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A second site specific endonuclease from Thermus thermophilus 111, Tth111II

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ABSTRACT

A second site specific endonuclease with novel specificity has been purified from *Thermus thermophilus* strain 111 and named *Tth*111II. The enzyme is active at temperature up to 80°C and requires Mg^{2+} or Mn^{2+} for endonuclease activity. *Tth*111II cleaves φX174RFDNA into 11 fragments and lambda DNA into more than 25 fragments. From the 5'-terminal sequences of *Tth*111II fragments of φX174RFDNA determined by the two dimensional homochromatography and the survey on nucleotide sequence of φX174RFDNA, it was concluded that *Tth*111II recognizes the DNA sequence 5'CAAPuCA(N)$_{11}$3' and cleaves the sites as indicated by the arrows. 3'GTTPyGT(N)$_9$5'

INTRODUCTION

Many endonucleases belonging to so-called TypeII restriction enzyme have been purified from various microorganisms [1] and used as an indispensable tool for dissection of genomes and DNA sequencing [2]. We also have been seeking Type II restriction enzymes in extremely thermophilic bacteria found in Japanese thermal spas for a few years. We have found so far *Tth*HB8I, an isoschizomer of *Taq*I, in *Thermus thermophilus* HB8 (ATCC27634) [3], and *Tth*111I in *Thermus thermophilus* 111 which recognizes a nucleotide sequence 5'-GACNNNGTC-3' 3'-CTGNNNCAG-5' and cleaves the sites as indicated by arrows [4]. During studying on *Tth*111I, we have found a second endonuclease, *Tth*111II, in *T. thermophilus* 111 cells, though its content in the cell is low.

We describe here the nucleotide sequence recognized and the sites cleaved by *Tth*111II. The purification procedure and some properties of the enzyme are also described.

MATERIALS AND METHODS

Bacterial strain and materials: A strain of *Thermus thermophilus* 111 isolated from a Japanese thermal spa was a generous gift of Dr. T. Oshima of this institute. The cells were grown at 75°C in 20 liters of culture medium

using a jar fermenter with vigorous aeration and harvested at the stationary phase as described for *T. thermophilus* HB8 [5,6]. About 850 g (wet weight) of cells were obtained from 80 liters of culture fluid and kept at -80°C.

*Hae*III, *Hin*fI, *Hpa*II and *Hin*dII were obtained from Bethesda Research Laboratories. *Tth*HB8I, an isoschizomer of *Taq*I, was prepared as described previously [3]. T4 polynucleotide kinase and hexokinase were purchased from Boehringer-Mannheim Corporation. Bacterial phosphatase (Type III-R) from Sigma Chemical Company was heated at 85°C for 15 min in the presence of 1 mM $MgCl_2$. Venom phosphodiesterase and pancreatic DNase were purchased from Worthington Biochemical Corporation. [γ-^{32}P]ATP was a product of Radiochemical Center, Amersham. Strips of cellulose acetate membrane (2.5 × 45 cm) were obtained from Schleicher and Shüll. DEAE-cellulose thin layer was a product of Machery-Nagel+Co (Polygram Cell 300 DEAE/HR-2/15). Yeast RNA for homomixture was purchased from P-L Biochemicals Co.

DNA preparation: Bacteriophage lambda was prepared by heat induction of $CI_{857}S7$ prophage and its DNA was obtained by phenol extraction of phage particles banded in a CsCl gradient [7]. Bacteriophage ϕX174am3 and its host were generous gifts of Dr. Y. Machida and its replicative form DNA (RFDNA) was prepared by modification of the method described by Altman and Denhalt[8].

