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A new site-specific endonuclease, *Sca*I, from *Streptomyces caespitosus*

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A new site-specific endonuclease has been isolated from *Streptomyces caespitosus* and named *Sca*I. Based on analysis of sequences around the restriction sites in pBR322 and pBR325, the recognition sequence of *Sca*I endonuclease was deduced to be a new hexanucleotide 5′-AGTACT-3′. The cleavage site was determined by comparing the *Sca*I-cleaved product of a primer-extended M13mp18-SCA DNA, which contains an AGTACT sequence, with dideoxy chain terminator ladders of the same DNA. *Sca*I was found to cleave the recognition sequence between the internal T and A, leaving flush ends to the cleaved fragments.

INTRODUCTION

A number of restriction endonucleases have been isolated from many species of procaryotes and their specificities documented (Roberts, 1985). However, the presently isolated restriction endonucleases are still only a portion of those in existence. The finding of new restriction endonucleases is still required for the study of gene structure and recombinant DNA techniques.

We have screened for site-specific endonucleases in *Streptomyces* strains and found that different *Streptomyces* strains produce endonucleases with different specificities (Takahashi *et al.*, 1979; Shimotsu *et al.*, 1980). This report describes purification and characterization of a new site-specific endonuclease from *Streptomyces caespitosus*, named *Sca*I.

MATERIALS AND METHODS

Bacterial strain and medium

Streptomyces caespitosus KCC-S-0438 was provided by Dr. A. Seino (Kaken Chemical Co., Tokyo, Japan). Cells were grown at 28 °C for 2 days with rotary shaking in a medium containing Nutrient broth (Difco), 10 g; yeast extract (Difco), 1 g; NaCl, 5 g; glucose, 2.5 g/l (pH 7.2), and were harvested by centrifugation.

DNA preparations

Phage DNAs, $\lambda CI_{857}Sam_7$, ϕX174 RFI and T4dC were prepared as described in previous papers (Takahashi *et al.*, 1979; Shimotsu *et al.*, 1980). pBR322 and pBR325 DNAs were purified by CsCl/ethidium bromide equilibrium centrifugation (Oka, 1978). Adenovirus 2 DNA and SV40 DNA were purchased from Bethesda Research Laboratories (Rockville, MD, U.S.A.). M13mp18 RF and single-stranded DNA were prepared from M13mp18 phage infected cells and the purified phage particles, respectively (Yanish-Perron *et al.*, 1985). Synthetic nucleotide linker (5′-AAGTACTT-3′) was provided by Takara Shuzo Co. (Kyoto, Japan). A universal primer (3′-TGACCGGCAGCAAAATG-5′) for the dideoxy chain-terminator method was provided by Dr. M. Kumagai (Yamasa Shoyu Co., Chosi, Japan).

Enzymes

Restriction endonucleases *Alu*I, *Hae*III, *Hinc*II, *Hinf*I, *Hpa*II, *Hind*III, *Eco*RI, *Pst*I and *Rsa*I were purchased from Takara Shuzo Co. T4 polynucleotide kinase, T4 DNA ligase and Klenow fragment were purchased either from Takara Shuzo Co. or from Amersham (Japan) (Tokyo, Japan).

Enzyme assay

In a typical assay, 1–5 μl aliquots of column fractions were incubated in 20 μl of reaction mixture containing 0.5 μg of $\lambda CI_{857}Sam_7$ DNA, 10 mM-Tris/HCl, pH 7.5, 100 mM-NaCl, 8 mM-$MgCl_2$ and 10 mM-2-mercaptoethanol at 37 °C for 1 h. Reactions were terminated by the addition of 2 μl of 0.2 M-EDTA and heated at 65 °C for 5 min followed by rapid cooling. The cleavage products were analysed by electrophoresis on a horizontal agarose gel in 36 mM-Tris/32 mM-NaH_2PO_4/1 mM-EDTA, pH 7.8 as described in previous papers (Takahashi *et al.*, 1978; Takahashi & Saito, 1982). For separation of short DNA fragments, acrylamide-gel electrophoresis was used.

