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Two new restriction endonucleases *Dra*II and *Dra*III from *Deinococcus radiophilus*

Rüdiger Grosskopf, Werner Wolf and Christoph Kessler

Boehringer Mannheim GmbH, Biochemical Research Center, Bahnhofstr. 9-15, D-8132 Tutzing, FRG

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ABSTRACT

In addition to recently characterized DraI (1), two new Type II restriction endonucleases, DraII and DraIII, with novel site-specificities were isolated and purified from Deinococcus radiophilus ATCC 27603. DraII and DraIII recognize the hepta- and nonanucleotide sequences

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          ↓
5'-Pu G G N C C Py-3'
3'-Py C C N G G Pu-5'        (DraII)
              ↑
```

and

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                ↓
5'-C A C N N N G T G-3'       (DraIII).
3'-G T G N N N C A C-5'
          ↑
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The cleavage sites within both strands are indicated by arrows.

The recognition sequences were established by mapping of the cleavage sites on pBR322 (DraII) and fd109 RF DNA (DraIII). The sequence specifities were confirmed by computer-assisted restriction analyses of the generated fragment patterns of the sequenced DNA's of the bacteriophages λ, ϕX174 RF, M13mp8 RF and fd109 RF, the viruses Adeno2 and SV40, and the plasmids pBR322 and pBR328.

The cleavage positions within the recognition sequences were determined by sequencing experiments.

INTRODUCTION

A large number of Type II restriction endonucleases have been isolated from eu- and archaebacterial organisms (2,3). More recently one of these enzymes, DraI recognizing the palindromic hexanucleotide sequence 5'-AAA↓TTT-3', has been purified from the gram-positive eubacterium Deinococcus radiophilus ATCC 27603 (1). We re-examined this organism for the presence of additional site-specific endonucleases and, in addition to DraI, we found two further Type II restriction endonucleases with completely different sequence specificities.

We report here the isolation and characterization of these two additional

enzymes. In accordance with the proposal of Smith and Nathans (4) we designated these new Type II restriction endonucleases as DraII and DraIII.

MATERIALS AND METHODS

Bacterial strain and culture conditions

D. radiophilus ATCC 27603 belongs to the family of Deinococcacae. The members of this family are characterized by their extreme resistance to the lethal effects of both ionizing and ultraviolet radiation. Cells were cultured aerobically at 30°C for 20 hrs in tryptone, 5.0 g/l, glucose, 1.0 g/l, yeast extract, 3.0 g/l, DL-methionine, 0.5 g/l, and harvested in the late logarithmic phase.

Enzymes and DNA's

DNA Polymerase I (Klenow Enzyme), T4 Polynucleotide Kinase and the restriction enzymes AccI, BamHI, DraI, EcoRI, EcoRV, HaeII, HindIII and ScaI were products of Boehringer Mannheim GmbH. Plasmids (pBR322 and pBR328) and phage DNA's (λ, M13mp8 RF and fd109 RF) were also supplied by Boehringer Mannheim GmbH. Adenovirus 2, SV40 and ϕX174 RF DNA's were from BRL.

Heparin-Sepharose CL-6B was purchased from Pharmacia, Ultrogel AcA 54 from LKB, and Cellulose-Phosphate P11 from Whatman. Agarose was from Seaplaque and Low Melting Temperature Agarose, Type VII from Sigma. $\gamma[^{32}P]$ATP, $\alpha[^{32}P]$dATP and $\alpha[^{32}P]$dGTP (all $\sim$3000 Ci/mmol) were from Amersham.

Isolation and purification of DraII and DraIII

Frozen cells (50 g wet weight) were suspended in 100 ml of buffer A (40 mM Tris-HCl, pH 8.0/4°C; 0.1 mM EDTA; 7 mM ß-mercaptoethanol; 10 % (v/v) glycerol) and disrupted by sonification. Cell debris was removed by centrifugation for 45 min at 27.000 x g. The supernatant was fractionated on a Heparin-Sepharose CL-6B column (3 x 10 cm, equilibrated with buffer A) by applying a 0 to 1 M NaCl linear gradient in buffer A. Enzyme activities were eluted at 0.1 - 0.3 M NaCl (DraII) and 0.5 - 0.7 M NaCl (DraIII).

