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A new restriction endonuclease from *Acetobacter pasteurianus*

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ABSTRACT

A restriction endonuclease, ApaI, has been partially purified from Acetobacter pasteurianus. This enzyme cleaves bacteriophage λ DNA and Simian virus 40 DNA at one site, adenovirus-2 DNA at more than nine sites, but it does not cleave ΦX174 DNA nor plasmid pBR322 DNA. This enzyme recognizes the sequence 5' GGGCC↓C 3' / 3' C↑CCGGG 5' and cuts at the sites indicated by the arrows.

INTRODUCTION

Over the years, a large number of site-specific restriction endodeoxyribonucleases have been isolated from a wide variety of microorganisms [1]. During a survey for new restriction enzymes with hitherto unknown recognition sequences we found that A. pasteurianus contains a type II restriction enzyme that recognizes

5' GGGCC↓C 3'
3' C↑CCGGG 5'

MATERIALS AND METHODS

Bacterial strain and medium

A. pasteurianus NCIB 7215 [2], was grown at 30°C in mannitol medium (0.5% yeast extract, 2.5% mannitol, 0.3% bactopeptone). The cells were harvested after 24 hours by centrifugation and stored at -20°C until use. The yield was about 5 g/litre.

DNA preparations

Bacteriophage λ DNA (strain 3110 λ C857S7) was prepared by phenol extraction of CsCl-banded phage.

pBR322 was purified from cleared lysate by CsCl-EtBr equilibrium centrifugation.

Adenovirus-2 (Ad2) and SV40 DNA (strain 776) were a gift of Prof.

W. Fiers (Gent, Belgium).

ΦX174 was obtained from New England Biolabs (Maryland, U.S.A.).

Enzymes

Bacterial alkaline phosphatase was obtained from Worthington (Bedford, MA, U.S.A.).

T4 polynucleotide kinase, and all other restriction endonucleases were from Boehringer (Mannheim, F.R.G.).

Enzyme assay

Samples (4 µl) of column fractions were incubated at 37°C for 2 hours in a reaction mixture (50 µl) containing 1 µg DNA, 6 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 6 mM β-mercaptoethanol. After digestion, 5 µl of a solution containing 50% (w/v) sucrose, 0.1% (w/v) bromophenol blue were added, and the mixture was loaded onto a 1.2% (w/v) agarose slab gel in Tris-acetate buffer (40 mM Tris, 20 mM Na-acetate, 2 mM EDTA, pH 7.5).

Electrophoresis was carried out at 80 Volt for 3 hours in the presence of 0.3 µl/ml EtBr.

RESULTS

Purification of ApaI

Frozen cells were resuspended in 20 ml of a buffer containing 0.01 M Tris-HCl (pH 7.5), 0.01 M β-mercaptoethanol, 1 mM EDTA, and were disrupted by sonication (15 min). Cell debris was removed by high-speed centrifugation at 100,000 g for 90 min at 5°C.

All subsequent steps were carried out at 0°C.

The supernatant was made 1.0 M NaCl and applied to a column (50 cm x 2.5 cm diameter) of Biogel A, 0.5 M (Biorad) which was eluted with a buffer containing 1.0 M NaCl, 0.01 M Tris-HCl (pH 7.5), 0.01 M β-mercaptoethanol. Fractions (5 ml) were collected and assayed for the presence of restriction enzyme. Positive fractions were combined and dialyzed overnight against a buffer containing 0.01 M potassium phosphate (pH 7.4), 0.01 M β-mercaptoethanol, 0.001 M EDTA, 10% (v/v) glycerol (PC buffer). Dialyzed fractions were applied to a column (10 cm x 2.5 cm) of DEAE cellulose (Whatman DE52) previously equilibrated with PC buffer. After washing with 50 ml of PC buffer, the column was eluted with a linear gradient (150 ml total volume) of 0 - 1 M KCl in PC buffer. ApaI eluted between 0.3 M and 0.5 M KCl. Fractions with enzyme activity were combined, dialyzed agains PC buffer, and applied to a column (10 cm x 0.9 cm) of phosphocellulose (Whatman P11). The column was washed with two column

