Nuclease TT1 from *Thermus thermophilus* HB8 has an endonuclease activity preferential to circular DNAs*

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ABSTRACT

Homogeneously purified nuclease TT1 from *Thermus thermophilus* HB8 is known as an exonuclease to produce 5'-mononucleotides. Besides the exonuclease activity, nuclease TT1 also possesses endonuclease activity preferential to superhelical (form I) and single-stranded circular DNA. Although the rate of cleavage is slower than that of form I, covalently closed circular DNA (form I') is also cleaved. Form I DNA was nicked to yield relaxed circles (form II) first, and was then nicked at the opposite site to yield unit length linear DNA (form III) which was subsequently hydrolyzed to 5'-mononucleotides exonucleolytically. Both endo- and exo-nuclease activities co-migrate on polyacrylamide gels. The general properties of the endonuclease activity are very similar to those of the exonuclease activity. The temperature optimum for endonuclease activity was 85°C. The pH-optimum was in pH-range from 7.5-9.1. The enzyme was active over a wide range of Mg^{2+} concentrations (2.5-125 mM), and was inhibited by EDTA. A linear substrate such as (dT)₈ was a competitive inhibitor for this endonuclease activity.

INTRODUCTION

An extracellular nuclease from the culture broth of an extreme thermophile, *Thermus thermophilus* HB8 was homogeneously purified (1), and its properties and substrate specificity toward linear DNA and RNA were reported (1,2). This nuclease was initially termed exonuclease HB8, because the enzyme liberated 5'-mononucleotides from the terminus of both DNA and RNA (1). However, it was unexpectedly found that the enzyme nicked form I DNA and single-stranded circular DNA. In this respect, this enzyme should possess an endonuclease activity in addition to exonuclease activity. The term exonuclease is not appropriate for this enzyme. Thus, it was renamed nuclease TT1 (2). The designation, TT1 derives from the initial letters of the two words, *Thermus thermophilus*.

Recently, it was reported that venom phosphodiesterase has an endonuclease activity specific for single-stranded and superhelical DNA in addition to the well-known exonuclease activity (3-5).

In this paper, we describe the properties of the endonuclease activity of nuclease TT1.

MATERIALS AND METHODS

Enzymes Nuclease TT1 was purified to homogeneity from the culture broth of *T. thermophilus* HB8 by a modification of the previous method (1), that is, as the final step of purification, affinity chromatography on 2',5'ADP-Sepharose 4B was applied. Restriction enzymes *Pst*I, *Eco*RI and *Hind*III were purchased from Boehringer Mannheim GmbH, *Hae*III from Biolabs Inc., *Hae*II, *Hpa*I, *Hpa*II and a superhelical DNA relaxing enzyme from Bethesda Research Laboratories Inc.

Substrates Calf thymus DNA, ϕ X174 RFI and the single-stranded circular DNA were prepared in this laboratory. ColEI, PM2 and λ DNA were purchased from Boehringer Mannheim GmbH, (pT)₈ from Collaborative Research Inc. PTF62 DNA (6) was a gift from Dr. Hishinuma of this institute.

Preparation of Form I' DNA Form I' DNA was prepared by incubation of PM2 form I DNA with a superhelical DNA relaxing enzyme. The reaction mixture (1.7 ml) consisted of 1.0 A₂₆₀ unit of PM2 DNA, 20 mM Tris-HCl, pH 7.8, 2 mM MgCl₂, 7 mM 2-mercaptoethanol and 25 units of the relaxing enzyme and was incubated for 4.5 h at 37°C. A sample of the reacted DNA was purified by centrifugation in a propidium diiodide/CsCl buoyant density gradient (7,8).

Assay of Nuclease TT1 Activity The exonuclease activity was measured in terms of the amount of acid-soluble products formed from calf thymus heat-denatured DNA according to the standard assay method described previously (1). One unit of the nuclease activity is defined as that amount of enzyme which releases 1.0 A₂₆₀ unit of acid soluble materials in 30 min at 60°C under standard assay conditions. The endonuclease activity was determined as the amount of enzyme which converts circular DNA into linear DNA. The reaction mixture (total volume 40 μ l) contained 0.25 M glycine-NaCl-NaOH buffer, pH 9.1, 25 mM MgCl₂, ca. 0.01 A₂₆₀ unit of circular DNA and an appropriate amount of nuclease TT1. The reaction proceeded for 30 min at 60°C and was then stopped by adding 15 μ l of B.J. solution (EDTA, 0.2 M; sucrose 60%(w/w); bromophenol blue, 0.05%). This sample was used for agarose gel electrophoresis. The conversion of circular DNA to linear DNA was manifested on agarose gels as a diminution of a band of form I or form II and the appearance of a band of form III.

