A site specific endonuclease from *Thermus thermophilus* 111, Tth111I

Takahisa Shinomiya and Showbu Sato

Mitsubishi-Kasei Institute of Life Sciences, Minamiooya, Machida, Tokyo 194, Japan

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ABSTRACT

A site specific endonuclease with novel specificity has been isolated from *Thermus thermophilus* strain 111 and named Tth111I. Tth111I cleaves λ DNA into three fragments of 23.5, 25.7 and 50.8 % of the total length, and ColEI DNA into two fragments of nearly equal length. The sequences around Tth111I cleavage sites of ColEI and λ DNA were determined by the Maxam and Gilbert method and the two dimensional mapping method. The results suggest that Tth111I recognizes the DNA sequence 5'-TGACNNNGTC-3' and cleaves the

3'-ACTGNNNCAG-5'

site as indicated by the arrows. Assuming that the first T.A pair in the sequence can be replaced for any base pair, the Tth111I recognition sequence has the symmetry with the two-fold axis as most type II restriction endonucleases do.

INTRODUCTION

Endonucleases that recognize specific base-paired sequences in DNA have been purified from various microorganisms[1]. Site specific endonucleases of this type (type II restriction endonucleases) are indispensable for dissection of genomes and for DNA sequencing[2]. Enzymes with new specificity are still required for these studies. Enzymes from an extreme thermophile, *Thermus thermophilus*, are exceptionally stable to heat and to protein denaturing reagents[3,4]. We have been screening thermostable restriction endonucleases from this species that would give aid in the study of DNA structure and of the mode of action of restriction endonucleases. We report here on the partial purification of a new site specific endonuclease, Tth111I, from *T. thermophilus* 111. The nucleotide sequences around the cleavage sites are also described.

MATERIALS AND METHODS

Materials and strain: *T. thermophilus* 111 isolated from a Japanese thermal spa was a generous gift from Dr. T. Oshima of this institute. The

cells were grown at 75°C in 20 liters of the culture medium using a jar fermenter with vigorous aeration and harvested at the late log phase as described for Thermus thermophilus HB8[5,6]. About 300g(wet weight) of cells were obtained from 80 liters of the culture fluid and kept at -80°C.

HaeIII, HhaI and HpaII were obtained from New England Biolab Inc. EcoRI, HindIII and T4 polynucleotide kinase were purchased from Boeringer-Mannheim Corp. Bacterial phosphatase(TypeIII-R) from Sigma Chemical Company was heated at 85°C for 10 min. Venom phosphodiesterase and pancreatic DNase were purchased from Worthington Biochemical Corporation. [γ -³²P]ATP was a product of Radiochemical Center, Amersham. Cellulose acetate membrane(2.5 × 40 cm) was obtained from Schleicher and Schüll. DEAE-cellulose thinlayer(Polygram cel 300 DEAE/HR-2/15) and PEI-cellulose thinlayer(Polygram cell 300 PEI) were products of Machery-Nagel+Co. Yeast RNA was purchased from P-L Biochemicals Inc.

DNA preparations: Bacteriophage λ was prepared by heat induction of CI₈₅₇S7 prophage and its DNA was obtained by phenol extraction from phage particles banded in a CsCl gradient[7]. ColE1 was isolated from E. coli JC411 thy⁻(favored by Dr. T. Tanaka) cultured in the presence of chloramphenicol[8].

Digestion of DNA and gel electrophoresis: DNA(1 μ g) was digested with Tth111I in 25 μ l of 8 mM Tris-HCl(pH7.4)/50 mM NaCl/8 mM 2-mercaptoethanol/8 mM MgCl₂ for 1 h at 65°C. Reactions were stopped by adding 10 μ l of stop mixture(100 mM EDTA in 30 % sucrose containing 0.1 % bromphenol blue). The samples were analysed by electrophoresis in Tris-borate-EDTA[9] on horizontal slabs consisting of 0.2 % [10] or 0.7 % agarose gel. One unit of enzyme activity was defined as the amount of enzyme required for complete digestion of 1 μ g of λ DNA in 1 h at 65°C. For separation of smaller fragments of DNA produced by digestion with restriction endonucleases, 5 % polyacrylamide slab gels (14 × 15 × 0.15 cm) in Tris-borate-EDTA were used. Gels were stained with ethidium bromide(0.5 μ g/ml in the Tris-borate-EDTA buffer) for 20-30 min and photographed under UV light with a red filter on the lens[11]. For autoradiography, gels were covered with Saranwrap and exposed to X-ray films.