Digestion of DNA and gel electrophoresis: DNA (1 µg) was digested with *Tth*111II in 30 µl of 6 mM Tris-HCl (pH7.4) / 120 mM NaCl / 6 mM 2-mercaptoethanol / 6 mM $MgCl_2$ for 1 h at 65°C. Reactions were stopped by adding 10 µl of stop mixture (100 mM EDTA / 0.4 % sodium heparin / 50 % glycerol / 0.01 % bromophenol blue (BPB)). The samples were analysed by electrophoresis in Tris-borate-EDTA [9] on cylindrical 0.7 % agarose gels or on 5 % polyacrylamide vertical slabs (14 × 16 × 0.15 cm). Gels were stained with ethidium bromide (0.5 µg/ml in Tris-borate-EDTA buffer) for 20-30 min and photographed under UV light with a red filter on the lens [10].

Isolation of restriction fragments: The digest of ϕX174RFDNA with restriction endonuclease was treated with water saturated phenol and precipitated with ethanol. The precipitate was dissolved in Tris-borate-EDTA buffer containing 10 % glycerol and 0.02 % BPB and electrophoresed on a 5 % polyacrylamide slab gel (14 × 35 × 0.15 cm). After staining with ethidium bromide, banded DNA fragments were eluted from the gel into dialysis bags by electrophoresis in plastic tubes (2 mA per tube for 12 h).

5' end labeling of restricted fragment: DNA fragments produced by *Tth*111II digestion were phosphorylated with ^{32}P at 5' ends as follows. The fragment of ϕX174RFDNA (5-10 pmol) was dissolved in 100 µl of 20 mM Tris-HCl

(pH8.9) containing 2 mM $MgCl_2$. One microliter of alkaline phosphatase (about 5 µg) 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 (12,000 *g* for 10 min) and dried in vacuum. The dephosphorylated DNA fragment was heated in boiling water for 3 min in 10 µl of 5 mM Tris-HCl (pH9.5) / 0.1 mM EDTA / 1 mM spermidine, chilled in ice water and supplemented with 5 µl of 200 mM Tris-HCl (pH9.5)/ 20 mM dithiothreitol and 1 µl of 200 mM $MgCl_2$. The DNA fragment was phosphorylated in a final volume of 20 µl by incubation at 37°C for 1 h with 40 pmol of [γ-^{32}P]ATP (specific activity about 1,000 Ci/mmol) and 1 unit polynucleotide kinase. After the reaction mixture was supplemented with 20 µl of 4 M ammonium acetate, the DNA fragment was precipitated with 120 µl of ethanol.

The sequence analysis of cleavage sites: The two dimensional homochromatography developed by Brownlee and Sanger [11] was used with some modification described by Tu et al. [12]. The DNA fragment ^{32}P-phosphorylated at one 5' end was digested with pancreatic DNase (5 µg) in 10 µl of 20 mM Tris-HCl (pH7.4) / 10 mM $MgCl_2$ for 30 min at 37°C. One tenth of the mixture was removed and incubated in a fresh mixture (3 µl) of 33 mM Tris-HCl (pH8.9) / 33 mM $MgCl_2$ containing 1 µg venom phosphodiesterase for 30 min at 37°C. The reaction mixture was heated at 90°C for 10 min to inactivate the enzyme and combined with the rest of the pancreatic DNase digest. An aliquot (2,000-10,000 cpm) of the mixture was electrophoresed on cellulose acetate at pH3.5 for the first dimension, followed by homochromatography on DEAE-cellulose thin layer using homomix VI [13] for the second dimension. The 5'-terminal sequences were deduced from the mobility shifts [12] comparing with the known 5'-terminal sequence of restriction DNA fragments.

Computer search for recognition nucleotide sequence: Possible nucleotide sequences recognized by *Tth*111II were searched on φX174RFDNA [2,14] by using a minicomputer (type NOVA 02/30, a product of Data General Japan) with programs in BASIC and the distribution of the specific nucleotide sequences was compared with that of *Tth*111II sites deduced from the experiments.

