Purification and characterization of endonucleases *Dra*II and III from *Deinococcus radiophilus*

C.M. de Wit, B.M.M. Dekker, A.C. Neele and A. de Waard

Department of Medical Biochemistry, Sylvius Laboratories, University of Leiden, Wassenaarseweg 72, 2333 AL Leiden, The Netherlands

Received 23 November 1984

Deinococcus radiophilus strain ATCC 27603 contains, apart from endonuclease *Dra*I [1], two additional sequence-specific endonucleases. These enzymes, designated *Dra*II and *Dra*III, recognize nucleotide sequences with novel specificities, PuG↓GNCCPy and CACNN↓GTG, respectively.

Restriction enzyme *Nucleotide sequence*

1. INTRODUCTION

During the preparation of endonuclease *Dra*I from *Deinococcus radiophilus*, strain ATCC 27603 (which is specific for the nucleotide sequence TTT↓AAA [1]) we noted in column eluates the presence of two additional sequence-specific endonucleases. These new enzymatic activities could not be attributed to bacterial contamination of our starting material. Here we report on the purification of endonucleases *Dra*II and III and the identification of their DNA-cleaving properties.

2. EXPERIMENTAL METHODS

2.1. *Growth of the organism*

The liquid medium (L-broth) contained per litre: tryptone, 10 g;yeast extract (Difco), 5 g; NaCl, 10 g. *D. radiophilus*, ATCC 27603 was grown at 37°C in 4.5 l L-broth to stationary phase (which was reached in approximately 24 h); the yield was 18 g. The cells were harvested by centrifugation and stored at -20°C after the cultures had been checked for possible bacterial contamination by plating aliquots on medium solidified with agar.

Abbreviations: Ad, adenovirus; FPLC, fast protein liquid chromatography; Pu, purine; Py, pyrimidine; mec, gene for deoxycytidylate methyl transferase

2.2. *Enzyme purification*

Frozen cells (9 g) were thawed, sonicated in 50 ml of buffer A (10 mM Tris-HCl (pH 7.4), 2 mM mercaptoethanol), and centrifuged first at 12 000 × *g* for 10 min and then at 70 000 × *g* for 40 min in a Spinco 30 rotor. These and all other operations were performed at approximately 0°. To the supernatant NaCl was added to 0.4 M, followed by a 10% solution of polyethylene imine (Polymin P, BASF, Ludwigshafen) at pH 7.9 which was stirred into a final concentration of 1%. The resulting turbid preparation was centrifuged at 12 000 × *g* for 20 min. The supernatant was dialysed against 4 l of buffer B (10 mM potassium phosphate (pH 7.4), 10 mM mercaptoethanol) with one change. The dialyzed solution was loaded onto a 15 × 2 cm column of phosphocellulose equilibrated with buffer B and chromatographed using a linear 500 ml gradient of 0–1.0 M NaCl in buffer B. Fractions of 6 ml were collected and assayed for endonuclease activity towards bacteriophage λ DNA by agarose gel electrophoresis [1]. Endonuclease *Dra*II, which eluted at 0.25 M NaCl, free from the other two nucleases, was purified further in 50% yield on hydroxyl apatite (Biorad), using a linear 200 ml gradient of 10–500 mM potassium phosphate (pH 7.6). Active fractions were dialyzed against storage buffer C (25 mM Tris-HCl (pH 7.4), 10 mM mercap-

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

toethanol, 1 mM EDTA, 100 mM NaCl and 50% glycerol). Endonucleases *Dra*I and *Dra*III eluting in the range of 0.50–0.65 M NaCl and 0.60–0.75 M NaCl, respectively, were not completely resolved. These fractions were then dialyzed against buffer A containing 10% glycerol and subsequently chromatographed on a Mono Q column in a Pharmacia FPLC apparatus (90 ml linear gradient from 0–0.5 M KCl in buffer A with 10% glycerol; duration 90 min). Endonucleases *Dra*I and *Dra*III eluted at 120 and 280 mM KCl, respectively. Care was taken to collect these fractions in tubes containing bovine serum albumin (special quality for molecular biology, Boehringer, Mannheim), final concentration 50 μg/ml. Both enzymes were dialyzed against storage buffer C but this caused an appreciable loss of endonuclease *Dra*III. The yield of the newly discovered enzymes before the final dialysis was approximately 2000 units per gram cells. Optimal assay conditions for endonuclease *Dra*II were 7 mM Tris-Cl (pH 7.5), 7 mM $MgCl_2$, 7 mM mercapto-ethanol while *Dra*III required also 50 mM NaCl. The substrate was the DNA of bacteriophage lambda obtained by temperature induction of *E. coli* HB129λ CI857SuS7 lysogen. One unit of enzyme is the amount that cleaves 1 μg of λ DNA completely in 1 h.

