Characterization of restriction-modification enzymes Cfr13 I from *Citrobacter freundii* RFL13

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Received 2 January 1985

This communicatiopn describes some properties of RCfr13 I and MCfr13 I, isolated from *Citrobacter freundii* RFL13. RCFfr13 I restriction enzyme recognizes the 5′-G↓GNCC sequence and cleaves, as indicated by the arrow. MCfr13 I methylase modifies the internal cytosine producing m^5C (5′-GGNm^5CC). RCfr13 I is sensitive not only to this type of substrate modification but also to hemimethylation in overlapping sites by MCfr10 I (internal cytosine of RCfr13 I recognition is methylated) and MHpa II (external cytosine is methylated). From these results the sensitivity of RCfr13 I to methylation by dcm methylase of *E.coli* in overlapping sites is deduced.

Site-specific endonuclease *DNA methylation* *Modification methylase*

1. INTRODUCTION

Out of 30 strains of *Citrobacter freundii* screened for site-specific endonucleases 16 were found to produce these enzymes [1–4]. Both isoschizomers and enzymes recognizing new nucleotide sequences were found when studying the substrate specificity of some of them. The characterization of restriction endonuclease RCfr13 I, an isoschizomer of RSau96 I [5], and of accompanying modification methylase MCfr13 I, is reported.

2. MATERIALS AND METHODS

MHpa II and MCfr10 I methylases were isolated in our laboratory. Spleen phosphodiesterase (SPDE) was obtained from P-L Biochemicals. dC and dpC were purchased from Serva, and dCpC was synthesized in our laboratory by the modified phosphotriester method [6]. DNA substrates, enzymes and other reagents used were as in [7,8].

C. freundii RFL13 strain, which was used as a source for specific endonuclease and methylase preparative isolation, was grown at 37°C as described in [1].

10 g of frozen cells were suspended in 20 ml buffer A (10 mM potassium phosphate, pH 7.5; 1 mM EDTA, 7 mM 2-mercaptoethanol), disrupted by sonication and centrifuged for 60 min at 48000 × *g*. The supernatant was applied on a phosphocellulose column (1.5 × 13 cm) and chromatographed with a 200 ml linear gradient of KCl (0–0.6 M) in buffer A. The fractions, which elute at 0.36–0.44 M KCl, contain both endonuclease and methylase activities. They were pooled and dialysed against buffer A. The enzyme solution was chromatographed on a 1.5 × 13 cm column of DEAE-cellulose using a 200 ml linear 0–0.3 M KCl gradient in buffer A. The fractions with RCfr13 I and MCfr13 I activities, eluted at 0.08–0.12 M, were then dialysed against buffer A. Further purification of enzymes was carried out by heparin-Sepharose column (1 × 10 cm) chromatography. The column was developed with a 100 ml KCl linear gradient (0–0.5 M). Endonuclease RCfr13 I eluted at 0.24–0.28 M KCl and methylase MCfr13 I at 0.33–0.38 M KCl. The active fractions were pooled and concentrated by dialysis against buffer A, containing 100 mM KCl

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Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

and 50% glycerol (v/v), and stored at −20°C.

To determine RCfr13 I activity and the influence of different factors (i.e., pH, cofactor requirement, concentration of NaCl) an aliquot of enzyme solution was added to 40 μl of appropriate reaction mixture containing 2 μg phage λDNA. Incubations were performed at 37°C for 1 h. Restriction fragments were separated by electrophoresis as in [7].

Methylase MCfr13 I activity was assayed in 25 mM Tris-HCl buffer (pH 8.0), 25 mM NaCl, 1 mM EDTA and 5 mM 2-mercaptoethanol as described in [4].

To determine RCfr13 I cleavage specificity, analysis of restriction fragment end structure was carried out as in [7].

Methylation of DNA with MCfr13 I, isolation of modified DNA, acid hydrolysis to bases and chromatographic identification of the radioactive base was performed as in [4,8]. For the determination of the position of the methylated base in the recognition sequence [*methyl*-^{3}H]DNA was subjected to depurinization according to Burton [9]. The resulting oligopyrimidines were dephosphorylated with alkaline phosphatase. dCpC, the radioactively labeled dinucleotide monophosphate, was then isolated by high-voltage electrophoresis in 0.05 M formate buffer (pH 2.7) on Whatman 3 MM. The position of the methylated base in dinucleotide dCpC was established after hydrolysis with snake venom phosphodiesterase (VPDE) and SPDE as in [10]. Electrophoretic separation of resulting products was performed in a mixture with standards dC, dCpC and pdC on Whatman 3 MM in 0.03 M triethylammonium bicarbonate buffer (pH 8.5).