The active fractions of DraII and DraIII were pooled separately and precipitated by adding solid ammonium sulphate to 80 % (w/v) saturation. The precipitates of the two pools were sedimented by centrifugation for 30 min at 27.000 g after standing for 16 hrs at 4°C.

The two precipitates were dissolved in buffer A and fractionated on two Ultrogel AcA 54 columns (2 x 100 cm) in buffer A containing 0.5 M NaCl. The active DraII and DraIII fractions were pooled and dialyzed against buffer A.

DraII was further purified by re-chromatography on a Heparin-Sepharose CL-6B column (1 x 10 cm, equilibrated with buffer A) by applying a 0 to 1 M

NaCl linear gradient in buffer A. DraII activity eluted at 0.1 - 0.3 M NaCl.

DraIII was fractionated on a Cellulose-Phosphate P11 column (1 x 10 cm, equilibrated with buffer A) by applying a 0 to 1 M NaCl linear gradient in buffer A. DraIII activity eluted at 0.3 - 0.5 M NaCl.

The two final pools of DraII and DraIII were dialyzed for 4 hrs against storage buffer (20 mM Tris-HCl, pH 8.0/4°C; 100 mM NaCl; 0.1 mM EDTA; 10 mM ß-mercaptoethanol; 50 % (v/v) glycerol). The enzyme preparations were stored at -20°C.

Starting from 50 g of wet cells the procedure yielded 15.000 U DraIII and 5.000 U DraIII.

Enzyme assay

Either 1-5 µl samples of the various column fractions or, for exact activity determination, appropriate dilutions of the final enzyme preparation were incubated for 60 min at 37°C with 1 µg pBR322 DNA (DraII) or λ DNA (DraI, DraIII) in 25 µl of the incubation mixture containing 10 mM Tris-HCl (pH 8.2/37°C), 10 mM $MgCl_2$, 7 mM ß-mercaptoethanol and 25 mM NaCl (DraI, DraII) or 100 mM NaCl (DraIII). Reactions were terminated by adding 5 µl of stop mixture (7 M urea, 20 % (w/v) sucrose, 60 mM EDTA, 0.01 % (w/v) bromophenol blue). Digests were resolved by electrophoresis on 1 % (w/v) agarose gels using 0.04 M Tris-acetate (pH 8.2/25°C), 2 mM EDTA and 1 µg/ml ethidium bromide as buffer.

One unit is defined as the amount of enzyme that digests 1 µg pBR322 DNA (DraII) or 1 µg λ DNA (DraIII) within 1 h at 37°C under the stated assay conditions.

DNA sequencing

Then nucleotide sequences at the cleavage sites were determined according to Maxam and Gilbert (5).

RESULTS

Optimal conditions for enzyme activity

The temperature optimum for DraII and DraIII is 37°C, whereas the pH optimum for both enzymes is at 8.2. The two enzymes are strictly dependent on Mg^{2+}-ions, but do not require S-adenosyl-methionine or ATP for activity. Optimal concentrations of $MgCl_2$, NaCl and ß-mercatoethanol were determined for the two new enzymes and compared with those for DraI in Table 1.

Determination of recognition sequences

The recognition sequences of DraII and DraIII were determined by mapping their cleavage sites on pBR322 (DraII) and fd109 RF DNA (DraIII) using double

Table 1 Optimal conditions for DraI, DraII and DraIII restriction activities

Parameter	DraI	DraII	DraIII
T (°C)	37	37	37
pH (at 37°C)	7.5	8.2	8.2
$MgCl_2$ (mM)	10	10	10
NaCl (mM)	50	25	100
ß-mercaptoethanol (mM)	7	7	7

digests with known restriction enzymes cutting around these sites.

The mapping data for DraII on pBR322 DNA obtained by analysis of double digestions with DraII and HindIII (pos. 29), ScaI (pos. 3848) or EcoRI (pos. 4361) localized the DraII recognition site 5'-AGGCCCT-3' at pos. 4343. The single DraIII recognition site 5'-CACGTAGTG-3' on fd109 RF DNA at pos. 7598 was determined by double digestion experiments with DraIII and HindIII (pos. 7241), EcoRV (pos. 7401) or HaeII (pos. 7447).