RESULTS

Isolation of *Tth*111II: Frozen cells (850 g) of *T. thermophilus* 111 were thawed in 1700 ml of buffer A (20 mM Tris-HCl (pH8.0) / 1 mM EDTA / 5 mM 2-mercaptoethanol) containing 0.15 M NaCl and disrupted by sonication. After centrifugation at 30,000 *g* for 3 h, the supernatant was added with

solid NaCl to make electric conductivity of the solution equivalent to that of 0.15 M NaCl. The solution was applied to a column (5 × 45 cm) of DEAE-cellulose (Whatman DE-23) previously equilibrated with buffer A containing 0.15 M NaCl. The DEAE-cellulose was washed with 2000 ml of buffer A containing 0.15 M NaCl, and with 2000 ml of 0.4 M NaCl in buffer A. The fractions eluted with 0.15 M in buffer A were pooled and applied to a column (4 × 40 cm) of phosphocellulose (Whatman P11) previously equilibrated with buffer A containing 0.15 M NaCl. The column was washed with 0.15 M NaCl / 0.01 M potassium phosphate buffer (pH7.4) / 5 mM 2-mercaptoethanol and then developed with 3000 ml of NaCl gradient (0.15 - 1.0 M) in 0.01 M potassium phosphate (pH7.4) containing 5 mM 2-mercaptoethanol. As shown in Fig. 1, two different site specific endonucleases were found in the fractions around fraction number 40 and 124. The first endonuclease is *Tth*111I, which was previously reported [4]. The second endonuclease eluted at 0.5 - 0.65 M NaCl was named *Tth*111II after the recommendation of Smith and Nathan [15]. The fractions of *Tth*111II were pooled and diluted with H_2O so that electric conductivity of the solution was equivalent to that of 0.15 M NaCl. The enzyme solution was applied to a column (3.2 × 25 cm) of heparin-Sepharose 4B (prepared according to the methods described by Iverius [16] and Bickel et al. [17]) previously equilibrated with buffer A containing 0.15 M NaCl. The column was eluted with a 2 liter linear gradient of NaCl (0.15 to 0.8 M). *Tth*111II activity was eluted around 0.6 M NaCl. The fractions eluted between 0.5 and 0.7 M NaCl were pooled, diluted with 5 volumes of H_2O and adsorbed on to a hydroxylapatite column (2.2 × 40 cm, BDH Chemicals, spheroidal)

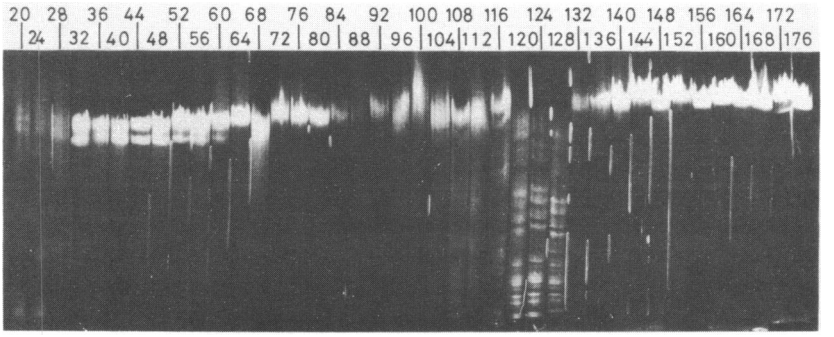

Fig. 1. Assay of endonuclease activity. Samples (5 µl) from the phosphocellulose column fractions (15 ml) were incubated with 1 µg of λDNA for 30 min at 65°C. Numbers above refer to fraction numbers.

previously equilibrated with 0.1 M NaCl in 1 mM potassium phosphate (pH7.4). The column was washed with a 300 ml of the same solution. The elution was carried out with a 1.5 liter linear gradient of potassium phosphate (pH7.4) from 0.001 to 1.0 M in the presence of 0.1 M NaCl. The *Tth*111II activity eluted at 0.03 to 0.1 M potassium phosphate. *Tth*111II fractions were pooled and adsorbed on to a small column (2-3 ml) of phosphocellulose. After washing the column with 10 ml of 0.3 M NaCl in buffer A containing 50 % glycerol, *Tth*111II was eluted with 1 M NaCl in buffer A containing 50 % glycerol and stored at -20°C. In this condition, *Tth*111II retained its activity after at least 2 months.

