I. However, a comparison between the *Acy*I and *Hae*II (or *Hind*III) sites:

5′ G Pu ↓ C G Py C 3′

3′ C Py G C ↑ Pu G 5′

(*Acy*I)

5′ Pu G C G C ↓ Py 3′

3′ Py ↑ C G C G Pu 5′

(*Hae*II)

reveals two differences. These enzymes cleave at different positions within their respective sequences (see arrows). In addition, the degeneracy in the *Hae*II site occurs at the bases in the first and sixth positions whereas that in the *Acy*I site occurs at the bases in the second and fifth positions.

We have investigated whether the first and sixth nucleotides of the *Acy*I recognition sequence could also be degenerate. Were this to be the case, *Acy*I would cleave at all *Hae*II sites. *Hae*II generates 11 fragments from plasmid pBR322 DNA [14]. The fragments obtained after *Acy*I digestion are precisely those predicted from cleavage at the six 5′ GPuCGPyC 3′ sites (positions 414, 435, 548, 1205, 3903 and 4285; J. G. Sutcliffe, personal communication). There was no evidence for cleavage at *Hae*II sites other than the four 5′ GGCGCC 3′ sites. In addition, SV40 DNA, which has one *Hae*II site of sequence 5′ AGCGCT 3′ and no 5′ GPuCGPyC 3′ sites [11,12], is not cleft by *Acy*I. Therefore, the first and sixth nucleotides of the *Acy*I recognition sequence are not degenerate.

Another endodeoxyribonuclease occasionally sharing a recognition site with *Acy*I is *Hha*I from *Haemophilus haemolyticus* [13] (5′ GCG ↓ C 3′). Whereas all *Hae*II sites are also sites for *Hha*I, only

those *Acy*I sites with the sequence 5′ GGCGCC 3′ are substrates for *Hha*I.

Thus endonuclease R.*Acy*I has a novel recognition sequence. The enzyme is, therefore a useful addition to the list of sequence-specific endodeoxyribonucleases.

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