ABSTRACT

In addition to recently characterized Dral (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

\[
\begin{align*}
5'\text{-Pu G G N C C Py-3'} & \quad (\text{DraII}) \\
3'\text{-Py C C N G G Pu-5'} \\
\end{align*}
\]

and

\[
\begin{align*}
5'\text{-C A C N N N G T G-3'} & \quad (\text{DraIII}) \\
3'\text{-G T G N N N C A C-5'} \\
\end{align*}
\]

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 specificities 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 Drall and DrallI.

MATERIALS AND METHODS

Bacterial strain and culture conditions

D. radiophilus ATCC 27603 belongs to the family of Deinococcaceae. 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, HaelI, HindIII and Scal 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. [^32P]ATP, [^32P]dATP and [^32P]dGTP (all ~3000 Ci/mmol) were from Amersham.

Isolation and purification of Drall and DrallI

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 β-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 (DrallI) and 0.5 - 0.7 M NaCl (DrallIII).

The active fractions of DrallI and DrallIII 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 DrallI and DrallIII fractions were pooled and dialyzed against buffer A.

Drall 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 Pll 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 β-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 DraII.

**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 MgCl₂, 7 mM β-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 hr 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 Mg²⁺-ions, but do not require S-adenosyl-methionine or ATP for activity. Optimal concentrations of MgCl₂, NaCl and β-mercaptoethanol 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 Dral, Drall and DraIII restriction activities

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Dral</th>
<th>Drall</th>
<th>DraIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>T (°C)</td>
<td>37</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>pH (at 37°C)</td>
<td>7.5</td>
<td>8.2</td>
<td>8.2</td>
</tr>
<tr>
<td>MgCl₂ (mM)</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>NaCl (mM)</td>
<td>50</td>
<td>25</td>
<td>100</td>
</tr>
<tr>
<td>B-mercaptoethanol (mM)</td>
<td>7</td>
<td>7</td>
<td>7</td>
</tr>
</tbody>
</table>

digests with known restriction enzymes cutting around these sites.

The mapping data for DraIl on pBR322 DNA obtained by analysis of double digests with Drall and HindIII (pos. 29), Scal (pos. 3848) or EcoRI (pos. 4361) localized the DraIl 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 Drall and DraIII, respectively, with the help of computer programs designed for the search of new recognition sequences on the basis of the physical mapping data discussed above (P.S. Neumaier, Boehringer Mannheim GmbH, personal communication):

\[
\begin{align*}
5'-'Pu & G & G & N & C & C & Py-3' \\
3'-'Py & C & C & N & G & G & Pu-5'
\end{align*}
\]  
(Dral)

\[
\begin{align*}
5'-'C & A & C & N & N & G & T & G-3' \\
3'-'G & T & G & N & N & C & A & C-5'
\end{align*}
\]  
(DraIII).

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 [32p]-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 Dral, Drall and Dralll. 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).

Comparison 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 Dral, Drall and Dralll on Adenovirus 2 DNA (Ad2; 10), Simian Virus 40 DNA (SV4C; 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 Dral, DraliI or DraII as indicated. EcoRI/HindIII-digested λ DNA was used as DNA molecular weight standard. 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 Drall or DraIII (E: experimentally determined; C: computer-derived; *: length determined in sequencing gels)

<table>
<thead>
<tr>
<th></th>
<th>Drall A DNA</th>
<th>pBR322 DNA</th>
<th>Drall pBR322 DNA</th>
<th>DraIII A DNA</th>
<th>pBR322 DNA</th>
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<tbody>
<tr>
<td>E</td>
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<td>1000</td>
<td>1022</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>68*</td>
<td>68</td>
<td></td>
<td></td>
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<td></td>
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</tbody>
</table>

Determination of cleavage sites

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

DraIII

pBR322 DNA was linearized with HindIII at position 29 and terminally

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

<table>
<thead>
<tr>
<th>DNA</th>
<th>Dral</th>
<th>Drall</th>
<th>DraIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ</td>
<td>13</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Ad2</td>
<td>12</td>
<td>44</td>
<td>10</td>
</tr>
<tr>
<td>SV40</td>
<td>12</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>φX174 RF</td>
<td>2</td>
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<td>1</td>
</tr>
<tr>
<td>fd109 RF</td>
<td>8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>M13mp8 RF</td>
<td>5</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>pBR322</td>
<td>3</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>pBR328</td>
<td>5</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 3 Fragments used for the determination of the cleavage sites within the Drall and DralII 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 Drall cleavage site. A represents [32P]-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 DralII cleavage site. G represents [32P]-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-[32P]dATP at the 3'-end and T4 Polynucleotide Kinase and δ-[32P]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 Drall 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·DralII/HindIII-fragment appears beneath the band of the 5'-sequence ladder representing the central G-residue at position 4347 within the Drall 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 αL-[32P]dATP (~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 αL-[32P]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 αL-[32P]dGTP (~3000 Ci/mmol) for 3'-end labeling. The labeled fragments were purified by electrophoresis using 1% (w/v) low melting temperature agarose gels. The sequencing procedure 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/AceI-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 triplet 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'.

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DISCUSSION

In addition to Dral, 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, Dral is the dominant enzyme species. In contrast, DralI and DralII are isolated in smaller quantities and therefore represent minor enzyme activities. However, as well as with Dral, the properties of the two new enzymes, DralI and DralII, 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 Dral, which recognizes the unambiguously defined hexanucleotide palindrome 5'-AAA'TTT-3', DralI and DralII both act on more complex recognition sequences characterized by longer extensions and internal $W_{x}$ sequences. Unlike the Dral recognition site only consisting of A:T base-pairs, DralI and DralII recognize sequences including A:T as well as G:C base-pairs. Furthermore, Dral-cleavage results in blunt-ended termini, whereas DralI and DralII create fragments with 5'- or 3'-protruding termini.

The G:C-rich heptanucleotide DralI recognition sequence

$$5'-'Pu G^*G N C C Py-3'$$

may be of special interest, because it is the first example of a recognition site of the type $A B C (N)xC'B'A'$ characterized by ambiguously defined base-pairs within the flanking trinucleotide sequences (2,3). Thus, the cleavage frequencies of DralII 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$^t$TNAG-3' and 5'-CC$^t$TNAGG-3' within unambiguously defined flanking di- and trinucleotides.

Because DralI 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 DralII recognition sequence

$$5'-C A C N N N^*G T G-3'$$

is similar to the type already described in the case of TtEI and TtII which
recognize the sequence 5′-GACNGTGTC-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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