Properties of the enzyme: Molecular weight of *Tth*111II was determined to be approximately 95,000 by gel filtration on a Sephadex G-200 column. The isoelectric point was determined to be about 7.7 by isoelectrofocusing using 1 % ampholine of the pH range 7 to 9 (a LKB product). However, this value had been roughly estimated since precipitate of the enzyme was formed during electrofocusing. The purified *Tth*111II was precipitated when the ionic strength of the enzyme solution was lowered less than 0.1. The enzyme activity was inhibited under the condition of low ionic strength to some extent. The maximum activity was obtained at ionic strength of 0.12 to 0.15. The optimal temperature for the activity was 65-70°C. The enzyme activity was less than one eighth of the maximum at 37°C. A divalent cation is necessary for the enzyme activity. The enzyme activity was measured in a solution of 0.12 M NaCl / 10 mM Tris-HCl (pH7.4) in the presence of 2 mM of any of various divalent cations. The highest activity was obtained with Mg^{2+}, or Mn^{2+}. The activity with $CoCl_2$ was less than one tenth of that with $MgCl_2$. The endonucleolytic activity was not observed in the presence of Ca^{2+}, Zn^{2+}, Sr^{2+}, Ba^{2+}, Cd^{2+} or Cu^{2+}. Heparin and sodium dodecyl sulfate completely inhibited at concentration of 0.01 % and glycerol slightly reduced the activity at the concentration more than 10 %.

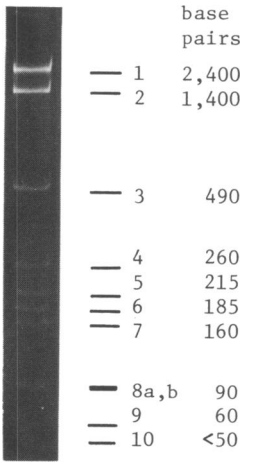

Fig. 2. Gel electrophoresis of *Tth*111II digest of φX174RFDNA. The samples was analysed on a slab of 5 % polyacrylamide gel.

Cleavage sites of *Tth*111II on ϕX174RFDNA: When the digest of ϕX174RFDNA with *Tth*111II was analysed on 5 % polyacrylamide slab gel, eleven bands (designated 1 to 10 from large to small fragment) appeared with stain of ethidium bromide (Fig.2). Size of the fragments was estimated by means of the plot of electrophoretic mobility vs. molecular size of known restriction fragments. In order to determine *Tth*111II cleavage sites on ϕX174RFDNA, double digestions with *Tth*111II and *Hin*fI or *Tth*HB8I were carried out (Fig.3). The band 4, 9, 10, 12 and 13 in the *Hin*fI digest disappeared and band 5 became faint, by the action of *Tth*111II. In the double digest of *Tth*111II and *Tth*HB8I, the band 1, 4, 6 and 8 in the *Tth*HB8I digest disappeared. These results indicate that *Tth*111II should cleave *Hin*fI fragment 4, 9, 10, 12, 13 and any of 5a, 5b and 5c, and *Tth*HB8I fragment 1, 4, 6 and 8. The double digestions with *Tth*111II and *Hin*dII or *Hae*III were also carried out. *Hin*dII fragment 2, 4, 6a and 7b, and *Hae*III fragment 1, 2, and 3 disappeared in each double