2.3. *Determination of cleavage specificity*

The techniques used to determine nucleotide sequences at or adjacent to cleavage sites for endonucleases *Dra*III and II were the chemical cleavage method of Maxam and Gilbert [2] and the wandering spot method [3].

As the labeling procedure with polynucleotide kinase presented difficulties when applied to DNA fragments generated by endonuclease *Dra*III, we resorted to the so-called fifth lane procedure, in which the enzymically cleaved fragment is run on a polyacrylamide gel next to a sequence ladder [4]. A schematic representation of our approach is given in fig. 1. This method enabled us to read the recognition (cleavage) site for *Dra*III directly from the gel.

3. RESULTS AND DISCUSSION

The three endonucleases from *D. radiophilus* eluted from the phosphocellulose column in the order II, I, III, the latter two being only partially separated. Further purification of these enzymes was achieved by FPLC (see section 2). Endonuclease *Dra*II cuts bacteriophage λ DNA infrequently (at three sites), only one fragment being discernible on standard agarose gels.

3.1. *Recognition sequence of endonuclease* Dra*II*

Plasmid pAT153 [5] (a derivative of pBR322) was cleaved by *Dra*II at three sites, and 5′-labeled with ^{32}P using T4 polynucleotide kinase. Two labeled fragments, 186 and 214 bp long, respectively, that were obtained after secondary digestion with *Taq*I, were selected for sequencing [2]. One of these sequence determinations is shown in fig. 2. Comparison of these sequences with the complete (corrected) nucleotide sequence of the plasmid [6,7] indicated that the termini were situated within a hyphenated symmetrical nucleotide sequence of the type PuGGNCCPy. In the example shown in fig. 2 this sequence reads GGGGCCT (coordinates 529–523, lower strand) and in the other example (not shown) GGGTCCT (coordinates 1486–1480, lower strand). The unique cleavage site of *Dra*II in SV40 DNA [8] (coordinates of the recognition site are 2258–2264) was studied with the wandering spot technique. Fig. 3 shows the nucleotide sequence GGCCAACAC (2262–2253) of the lower strand, thus indicating that the point of cleavage in the *Dra*II recognition site is as indicated by the arrow: PuG↓GNCCPy.

It was noted that the sequence GGGTCCTGG in pAT153 (1438–1446) was resistant to the action of *Dra*II. This sequence harbors a potential *Dra*II cleavage site as well as an *Eco*RII site, CCTGG. It is known that in plasmids grown on mec^+ strains

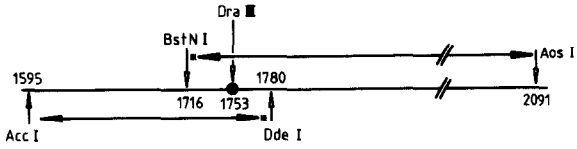

Fig.1. Schematic representation of experimental approach to elucidate the recognition sequence of endonuclease *Dra*III. An Ad12 DNA fragment was sequenced [2] across a *Dra*III cleavage site, in two directions. Termini labeled at their 5′-ends are indicated by an asterisk. The sequencing gel in fig.4B represents the *Dde*I/*Acc*I fragment depicted above.