The sequence analysis of cleavage sites: For the analysis of the DNA sequences around cleavage sites of Tth111I, we used the method of Maxam and Gilbert[12]. The two dimensional homochromatography developed by Brownlee and Sanger[13] was also used to identify 5' terminal sequences with some modifications described by Tu et al.[14]. Details of these procedures are given in Fig. 4 through 5 with interpretation of the results.

5' end labeling of restricted fragment: For the analysis of the recognition sites, DNAs were digested with restriction enzymes and the 5' end was phosphorylated as follows. The fragment of DNA(5-50 μ g) was dissolved in 100 μ l of 20 mM Tris-HCl(pH8.9)/1 mM $MgCl_2$. One microliter of alkaline phosphatase(6 μ g) was added and the reaction mixture was incubated for 1 h at 37°C. After extraction with aqueous phenol, the DNA fragment was precipitated with two volumes of ethanol, chilled in dry-ice ethanol for 10 min, recovered by centrifugation(12,000g for 10 min), and dried under vacuum. The dephosphorylated DNA fragment was dissolved in 50 μ l of 5 mM Tris-HCl(pH9.5)/0.1 mM EDTA /1 mM spermidine, heated at 100°C for 3 min and chilled in ice water. Then 10 μ l of 500 mM Tris-HCl(pH9.5)/50 mM dithiothreitol and 1 μ l of 1 M $MgCl_2$ were added. The DNA was phosphorylated in a final volume of 100 μ l by addition of 200 pmol of [γ - 32 P]ATP(specific activity 800 Ci/mmol) and 2 units polynucleotide kinase. After incubation at 37°C for 1 h, the reaction mixture was supplemented with 100 μ l of 4 M ammonium acetate and the DNA was precipitated with 600 μ l of ethanol.

RESULTS

Isolation of Tth111I: Frozen cells(120 g) of *T. thermophilus* 111 were thawed in 240 ml of 20 mM Tris-HCl buffer(pH 8.0) containing 1 mM EDTA and 1 mM 2-mercaptoethanol(buffer A) and disrupted by sonication. After centrifugation at 100,000 g for 90 min, the supernatant was fractionated with ammonium sulfate precipitation. The precipitate between 30-85 % saturation was collected by centrifugation and dialyzed against buffer A. The dialysate was applied to a column(2.6 $\times$ 40 cm) of DEAE-cellulose(Whatman DE-23) previously equilibrated with buffer A. The DEAE-cellulose was washed with 300 ml of buffer A containing 0.1 M NaCl and the endonuclease activity was eluted with 0.4 M NaCl in buffer A. The eluate was brought to 85 % saturation with solid ammonium sulfate. The precipitate was collected by centrifugation and dialysed against buffer A. The dialysate was applied to a column(2 $\times$ 30 cm) of Heparin-Sepharose 4B(prepared according to the method described by Iverius [15] and Bickel et al.[16]) previously equilibrated with buffer A. The column was developed with 1,000 ml of NaCl gradient (0.0-1.0 M in buffer A). The enzyme activity appeared as a single peak at about 0.7 M NaCl. The fractions of the endonuclease were pooled(50 ml), dialysed against 30 mM sodium phosphate buffer(pH7.5) and subjected to electrofocusing in a LKB column(110 ml) of 1 % Ampholine(pH8.0-9.5, LKB carrier ampholite) with a glycerol density gradient. Electrofocusing was run at 200 V for 14 h and then at 600V for

32 h. Active fractions were pooled, dialysed against buffer A containing 50 % glycerol and 0.2 M NaCl, and stored at -20°C . The preparation of the enzyme at this step was free from contamination of other non-specific nucleases, judging from clear and unchanged banding pattern of ColE1 DNA after incubation with an excess of the enzyme for prolonged period. Nicking activity was not observed when the super helical ϕX174 RFDNA was incubated with the purified enzyme preparation, while the DNA had no cleavage site of the enzyme. The enzyme was named Tth111I after the recommendation of Smith and Nathan[17].