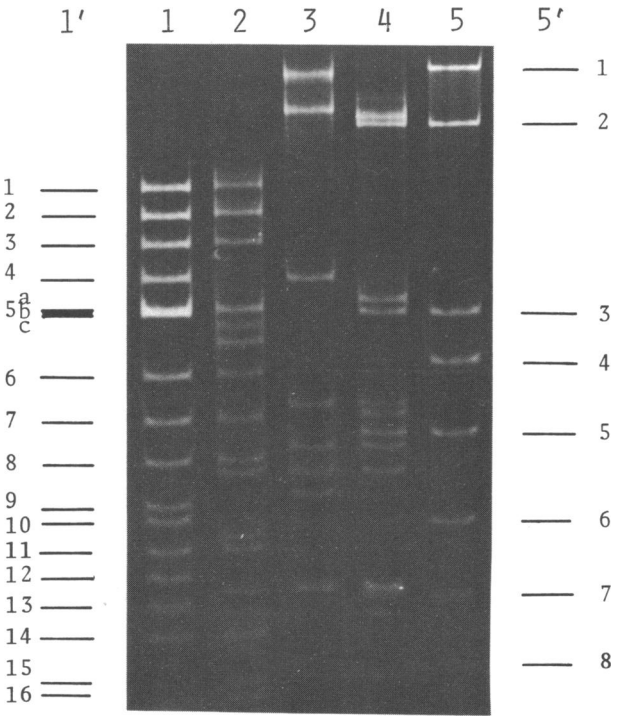

Fig. 3. Double digestion of ϕX174RFDNA. The samples were analysed on a slab of 5 % polyacrylamide gel. Lane 1 and 1', *Hin*fI; lane 2, *Hin*fI plus *Tth*111II ; lane 3, *Tth*111II; lane 4, *Tth*HB8I plus *Tth*111II; lane 5 and 5', *Tth*HB8I.

digest. In consideration of molecular size of *Tth*111II fragments and the results of double digestions, we have roughly estimated the positions of *Tth*111II cleaving sites as illustrated in the 6th row in Fig. 4.

Identification of the 5'-terminal mononucleotides of *Tth*111II fragments of φX174RFDNA. φX174RFDNA (20 μg) was digested with 40 units of *Tth*111II at 65°C for 1 h in a reaction mixture (100 μl) of 8 mM Tris-HCl (pH7.4) / 8 mM $MgCl_2$ / 8 mM 2-mercaptoethanol / 150 mM NaCl. The resulting fragments were labeled with ^{32}P at 5' ends as described in Materials and Methods. Remaining unreacted [γ-^{32}P]ATP was transferred to glucose with hexokinase [18] as follows; the ethanol precipitate of the DNA fragment was dissolved in 15 μl of 10 mM Tris-HCl (pH7.4) / 10 mM $MgCl_2$ / 10 mM 2-mercaptoethanol / 1 mM glucose containing 0.02 unit of hexokinase and incubated at 37°C for 5 min. After a further incubation for 10 min with 5 nmol of non-radioactive ATP, the reaction mixture was heated to inactivate the enzyme. The ^{32}P-labeled fragments were digested with 1 μg of pancreatic DNase, followed by digestion with venom phosphodiesterase. The digest was added with standard four 5'-deoxymononucleotides and electrophoresed on a strip of cellulose acetate membrane at pH 3.5. ^{32}P-labeled mononucleotides were identified by comparing UV absorbance of the standard nucleotides. The spots were cut out and their radioactivity was measured in toluene based scintillation fluid. The radioactivity was distributed as follows, 8.7 % in pdC, 19.0 % in pdG, 25.3 % in pT and 47.0 % in pdA. The results indicate that 5'-terminal nucleotides of the *Tth*111II fragments are heterogeneous, suggesting that *Tth*111II should recognize a complicated nucleotide sequence as seen in *Mbo*II, *Hga*I or *Mnl*I.

