A new endonuclease recognizing the deoxynucleotide sequence C[^]CNNGG from the cyanobacterium *Synechocystis* 6701

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ABSTRACT

A new sequence-specific endonuclease from the cyanobacterium *Synechocystis* species PCC 6701 has been purified and characterized. This enzyme, SecI, is unique in recognizing the nucleotide sequence:

and cleaves it at the position indicated by the [^] symbol. Two other restriction endonucleases, SecII and SecIII, found in this organism are isoschizomers of MspI and MstII, respectively.

INTRODUCTION

The number of Type II restriction endonucleases found in cyanobacteria that recognize unique nucleotide sequences is growing steadily. *Synechocystis* 6701 is a unicellular cyanobacterium isolated by J. Hauxhurst and originally named Aphanocapsa (1). This strain is capable of synthesizing phycoerythrin, a red-colored phycobiliprotein and belongs to chromatic adapters of group II as described by N. Tandeau de Marsac (2). We report here the isolation and characterization of a new 6-base site-specific restriction endonuclease from this organism, as well as two isoschizomers of enzymes which have been previously found in other (cyano)-bacteria.

MATERIALS AND METHODS**Culture conditions.**

Synechocystis species PCC 6701 (ATCC 27170) cells were grown at room temperature in mineral medium BG11 (1), under illumination with cool-white fluorescent light at 1000 lux. Continuous agitation and CO₂ were provided by bubbling 5% CO₂/95% air through the culture.

Chemicals.

Cellulose phosphate P11 and chromatography paper 1 were obtained

from Whatman; hydroxylapatite, Bio-Gel HTP from Bio-Rad Laboratories and agarose Type II: medium EEO from Sigma. Cellulose acetate strips (30x550 mm) were purchased from Schleicher and Schull, and DEAE-Cellulose (MN 300 DEAE- MN 300 HR) thin layer plates (20x20cm) from Macherey-Nagel and Co. The homo-mixtures were prepared as described previously (3).

Enzymes and DNA molecules.

Bacteriophage lambda DNA, and restriction enzymes TaqI and HincII were obtained from Promega-Biotec, T4 polynucleotide kinase and DNA polymerase I (Klenow fragment) from Anglian Biotechnology Ltd, snake venom exonuclease and pancreatic DNase were from Boehringer Mannheim. Nucleoside triphosphates (α - or γ -labelled with ^{32}P ; 3000 Ci/mmol) were obtained from Amersham. Bacteriophage X174 RF DNA was a gift from Drs. P.D. Baas and A.D.M. van Mansfeld of the University of Utrecht.

Isolation and purification of SecI.

10 g of frozen cells were thawed at 30°C, and refrozen in liquid nitrogen; this procedure was repeated three times. The cells were then disrupted in a frozen Eaton press (4) with a pressure of 6000 kg/cm² suspended in 60 ml of buffer A (10 mM Tris-HCl, pH 7.4 at 4°C; 0.2 mM MgCl₂; 0.1 mM EDTA, 2 mM mercaptoethanol) and sonicated at full power for three 3-min periods in a Branson apparatus. The temperature was kept below 8°C by using a stainless-steel beaker immersed in melting ice. The extract was centrifuged and processed up to a dialysis step after precipitation of the proteins with ammonium sulfate as described (5).

The dialyzed solution was clarified by centrifugation, loaded onto a 16x2.5 cm column of phosphocellulose equilibrated with buffer B (20 mM KH₂PO₄, pH 7.4 at 4°C; 0.2 mM MgCl₂; 0.1 mM EDTA; 2 mM 2-mercaptoethanol) and washed in with the same buffer until the effluent becomes colourless. To elute the endonucleases, a 150 ml linear gradient of 0-0.25 M NaCl in buffer Bg (buffer B with 10% glycerol) was applied. This choice of gradient permitted us to eliminate in part two other sequence-specific endonucleases also present in this strain of Synechocystis, SecII and SecIII. Those fractions of SecI that eluted at 0.10-0.18 M NaCl and were least contaminated with SecII were purified further on a hydroxylapatite column (3x1.5 cm), using a linear 20 ml gradient of 20-500 mM potassium phosphate buffer Bg, pH 7.6 at 4°C. SecI emerged at 80 mM potassium phosphate completely free from other nucleases as judged by gel-electrophoretic patterns of incubations with Φ X174 RF DNA and SV40 DNA.