The total amount of the enzyme in the final step of the purification was 600,000 units, that is, an amount enough to digest 600 mg of λ DNA for 1 h at 65°C completely. The optimal temperature for the enzyme activity was between 60°C and 70°C . The enzyme activity decreased at 75°C , and was less than one tenth of the maximum at 37°C . The optimal condition for enzyme activity was 6-10 mM MgCl_2 and 50-100 mM NaCl. The enzyme activity was lost when the reaction mixture was depleted with MgCl_2 or supplemented with NaCl of 0.2 M or more. The enzyme did not lose the activity after storage for at least three months at -20°C or 4°C . Repetition of freezing and thawing decreased the activity. Heat treatment at 100°C for 3 min inactivated Tth111I completely.

Cleavage site of Tth111I on λ DNA and ColE1 DNA: When λ DNA digested with Tth111I was analysed on 0.2 % agarose gel(Fig. 1a), three bands appeared. Sizes of the fragments were estimated to be about 15-16, 8 and 7 megadaltons by comparing the electrophoretic mobility with λ DNA fragments produced by HindIII digestion. To determine Tth111I cleavage sites on λ DNA, double digestion with EcoRI or HindIII was carried out. In the double digest with EcoRI and Tth111I(Fig. 1b), EcoRI A and B fragments(14 and 4.5 megadaltons [21]) and Tth111I A and B fragments (the largest and the second largest fragments) disappeared and three new fragments, b, f and h, appeared. The sizes of the three fragments were 6.8, 2.8 and 1.9 megadaltons. The two smaller fragments, f and h, seemed to be derived from the EcoRI B fragment. The fragment b derived from the EcoRI A fragment and its counterpart in EcoRI A fragment had the identical mobility with the smallest fragment of the single Tth111I digest. When λ DNA was digested with both HindIII and Tth111I (Fig. 1c), the HindIII A and B fragments disappeared. It seems that the HindIII B fragment was cleaved by Tth111I into the fragment c and a small fragment not observed in the figure (less than one megadaltons) and that the HindIII A fragment was cleaved into two fragments, a and b, in the double

Fig. 1. Gel electrophoresis of λ DNA digests. (a) TthlIII digest of λ DNA, lane 1 and 1'. HindIII digest of λ DNA, lane 2 and 2'. The samples were analysed on a slab of 0.2 % agarose gel. (b) Double digestion with TthlIII and EcoRI. Lane 3, without enzyme; lane 4, TthlIII; lane 5 and 5', TthlIII plus EcoRI and lane 6 and 6', EcoRI. (c) Double digestion with TthlIII and HindIII. Lane 7, TthlIII; lane 8 and 8', TthlIII plus HindIII and lane 9 and 9', HindIII. In (b) and (c), the samples were analysed on a slab of 0.7 % agarose gel.

digest. The fragment b had similar mobility with the smallest fragment in the single TthlIII digest. Taking into account the sizes of the TthlIII fragments and the results of double digestion, we have concluded that the cleavage sites of TthlIII are positioned at 23.5 and 74.3 % of the total length of λ DNA (Fig. 3a).

When ColE1 DNA was digested with TthlIII, two close bands, A and B, corresponding to fragments of 2.2 and 2.1 megadaltons, were observed (Fig. 2a). In the double digest with TthlIII and EcoRI, two fragments of 1.1 and 0.9 megadaltons (B and C) came out instead of the smaller fragment in the single TthlIII digest. When ColE1 was doubly digested with TthlIII and HaeIII, fragments A and E in the digest with HaeIII only disappeared and four new fragments, A1, A2, E1 and E2, appeared, instead (Fig. 2b). The molecular sizes of the fragments A, E, A1, A2, E1 and E2 were estimated to be 1,100,

Fig. 2. Double digestion of ColE1 DNA. The samples were analysed on a slab of 0.7 % agarose gel in (a) and 5 % polyacrylamide gel in (b) through (d). Lane 1, EcoRI; lane 2 and 2', TthlIII plus EcoRI; lane 3 and 3', TthlIII; lane 4 and 4', TthlIII plus HaeIII; lane 5 and 5', HaeIII; lane 6 and 6', TthlIII plus HpaII; lane 7 and 7', HpaII; lane 8 and 8', TthlIII plus HhaI; lane 9 and 9', HhaI.