Two dimensional mapping of 5'-terminal sequence of *Tth*111II cleavage sites of φX174RFDNA. φX174RFDNA (50 μg) was digested with 100 units of

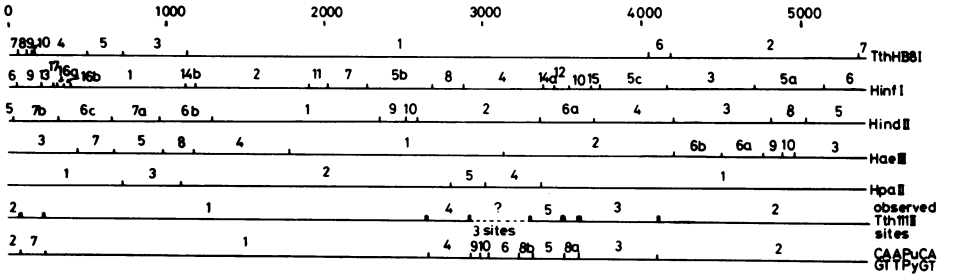

Fig. 4. Cleavage map of φX174RFDNA. The positions of *Tth*111II sites determined in this paper are shown in relation to the known restriction fragment maps of other enzyme [2]. The scale is in nucleotides.

*Tth*111II for 1 h at 65°C in 500 µl of the reaction mixture. *Tth*111II fragment 3, 4 and 5 (see Fig. 2) were isolated and phosphorylated with ^{32}P at the 5' ends. In order to obtain a fragment labeled at one 5'-terminal end, the ^{32}P-labeled fragment was incubated with 10 units of an appropriate restriction endonuclease in 20 µl of the digestion mixture (6 mM Tris-HCl, pH 7.4 / 6 mM $MgCl_2$ / 6 mM NaCl / 6 mM 2-mercaptoethanol) for 1 h at 37°C. *Hin*dII was used for the fragment 3 and 5. and *Hpa*II for the fragment 4. The

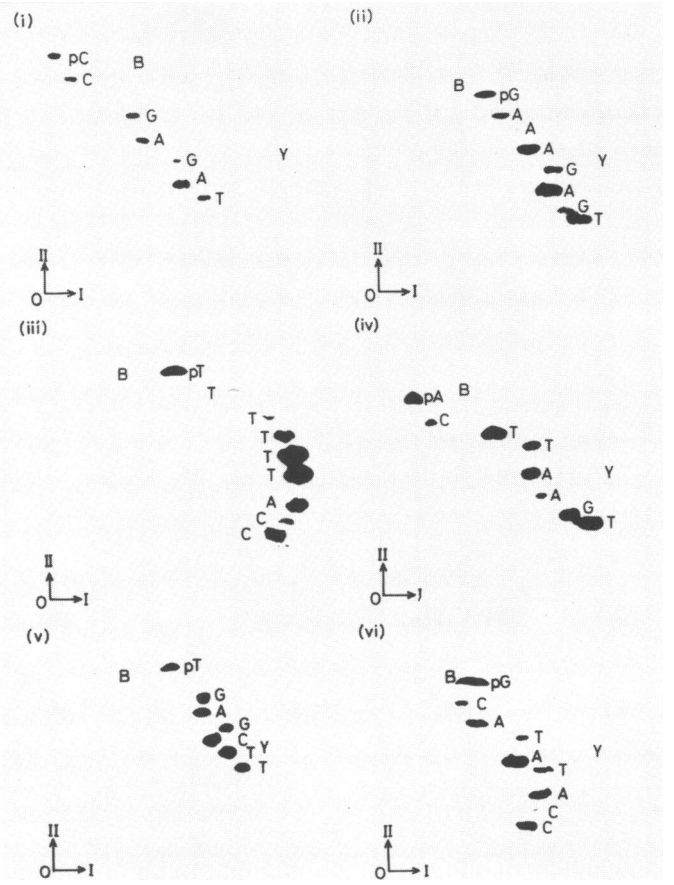

Fig. 5. Determination of 5'-terminal sequences of *Tth*111II fragments of ϕX174RFDNA by two dimensional maps. (i) and (ii), small and large fragments produced from *Tth*111II fragment 3 by *Hin*dII digestion, (iii) and (iv), small and large fragments produced from *Tth*111II fragment 4 by *Hpa*II digestion, and (v) and (vi), small and large fragments produced from *Tth*111II fragment 5 by *Hin*dII digestion. B and Y represent the blue (Xylene cyanol FF) and yellow (Orange G) dye markers, and O represents the origin of electrophoresis.