Double Digestion of ϕ X174 RFI DNA by Nuclease TT1 and *Pst*I The reaction mixture (30 μ l) consisting of ca. 0.02 A₂₆₀ unit of ϕ X174 RFI DNA, 0.25 M of

glycine-NaCl-NaOH buffer, pH 9.1, 25 mM $MgCl_2$ and 1.2 units of nuclease TtI was incubated for 1 h at 60°C. Under these conditions, all of form I and form II was converted into form III. The mixture was then treated with phenol, followed by ether and 2-butanol respectively, and precipitated with ethanol. The precipitate (form III) was dissolved in 18 μ l of 10 mM Tris-HCl buffer, pH 7.0, containing 50 mM NaCl and 10 mM $MgCl_2$, and to this DNA solution, 2 μ l of *Pst*I (20 units) were added. After incubation for 1 h at 37°C, the products were precipitated with ethanol, redissolved in 10 μ l of B.J. solution and used for agarose gel electrophoresis.

Agarose Gel Electrophoresis DNA samples were electrophoresed on 0.7% or 1.2% (w/v) agarose gels in tubes (12 x 0.6 cm) or in a vertical slab gel system (17 x 16.7 x 0.15 cm) using a buffer of 40 mM Tris-acetic acid, pH 8.0, 20 mM sodium acetate, 10 mM EDTA and ethidium bromide (0.5 μ g/ml) (9). The electrophoresis was performed at 50-60 V for 3-4 h at room temperature. The gels were irradiated with a UV lamp and photographed with Polaroid film type 107 (Polaroid Ltd.).

Acrylamide Gel Electrophoresis Nuclease TtI was electrophoresed on a tube (17 x 0.6 cm) of 7.0% polyacrylamide gel at pH 8.9, according to the previously described method (1).

RESULTS

Digestion of ϕ X174 Single-Stranded Circular DNA ϕ X174 single-stranded circular DNA was digested with nuclease TtI (Fig. 1). When ca. 0.03 A_{260} unit of this DNA was incubated with 0.1-0.2 unit of the enzyme for 30 min at 60°C, the band of this DNA on agarose gels disappeared completely. By the same amount of the enzyme, complete hydrolysis of calf thymus native DNA was achieved and half this amount of enzyme was required to hydrolyze the heat-denatured DNA to 5'-mononucleotides.

Cleavage of Various Kinds of Form I DNA Various kinds of form I DNA were cleaved by nuclease TtI. Figure 2 shows the time course of degradation of ϕ X174 RFI DNA at 70°C. This form I DNA was converted into a unit length linear DNA (form III) within 5 min. As a result of the cleavage of form I DNA with various amounts of nuclease TtI, about 0.01 A_{260} unit of PM2 form I was nicked with 0.005 unit of the enzyme and converted into form III (6.3×10^6 dalton) with 0.5 unit of the enzyme. ϕ X174 RFI and ColE1 form I DNA were also nicked to produce form III (3×10^6 and 4.2×10^6 dalton, respectively) with 0.5 unit of the enzyme. This means that form I and form II DNA were converted into form III DNA by an amount of enzyme 10-20 times greater than

Fig. 1 (left) Digestion of ϕ X174 single-stranded circular DNA (0.03 A_{260} unit) by nuclease T1. Units of nuclease T1: (1) 0, (2) 0.0125, (3) 0.025, (4) 0.05, (5) 0.1, (6) 0.2.

Fig. 2 (right) Time course of degradation of ϕ X174 RFI DNA by nuclease T1. (a) Products of ϕ X174 RFI DNA (ca. 0.01 A_{260} unit) cleaved by 0.4 unit of nuclease T1 at intervals of 0, 2, 5, 15, 37, 60, 95 and 130 min at 70°C; (b) unit length ϕ X174 DNA (3×10^6 dalton) generated by *Pst*I digestion; (c) marker, λ DNA fragments generated by *Hae*III digestion (14.63, 6.13, 4.05, 2.85, 1.45 and 1.26×10^6 dalton, from the top).

that required for the complete hydrolysis of native DNA to 5'-mononucleotides.

Cleavage of Open Circular DNA Not only form I, but also form II was cleaved to form III (Fig. 2). PTF62 form II DNA was cleaved into form III (6.6×10^6 dalton) with a similar amount of nuclease T1 as was required for cleaving of form I. These results indicate that endonuclease activity introduces single-strand scission opposite a pre-existing nick in one of the strands. Form III DNA produced by nuclease T1 has no other nick inside of the chain, since the size of single-stranded DNA obtained by alkaline denaturation of ϕ X174 form III DNA produced by the enzyme was the same as that of *Pst*I product, which is known to undergo a single specific cleavage (Fig. 3). Following the conversion of form I or form II into form III, the unit length linear DNA was reduced to a smaller size molecule by the exonuclease activity of nuclease T1.

Cleavage of Covalently Closed Circular DNA This enzyme also cleaves PM2 form I' DNA which was prepared by a superhelical DNA relaxing enzyme (Fig. 4). Although an accurate amount of form I' could not be determined, ca. 0.001 A_{260} unit of form I' was cleaved into form III with 0.125 unit of the enzyme

Fig. 3 (left) Alkaline denaturation of ϕ X174 form III DNA produced by nuclease TT1 (a) or *Pst*I (b). (1) Products denatured with 0.3 N NaOH, (2) Form III.

