Volume 11 Number 16 1983 Nucleic Acids Research

Isolation and characterisation of DraI, a type II restriction endonuclease recognising a sequence containing only A:T basepairs, and inhibition of its activity by uv irradiation of substrate DNA

I.J.Purvis and B.E.B.Moseley

Department of Microbiology, University of Edinburgh, School of Agriculture, Edinburgh EH9 3JG, UK

Received 11 May 1983; Revised and Accepted 29 July 1983

ABSTRACT

A type II restriction endonuclease, DraI, isolated from *Deinococcus radiophilus* ATCC 27603 recognises the palindromic hexanucleotide sequence

5'-T-T-T↓-A-A-A-3'
3'-A-A-A-↑T-T-T-5'

and cleaves it, as indicated by the arrows, to produce blunt-ended fragments. The yield of enzyme is 100 to 1000 times that of the only other known type II restriction endonuclease that recognises a sequence composed solely of A:T basepairs, the isoschizomer AhaIII (1). Ultraviolet irradiation of the DNA substrate at relatively low doses inhibits the activity of DraI by "protecting" the recognition sequence and this may be exploited to give control of partial digestion of DNA by DraI.

INTRODUCTION

D. radiophilus belongs to the Family Deinococcaceae, the major characteristic of members of this group being their extreme resistance to the lethal effects of both ionising and ultraviolet radiations (2). The type species of the group *D. radiodurans* ATCC 13939, has been shown to possess a type II restriction endonuclease (3), MraI, recognising the sequence

5'-C-C-G-C-G-G-3'
3'-G-G-C-G-C-C-5'

We report here a description of the isolation and characterisation of the site-specific endonuclease DraI, and a method by which partial digestion of DNA molecules by DraI may be controlled.

METHODS

Assay for Restriction

Enzyme activity was estimated by incubating a sample (usually 1 µl) with either 1 µg λ-DNA or ColE1:Tn5 ccc DNA. One unit of enzyme represents that activity which completely digests 1 µg of DNA during 1 hour's incubation at 37°C in restriction buffer (10 mM Mg, 10 mM Tris-HCl, pH 8.0).

Protein estimation

Protein concentration was measured both by comparative absorption at 260 nm and 280 nm wavelength light and using a Bio-Rad protein assay kit.

Isolation of DraI

Three one-litre cultures of *D. radiophilus* in nutrient broth No 2 (Oxoid) were grown to early stationary phase by shaking at 37°C. It should be noted that protease activity increases greatly in late stationary and decline phase. About 15 g wet weight of cells was harvested by centrifugation at 10,000 g for 10 min. The bacteria were resuspended in 30 ml 10 mM Tris-HCl, 2 mM 2-mercaptoethanol (MSH), 0.1 mM phenylmethylsulphonylfluoride (PMSF), pH 7.5 and broken open in a French pressure cell at 3,000 psi. Following centrifugation at 10,000 g for 10 minutes to remove undamaged cells and large fragments, the lysate was centrifuged at 100,000 g for 2 hours. Most of the DNA present in the supernate was removed using polyethylene glycol 6,000 (PEG): dextran T500 phase partition (4). This procedure also removed significant amounts of non-specific exonuclease activity. The enzyme preparation was dialysed (10-20 hours) against column buffer 1 (CB1 - 10 mM Tris-HCl, 2 mM MSH, 0.1 mM PMSF and 0.075 M NaCl, pH 8.0) and applied to a 20 x 2.6 cm DEAE-sephacel (Bio-Rad) column, previously equilibrated with CB1. After washing, the column was developed with a linear