Purification of *Sca*I endonuclease

Procedures for purification of *Sca*I were essentially the same as the methods described previously (Takahashi *et al.*, 1979; Shimotsu *et al.*, 1980). Briefly, they were as follows. Cells were disrupted by sonication and, after high speed centrifugation, the supernatant was treated with streptomycin sulphate to a final concn. of 2% to remove nucleic acids. After removal of the precipitate, the supernatant was fractionated by $(NH_4)_2SO_4$ and the 40–80% fraction, where most of the site-specific endonuclease activity was found, was dissolved in a minimal volume of PC buffer [10 mM-potassium phosphate (pH 7.4)/10 mM-2-mercaptoethanol/0.1 mM-EDTA/10% (v/v) glycerol] and dialysed against the same buffer. The solution was applied to a DEAE-cellulose (DE52, Whatman) column and developed with a linear gradient of 0–1.0 M-KCl in PC buffer. Active fractions, appearing at around 0.25 M-KCl, were pooled and dialysed against DE buffer [10 mM-Tris/HCl (pH 7.4)/10 mM-2-mercaptoethanol/0.1 mM-EDTA/10% (v/v)

Abbreviations used: bp, base pair; kb, kilobase; RF, replicative form; T4dC, cytosine-substituted T4 phage.

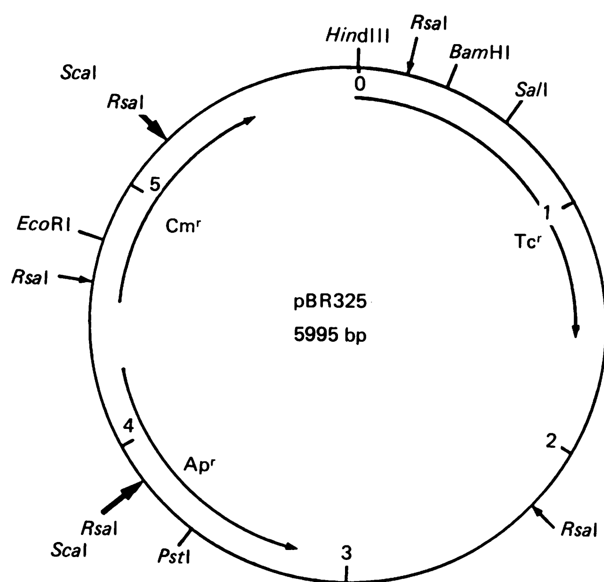

Fig. 1. Restriction map of pBR325

Among five *Rsa*I sites on pBR325, two sites coincide with *Sca*I sites, shown by thick arrows. Three drug-resistant genes are taken from Prentki *et al.* (1981).

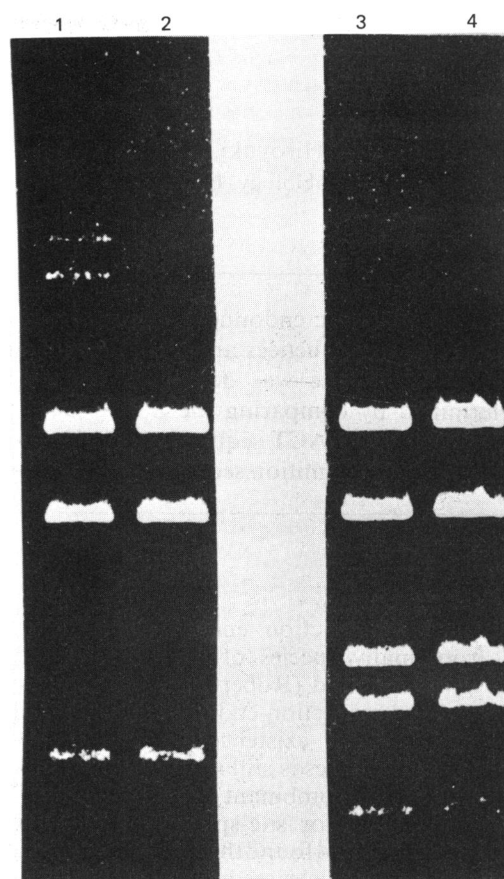

Fig. 2. Agarose gel patterns of pBR322 and pBR325 with *Rsa*I single- and *Rsa*I–*Sca*I double-digestion products

Lanes 1 and 3, *Rsa*I-digested pBR322 and pBR325, respectively; lanes 2 and 4, *Rsa*I–*Sca*I double digestion of pBR322 and pBR325, respectively.

glycerol]. Then the solution was loaded onto a DEAE-Sephacel (Pharmacia) column, which had been equilibrated with DE buffer, and eluted with a linear gradient of 0–1.0 M-KCl in DE buffer. *Sca*I activity was eluted at between 0.15 and 0.25 M-KCl. Active fractions were combined and dialysed against PC buffer and applied to a heparin–Sepharose CL-6B (Pharmacia) column, previously equilibrated with PC buffer. The column was developed with a linear gradient of potassium phosphate (0.01–0.50 M) in PC buffer. The active fractions, eluted at around 0.3 M-potassium phosphate, were pooled and concentrated by dialysis against a storage buffer [50 mM-Tris/HCl (pH 7.6)/0.1 M-KCl/0.1 mM-EDTA/1 mM-NaN_3/10 mM-2-mercaptoethanol/50% (v/v) glycerol].