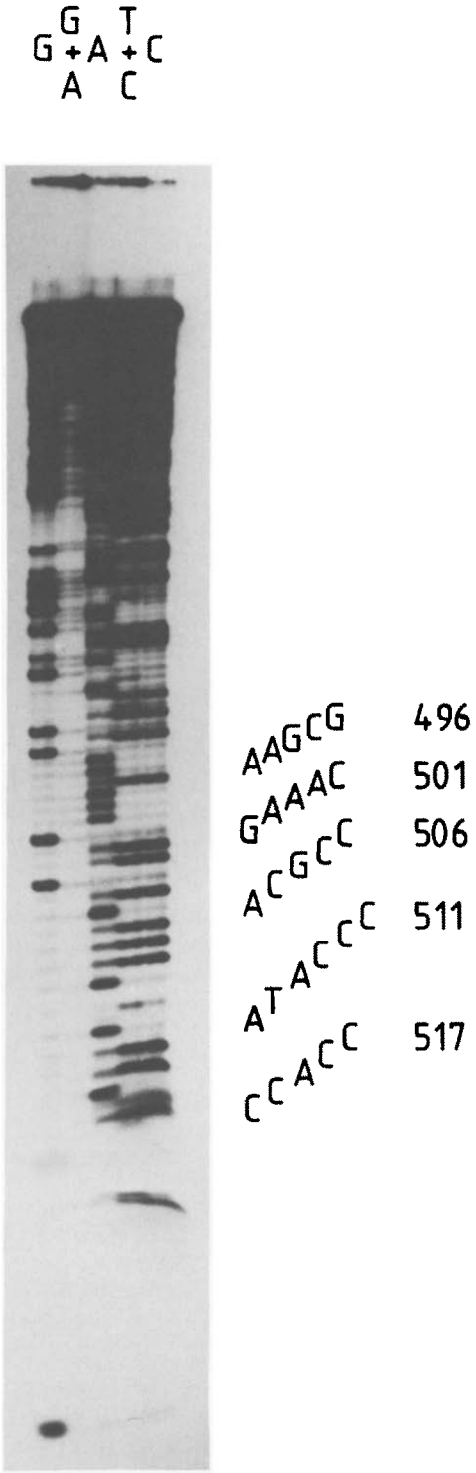

Fig.2. Sequencing gel of a 186 bp *Dra*II/*Taq*I fragment of plasmid pAT153 adjacent to the *Dra*II cleavage site at coordinates 529–523.

of *E.coli* (such as DH1 [14]) *Eco*RII sites are methylated at the internal cytidylate residues [9,10]. We consider it likely therefore, that endonuclease *Dra*II is inhibited by the presence of 5-methylcytosine in a potential cleavage site at the position marked by an asterisk.

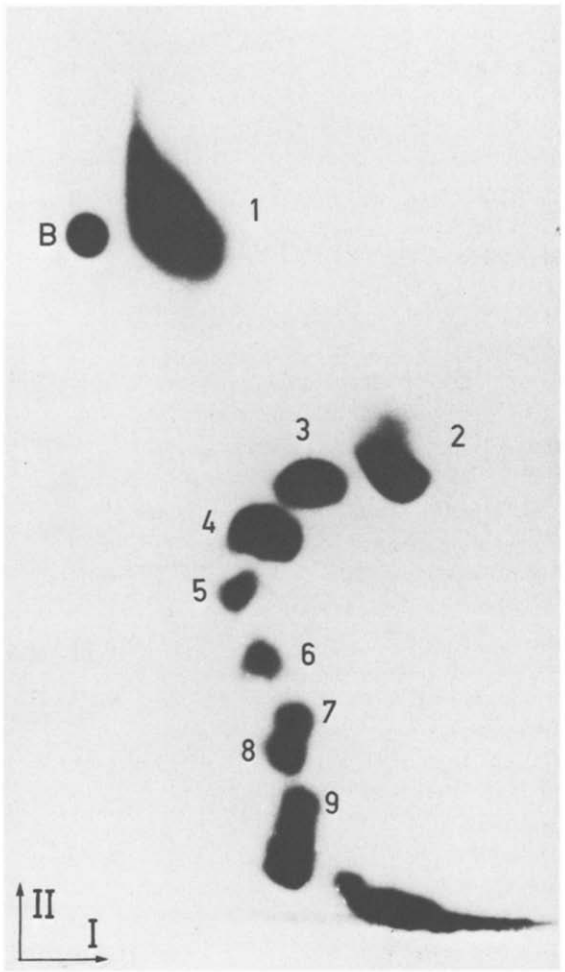

Fig.3. Wandering spot sequence analysis of *Dra*II cleavage site in SV40 DNA (coordinates 2262–2258) and neighbouring nucleotides. A single-stranded DNA fragment that had been 5′-labeled at the *Dra*II site was degraded with pancreatic DNase and snake venom exonuclease. The resulting oligonucleotides were fractionated according to [3]. Spot 1, pG (identified by paper electrophoresis); spot 9, pGpGpCpCpCpApApC-pA. I, direction of electrophoresis; II, homochromatography; B, blue marker (xylene cyanol FF).