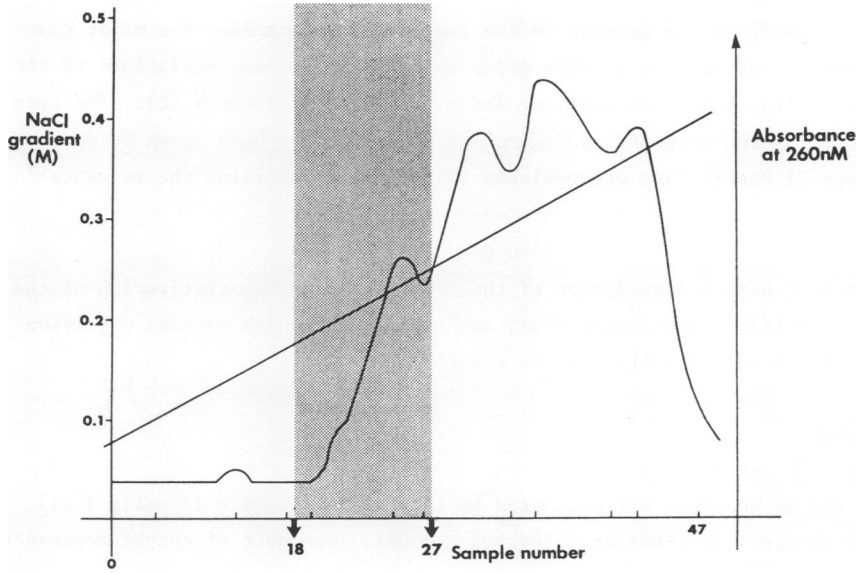

Fig. 1. Protein Elution profile of D.radiophilus cell extraction DEAE-Sephacel

0.075-0.4 M NaCl gradient, DraI activity eluting in the 0.18-0.24 NaCl region (Fig 1, shaded area represents region of 'unit' enzyme activity).

Active fractions were pooled, dialysed against column buffer 2 (CB2 - 0.01 M phosphate buffer, 2 mM MSH, 0.05 M NaCl, pH 7.5) and then applied to a 10 x 2.6 cm hydroxylapatite (Bio-Rad) column previously equilibrated with CB2. The column was washed with CB2 and then eluted with a linear gradient of 0.01 to 0.4 M phosphate buffer. DraI activity eluted between 0.20 and 0.32 M phosphate. Each collecting tube contained enough restriction enzyme-grade bovine serum albumin (BRL) to ensure that the total protein concentration did not fall below 750 $\mu g\ ml^{-1}$. Fractions showing greater than 1,000 units ml^{-1} of enzyme activity were pooled and dialysed against storage buffer (50% glycerol, 10 mM Tris-HCl, 2 mM MSH, 0.05 M NaCl, pH 8.0). DraI is stable in this buffer for > 12 months at - 20°C.

RESULTS

The yield and specific activity of DraI during various stages of isolation are shown in Table 1. The final preparation was considered to be free of contaminating 5' and 3' exonucleases since there was no alteration of cleavage patterns upon extensive incubation and because of the success of sequencing techniques which are highly sensitive to such contamination.

Optimal Conditions for DraI Activity

DraI has an absolute requirement for Mg^{2+}, a feature not uncommon in type II restriction endonucleases (5). Maximum DraI cleavage was obtained at 37°-39°C in a buffer containing 10 mM Mg^{2+} and 10 mM NaCl at pH 8.0. Concentrations of > 100 mM NaCl, > 30 mM Mg^{2+}, > 1 mM Mn^{2+} and > 0.1 mM Ca^{2+} were each inhibitory to enzyme activity. In general, conditions for maximum activity were similar to those found for MraI (3).

Table 1. Stages of DraI Purification

Stage	Total protein (mg)	Total enzyme (units)	Specific activity (units mg^{-1} protein)	Yield (%)
High speed centrifugation	1.86×10^3	8.0×10^5	4.32×10^2	100
PEG:dextran phase partition	1.22×10^3	7.5×10^5	6.15×10^2	93.75
DEAE-sephacel	3.15×10^1	4.1×10^5	1.30×10^4	51.25
Hydroxylapatite	0.57	1.4×10^5	2.46×10^5	17.50

Table 2. Specificities of Type II Restriction Enzymes DraI and AhaIII

Enzyme	Number of cleavage sites				
	pBR322	Ade 2	λ	SV40	ØX174
AhaIII	3	16	13	12	2
DraI	2 (3[a])	> 10	13	> 10	2

[a]From sequence data, see text.

Mapping of DraI cleavage sites

Cleavage of a variety of different DNA species, ie λ; Ade-2; ØX174 Rf; SV40 and pBR322 (6-9) produced a pattern of bands, after polyacrilamide gel electrophoresis (7-12% gradient), only previously seen in the case of AhaIII (1) digestions (Table 2).

