A new site-specific endodeoxyribonuclease from *Citrobacter freundii*

A. Janulaitis, P. Stakenas, Yu.A. Berlin*

Institute of Applied Enzymology, 232028 Vilnius, Fermentu 8, Lithuanian SSR and **Shemyakin Institute of Bioorganic Chemistry, USSR Academy of Sciences, Moscow 117988, USSR*

Received 11 July 1983

*Cfr*10 I, a site-specific endonuclease from *Citrobacter freundii* strain RFL10, was isolated. It recognizes and cleaves the family of related sequences: 5'Pu↓CCGGPy to generate DNA fragments with 5' tetranucleotide extensions. *Cfr*10 I may be useful in molecular cloning experiments, especially in conjunction with other enzymes which generate the same terminal extensions.

Citrobacter freundii *site-specific endonuclease* *molecular cloning*

1. INTRODUCTION

Thirty strains of *Citrobacter freundii* were screened, and of these 16 were found to produce restriction endonucleases [1,2]. Both isoschizomers and enzymes recognizing new nucleotide sequences were found when studying the substrate specificity of some of these enzymes.

The characterization of a new site-specific endonuclease *Cfr*10 I recognizing a hexanucleotide sequence 5'Pu↓CCGGPy and cleaving, as indicated by the arrow, is reported.

2. MATERIALS AND METHODS

Citrobacter freundii strain RFL10 was cultivated as in [2]. Restriction enzymes *Eco*RI and *Msp*I were isolated in our laboratory. DNA of phages λc1857 s7, φX174, fd and plasmid pBR322 were a kind gift of K. Sasnauskas. DNA of PCS7 plasmid (modified pBR322) was provided by G.V. Shpakovsky. DNA polymerase I (Klenow fragment) was obtained from Boehringer (Mannheim), [α-^{32}P]dATP >2000 Ci/μm from Amersham (Bucks), dTTP from Calbiochem, Sephadex G-50 from Pharmacia Fine Chemicals, agarose from Bio Rad. All other reagents were analytical grade commercial products.

2.1. *Isolation of* Cfr*10 I*

*Cfr*10 I was partially purified until essentially free of contaminating nuclease activities by chromatography on phosphocellulose P11 (Whatman) and heparin-Sepharose (Pharmacia Fine Chemicals). Full details of the purification of *Cfr*10 I will be presented elsewhere. In two separate preparations the yield of *Cfr*10 I was 250 units/g of wet packed cells. The enzyme preparation containing 50% glycerol is stable for at least 6 months when stored at −20°C.

2.2. *Enzymatic reactions*

Endonuclease activity was assayed by adding 1–10 μl enzyme solution to 40 μl reaction mixture: 20 mM Tris–HCl (pH 8.5), 75 mM NaCl, 3 mM $MgSO_4$, 5 mM 2-mercaptoethanol, 0.02% Triton X-100, 2 μg DNA. Incubations were routinely performed at 37°C for 1 h and terminated by adding 20 μl of a solution of 60% sucrose, 60 mM EDTA and 0.025% bromphenol blue. Restriction fragments were separated by electrophoresis in 1% agarose as in [3].

2.3. *Determination of enzyme specificity*

*Cfr*10 I recognition sequence was deduced on the basis of the tables given in [4] and the results of restriction enzyme digests of some DNAs with known sequence.

Published by Elsevier Science Publishers B.V.
00145793/83/$ 3.00

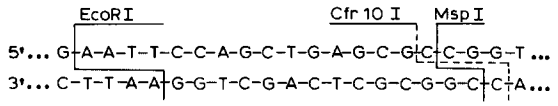

Fig.1. Independently determined DNA sequence of PCS7 plasmid. A continuous line indicates cleavage sites of *Eco*RI and *Msp*I and a dashed line indicates cleavage site of *Cfr*10 I. See fig.2 for the determination of the *Cfr*10 I cleavage site.

PCS7 plasmid was used to determine the cleavage site. Cleavage of DNA by *Eco*RI was carried out in buffer solution (20 mM Tris–HCl, pH 7.8, 10 mM $MgCl_2$, 50 mM NaCl, 2 mM DTT) at 37°C. Protruding DNA ends were repaired using DNA polymerase I, an equimolar quantity of [α-^{32}P]dATP and a 5-fold quantity of dTTP. Incubations were performed for 15 min at 20°C. The DNA was freed from precursors by gel filtration and cleaved with *Cfr*10 I and *Msp*I. Resulting *Eco*RI–*Cfr*10 I and *Eco*RI–*Msp*I fragments were subjected to electrophoresis in 20% polyacrylamide gel (acrylamide:methylene-bisacrylamide 30:1, 50 mM, Tris–borate, pH 8.3, 1 mM EDTA, 7 M urea). *Eco*RI–*Msp*I and *Eco*RI–*Cfr*10 I fragments were extracted and sequenced after base-specific chemical DNA cleavage [5].