10 g of frozen cells yielded 400 U of SecI, one unit being the amount

of enzyme that cleaves 1 μ g X174 RF DNA per hour under optimal assay conditions, which are 10 mM Tris-HCl, pH 7.5 measured at 37°C; 10 mM $MgCl_2$; 10 mM NaCl; 1 mM dithiothreitol; 100 μ g/ml BSA at 37°C. Prolonged incubation (16 hours) caused no alteration of the electrophoretic pattern.

Determination of nucleotide recognition pattern.

Nucleotide sequences at the cleavage sites were determined using the chemical degradation method of Maxam and Gilbert (6) and the wandering spot technique (7). The substrate used was bacteriophage ϕ X174 RF DNA (8,9).

RESULTS AND DISCUSSION

Recognition sequence of *SecI*.

Fragments resulting from cleavage of X174 RF DNA with *SecI* were 3'-labelled with Klenow DNA polymerase I, $\alpha[^{32}P]$ -dATP and $\alpha[^{32}P]$ -dCTP,

Table I. Cleavage sites of *SecI* on ϕ X174 RF DNA

880
CTCGCCCTGGTCGT
1415
AGCGCCGTGGATGC
1434
CGTACCGAGGCTAA
2859
AATGCCGCGGATTG
4681
GCTGCCGAGGGTCG
5182
CTGTCCACGGAGTG

Fig.1. Size of fragments generated by incubation of ϕ X174 RF DNA and SV40 DNA with endonuclease SecI (left and middle column, respectively). The numbers in those two columns are predicted values assuming that SecI uniquely recognizes the sequence CCNNGG. Size markers (a Hinf digest of pAT153) are shown in the right column (m). There is complete agreement between the experimental values and those which follow from the above assumption.

then cleaved with TaqI. After electrophoresis on a 5% polyacrylamide gel, fragments of 159 bp, 173 bp, 245 bp, 290 bp and 501 bp were isolated and sequenced (6). By computer-aided analysis (10,11) we found that these fragments were all flanked by the common sequence 5'-CCNNGG-3', where N stands for any of the four possible nucleotides, see Table I. The frequency of cleavage of ϕ X174 RF and SV40 DNA by SecI and the size of the frag-

Fig.2. Nucleotide sequence analysis of a 269-bp SecI/HincII fragment (labeled at the 5' terminus with ^{32}P) of ϕX174 RF DNA. Spot 1: *pC (confirmed by paper electrophoresis); spot 2: *pCpG; spot 3: *pCpGpC, etcetera; spot 10: *pCpGpCpGpGpCpApTpTpT. This sequence coincides with position 2863-2854 of ϕX174 RF DNA, (8,9).

I: direction of electrophoresis; II: direction of homochromatography; B: Blue marker (xylene cyanol FF).

ments generated were determined on agarose gel with the help of markers (DNA fragments of known size), see Fig. 1. There is complete agreement between the experimental values and the predictions based on the assumption that the recognition sequence of SecI is CCNNGG.

Cleavage sites of SecI in recognition sequence.

Fragments of ϕX174 RF DNA cleaved by SecI were labelled at the 5'-end with $\gamma[^{32}\text{P}]\text{-ATP}$ using T4 polynucleotide kinase and cut with HincII. Two fragments of 269 bp and 131 bp were isolated and purified from polyacrylamide gel. Partial digestion of these fragments with pancreatic DNase/snake venom exonuclease and wandering spot analysis (7) of the 5'- ^{32}P oligonucleotides yielded the result depicted in Fig. 2.

The 5'- ^{32}P mononucleotide as determined by paper electrophoresis was in all cases deoxycytidylate.

Action of SecI on various sequences.