3. RESULTS AND DISCUSSION

The wide use of sequence-specific endodeoxyribonucleases (type II restriction endonucleases) as analytical reagents in DNA research and genetic engineering has stimulated their extensive investigation. To date, a great number of site-specific endonucleases have been isolated [11]. For many practical purposes it is the recognition sequence which is the fundamental property that needs to be known about a restriction endonuclease. The characterization of other substrate specificity manifestations (i.e., cleavage site, sensitivity to the specific modification of substrate, catalytic properties) is also desirable.

3.1. *Optimal conditions for RCfr13 I activity*

The restriction endonuclease RCfr13 I is strictly dependent on Mg^{2+}, but does not require *S*-adenosylmethionine or ATP for its activity. Bearing this in mind as well as the dyad symmetry of the recognition site, RCfr13 I was classified as a type II restriction endonuclease. The activity of the enzyme is maximal at 5 mM Mg^{2+}. RCfr13 I is active in a wide pH range,from 7.5 to 9.0. The maximal activity is observed in 10 mM Tris-HCl buffer, pH 9.0 (pH of buffer solutions was measured at 25°C). At pH 9.0 concentrations of NaCl up to 50 mM stimulate and at greater concentrations inhibit enzyme activity. The addition of serum albumin (100 μg/ml) and Triton X-100 (0.02%) to the reaction mixture slightly activates (or stabilizes) the enzyme. When the enzyme activity was measured under the optimal conditions found (10 mM Tris-HCl buffer, pH 9.0, 50 mM NaCl, 5 mM $MgCl_2$, 100 μg/ml albumin and 2 μg phage λ DNA/40 μl reaction mixture), the yield of purified enzyme equalled 20000 units/g wet packed cells. One unit is the amount required to digest completely 1 μg phage λ DNA in 1 h at 37°C. The enzyme preparation is essentially free of non-specific nucleases. DNA fragments obtained with a 10-fold excess of RCfr13 I for 16 h are efficiently ligated with T4-ligase DNA and then completely recut with the restriction enzyme. The storage of the purified enzyme in 10 mM potassium phosphate buffer (pH 7.5), 100 mM KCl, 1 mM EDTA, 7 mM 2-mercaptoethanol, 200 μg/ml albumin and 50% glycerol at −20°C did not result in any decrease of the initial activity for at least 6 months.

3.2. *Determination of RCfr13 I substrate specificity*

Simultaneous cleavage of phage λ DNA with RCfr13 I and RSau96I [4] proved RCfr13 I to be an isoschizomer of Sau96 I, thus recognizing the 5′-GGNCC nucleotide sequence [4,5]. To locate the cleavage site in the above recognition sequence, restriction fragments obtained by cleaving pBR322 DNA with RCfr13 I and subsequently labelled at their 5′-ends with [^{32}P]phosphate were subjected to hydrolysis with pancreatic DNase and VPDE

and then analyzed. After exhaustive digestion approx. 98% of radioactivity input was found in the pdG spot after 5′-^{32}P-labelled mononucleotide electrophoretic separation on Whatman 1 paper. The oligonucleotides derived from partial hydrolysis were analyzed by two-dimensional homochromatography [12]. From the pattern obtained a 5′-terminal nucleotide sequence 5′-GNCC could be deduced (fig.1). This establishes the RCfr13 I cleavage site and confirms the 5′-G↓GNCC recognition sequence in which the central position can be occupied by any of the 4 nucleotides. Both manifestations of RCfr13 I specificity are identical to those of endonucleases RSau96 I [5] and RAsu I [13].