Double digestions of pBR322 using each of the commercially available enzymes EcoRI, BamHI, PstI, HindIII (all from Miles Laboratories) and SalI (NBL Enzymes Ltd) with DraI indicated the presence of two DraI recognition sites in this plasmid at the map positions 3250 and 3900 respectively. These data suggested that a DNA sequencing procedure closely based upon that described for AhaIII(1) should be used. The Sau3AI fragment 9 of pBR322 was cloned into the M13mp7Rf1 BamHI site (1, 10-12), sequenced using the chain-terminator method (13) and the DraI cleavage sites determined (1 and 14). As was the case with AhaIII, during the course of sequencing a second DraI site was identified within the cloned fragment (3 in the whole pBR322 molecule) producing upon cleavage a 19bp fragment.

From the above data it was concluded that *D. radiophilus* contains a type II restriction endonuclease recognising the palindromic hexanucleotide sequence

5'-T-T-T-A-A-A-3'
3'-A-A-A-T-T-T-5'.

Inhibition of DraI activity by uv irradiation of the DNA substrate

Cleaver *et al* (15) and Hall and Larcom (16) have shown that the activity of type II sequence specific endonucleases is inhibited by thymine-thymine dimer, and possibly cytosine-thymine dimer, production within or adjacent to the enzyme recognition sequence. In those studies the type II restriction enzyme most sensitive to such inhibition was HindIII, the recognition sequence of which is

5'-A-A-G-C-T-T-3'
3'-T-T-C-G-A-A-5'

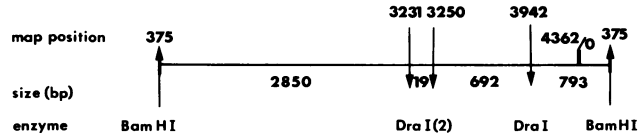

Fig 2. Schematic representation of the pBR322 molecule showing cleavage sites of DraI and BamHI.

However DraI activity is much more sensitive to inhibition following uv irradiation of the DNA substrate than HindIII. By linearising cccpBR322 (1 μg) with the type II restriction endonuclease BamHI, (map position 375 on the pBR322 molecule), followed by uv irradiation at an incident dose rate of 1.05 Jm^{-2} in a uv transparent buffer (10 mM Tris-HCl pH 8.0), the effect of different radiation doses upon DraI activity was observed (Figs 2 and 3). Unfortunately the presence in pBR322 of two DraI sites only 19 base-pairs apart does complicate interpretation of the results. The appearance of a 1489 basepair fragment allied to the simultaneous removal of both 692 and 793 basepair fragments indicates the inhibition of activity at the DraI recognition site positioned at 3942 on the pBR322 molecule. Inhibition of activity at either of the other two sites singly will not produce any new bands as the fragment size resolution of a 1% agarose gel will not allow

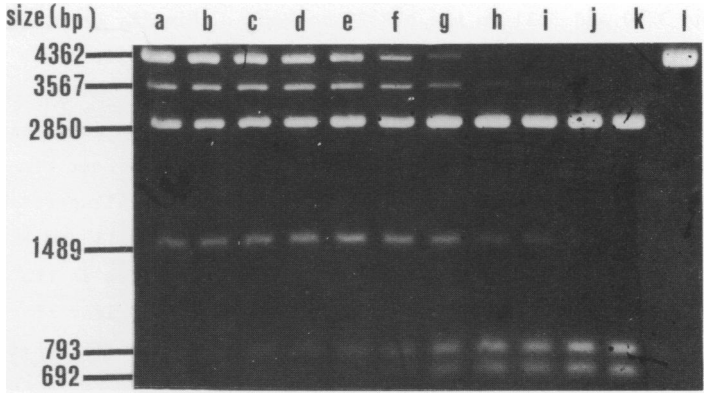

Fig 3. DraI digestion of uv treated, BamHI linearised pBR322.
Lanes *a-k* illustrate 1 μg pBR322 linearised with BamHI and given uv radiation doses of 4200; 3600; 3000; 2400; 1800; 1200; 600; 300; 180; 60 and 0 Jm^{-2} respectively before 1 hr DraI digestion. Lane *l* shows BamHI linearised pBR322 plasmid DNA. Fragment separation was by agarose gel electrophoresis (1%) and visualisation by EtBr staining.