410, 1030, 70, 210 and 190 base pairs, respectively, by means of the plot of electrophoretic mobility vs. molecular size of known restriction fragments. From the molecular size, it is apparent that the HaeIII fragment A was cut by TthlIII into the fragments A1 and A2, and the HaeIII fragment E into the fragment E1 and E2. The cleavage sites of TthlIII on ColE1 DNA were determined from these data and shown in Fig. 3b.

In the double digest with HpaII and TthlIII(Fig. 2c), the fragment D in the HpaII single digest disappeared and a new fragment which was about 30 base pairs smaller than the fragment D appeared. However, the other HpaII fragments cleaved by TthlIII could not be detected in this system. As for the double digestion with HhaI and TthlIII(Fig. 2d), the fragment H and K in the HhaI single digest were cleaved by TthlIII. The fragmented products could not be identified, because they were overlapped by the fragments produced by the single digestion with HhaI.

SacI, SalGI and KpnI out of all known restriction enzymes gave two cuts in λ DNA. SacI or SalGI cleaves λ DNA at different sites[19] from the TthlIII sites and there is no KpnI site in ColE1 DNA[20]. The results described above show that TthlIII is an enzyme with new specificity.

Identification of the 5' terminal mononucleotides of TthlIII fragments of ColE1 DNA: ColE1 DNA(40 μ g) was incubated in a reaction mixture(700 μ l)

Fig. 3. TthlIII cleavage sites on λ and ColE1 DNA. Cleavage sites by EcoRI or HindIII for λ DNA and EcoRI or HaeIII for ColE1 DNA are also shown for reference.

containing 8 mM 2-mercaptoethanol, 8 mM Tris-HCl(pH 7.4), 8 mM MgCl₂ and 100 units TthIII for 1 h at 65°C. The resulting fragments were labeled with ³²P at 5' ends as described in Materials and Methods and subjected to electrophoresis on a 5 % polyacrylamide slab gel to remove unreacted [γ -³²P]ATP. The two labeled fragments (corresponding to bands A and B in Fig. 2a lane 3) were eluted together by electrophoresis (2 mA per tube for 12 h) from the excised segments of the gel into a dialysis bag. The ³²P-labeled DNA fragments were digested with pancreatic DNase I, followed by digestion with venom phosphodiesterase. The reaction mixture was mixed with standard four 5'-deoxymononucleotides and chromatographed on a PEI-cellulose thin layer with 10 % acetic acid/1.4 % pyridine(pH 3.6)[22]. ³²P-labeled nucleotides were identified by comparing UV absorbance of the standard nucleotide with the spots on the autoradiogram. No spot other than 5'-deoxymononucleotides was detected on the autoradiogram. The spots were cut out and measured in radioactivity by G-M counter. The percentage of radioactivity found in pdC, pdG, pdT and pdA was 42, 54, 3 and 1, respectively. This indicated that 5'-terminal nucleotide of TthIII restriction fragments of ColE1 DNA was pdC or pdG, suggesting a complicated sequence specificity of TthIII.

Determination of nucleotide sequence around TthIII cleavage sites of ColE1 DNA using the technique of Maxam and Gilbert: TthIII cleaves the HpaII-D fragment and the HhaI-K fragment as shown above(Fig. 2c and 2d). We have applied the method of Maxam and Gilbert to these fragments for determination of the nucleotide sequence recognized and cleaved by TthIII.

ColE1 DNA(50 μ g) was digested with HpaII(100 units) for 1 h at 37°C and separated by 5 % polyacrylamide gel electrophoresis. The fragment D was eluted from the gel, labeled with ³²P at both 5' ends and digested with HhaI. Two radioactive fragments labeled with ³²P at one end were obtained by gel electrophoresis, one of which changed in electrophoretic mobility by the action of TthIII. The radioactive fragment containing the TthIII site was subjected to the chemical degradation for sequencing and analysed by electrophoresis(Fig. 4a). The sequence, 5'--AGTGACAGTGTCCCATAG--3', can be read out around the site corresponding to the molecular size of the TthIII fragment (Fig. 4a, lane 1). The TthIII product had nearly the same mobility as a fragment cleaved at the 5' side of the G in the center of the sequence, 5'--GACAGTGTG--3'. A fragment which appeared as a result of chemical cleavage at G has lost that G and ended with the nucleotide to the 5' side of that G in this case A. Therefore TthIII was suggested to cleave this strand at the 5' side of the central G of this sequence. The TthIII product moved more