Determination of the *Sca*I cleavage site

A M13mp18 derivative (M13mp18-SCA) containing an AGTACT palindromic sequence was constructed by inserting a synthetic nucleotide 5′-AAGTACTT-3′ into the unique *Hin*cII (*Sal*I) site of M13mp18. Single-stranded DNA prepared from purified phage particles was annealed with the universal primer and labelled with [α-^{32}P]dATP in the presence of the other three dNTP and Klenow fragment. The primer-extended DNA was cleaved with *Sca*I, analysed on an 8% acrylamide/8 M-urea gel (Sanger & Coulson, 1978), and compared with the dideoxy sequencing ladders. Dideoxy sequencing was carried out by using a modified Sanger method as described in the User Manual from Amersham (Japan). [α-^{32}P]dATP (400 mCi/mmol) was purchased from Amersham.

RESULTS AND DISCUSSION

Purification and properties of *Sca*I endonuclease

After DEAE-cellulose and DEAE-Sephacel column chromatography, the *Sca*I enzyme preparation still contained a trace amount of non-specific nucleases, which were removed by heparin–Sepharose column chromatography. Elution of the adsorbed proteins by increasing potassium phosphate concentration was quite effective in removing the non-specific nucleases, since *Sca*I activity appeared after the bulk of proteins had been eluted.

At this stage, the *Sca*I preparation was essentially free of non-specific nucleases and contained no detectable phosphatase activity. This enzyme preparation was sufficient to use for characterization, since prolonged incubation of DNA with excess of enzyme preparation did not change the gel electrophoretic patterns. Comparison of the restriction patterns obtained from λ, adenovirus 2, T4dC and plasmid DNAs revealed that the specificity of the enzyme is different from those of known restriction endonucleases.

*Sca*I was active in a broad pH range around 7.5. The optimal Mg^{2+} concentration was 5–20 mM. *Sca*I activity was stimulated by the addition of NaCl and the maximum activity was obtained at 0.1–0.13 M-NaCl.

Determination of recognition sequence of *Sca*I

Digestion of pBR322, pBR325, adenovirus 2, and T4dC DNAs with *Sca*I gave one, two, four, six and at least eight bands, respectively, on a 1.0% agarose gel. No

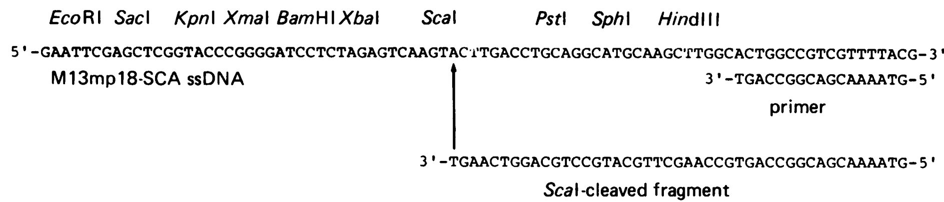

Fig. 3. Relevant sequences around the primer-attached and polylinker regions of M13mp18-SCA DNA.

The arrow indicates the *Sca*I cleaving point.

cleavage site in ϕX174 RF DNA or SV40 DNA was found.

Based on these digestion data and on the tabularized sequencing data for several DNAs in Fuchs *et al.* (1980), it was deduced that the recognition sequence for *Sca*I could be either 5′-AGTACT-3′ or 5′-TCCGGA-3′, since the other candidates do not occur in the *cat* gene or its flanking sequences in pBR325 (Prentki *et al.*, 1981). The sequences AGTACT and TCCGGA occur in neither SV40 or ϕX174 RF DNA (Reddy *et al.*, 1978; Sanger *et al.*, 1978).

The palindromic AGTACT sequence occurs only once in pBR322 DNA at position 3845 (Sutcliffe, 1979). Also the sequence occurs in the *cat* gene on pBR325 DNA at position 5195 (Prentki *et al.*, 1981) while the TCCGGA sequence occurs at a position 4776 which is only five bases apart from the *Eco*RI site. The *Sca*I sites on pBR322 and pBR325 were analysed by digesting the DNA with *Sca*I in combination with *Hin*dIII, *Pst*I, *Eco*RI, *Alu*I, *Hae*III or *Hin*fI, and comparing the gel patterns with those obtained when *Sca*I was omitted. As the result, the unique *Sca*I site on pBR322 was mapped on *Hae*III-A (0.50 kb), *Alu*I-B (0.67 kb) and *Hin*fI-A (1.6 kb) and the second *Sca*I site on pBR325 was located in the *cat* gene at around position 5200 (Fig. 1). These results are consistent with the notion that *Sca*I recognizes 5′AGTACT-3′ sequences.