The following palindromic sequences have been identified for DraII and DraIII, respectively, with the help of computer programs designed for the search of new recognition sequences on the basic of the physical mapping data discussed above (P.S. Neumaier, Boehringer Mannheim GmbH, personal communication):

```
        ↓
5'-Pu G G N C C Py-3'        (DraII)
3'-Py C C N G G Pu-5'
           ↑

              ↓
5'-C A C N N N G T G-3'       (DraIII).
3'-G T G N N N C A C-5'
         ↑
```

N stands for any nucleotide, Pu for purines (A,G) and Py for pyrimidines (T,C).

Possible ambiguities in the DraII and DraIII recognition sequences (especially concerning the central N's in both recognition sequences) were resolved by detection of the 42 bp pBR322 DraII-fragment, the 68 bp λ·DraII-fragment as well as the 28 bp λ·DraIII-fragment after digestion of [^{32}P]-labeled pBR322 or λ DNA with DraII and DraIII and subsequent separation of the fragment mixtures on sequencing gels (data not shown).

The results of the double digestion experiments could be confirmed by com-

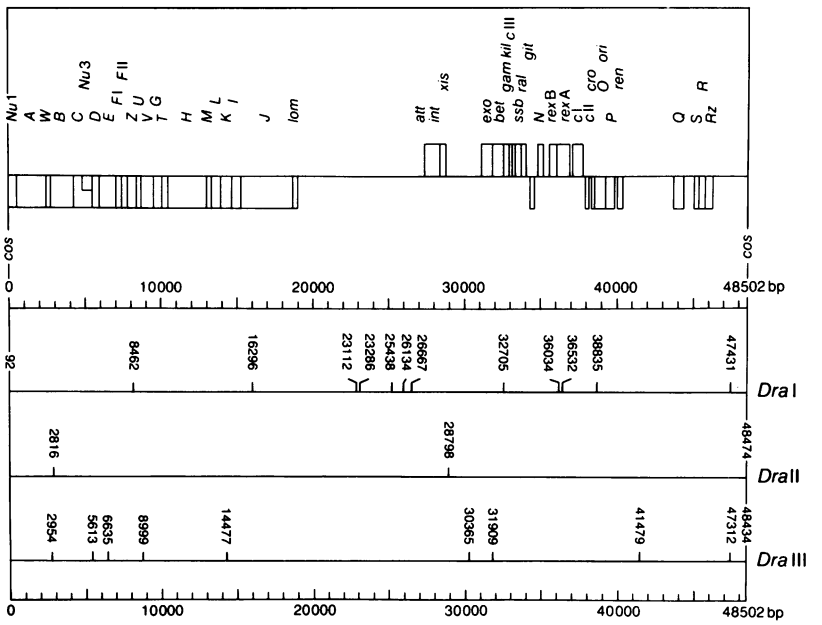

Figure 1 Physical map of bacteriophage λ DNA with the recognition sites of DraI, DraII and DraIII. The coordinate numbers refer to the positions of the nucleotides within the recognition sequences of the (+)-strand 5'-adjacent to the cut. The first 5'-base G of the left single-stranded end 5'-GGGCGGCGACCT-3' of the (+)-strand (1) of the λ genome is counted as position 1. The physical map is headed by genetic information including the reading frames of the 45 genes of presently known functions according to Daniels et al. (22).

parison of the experimentally determined restriction patterns of λ (6) and pBR322 DNA (7,8) with corresponding data derived by computer analyses of the known sequences (9; for experimental data see Fig. 1,2 and Table 2,3).

The sequence specificities were further analyzed by the determination of the frequency of cleavage sites and the resulting fragment lengths generated by DraI, DraII and DraIII on Adenovirus 2 DNA (Ad2; 10), Simian Virus 40 DNA (SV40; 11,12) and fd109 RF DNA (13), as shown in Fig. 2 and Table 3. In addition, the numbers of cleavage sites were determined for the DNA's of ϕX174 RF (14,15), M13mp8 RF (16) and pBR328 (17) as shown in Table 3. As with λ and pBR322 DNA discussed above, the experimentally determined numbers of cleavage sites and the observed fragment lengths are identical with those obtained by computer analyses of the published sequences (17).