digests were separated by electrophoresis on a 5 % polyacrylamide slab gel. The gel was stained with ethidium bromide and exposed to a sheet of X-ray film. Each fragment had been cleaved into two radioactive fragments of the following sizes: fragment 3 / *Hin*dII, 100 and 380 base pairs; fragment 4 / *Hpa*II, 120 and 150 base pairs and fragment 5 / *Hin*dII, 60 and 140 base pairs.

Each of the above six fragments labeled at one 5' end was collected again by electrophoresis and subsequently analysed by the two dimensional homochromatography. Resulting finger prints are shown in Fig. 5. It can be read as follows; (i), 5'pCCGAGAT--; (ii), 5'pGAAAGAGT--; (iii), 5'pTTTTTTACC--; (iv), 5'pACTTAAGT--; (v), 5'pTGAGCTT-- and (vi), 5'pGCATATACC--. A specific nucleotide sequence common to the all was searched on nucleotide sequences around the above six cleavage sites. The results were arranged for the help for understanding and shown in Fig. 6. The sequences, 5'CAAGCA3' and 5'CAAACA3' are found at a distance of 11 bases apart from the cleavage point (Fig. 6, i, ii, iv and vi). Their complementary sequences, 5'TGTTTG3' and 5'TGCTTG3', exist at a distance of 9 bases apart from the cleavage points as well (Fig. 6, iii and v). These results suggest that *Tth*111II should

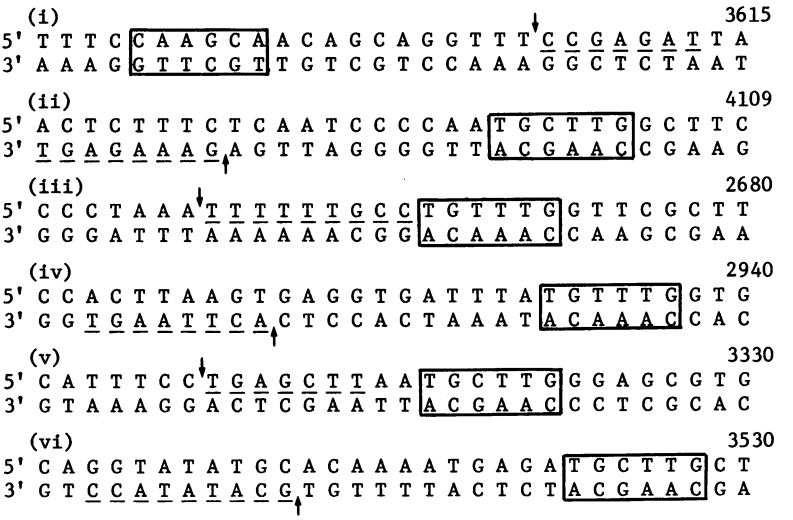

Fig. 6. Sequences around the six *Tth*111II cleavage sites. Arrows point at the *Tth*111II cleavage sites. The sequences determined by the two dimensional homochromatography are indicated by broken underlining and the common sequences are boxed. The sequences are presented with the viral DNA sequence in the upper line. The numbers refer to the distance of the 3' end of the viral DNA sequence from the *Pst*I site [2,14].

recognize a sequence $\overset{5'CAAPuCA(N)_{11}\downarrow 3'}{3'GTTPyGT(N)_{9}\uparrow 5'}$ and cleave at the sites indicated by arrows.