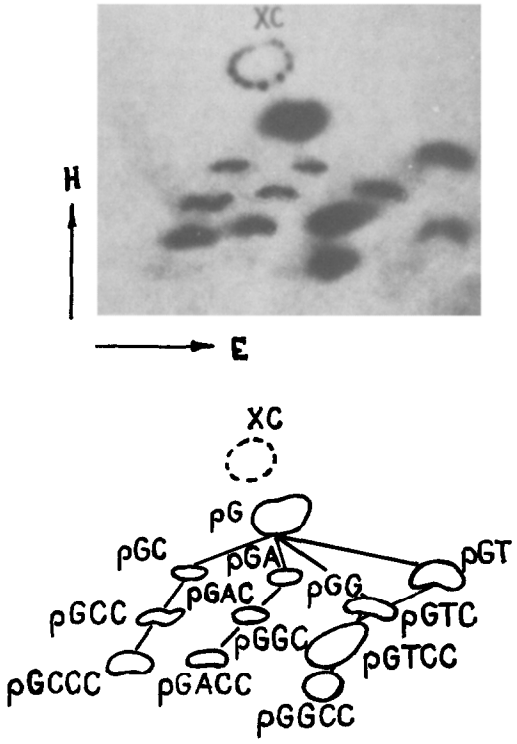

Fig.1. Autoradiogram and schematic representation of the two-dimensional fractionation of oligonucleotides obtained after partial pancreatic DNase and snake venom phosphodiesterase digestion of pBR322 DNA 5′-terminally labeled fragments produced by RCfr13 I. E, electrophoresis on cellulose acetate strip in pyridine acetate at pH 3.5; H, homochromatography on a DEAE-cellulose thin-layer plate in homomixture VI [12]. XC, xylene cyanol FF.

3.3. *Determination of MCfr13 I specificity*

Substantially less attention as compared to restriction endonucleases has been paid to the study of accompanying modification methylases [11,14]. Determination of their substrate specificity is an important characteristic per se, and a prerequisite for the investigation of cognate restriction endonuclease specificity in regard to substrate modification. It was demonstrated that DNA, preincubated with MCfr13 I in the presence of SAM, is resistant to subsequent RCfr13 I action (fig.2). The absence of cleavage of modified substrate should not be treated as an experimental artefact, since methylated DNA served as a substrate for another enzyme (Sau3A I) (track 6) and, furthermore, unmodified DNA was sensitive to RCfr13 I cleavage in a mixture of modified and unmodified substrate (track 5). These results allow us to assume that MCfr13 I methylase recognizes either the whole RCfr13 I recognition sequence or part of it. The identity of RCfr13 I and MCfr13 I recognition sequence was proved in experiments showing that pBR322 DNA, predigested with RCfr13 I, did not serve as a substrate for MCfr13 I (no radioactivity incorporation in the presence of [^{3}H]SAM and MCfr13 I was observed as compared to that of untreated substrate). Thus, we may conclude that MCfr13 I, like RCfr13 I,

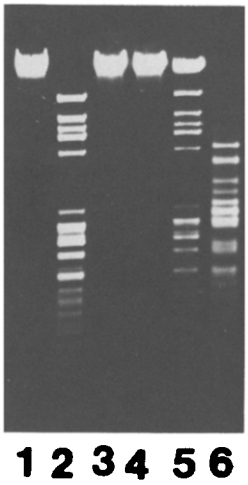

Fig.2. Protection of λ DNA methylated with MCfr13 I from RCfr13 I action. (1) DNA; (2) DNA + RCfr13 I; (3) DNA modified with MCfr13 I; (4) modified DNA + RCfr13 I; (5) unmodified DNA + modified DNA + RCfr13 I; (6) modified DNA + RSau3A I.

recognizes the 5′-GGNCC nucleotide sequence.

To identify the nature of the modified base, chromatographic systems discriminating m^4C and m^5C (to be published) were used for the separation of [^{3}H]DNA (methylated with MCfr13 I and [^{3}H]SAM) acid hydrolysis products. From the data obtained it can be concluded that the base under investigation is 5-methylcytosine (table 1). The position of the modified base was established following the analysis of [^{3}H]methylated dinucleotide dCpC, isolated from [*methyl*-^{3}H]-DNA. This dinucleotide was not the only ^{3}H-labeled oligopyrimidine product resulting from the depurinization reaction. It was separated from other pyrimidine oligonucleotides by electrophoresis on Whatman 3 MM (pH 2.7) and digested separately by VPDE and SPDE. Analysis of the resulting nucleoside and mononucleotide products for ^{3}H radioactivity allows unequivocal assignment of a structure for the dinucleotide. The results given in table 2 enable us to conclude that the internal cytosine of the recognition sequence is the acceptor of the methyl group, which is transferred by methylase MCfr13 I as follows: 5′-GGNm^5CC.