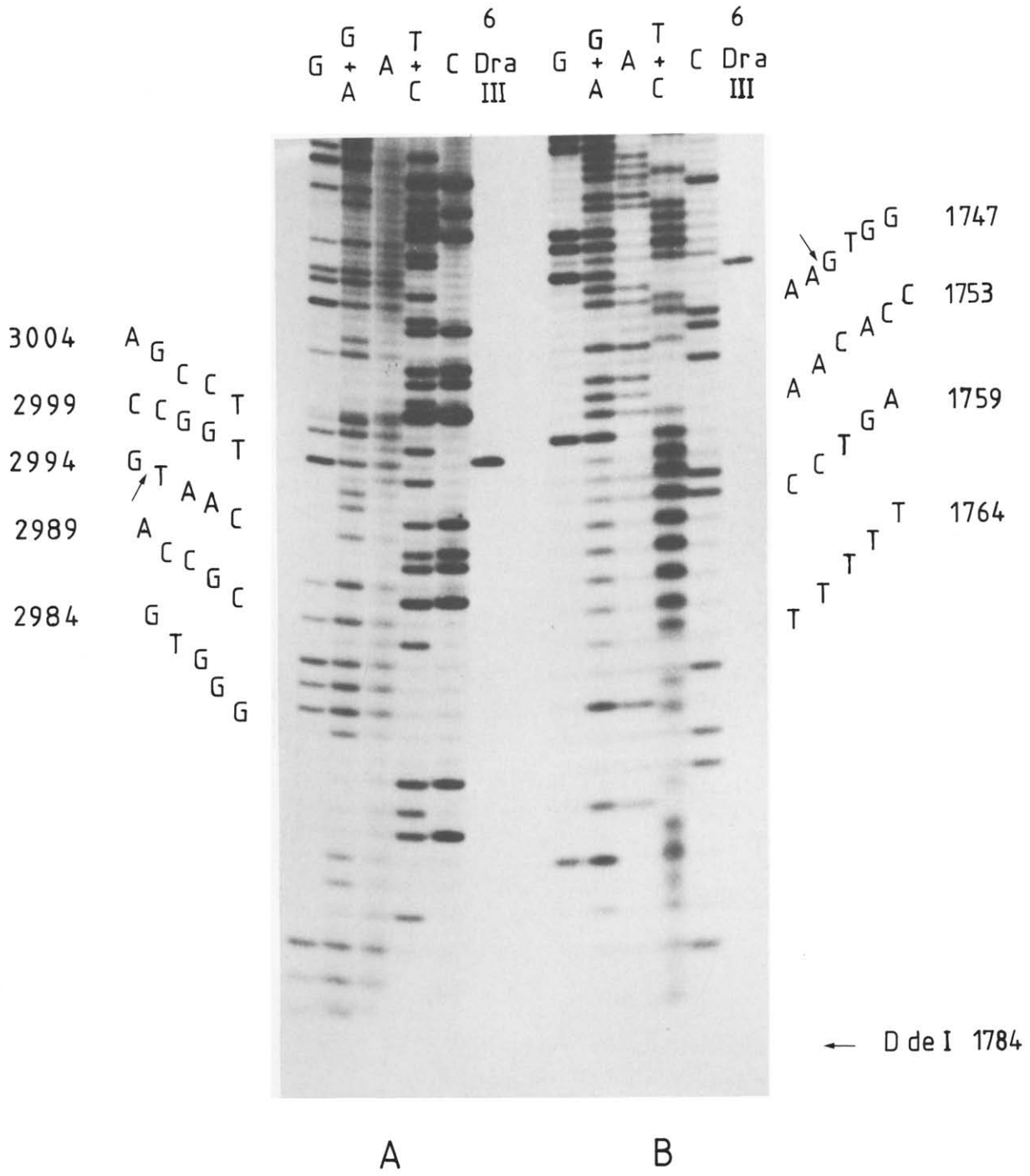

Fig.4. Sequence gels of 'sixth lane' experiments. (A) An Ad5 DNA fragment containing a *Dra*III cleavage site CACNNN↓GTG at coordinates 2988–2996, CACAAT↓GTG. (B) An Ad12 DNA fragment containing a *Dra*III cleavage site CACNNN↓GTG at coordinates 1756–1748, CACCAA↓GTG. This is the *Dde*I/*Acc*I fragment of fig.1. Note that the structure of the enzymatic product(*Dra*III lane) is of the nature 5′ *p...CACNNN-OH 3′ which migrates slightly slower than the chemically degraded product of the same sequence 5′ *p...CACNNN-p 3′ carrying a 3′ terminal phosphate. The point of cleavage within the *Dra*III recognition site is therefore between the third N and G. This conclusion was proven rigorously by wandering spot analysis (not shown).

3.2. *Recognition sequence of endonuclease* Dra*III*

The recognition sequence from this enzyme was determined directly using a 'fifth lane' (actually a sixth lane) procedure (cf. fig. 1, [5] and section 2). This method was applied to two overlapping segments of a cloned Ad12 DNA fragment and to a fragment of Ad5 DNA, both of known sequence [11,12]. The results are presented in fig.4. A close inspection of the gel patterns reveals both the recognition sequence and the location of the point of cleavage (see legend). The latter point was confirmed by an independent method. A 5′-^{32}P-terminally labeled Ad12 DNA fragment generated with *Dra*III was subjected to a wandering spot analysis (not shown) which gave 5′-^{32}pGpTpG. Thus the recognition sequence of endonuclease *Dra*III is CACNNN↓GTG.

The authors of [1] did not report the presence of endonucleases *Dra*II and III in their preparations. When we adopted their phase partition method we did observe these new enzymes. When our sequence studies on the characterization of *Dra*II and III enzymes were well in progress, we learned that R. Hansen (quoted in [13]), had also observed these additional nucleases.