The degenerate nature of the recognition sequence of SecI allows the

Table II. Cleavage sites of SecI in various DNAs

Ad2	λ	SV40	pBR322	pAT153	X174	M13mp7
(13)	(14)	(15)	(16,17)	(18)	(8,9)	(19)
234	105	16	8	8	6	9

use of this enzyme to cut the sequences recognized by the following endonucleases: NcoI (C[^] CATGG), XmaI (C[^] CCGGG), AvrII (C[^] CTAGG), SacII (CCGC[^]GG), and StyI (C[^]CA^{TA}TGG) cited in (12).

For the convenience of the reader the number of cleavage sites of endonuclease SecI in various DNAs has been tabulated in Table II.

Other endonucleases present in Synechocystis PCC 6701: SecII and SecIII.

The peak fractions of SecII and SecIII after chromatography on phosphocellulose eluted at 0.29 M and 0.42 M NaCl, respectively. SecII covered a large part of the eluate thus contaminating the two other enzymes. SecII and SecIII were purified by Fast Protein Liquid Chromatography (FPLC system, Pharmacia) on a Mono Q column; SecII eluted at 0.22 M KCl while a large part of SecIII appeared in the pass-through. When SecII and SecIII were incubated with three different DNAs, they gave the same restriction patterns as the known endonucleases MspI and MstII, which recognize the nucleotide sequences CCGG and CCTNAGG, respectively. Thus all three endonucleases SecI, SecII and SecIII recognize nucleotide sequences of the type 5'-CC...GG-3'; the functional meaning of this fact remains unclear, although it could possibly be related to the very low G/C content (35.7%) encountered in that strain (20), implying that its DNA contains very few, if any, sites for these endonucleases.

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REFERENCES

1. Rippka, R., Deruelles, J., Waterbury, J.B., Herdman, M. and Stanier, R. (1979) J.Gen.Microbiol. 111, 1-61.
2. Tandeau de Marsac, N. (1977) J.Bact. 130, 82-91.
3. Ling, V. (1972) J.Mol.Biol. 64, 87-102.
4. Eaton, N.R. (1962) J. Bacteriol. 83, 1359-1360.
5. Duyvesteyn, M. and De Waard, A. (1980) FEBS Lett. 111, 423-426.

6. Maxam, A.M. and Gilbert, W. (1980) *Methods Enzymol.* 65, 499-560.
7. Tu, C.-P.D. and Wu, R. (1980) *Methods Enzymol.* 65, 620-638.
8. Sanger, F., Air, G.M., Barrell, B.G., Brown, N.L., Coulson, A.R., Fiddes, J.C., Hutchinson, C.A. III., Slocombe, P.M. and Smith, M. (1977) *Nature* 265, 687-695.
9. Sanger, F., Coulson, A.R., Friedman, T., Air, G.M., Barrell, B.G., Brown, N.L., Fiddes, J.C., Hutchinson, C.A. III., Slocombe, P.M. and Smith, M. (1978) *J. Mol.Biol.* 125, 225-246.
10. Staden, R. (1977) *Nucl.Acids Res.* 4, 4037-4051.
11. Staden, R. (1978) *Nucl.Acids Res.* 5, 1013-1015.
12. Kessler, C., Neumaier, P.S. and Wolf, W. (1985) *Gene* 33, 1-102.
13. From: EMBL DNA Data Bank, release 5, accession number J01917 (1985).
14. Sanger, F., Coulson, A.R., Hong, G.F., Hill, D.F. and Peterson, G.B. (1982) *J.Mol.Biol.* 162, 729-773.
15. Buchman, A.R., Burnett, L. and Berg, P. (1981) in: *Molecular Biology of Tumor Viruses (revised)* pp. 799-841, Cold Spring Harbor.
16. Sutcliffe, J.G. (1979) *Cold Spring Harbor Symp.Quant.Biol.* 43, 77-90.
17. Peden, K.W.C. (1983) *Gene* 22, 277-280.
18. Maniatis, T., Fritsch, E.F., Sambrook, J. (1982) in *Molecular cloning: A Laboratory Manual*, pp.4.
19. Messing, J., Crea, R. and Seeburg, P.H. (1981) *Nucl. Acids. Res.* 9, 309-321.
20. Herdman, M., Janvier, M., Waterbury, J.B., Rippka, R. and Stanier, R.Y. (1979) *J.Gen.Microbiol.* 111, 63-71.