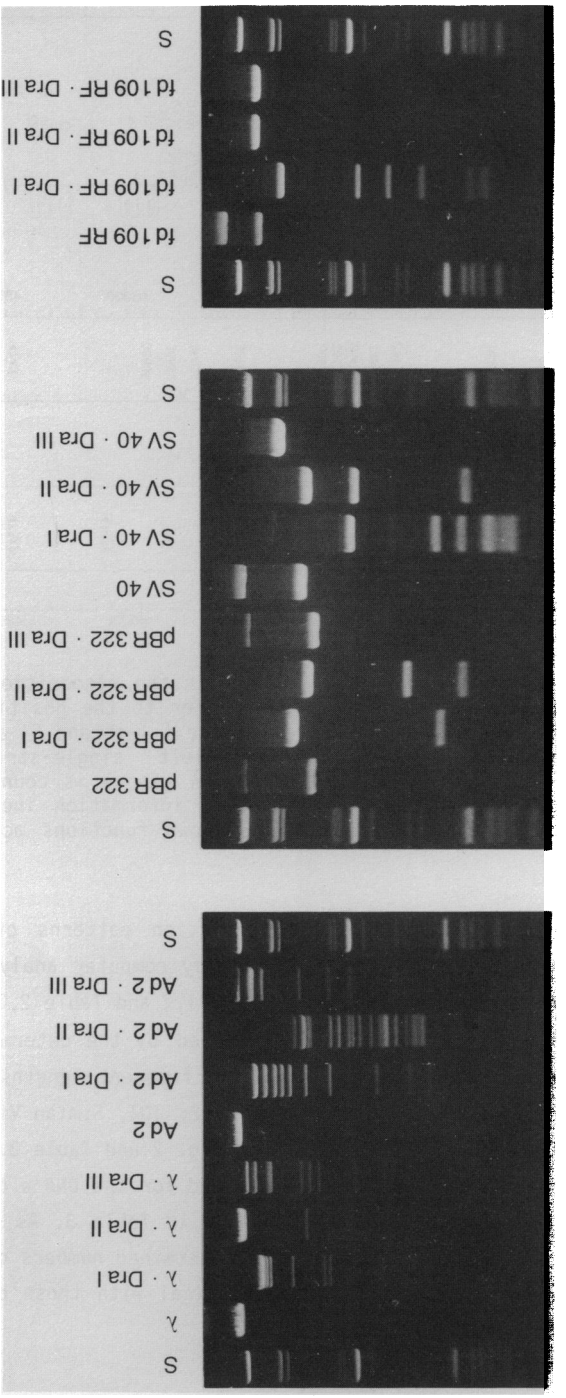

Figure 2 Restriction fragment patterns of various DNA's after digestion with DraI, DraII or DraIII as indicated. EcoRI/HindIII-digested λ DNA was used as DNA molecular weight standard (S). Fragment separation was carried out by electrophoresis under native conditions for 2.5 hrs at 100 V in 1 % (w/v) agarose gels.

