A new sequence specific endonuclease *Esp*I, of cyanobacterial origin

F. Calléja, B.M.M. Dekker, T. Coursin* and A. de Waard

Department of Medical Biochemistry, Sylvius Laboratories, University of Leiden, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands, and **Unité de Physiologie Microbienne, C.N.R.S. ERA 398, Département de Biochemie et Génetique Moléculaire, Institut Pasteur, Rue du Dr. Roux, 75724 Paris Cedex 15, France*

Received 3 September 1984

The isolation of a new sequence-specific endonuclease from a unicellular cyanobacterium is described. This enzyme specifically cleaves the nucleotide sequence GC↓TNAGC.

Restriction enzyme *Nucleotide specificity*

1. INTRODUCTION

A screening programme of unicellular cyanobacteria (grown in pure culture) for identifying sequence-specific endonucleases with novel recognition properties is being carried out in our laboratories. We encountered a strain, PCC 6906 in the Pasteur Culture Collection, which appeared to contain an endonuclease showing a unique gel-electrophoretic banding pattern when incubated with bacteriophage λ DNA. This strain was named *Eucapsis species* by its discoverer J. West and classified as Synechocystis species 6906 by authors in [1]. We have studied the cleaving properties of endonuclease *Esp*I and report here on its nucleotide recognition pattern.

2. EXPERIMENTAL PROCEDURES

2.1. *Growth of the organism*

Medium MN contained 3 parts of autoclaved sea water and one part of distilled water. The following ingredients were added per litre: $MgSO_4 \cdot 7H_2O$, 38 mg; $CaCl_2 \cdot 2H_2O$, 18 mg; $NaNO_3$, 750 mg; Na_2CO_3, 20 mg; $K_2HPO_4 \cdot 3H_2O$, 20 mg; $Fe_2(SO_4)_3$, 8 mg (complexed with EDTA); H_3BO_3, 3 mg; $MnCl_2 \cdot 4H_2O$, 2 mg; $ZnSO_4 \cdot 7H_2O$, 200 μg; $Na_2MoO_4 \cdot 2H_2O$, 40 μg, $CuSO_4 \cdot 5H_2O$, 80 μg; $CoSO_4 \cdot 6H_2O$, 50 μg; vitamin B12, 100 μg. The medium was buffered with 30 mg/l Hepes buffer at pH 7.8 while sterile filtered air enriched with 5% CO_2 was blown through the culture. Illumination by fluorescent light was at 1000 lux.

Abbreviations: Ad, adenovirus; FPLC, fast protein liquid chromatography

2.2. *Enzyme purification*

Frozen cells (10 g) were disrupted in an Eaton press [2] in which they are forced under hydraulic pressure (6000 kg/cm^2) through a narrow hole. The broken cells were suspended in 100 ml of buffer A (10 mM Tris–HCl, pH 7.4; 2 mM mercaptoethanol) and subsequently sonicated (Branson sonifier) in a stainless-steel beaker immersed in melting ice for 4 1-min periods. The extract was centrifuged and processed as described [3]. Endonucleolytic activity was eluted from a phosphocellulose column at approximately 0.5 M NaCl (a 400 ml linear gradient from 0 to 0.7 NaCl in 20 mM potassium phosphate (pH 7.4)/2 mM mercaptoethanol/10% glycerol was applied). The *Esp*I activity, being the main endonuclease in the enzyme peak, was rid of two contaminating minor nucleases (which cleave pBR322 DNA) on

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

heparin–Sepharose. To that end fractions with the highest endonucleolytic activity towards bacteriophage λ DNA were dialyzed for 4 h against buffer A, also containing 10% glycerol and 100 mM KCl (the purified enzyme is rendered inactive if the latter is omitted). The heparin–Sepharose column (5 × 1.5 cm) was eluted using a 200 ml gradient of 0.10–0.50 M KCl in buffer A containing 10% glycerol. Endonuclease *Esp*I emerged at approximately 0.35 M KCl free from other nucleases. It could be concentrated on a small column of heparin–Sepharose or by fast protein liquid chromatography (Pharmacia FPLC system) using a Mono Q column (from which it elutes at 0.27 M KCl) and by ultrafiltration. The enzyme was stored at −8°C in an ice-salt bath.

2.3. *Determination of cleavage specificity*

This was done by nucleotide sequence analysis of fragments terminating at *Esp*I cleavage sites according to [4] and by the wandering spot technique [5], similar to the way described in [6].