ACKNOWLEDGEMENTS

The authors are grateful to Dr H. van Ormondt for his valuable help in supplying data on nucleotide sequences and to Dr R. Bernards for a gift of a cloned Ad12 DNA fragment. This work was supported in part by the Netherlands Organization for the Advancement of Pure Research (ZWO) through the Foundation for Fundamential Medical Research (FUNGO) and by the Foundation for the Technical Sciences (STW).

REFERENCES

[1] Purvis, I.J. and Moseley, B.E.B. (1983) Nucleic Acids Res. 11, 5467–5474.
[2] Maxam, A.M. and Gilbert, W. (1980) Methods Enzymol. 65, 499–560.
[3] Tu, C.-P.D. and Wu, R. (1980) ibidem 65, 620–638.
[4] McConnell, D.J., Searcy, D.G. and Sutcliffe, J.G. (1978) Nucleic Acids Res. 5, 1729–1739.
[5] Old, R.W. and Primrose, S.B. (1981) in: Principles of Gene Manipulation, 2nd ed., Blackwell, Oxford.
[6] Sutcliffe, J.G. (1978) Cold Spring Harbor Symp. Quant. Biol. 43, 77–90.
[7] Peden, K.W.C. (1983) Gene 22, 277–280.
[8] Buchman, A.R., Burnett, L. and Berg, P. (1981) in: Molecular Biology of Tumor Viruses, 2nd ed., DNA Tumor Viruses (revised), Cold Spring Harbor, pp. 799–841.
[9] Boyer, H.W., Chow, L.T., Dugaiczyk, A., Hedgpeth, J. and Goodman, H. (1973) Nat. New Biol. 244, 40–43.
[10] May, M.S. and Hattman, S. (1975) J. Bacteriol. 123, 768–770.
[11] Bos, J.L., Polder, L.J., Bernards, R., Schrier, P.I., Van den Elsen, P.J., Van der Eb, A.J. and Van Ormondt, H. (1980) Gene 10, 27–38.
[12] Maat, J., Van Beveren, C. and Van Ormondt, H. (1980) Gene 10, 27–38.
[13] Roberts, R.J. (1984) Nucleic Acids Res. 12, r167–204.
[14] Maniatis, T., Fritsch, E.F. and Sambrook, J. (1982) Molecular Cloning, A Laboratory Manual (Appendix), Cold Spring Harbor, NY.