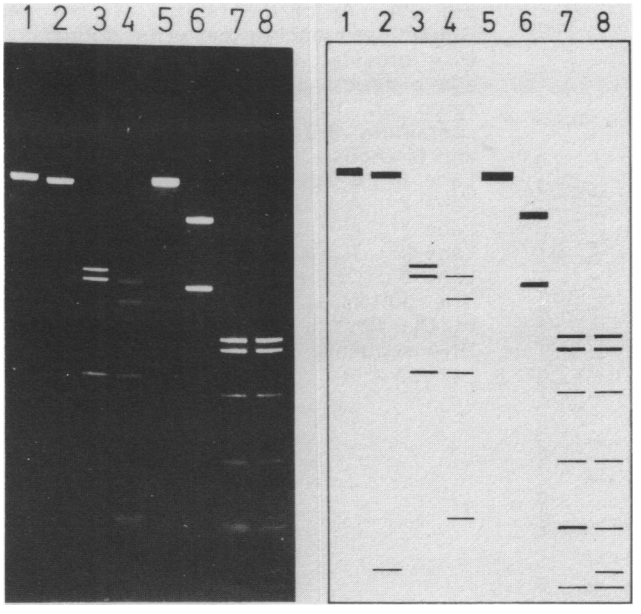

Fig. 1. Double digestion patterns of ApaI restriction enzymes and other on SV40 DNA.
Lane 1 : SV40 DNA + BamHI
Lane 2 : SV40 DNA + BamHI + ApaI
Lane 3 : SV40 DNA + HpaI
Lane 4 : SV40 DNA + HpaI + ApaI
Lane 5 : SV40 DNA + HpaII
Lane 6 : SV40 DNA + HpaII + ApaI
Lane 7 : SV40 DNA + MboI
Lane 8 : SV40 DNA + MboI + ApaI

volumes of PC buffer, and a 200 ml (total volume) linear gradient from 0 - 1 M KCl was applied. ApaI activity eluted at approximately 0.4 M KCl. The enzyme fractions were pooled, concentrated by dialysis against PC buffer containing 50% glycerol, and bovine serum albumin was added to a concentration of 100 µg/ml. This enzyme preparation was stored at -20°C for more that two years without significant loss of activity. At this stage, ApaI was essentially free of non-specific nucleases because Ad2 DNA digested with an excess of enzyme gave sharp bands on agarose gels.

Recognition sequence of ApaI

Preliminary experiments with ApaI on SV40 DNA and double digestions of this DNA with ApaI and a variety of other restriction enzymes show that a unique cleavage site with ApaI can be assigned on SV40 DNA between

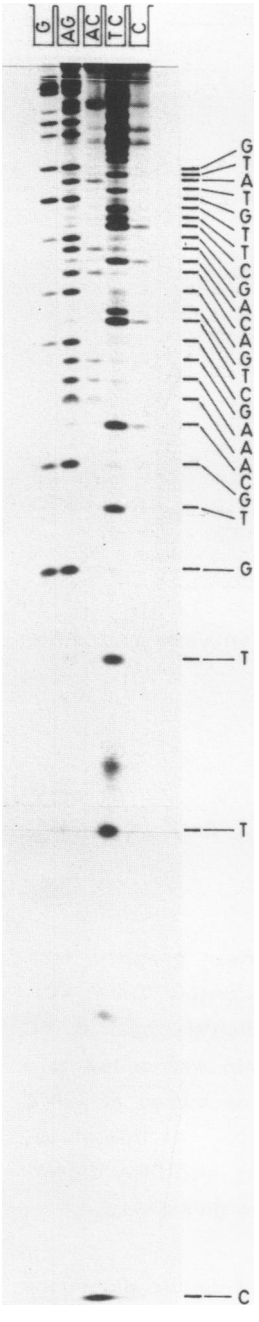

Fig. 2A. Autoradiography of the Maxam and Gilbert sequencing patterns obtained with SV40 DNA digested with ApaI (see text). The cleavage products of the 271 bp fragment were separated on 15% acrylamide gel (0.3 mm x 90 cm) containing 8.3 M urea as described in Materials and Methods.
Lane 1 : G-specific degradation
Lane 2 : A + G-specific degradation
Lane 3 : A + C-specific degradation
Lane 4 : T + C-specific degradation
Lane 5 : C-specific degradation.
The bottom spot in the T + C lane corresponds to the first 5' nucleotide. The published SV40 DNA sequence identifies it as C.