3. RESULTS AND DISCUSSION

The purified enzyme degraded bacteriophage λ DNA into 7 fragments which gave a unique banding pattern in agarose gel electrophoresis (fig.3). The recognition sequence for endonuclease *Esp*I was shown to be GC↓TNAGC. This conclusion was arrived at by cleaving SV40 DNA and a number of cloned adenovirus DNA fragments of known sequence with *Esp*I, determining the terminal sequences (e.g., fig.1) and comparing these (table 1). The adenovirus sequences all had the sequence GCT$^{G}_{C}$AGC in common. However, the results with simian virus (SV)40 DNA make clear that the symbol N in the recognition site indeed must stand for any nucleotide and not only for the G/C pair. SV40 DNA was found to be cleaved once by *Esp*I. According to its published sequence [10], this DNA contains the sequence GCT$^{A}_{T}$AGC at positions 1710–1716. Fig.2 shows that the *Esp*I cleavage site coincides with this sequence. From the inferred recognition site the number and size of the fragments generated by *Esp*I in bacteriophage λ DNA can be predicted. The results presented in fig.3 completely confirm this prediction. The cleavage sites (which are all of the N = G/C type) at coordinates 10,298; 10,683; 11,662; 16,519;

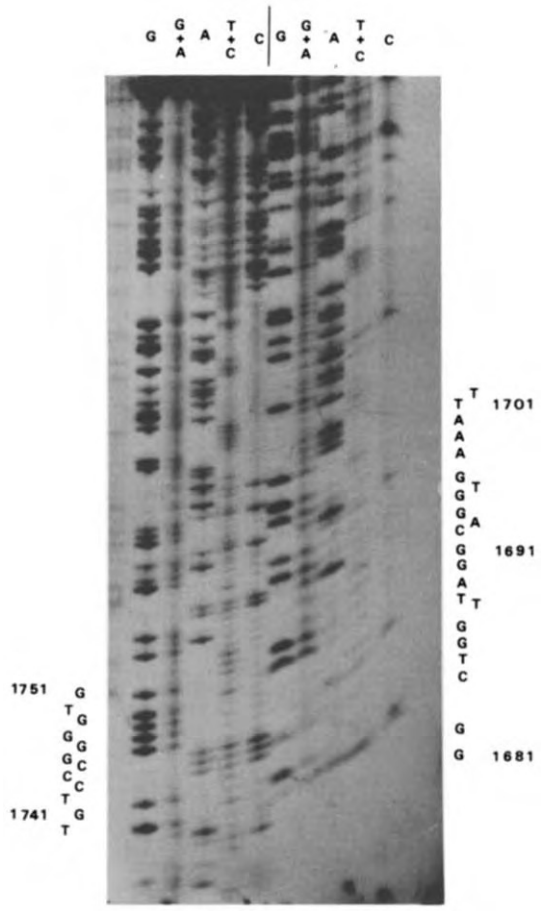
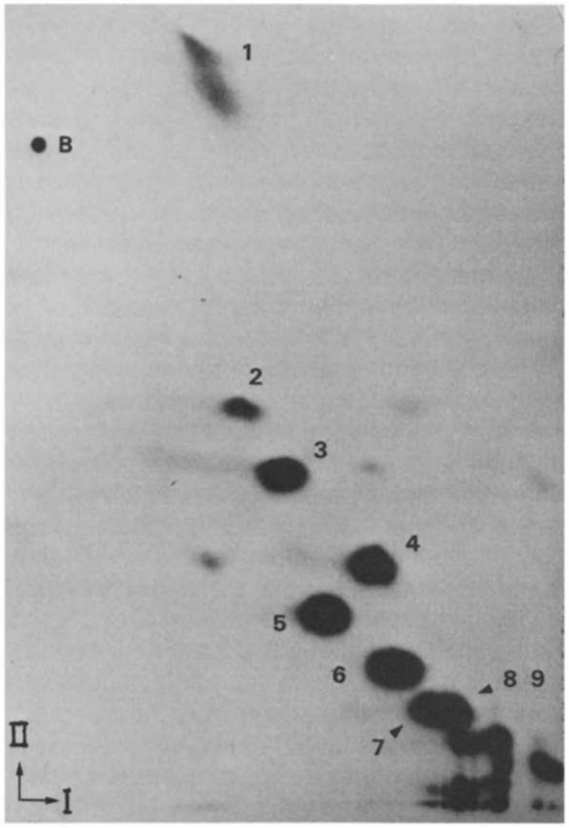


Fig.1. Nucleotide sequence of simian adenovirus 7P DNA flanking the cleavage site for endonuclease *Esp*I at nucleotide 1674. The sequence from nucleotide 1673–1690 reads: GC↓TCAGCCGGCTGGTTAG [8]. The site of cleavage is as indicated by the arrow (not shown; but see fig.2). The two electrophoretic runs shown cover well over 100 nucleotides. Base-specific cleavages are as indicated at the top of the 5 lanes of each run.