Table 2 Comparison of the experimentally determined and computer-derived lengths of fragments (bp) after digestion of λ and pBR322 DNA with DraII or DraIII (E: experimentally determined; C: computer-derived; *: length determined in sequencing gels)

DraII				DraIII			
λ DNA		pBR322 DNA		λ DNA		pBR322 DNA	
E	C	E	C	E	C	E	C
15500	15888	2850	2863	26000	25982	-	-
9600	9570	900	915	20000	19676		
5800	5833	550	543	2800	2816		
5500	5478	42*	42	28*	28		
3000	3022						
2950	2954						
2650	2659						
2350	2364						
1550	1544						
1100	1122						
1000	1022						
68*	68						

Determination of cleavage sites

The cleavage sites of DraII and DraIII were determined according to McConnel at al. (18) as follows (Fig.3):

DraII

pBR322 DNA was linearized with HindIII at position 29 and terminally

Table 3 Number of cleavage sites of the three DraI, DraII and DraIII restriction enzymes on various DNA's

DNA	DraI	DraII	DraIII
λ	13	3	10
Ad2	12	44	10
SV40	12	3	1
ϕX174 RF	2	0	1
fd109 RF	8	1	1
M13mp8 RF	5	0	1
pBR322	3	4	0
pBR328	5	2	0

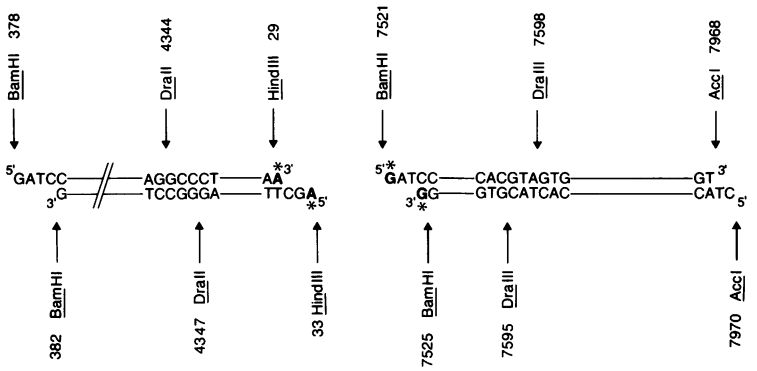

Figure 3 Fragments used for the determination of the cleavage sites within the DraII and DraIII recognition sequences. In both cases, the restriction sites of the applied enzymes are marked by an arrow. The position numbers refer to the nucleotides within the (+)-strand 5'-adjacent to the cuts. Position 0 of pBR322 DNA is the center of the single EcoRI restriction site; position 0 of fd109 RF DNA is the center of the single HpaI restriction site.
Left: pBR322·HindIII/BamHI-fragment used for the determination of the DraII cleavage site. **A** represents [^{32}P]-labeled deoxyadenosine nucleotides at either the 5'-end of the (+)-strand or the 3-end of the (-)-strand of the 4014 bp pBR322·HindIII/BamHI-fragment (fragment length refers to the length of the (+)-strand).
Right: fd109 RF·BamHI/AccI-fragment used for the determination of the DraIII cleavage site. **G** represents [^{32}P]-labeled deoxyguanosine nucleotides at either the 5'-end of the (-)-strand or the 3'-end of the (+)-strand of the 447 bp fd109 RF·BamHI/AccI-fragment (fragment length refers to the length of the (+)-strand).

labeled in two parallel reactions by using Klenow DNA Polymerase I and α[^{32}P]dATP at the 3'-end and T4 Polynucleotide Kinase and γ[^{32}P]ATP at the 5'-end. Each of the labeled fragments was then cut with BamHI at position 378 and the resulting larger 5'- or 3'-labeled pBR322·HindIII/BamHI-fragments (4014 bp) were isolated. Aliquots of the 5'-labeled pBR322·HindIII/BamHI-fragments were chemically modified, cleaved base-specifically, and then subjected to electrophoresis in a denaturing gel. In parallel, the gel was loaded with two samples obtained by additional DraII digestion of both the 5'- or 3'-labeled fragments at position 4344. The results are shown in the left section of Figure 4.

Both the 5'- and 3'-labeled strands of the pBR322·DraII/HindIII-fragment appears beneath the band of the 5'-sequence ladder representing the central G-residue at position 4347 within the DraII recognition sequence 5'-AGGGCCT-3'. However, the chemically derived DNA chain has lost its 3'-terminal G-residue as a consequence of the chemical cleavage. Therefore,