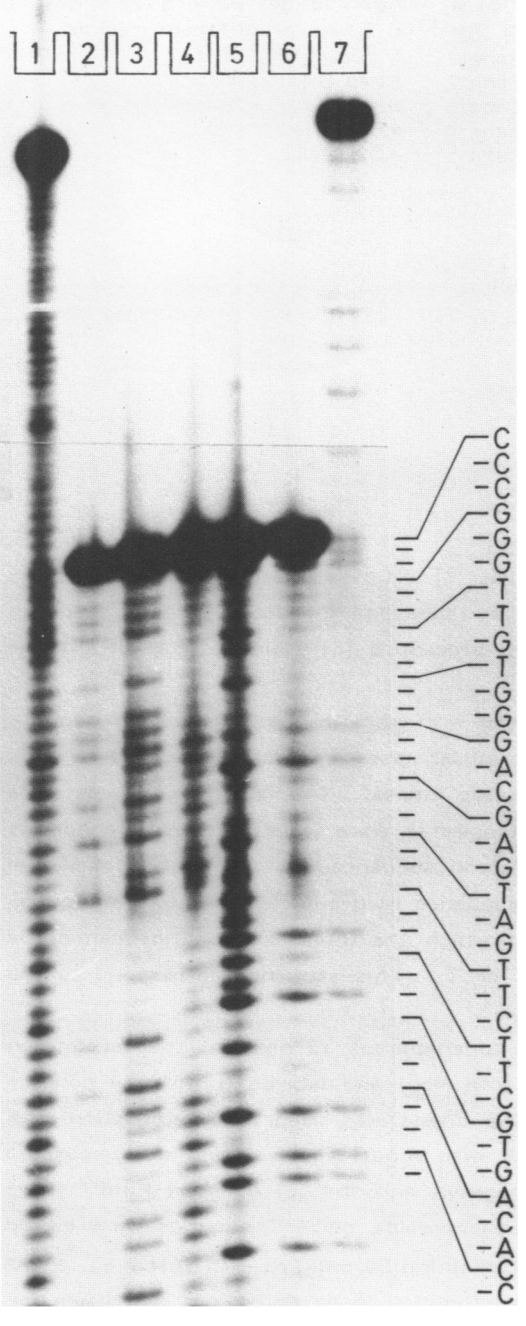

Fig. 2B. Autoradiography of the Maxam and Gilbert sequencing patterns obtained with SV40 DNA digested with MboI and ApaI (see text).
Lane 1 : a mixture of the samples of the A + G and C + T reaction on a HindII-MboI SV40 fragment (control lane).
Lanes 2 - 6 : reactions on a MboI-ApaI SV40 fragment
Lane 2 : G-specific reaction
Lane 3 : A + G-specific reaction
Lane 4 : A + C-specific reaction
Lane 5 : T + C-specific reaction
Lane 6 : C-specific reaction
Lane 7 : C-specific reaction on the HindII-MboI fragment of SV40.
The ultimate band (undegraded material) co-migrates with the second C. So the sequence reads 5' GGGCC 3'.

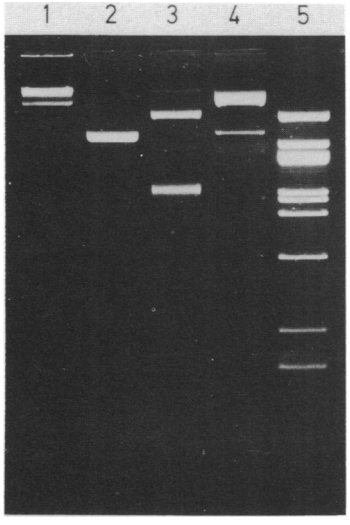

Fig. 3. Agarose gel pattern of digestions of ApaI on DNAs of different origin.
Lane 1 : λ DNA.
Lane 2 : SV40 DNA.
Lane 3 : pBR322.
Lane 4 : ΦX174.
Lane 5 : AD2 DNA.

bases L 2229 to L 2289 (Fig. 1) [3, 4]. Consequently, we used two methods to determine the recognition and cleavage specificity of ApaI based on the Maxam and Gilbert sequencing procedure [5] starting from DNA fragments labeled at different ends.

At first, SV40 DNA was cleaved with ApaI to linear molecules, dephosphorylated with bacterial alkaline phoshatase, and labeled at the 5' ends with [γ^{32}P]ATP and T4 polynucleotide kinase. The labeled molecules were then digested with BamHI. The fragments were isolated by electrophoresis on a 1.6% low gelling agarose gel, and sequenced by the method of Maxam and Gilbert [5] (Fig. 2A). The sequence read-out clearly shows a sequence TTCGACAGTCGAAACGTGTT in which the ultimate nucleotide cannot be determined but must be either C or T. This sequence correspond to the SV40 sequence from L 2283 to L 2262.

To confirm this result, and to enable us to pinpoint the recognition sequence we digested SV40 DNA with MboI and labeled it with T4 polynucleotide kinase. Two-thirds of the digest was used in a redigestion with ApaI and one-third was redigested with HindII enzyme, and was used as a control. The 124 bp MboI-ApaI fragment and the 161 bp MboI-HindII fragment were separated on a 6% polyacrylamide gel. The bands were eluted overnight by 37°C with a buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 0.01% SDS. After centrifugation in an Eppendorf centrifuge for 15 min, the supernatant was applied to a small DE52 column, and eluted

with 1.5 M NH_4OAc pH 10.4. The fractions containing the labeled DNA were collected and precipitated with isopropanol. After washing two times with ether and drying, the labeled fragments were again sequenced by the method of Maxam and Gilbert [5].

Comparison of the ladders of the MboI-ApaI fragment with the control ladders of the MboI-HindII fragment indicates that the site of cleavage lies between the two C's indicated by an arrow of the sequence 5' GGGCC$^{\downarrow}$C 3' (L 2258 to L 2263) (Fig. 2B).

A computer search through the sequences of pBR322 and ΦX174 indicates that this sequence should not occur in pBR322 or ΦX174, and indeed should occur once in SV40 DNA. These results are confirmed by the digestion patterns of these DNAs with ApaI, as shown in Fig. 3. Our results also indicate that λ DNA is cut only once, but that Ad2 DNA contains at least 9 recognition sites.

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