The proposed sequence 5′-AGTACT-3′ for *Sca*I contains the internal tetranucleotide 5′-GTAC-3′, which is the recognition sequence for *Rsa*I (Lynn *et al.*, 1980). To see whether the *Sca*I recognition sequence shares that of *Rsa*I, double digestion products of pBR322 or pBR325 DNAs with *Rsa*I and *Sca*I were compared with fragments obtained with *Rsa* I alone on agarose gel (Fig. 2), indicating that the cleavage sites for *Sca*I are coincident with those for *Rsa*I.

Based on these data, we have concluded that the recognition sequence for *Sca*I is a palindromic hexanucleotide 5′-AGTACT-3′.

Determination of cleavage site for *Sca*I

To determine the cleavage site of *Sca*I endonuclease, a M13mp18 derivative into which had been inserted an AAGTACTT octanucleotide linker in the *Hin*cII (*Sal*I) site was constructed and named M13mp18-SCA. Single-stranded M13mp18-SCA DNA was annealed with a primer and the primer-attached DNA was divided into five portions. Four portions were used for the dideoxy chain-terminator method (A, C, G and T ladders). The remaining portion was labelled without chain terminator and used for cleavage by *Sca*I. The relevant sequences of M13mp-SCA and the *Sca*I-cleaved fragment are shown in Fig. 3.

The *Sca*I-cleaved product of the primer-extended M13mp18-SCA DNA was analysed by electrophoresis on a polyacrylamide sequencing gel (Fig. 4). The primer-extended whole labelled DNA gave a high-M_r DNA band without *Sca*I-cleavage and gave an additional band at low M_r after cleavage with *Sca*I as shown by an arrow (lane 1), corresponding to one band in the T ladder (lane 5). Since the M13mp18-SCA DNA has only one AGTACT sequence in the polylinker region, which is about 50 bases from the primer end, the band should have derived from the 5′ primer end. This result clearly indicates that the cleavage site for *Sca*I within 5′-AGTACT-3′ is between the internal T and A as shown by an arrow in the right-hand side of Fig. 4.

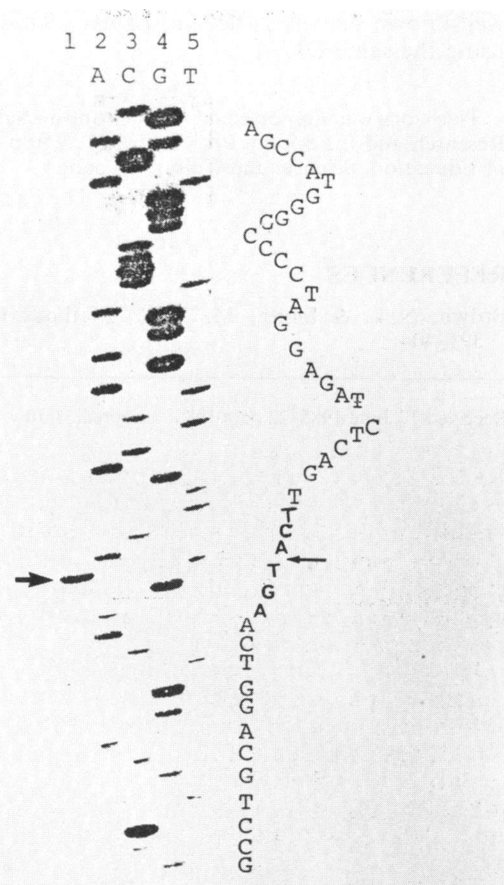

Fig. 4. Sequencing gel analysis of *Sca*I-cleaved DNA fragment

Lane 1, *Sca*I-cleaved fragment of the primer-extended M13mp18-SCA DNA; lanes 2–5 correspond to A, C, G and T chain-terminated ladders, respectively. The arrow in lane 1 shows a band produced by *Sca*I cleavage. The arrow on the right-hand side indicates the site attacked by *Sca*I.

Thus we conclude that *Sca*I recognizes a hexanucleotide palindromic sequence, 5′-AGT↓ACT-3′, and cleaves it in the middle, producing blunt-ended fragments as indicated by the arrows.

The procedure used for *Sca*I cleavage site determination is a modification of the method described by Brown & Smith (1980). Now that we can use various types of M13mp vectors containing polylinkers for the dideoxy sequencing, and also easily obtain synthetic oligonucleotides, the procedure described in this report will be useful for determination of site-specific endonucleases. The reliability of the method was checked by using other well-known enzymes such as *Eco*RI, *Bam*HI and *Pst*I using the same DNA.

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