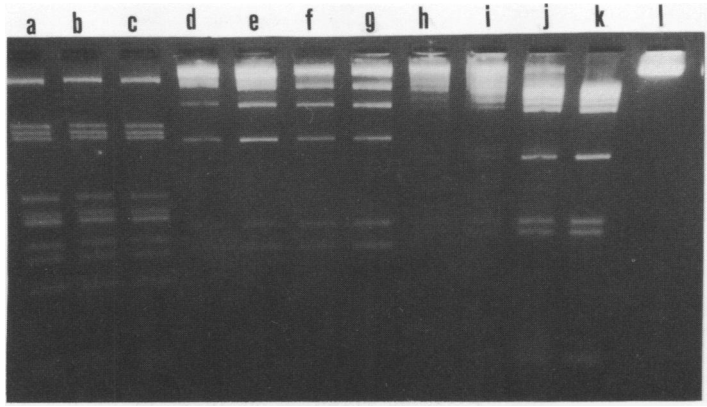

Fig 4. Cleavage of uv irradiated λ-DNA by DraI, HindIII or PstI.
Lanes *a-c* show digestion of 1 μg λ-DNA by PstI after uv radiation doses of 3600; 1800 and 0 Jm^{-2} respectively. Lanes *d-g* show cleavage of 1 μg λ-DNA by HindIII after uv doses of 3600; 1800; 300 and 0 Jm^{-2} respectively. Lanes *h-l* show digestion of 1 μg λ-DNA by DraI after uv doses of 3600; 1800; 300 and 0 Jm^{-2}. Fragment separation was by agarose gel electrophoresis (1%) and visualisation by EtBr staining.

distinctive separation of DNA molecules differing in size by only 19 basepairs. Initial alteration in the digestion pattern occurs at a uv dose of ~ 60 Jm^{-2} while above a dose of 180 Jm^{-2} a fragment of 3567 basepairs appears due to the inhibition of enzyme action at both the DraI recognition sequences at positions 3250 and 3231 on the pBR322 molecule. Finally at doses > 300 Jm^{-2} complete linear molecules of pBR322 (4362 basepairs) can be seen due to the inactivation of all DraI recognition sites.

Comparative studies using HindIII and DraI showed that inhibition of DraI activity at individual recognition sites was three to four times more sensitive to uv inhibition than that of HindIII. This is illustrated by comparative digestions of uv-damaged λ-DNA with DraI, HindIII and PstI (Fig 4). In spite of differing numbers of restriction sites in the λ-DNA, (13 for DraI, 7 for HindIII and 18 for PstI), it is still clear that DraI activity is inhibited to a greater degree than that of HindIII, whilst irradiation of the DNA has little effect upon the action of PstI, the recognition sequence of which contains no adjacent thymine residues.

DISCUSSION

In contrast to the blue green alga *Aphanothece halophytica* from which AhaIII is isolated, *Deinococcus radiophilus* is easily grown and gives a

relatively high yield of the AhaIII isoschizomer, DraI. *D. radiophilus* is also extremely radiation resistant and this property provides an excellent screen against contamination. These properties make it likely that DraI will become a commercially important restriction endonuclease.

Since DraI recognises a sequence containing only A:T basepairs it may be possible to expand studies on DNA-DNA and DNA-protein interactions which were being delayed by a dearth of suitable, easily available enzymes. DraI will also be useful in the study of eukaryotic DNA where, due to the marked asymmetry of A:T distribution, large regions of G:C rich DNA may be produced.

Difficulties in controlling partial digestion of DNA molecules during restriction may, to some extent, be overcome by utilising the inhibition of DraI activity by uv irradiation of the substrate DNA. Although DraI digestion produces blunt-ended fragments, this disadvantage can be removed by poly (dA) "tailing" of the restricted DNA to be cloned, followed by insertion into a poly (dT) "tailed" vector regenerating DraI sites either side of the cloned fragment. To allow gene expression within the irradiated insert, multiplication of the recombinant molecule in a uv repair-proficient strain of bacterium, yeast or tissue culture line must occur. However this process will 'reactivate' any DraI recognition sequences present within the DNA insert.

ACKNOWLEDGEMENTS

We wish to thank Dr N Brown of the University of Bristol who provided both instruction and assistance with the DNA sequencing. Special note should be made of the help and support of other members of our research group, M Mackay, D Evans and S Whyte.

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