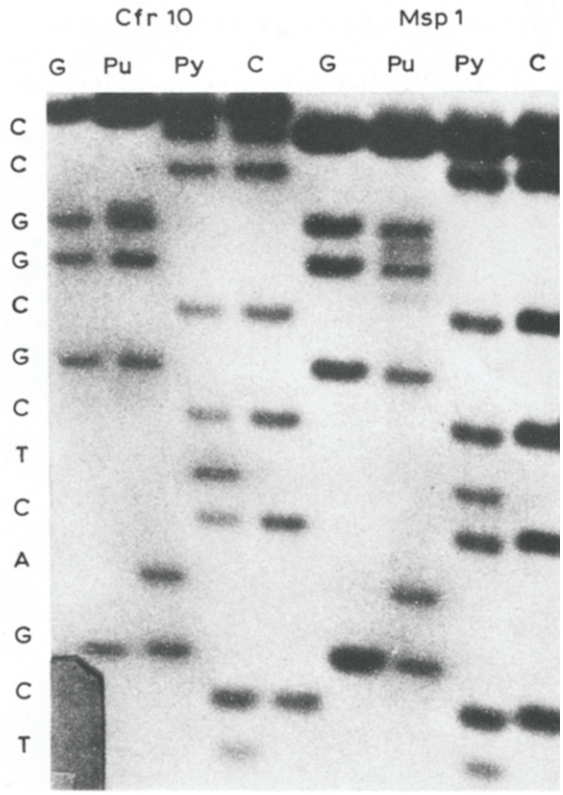

Fig.2. Autoradiograph of sequencing gel. Determination of the *Cfr*10 I cleavage site by sequencing of *Eco*RI–*Cfr*10 I and *Eco*RI–*Msp*I fragments.

3. RESULTS AND DISCUSSION

Digestion of various DNAs of known nucleotide sequence with *Cfr*10 I yielded not less than 6 fragments in pBR322 and no fragments in ϕX174 and fd. Comparison of these data with the tables given in [4] allowed us to predict that *Cfr*10 I recognizes the nucleotide sequence 5'PuCCGGPy.

To confirm the recognition site structure of *Cfr*10 I and determine its cleavage point we used PCS7 plasmid. The *Cfr*10 I site GCCGGT in this plasmid is located near the *Eco*RI site (fig.1). *Eco*RI–*Cfr*10 I and *Eco*RI–*Msp*I fragments of PCS7 plasmid ^{32}P-labeled at 3'-ends and isolated as described in section 2 were analyzed by the Maxam-Gilbert method (fig.2). As can be seen, the *Eco*RI–*Cfr*10 I fragment is longer at its 5'-end by one C residue as compared to the *Eco*RI–*Msp*I fragment. It can be concluded that *Cfr*10 I cleaves the 5'Pu↓CCGGPy sequence between Pu and C residues. This means that *Cfr*10 I fragments possess a 5'-tetranucleotide extension. As expected, *Cfr*10 I can be easily ligated with T4 DNA ligase (not shown).

As far as we know no other specific endodeoxyribonuclease has been described with the specificity of *Cfr*10 I and this constitutes a new addition to the collection of restriction enzymes. *Cfr*10 I contains cohesive termini identical to those of *Xma*I fragments [6]. Therefore, it should be possible to form recombinants in vitro between *Xma*I vectors and *Cfr*10 I fragments, which contain a hybrid junction resistant to both *Xma*I and *Cfr*10 I. It should be noted that the central tetranucleotide (CCGG) of the *Cfr*10 I recognition sequence is identical to the recognition sequence of *Msp*I. *Cfr*10 I should cleave DNA at all *Nae*I (GCCGGC) sites.

ACKNOWLEDGEMENTS

We wish to thank Dr K. Sasnauskas and Dr G.

Shpakovsky for their kind gifts of DNA and Dr R. Marcišauskas for T4 polynucleotide kinase. The authors thank R. Lukavičiuté for her help in typing and translating the text.

REFERENCES

[1] Janulaitis, A.A., Stakénas, P.S., Jaskelevičiené, B.P., Lebedenko, E.M. and Berlin, Yu.A. (1980) Bioorg. Khim. 6, 1746–1747.

[2] Janulaitis, A.A., Stakénas, P.S., Bitinaité, J.B. and Jaskelevičiené, B.P. (1983) Dokl. Akad. Nauk. SSSR, in press.

[3] Janulaitis, A.A., Stakénas, P.S., Lebedenko, E.M. and Berlin, Yu.A. (1982) Nucleic Acids Res. 10, 6521–6530.

[4] Fuchs, C., Rosenvold, E.C., Honigman, A. and Szybalski, W. (1980) Gene 10, 357–370.

[5] Maxam, A.M. and Gilbert, W. (1977) Proc. Natl. Acad. Sci. USA 74, 560–564.

[6] Endow, S.A. and Roberts, R.J. (1977) J. Mol. Biol. 112, 521–529.