Fig. 4. The sequence around the TthIII cleavage site in the HpaII E fragment of ColEI DNA (a), and in the HhaI K fragment (b). A single fragment in lane 1 and 6 was produced by TthIII. Fragments in lane 2, 3, 4, 5, 7, 8, 9 and 10 were produced by chemical cleavage, A>G, G>A, C, C>T, A>G, G>A, C and C>T respectively.

slowly than the corresponding chemical product, presumably because it lacked the 3' terminal phosphate which remains after chemical cleavage.

A similar experiment was carried out for the K fragment of ColE1 DNA produced by HhaI. In this case HpaII was used to obtain the fragment labeled with ³²P at one of its two 5' ends. The results shown in Fig. 4b suggest that TthIII cleaves the sequence 5'--CTGACCCAGTTCAC--3' at the site indicated

by the arrow. The sequence around the TthlIII site on the HpaII D fragment represents one of the two TthlIII cleavage sites on ColE1 DNA and that on the HhaI K fragment, the other. Taking account of base pairing and the results of the experiment of 5' terminal nucleotides of these fragments, TthlIII seems to cleave the sequences at the sites,

indicated by the arrows. To confirm this conclusion, the two dimensional homochromatography method was carried out.

Two dimensional mapping of 5' terminal sequence of TthlIII restriction fragments: HaeIII fragment A and E are cleaved by TthlIII into fragment A1 and A2, E1 and E2, respectively (see Fig. 3b). 5' terminal sequences of fragment A1, A2, E1 and E2 were analysed by two dimensional homochromatography.

ColE1 DNA (50 μ g) was digested with 100 units of TthlIII for 1 h at 65°C in 750 μ l of the reaction mixture. The DNA fragments were labeled with ^{32}P at the 5' ends and digested with 100 units of HaeIII in 100 μ l of the mixture (6 mM Tris-HCl (pH 7.4)/6 mM 2-mercaptoethanol/6 mM MgCl_2 /6 mM NaCl) for 2 h at 37°C. The digest was electrophoretically separated on a 5 % acrylamide slab gel. The gel was stained with ethidium bromide and exposed to a sheet of X-ray film. The radioactivity was found in only the bands corresponding to fragment A1, A2, E1 and E2. Each ^{32}P -labeled fragment was eluted by electrophoresis and subsequently digested with pancreatic DNase (5 μ g) in a reaction mixture (10 μ l) containing 20 mM Tris-HCl (pH 7.4) and 10 mM MgCl_2 for 30 min at 37°C. One fifth of the mixture was removed and incubated in fresh mixture (3 μ l) containing 33 mM Tris-HCl (pH 8.9), 33 mM MgCl_2 and 1 μ g venom phosphodiesterase for 30 min at 37°C. The reaction mixture was heated at 90°C for 10 min to inactivate the enzyme. One half of this mixture was analyzed for determination of 5' terminal nucleotide by the PEI-cellulose thin layer chromatography as mentioned above. The other half was combined with the rest of the pancreatic DNase digest and an aliquot (5,000-10,000 cpm) was electrophoresed on cellulose acetate at pH 3.5 for the first dimension, followed by homochromatography on DEAE-cellulose thin layer using homomix V [26] for the second dimension. The autoradiograms of the resulting fingerprints are shown in Fig. 5. 5' terminal nucleotide analysis showed that fragments A1, A2, E1 and E2 had radioactive pdG, pdC, pdG and pdC, respectively. 5' terminal sequences were deduced from the mobility shifts [14] of 5' ^{32}P -labeled oligonucleotides. A common sequence GTC is found in each fragment as follows; A1, 5'pGTGTCC-; A2, 5'pCTGTCACTT-; E1, 5'pGGGTCA-; E2,