20,745; and 39,451 [11] are fully in line with those observations. Although in all cases studied, except one, the recognition sequence of *Esp*I turned out to be GC↓T$^{G}_{C}$AGC, we have concluded that the sequence is GC↓TNAGC because the single cleavage site for *Esp*I in SV40 DNA is GC↓T$^{A}_{T}$AGC [10]. One might argue that the SV40 used in our experiments could have a point mutation at nucleotide 1713, but fig.2 shows that it is A and thus invalidates that assumption. The *Esp*I endonuclease described here uniquely recognizes the

Table 1
Nucleotide sequences containing cleavage sites for endonuclease *Esp*I

DNA species	Sequences determined	Reference
Human Ad5	7637 GGGGAGCTGAGCCCGTG	7
Human Ad5	9739 GGTAGGCTGAGCACCGT	7
Simian Ad SA7P	1674 ACTCCGCTCAGCCGGCT	8
Human Ad12	3725 TAACTGCTCAGCTGGAA	9
SV40	1711 TAAAAGCTTAGCAGCTG	10



nucleotide sequence GC↓TNAGC, thus furnishing a new tool to the molecular biologist. This site constitutes an extended *Dde*I site and in that respect resembles the recognition sequence of *Mst*II [12] and an isoschizomer of it, *Aoc*I (from a poorly described strain of *Anabaena oscillarioides*, unpublished), CC↓TNAGG.

ACKNOWLEDGEMENTS

The authors are grateful to Dr H. van Ormondt for his valuable help in supplying data on nucleotide sequences, to Dr R. Bernards for cloned Ad12 DNA and to A.C. Neele for performing FPLC.

←

Fig.2. Nucleotide sequence analysis of the lower strand of simian virus (SV)40 DNA at its unique *Esp*I cleavage site. A 203-bp *Esp*I-*Hae*III fragment 5′-labeled at the *Esp*I site was degraded with pancreatic DNase and snake venom exonuclease. The resulting oligonucleotides were fractionated according to [5]. Spot 1 = pT (confirmed by paper electrophoresis); spot 8 = pT-A-A-G-C-T-T-T. This sequence coincides with positions 1714–1707 of SV40 DNA [10]. I = direction of electrophoresis; II = homochromatography; B = blue marker (xylene cyanol FF).

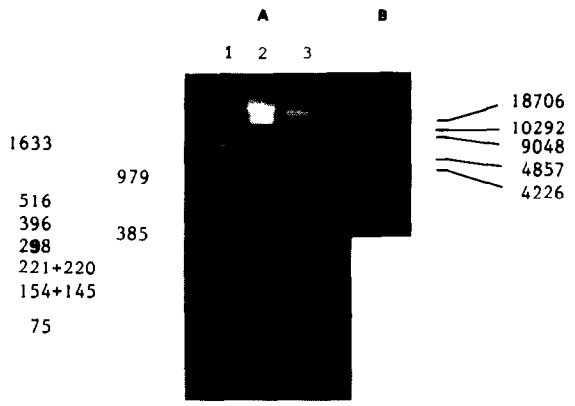


Fig.3. Agarose gel electrophoretic pattern of *Esp*I digest of bacteriophage λ DNA. A. Determination of the size of the smallest two fragments of 979 and 385 bp (lane 2) on a 1.5% gel. A calibration digest of plasmid pAT 153 (a derivative of pBR322) by endonuclease *Hin*fI is shown in lane 1. Lane 3 of the same gel shows the 5 remaining larger fragments which can be resolved on a 0.7% gel as shown in B, duplicate lanes. The size of those 5 fragments is given as number of base pairs. The two small fragments have run off this gel.

REFERENCES

[1] Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M. and Stanier, R. (1979) J. Gen. Microbiol. 111, 1–61.
[2] Eaton, N.R. (1962) J. Bacteriol. 83, 1359–1360.
[3] Duyvesteyn, M. and De Waard, A. (1980) FEBS Lett. 111, 423–426.
[4] Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. 65, 499–560.
[5] Tu, C.-P.D. and Wu, R. (1980) Methods Enzymol. 65, 620–638.
[6] Duyvesteyn, M.G.C., Korsuize, J. and De Waard, A. (1981) Plant Mol. Biol. 1, 75–79.
[7] Dekker, B.M.M. and Van Ormondt, H. (1984) Gene 27, 115–120.
[8] Dekker, B.M.M., Konings, D.A.M., Denisova, T.S., Gibadulin, R.A. and Van Ormondt, H. (1984) J. Gen. Virol. 65, in press.
[9] Bos, J.L., Polder, L.J., Bernards, R., Schrier, P.I., Van den Elsen, P.J., Van der Eb, A.J. and Van Ormondt, H. (1980) Cell 27, 121–131.
[10] Buchman, A.R., Burnett, L. and Berg, P. (1981) in: Molecular Biology of Tumor Viruses, 2nd edn, DNA Tumor Viruses (revised) pp.799–841, Cold Spring Harbor.
[11] Sanger, F., Coulson, A.R., Hong, G.F., Hill, D.F. and Petersen, G.B. (1982) J. Mol. Biol. 162, 729–773.
[12] New England Biolabs Catalog 1983/1984, p.16.