DISCUSSION

The enzyme purified as described above gave a single, symmetrical protein peak superposed with endonuclease activity on gel filtration as well as isoelectrofocusing. Therefore, the enzyme seems to have been purified to a high degree. Nevertheless, it remained to have a low specific activity as an endonuclease, about 1 unit per microgram of protein, compared with other restriction endonucleases. The purified enzyme preparation revealed DNA binding activity as well. The use of a usual stop mixture consisting of glycerol, EDTA and dye gave obscure bands of DNA fragments at the upper part of the gel on analysis of *Tth*111II digest by polyacrylamide gel electrophoresis, and an addition of heparin to the stop mixture cleared the DNA bands. The low specific activity of *Tth*111II enzyme preparation may be partly because concomitant DNA binding activity inhibits the endonucleolytic activity. Now, we make an effort to separate both activity each other.

About 20-50 units of the purified *Tth*111II per *g* of cell were obtained. Although *Tth*111II might be a useful enzyme for DNA studies, there are difficulties in preparing it because of its low content in the cell. It is necessary to seek other strains carrying *Tth*111II activity.

Known type II restriction endonucleases can be classified into two groups according to whether their recognition sequences have a symmetry or not. Most of type II restriction enzymes recognize symmetrical sequences with a two fold axis and symmetrically cleave the sites included in the sequences, as seen in *Hae*III, *Hha*I, *Pst*I and so on. Fuchs et al. examined the distribution of all possible tetra- penta- and hexanucleotide duplexes of two fold rotatory symmetry, on ϕX174RFDNA and SV40DNA, and estimated molecular size of fragments produced and sites cleaved by supposed restriction endonucleases [19]. We could easily rule out the possibility that *Tth*111II might recognize a symmetrical nucleotide sequence, by referring to their results on ϕX174RFDNA. On the other hand, the enzymes recognizing an unsymmetrical sequence cleave the DNA at the sites 5 to 10 nucleotides apart from the recognition sequence, e.g. $\overset{5'GAAGA(N)_{8}\downarrow 3'}{3'CTTCT(N)_{7}\uparrow 5'}$ for *Mbo*II and $\overset{5'GACGC(N)_{5}\downarrow 3'}{3'CTGCG(N)_{10}\uparrow 5'}$ for *Hga*I. *Tth*111II recognizes an unsymmetrical sequence as $\overset{5'CAAPuCA3'}{3'GTTPyGT5'}$, and cleave DNA at the sites of 9 and 11 base pairs from the recognition sequence. The recognition sequence is over six base pairs and the fourth base pair is G:C or A:T pair. The *Tth*111II recognition sequence was searched on

ϕX174RFDNA sequence by using a minicomputer to amount to 11, which is coincident with the number of *Tth*111II fragments. The molecular size of the fragments deduced from the distribution of the above recognition sequence is as follows, 1, 2,411; 2, 1,378; 3, 483; 4, 270; 5, 206; 6, 186; 7, 160; 8a, 93; 8b, 91; 9, 60 and 10, 48 (expressed in base pairs). These values are in agreement with the molecular sizes of *Tth*111II fragments estimated by the electrophoretic mobility (Fig. 2). The distribution of the nucleotide sequence, $\begin{matrix}5'\text{CAAPuCA}3'\\3'\text{GTTPyGT}5'\end{matrix}$, coincides the *Tth*111II fragment map except for the undetermined region assumed to have three cleavage sites (Fig. 4). A sequence, $\begin{matrix}5'\text{CAANCA}3'\\3'\text{GTTNGT}5'\end{matrix}$, is excluded for the recognition sequence of *Tth*111II because it numbers 23 on ϕX174RFDNA. Therefore, we have concluded that *Tth*111II recognizes a sequence, $\begin{matrix}5'\text{CAAPuCA(N)}_{11}\downarrow 3'\\3'\text{GTTPyGT(N)}_{9}\uparrow 5'\end{matrix}$, and cleaves as indicated by the arrows.

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