From the results it could be inferred that internal cytosine modification in the recognition sequence renders the substrate non-susceptible to accompanying RCfr13 I endonuclease action. It was determined that sequence specific endonucleases differ in their sensitivity to the modification pattern within its recognition sequence. There are examples of enzymes specifically inhibited by modifications anywhere within the recognition sequence as well as those which are insensitive to methylation at non-cognate sites [14]. Some additional experiments were undertaken to characterize RCfr13 I from this point of view. There are some sites in DNA where the Cfr13 I sequence overlaps with that of 5′-Cm^5CGG [15], methylated by MHpa II and 5′-Pum^{5}CCGGPy, methylated by MCfr10 I (unpublished). Both methylases yield 5-methylcytosine. The sequence of RCfr13 I should be methylated in overlapping sites to give 5-methylcytosine as shown in fig.3. These methylations appear to prevent RCfr13 I cleavage (fig.4). The results with MCfr10 I prove that hemimethylation in the cognate site of the recognition sequence is sufficient for substrate protection from RCfr13 I action (fig.4A). This is also true

Table 2

Localization of ^{3}H-labeled methyl group in d(CpC) by phosphodiesterase digestions

No.	Phosphodiesterase used	Distribution of radioactivity, between (%)			Type of modification
		dC	d(CpC)	pdC	
1.	–	4	96	–	–
2.	VPDE	94	6	–	m CpC
3.	SPDE	6	8	86	m CpC

Table 1

Chromatographic and electrophoretic identification of [^{3}H]methylated base

No.	Separation method	Mobility of bases, R_f			
		[^{3}H]methylated base	Standards		
			m^4C	m^5C	m^6A
1.	TLC on silica gel: acetonitrile-ethyl acetate-triethylamine-ethanol-water (15:5:5:5:1)	0.21	0.37	0.21	0.55
2.	Descending paper chromatography: butanol-water-ammonia (84:16:0.2)	0.29	0.41	0.29	0.53
3.	Paper electrophoresis[a] 0.08 M ammonium formate	0.97	0.82	0.97	–

[a] Represented R_c-values, relative mobility of the base to the cytosine

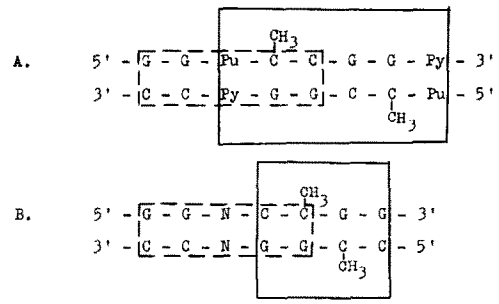

Fig.3. Overlapping sequences recognized by RCfr13 I and MCfr10 I (A) and by RCfr13 I and MHpa II (B). Recognition sequence of RCfr13 I boxed as (---). Recognition sequences of methylase MCfr10 I (A) or MHpa II (B) boxed as (——).

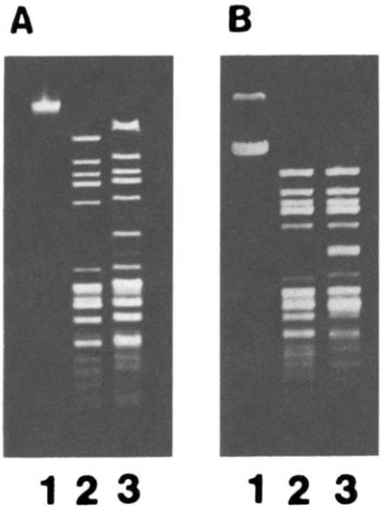

Fig.4. Electrophoretic comparison of cleavage patterns of RCfr13 I on unmodified and modified λ DNA. (A) DNA modified with MCfr10 I; (B) DNA modified with MHpa II; (1) DNA; (2) unmodified DNA + RCfr13 I; (3) modified DNA + RCfr13 I.

when external cytosine of the recognition sequence is methylated by MHpa II (fig.4B). Due to overlapping the external cytosine in the RCfr13 I recognition sequence should be methylated by dcm methylase of *E. coli*, which recognizes 5′-Cm^{5}C(A/T)GG [16]. From the data it can be concluded that this type of methylation should also interfere with substrate cleavage by RCfr13 I.

ACKNOWLEDGEMENTS

The authors are indebted to Drs P. Stakėnas and S. Puntežis for their kind gifts of MCfr10 I and MHpa II methylases, respectively, Dr K. Sasnauskas for pBR322 DNA and Dr R. Marcišauskas for polynucleotide kinase. The authors also thank R. Lukavičiute for her help in translating and typing the text.

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