Fig. 5. Two dimensional maps of the partial pancreatic DNase and venom phosphodiesterase digestion products of 5' terminal labeled fragments. (a) to (d), Tth1111 cleavage sites on HaeIII A and E fragments of ColE1 DNA. (a), fragment A1; (b), fragment A2; (c), fragment E1; (c) fragment E2. (e) and (f), Tth1111 cleavage sites on EcoRI B fragment of λ DNA.

pCAGTCACGT-. On the basis of 5' terminal sequences of A1, A2, E1 and E2 fragments, it is deduced that Tth1111 cleaves the fragment A and fragment E at the sites in the following sequences indicated by the arrows,

5'-AGTGACAGTGTCC-3' and 5'-ACGTGACTGGGTCA-3' , respectively.
 3'-TCACTGTTCACAGG-5' 3'-TGCACTGACCCAGT-5'

This conclusion is consistent with the above results obtained by the method of Maxam and Gilbert.

To make the Tth111I recognition sequence more unequivocal, one of the two cleavage sites of λ DNA was sequenced by the two dimensional mapping. λ DNA(50 μ g) was digested with EcoRI(200 units) at 37°C for 1 h. The fragment B (see Fig. 1b) was isolated by electrophoresis on a 0.7 % agarose gel. The gel containing fragment B was dissolved in 1.5 volumes of 5 M NaClO₄ at 65°C 30 min and purified by hydroxylapatite column chromatography[27]. The fragment B was digested with Tth111I, labeled with ³²P and completely digested with HaeIII. The resulting four radioactive fragments were isolated by electrophoresis and subjected to the two dimensional mapping as described above. Two of the four fragments had 5' terminal sequences of EcoRI site. 5' terminal sequences of other two fragments were pATGTCG- and pTTGTCA-(Fig. 5 e and f). This suggests that Tth111I cleaves the sequence 5'-TGACAATGTCG-3' as indicated by the arrows. From the above three examples of sequencing around the Tth111I cleavage site, we concluded that Tth111I recognizes the following sequence and cleaves the site indicated by the arrows, 5'-NNTGACNNNGTCNN-3' .

DISCUSSION

A new site specific endonuclease Tth111I which can be classified into type II restriction endonuclease according to its characteristics and recognition sequence, has been purified from Thermus thermophilus strain 111. The yield is about 5×10^3 units of purified enzyme per g of cell. Tth111I is moderately abundant in the cell, compared with other restriction enzymes, and will become a member of endonucleases useful for DNA studies. The enzyme preparation did not give any degradation of super helical ϕ X174 RFDNA. It was also shown in the experiment of Maxam and Gilbert method that neither 3'-endonuclease nor non-specific endonuclease is contaminating the enzyme preparation.

Known type II restriction endonucleases are classified into two groups according to whether their recognition sequences have the symmetry with two fold axis or not. The enzymes recognizing an unsymmetrical sequence cleave the DNA at the site 5 to 10 base pairs apart from the recognition sequence as seen in HgaI, HpaI, MboI or MnlI. On the other hand, most of type II restriction enzymes recognize the symmetrical sequence with two fold axis

and cleave the site located in that sequence. In some cases, the symmetrical sequences contain indefinite base pairs in the middle, e.g. GTNAC for HinFI and GTPyPuAC for HindII. The TthIII recognition sequence was deduced from its two sensitive sites in ColE1 DNA and one of the two sites in λ DNA was 5'-NNTGACNNNGTCNN-3' and the enzyme cleaves the site situated in the sequence. 3'-NNACTGNNNCAGNN-5'

In consideration of the above two groups of restriction endonucleases, TthIII probably belongs to the major group and the first AT pair in the sequence may be unnecessary for the recognition of TthIII. If so, the TthIII recognition sequence turns a palindromic one where three indefinite base pairs intervene. However, analyses of the sequence around many TthIII cleavage sites are necessary before that conclusion.

An endonuclease of an identical specificity with TthIII has also been isolated from Thermus thermophilus strain 110 and strain 23.

TthIII is stable and active at temperatures up to 70°C. Contaminating thermolabile nonspecific exo- and endonuclease are inactivated at this temperature and the availability of a thermostable restriction enzyme greatly extends the range of conditions that may be used to study DNA structure and the mode of action of restriction endonucleases.

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