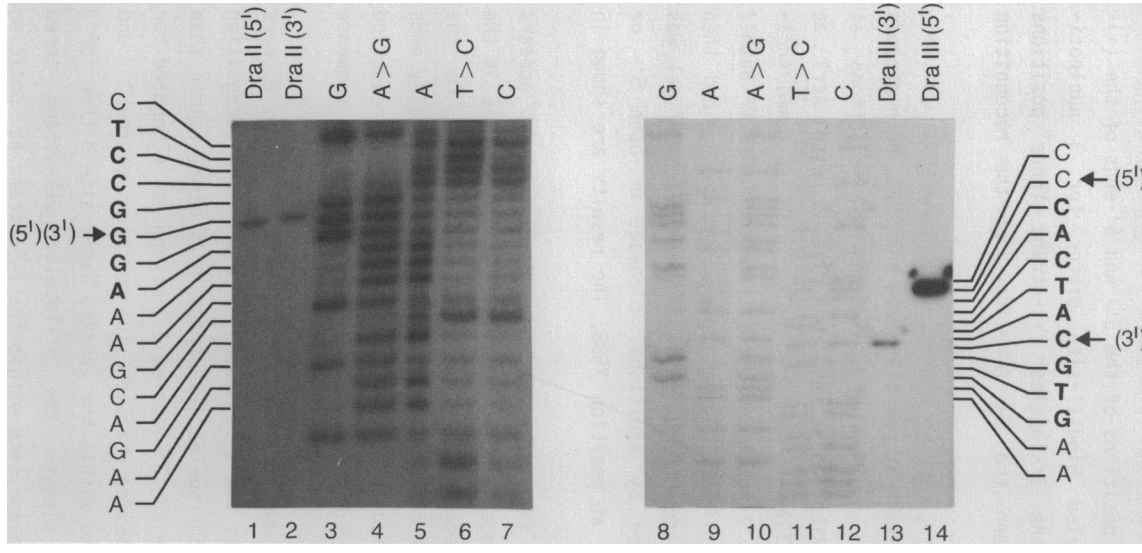

Figure 4 Determination of the cleavage position of DraII on pBR322 DNA (left) and DraIII on fd109 RF DNA (right). Autoradiographs show sequence ladders around DraII (left) and DraIII (right) recognition sites (drawn in bold letters). The aligned 5'- and 3'-labeled fragments have been cleaved either with DraII or DraIII. Labeling of the protruding 5'-ends of the (-)-strand of the pBR322•HindIII-fragment was performed with 2 units of T4 Polynucleotide Kinase and 10 pmol $\gamma[^{32}P]$ATP (~3000 Ci/mmol) per µg DNA according to Maxam and Gilbert (5); labeling of the recessed 3'-ends of the (+)-strand was obtained with 1 unit of Klenow Enzyme and 3 pmol $\alpha[^{32}P]$dATP (~3000 Ci/mmol) per µg DNA according to Rigby et al. (23). Labeling of both strands of the fd109 RF BamHI-fragment was performed under identical conditions with the exception of using $\alpha[^{32}P]$dGTP (~3000 Ci/mmol) for 3'-endlabeling. The labeled fragments were purified by electrophoresis using 1 % (w/v) low melting temperature agarose gels. The sequencing prodedure followed the protocol of Maxam and Gilbert (5). Electrophoresis under denaturing conditions was performed in 6 % (w/v) polyacrylamide gels containing 7 M urea. **Left:** The length of both the 5'- (lane 1) and 3'-labeled strands (lane 2) of the pBR322•HindIII/DraII-fragment was determined by comparing their migration distances with the positions of the bands of the sequence ladder of the 5'-labeled (-)-strand of the pBR322•HindIII/BamHI-fragment (lanes 3-7; see also Fig. 3, left). **Right:** The length of both the 3'- (lane 13) and 5'-labeled strands (lane 14) of the fd109 RF • BamHI/DraIII-fragment were determined by comparing their migration distances with the positions of the bands of the sequence ladder of the 3'-labeled (-)-strand of the fd109 RF•BamHI/AccI-fragment (lanes 8-12; see also Fig. 3, right).

this DNA chain actually ends with its 5'-neighbouring G-residue at position 4348. DraII thus cleaves the 5'-labeled (-)-strand at position 4347 between the two G-residues in the recognition sequence 3'-TCCCG↑GA-5'.

Because the 3'-labeled (+)-strand was elongated for a single A-residue during the labeling reaction, the positions of the 3'- and 5'-ends of the (+)- and (-)-strand within the labeled HindIII-site differ for 3 nucleotides. DraII therefore cleaves the 3'-labeled (+)-strand three positions upstream at position 4344 between the two G-residues of the recognition sequence 5'-AG↓GGCCT-3'.

DraIII

fd109 RF DNA was cleaved with BamHI at position 2220 and 7521. Both strands of the isolated 2983 bp fragment were labeled in two parallel reactions at both the 5'- and 3'-ends as described above, and then cleaved with AccI at position 7967. The resulting smaller 5'- and 3'-labeled fd109 RF•BamHI/AccI-fragments (447 bp) were isolated. Aliquots of the 3'-labeled fd109 RF•BamHI/AccI-fragment were chemically modified, cleaved base-specifically, and then subjected to electrophoresis in denaturing gels. In parallel, the gel was loaded with two samples obtained by additional cleavage of both 5'- or 3'-labeled fragments with DraIII at position 7598. The results are shown in the right section of Figure 4.

The 3'-labeled (-)-strand of the fd109 RF•DraIII/BamHI-fragment appears beneath the band of the 3'-sequence ladder representing the C-residue in the central triplett of the DraIII recognition sequence 3'-GTGCATCAC-5'. For the reasons discussed above, this DNA chain of the sequence ladder actually ends with its 3'-neighbouring G-residue. DraIII thus cleaves the 3'-labeled (-)-strand at position 7595 between the G and C of the recognition sequence 3'-GTG↑CATCAC-5'.

The 5'-labeled (+)-strand of the fd109 RF•DraIII/BamHI-fragment appears beneath the band, which represents the C-residue flanking of the recognition sequence. This means, that the 5'-labeled (+)-strand is 6 bases longer than the 3'-labeled (-)-strand. Because the 3'-labeled (-)-strand was elongated for a single G-residue during the labeling reaction, the positions of the 3'- and 5'-ends of the (+)- and (-)-strand within the labeled BamHI-site differ for 3 nucleotides. DraIII therefore cleaves the 5'-labeled (+)-strand three positions downstream of the cleavage site within the (-)-strand at position 7598 between the C- and G-residue of the recognition sequence 5'-CACGTA↓GTG-3'.

DISCUSSION

In addition to DraI, described earlier, two new Type II restriction endonucleases with novel site-specificities have been purified from Deinococcus radiophilus ATCC 27603.

Due to the amount of activity present in the final enzyme preparation, DraI is the dominant enzyme species. In contrast, DraII and DraIII are isolated in smaller quantities and therefore represent minor enzyme activities. However, as well as with DraI, the properties of the two new enzymes, DraII and DraIII, are characteristic for Type II restriction endonucleases. These characteristics are their absolute dependence on Mg^{2+}-ions, their independence of the cofactors ATP and S-adenosyl-methionine, and their symmetrical cleavage within recognition sequences of dyad symmetry.

In contrast to DraI, which recognizes the unambiguously defined hexanucleotide palindrome 5'-AAA$^{\downarrow}$TTT-3', DraII and DraIII both act on more complex recognition sequences characterized by longer extensions and internal $(N)_x$-sequences. Unlike the DraI recognition site only consisting of A:T base-pairs, DraII and DraIII recognize sequences including A:T as well as G:C base-pairs. Furthermore, DraI-cleavage results in blunt-ended termini, whereas DraII and DraIII create fragments with 5'- or 3'-protruding termini.

The G:C-rich heptanucleotide DraII recognition sequence

5'-Pu G$^{\downarrow}$G N C C Py-3'

may be of special interest, because it is the first example of a recognition site of the type A B C $(N)_x$C'B'A' characterized by ambiguously defined base-pairs within the flanking trinucleotide sequences (2,3). Thus, the cleavage frequences of DraII on DNA's of bacteriophage λ or plasmid pBR322 are between those of e.g. DdeI (19) and SauI (20) recognizing the penta- and heptanucleotide sequences 5'-C$^{\downarrow}$TNAG-3' and 5'-CC$^{\downarrow}$TNAGG-3' within unambiguously defined flanking di- and trinucleotides.

Because DraII generates fragments with 5'-protruding ends, these termini may serve as good substrates for the effective incorporation of a radioactive label at the 5'-ends with T4 Polynucleotide Kinase or at the 3'-ends with Klenow DNA Polymerase I. Alternative end-labeling of both DNA strands may be of advantage in chemical DNA sequence analysis, because both strands of a DNA fragment can be sequenced in parallel.

The DraIII recognition sequence

5'-C A C N N N$^{\downarrow}$G T G-3'

is similar to the type already described in the case of TteI and TtrI which

recognize the sequence 5'-GACNNNGTC-3' (21). However, unlike with DraIII the cleavage position of these enzymes is still unknown. The 3'-termini of DraIII-fragments may be efficiently labeled by tailing the single-stranded protruding ends with the aid of Terminal Transferase.

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