Fig. 4 (right) Cleavage of PM2 form I' DNA by nuclease TT1. (a) Products of PM2 form I' DNA (ca. 0.001 A_{260} unit) cleaved by nuclease TT1 (0.125 unit) at intervals of 0, 5, 10, 20 and 30 min at 60°C; (b) Products of PM2 form I and form II DNA (0.005 A_{260} unit) cleaved under the same conditions as (a).

for 30 min at 60°C. Under the same conditions, 0.005 A_{260} unit of PM2 form I DNA was converted into form II for 5 min and to form III for 30 min.

Location of Nicking Sites of Form I A series of experiments were carried out to determine whether the nicking site by nuclease TT1 was specific or not. ϕ X174 RFI DNA was cleaved into form III by nuclease TT1. Then the phenol-treated form III was digested by *Pst*I which is known to cut ϕ X174 RFI DNA at a single site. Analysis of these double digestion products serves to indicate the location of nicking sites. That is, when a nicking site by nuclease TT1 is unique, only two fragments may be expected. But when the nicking site is random the products will certainly be numerous and undetectable as discrete bands on agarose gels. Figure 5 shows that the double digestion products gave a series of discrete fragments. This result indicates that nuclease TT1 nicks ϕ X174 RFI DNA at a limited number of sites. The size of these fragments was determined by a standard curve with known products obtained by several kinds of restriction enzymes. These fragments can be paired and the sum of each molecular weight pairs corresponds to the unit length. The distance of these fragments from the *Pst*I cleavage site was calculated (Table I). Although assignment of short fragments and the corresponding large fragments is not clear, the estimated cleavage sites are at

Fig. 5 Double digestion products of ϕ X174 RFI DNA by nuclease T1 and *Pst*I and markers. ϕ X174 DNA fragments (base pairs) by restriction enzymes: (a) *Hae*III (1342, 1078, 872, 606, 310, 278); (b) *Hae*II (2310, 1557, 783, 269); (c) double digestion products by nuclease T1 and *Pst*I; (d) *Hpa*II (2745, 1690, 374, 348, 218); (e) *Hpa*I (3719, 1264, 192); (f) *Pst*I (5375).

TABLE I Location of Nuclease T1 Cleavage Site

Fragment No.	Number of base-pairs ^{a)}	Possible site location ^{b)}		Possible paired fragment No. ^{c)}
1	700-720	13.2	or 86.8	22
2	805	15.0	" 85.0	
3	870	16.2	" 83.8	
4	920	17.1	" 82.9	
5	1030	19.2	" 80.8	
6	1075-1090	20.2	" 79.8	21
7	1200	22.3	" 77.7	
8	1230	22.9	" 77.1	
9	1270	23.6	" 76.4	
10	1345	25.0	" 75.0	
11	1410	26.2	" 73.8	
12	1495	27.8	" 72.2	
13	2090	38.9	" 61.1	20
14	2260	42.0	" 58.0	19
15	2390	44.5	" 55.5	18
16	2500-2560	46.5-47.6	" 53.5-52.4	17
17	2765	51.4	" 48.6	16
18	2940	54.7	" 45.3	15
19	3220	59.9	" 40.1	14
20	3330	62.0	" 38.0	13
21	3690-3990	68.7-74.2	" 31.3-25.8	10-12
22	4400-4850	81.9-90.2	" 18.1-9.8	1-4

a) The nuclease T1 and *Pst*I-generated fragments shown in Fig. 5.

b) Two possible (clockwise and counterclockwise) locations of the nuclease T1 cleavage sites were calculated from the number of base-pairs of the fragments and expressed as per cent of the genome from the *Pst*I site. As the total length of ϕ X174 DNA, 5375 base-pairs were applied (10).

c) Possible paired fragments making up the total length of the DNA.

least 16. This result suggests that nuclease T1 cleavage sites on ϕ X174 RFI DNA are located in several definite regions.

Identity of Exo- and Endo-nuclease Activities as a Single Protein It has been reported that nuclease T1 is an enzyme that shows multi-molecular weight and hydrolyzes native and denatured DNA exonucleolytically (1,2). On the other hand, in this paper we have shown that the same enzyme cleaves circular DNAs. To prove that this endonuclease activity is identical with nuclease T1 which has an exonuclease activity, it was electrophoresed on polyacrylamide gels. As shown in Fig. 6, every protein band has both exo- and endo-nuclease activities and none of the proteins shows alternatively any one of both activities.

General Properties of the Endonuclease Activity A pH optimum was observed in a wide pH range from 7.5-9.1. The temperature-dependence of the endonuclease activity showed that the activity increased with temperature. Below 85°C, the temperature had no effect on the pattern of forms I, II and III DNA on agarose gels. At 92°C, since a portion of the substrate was already denatured without the enzyme, the data were not comparable to those at other temperatures. The endonuclease activity required the presence of Mg^{2+} (2.5 mM - 125 mM) and was inhibited completely with EDTA.

Effect of a Linear Substrate on the Endonuclease Activity The effect of a linear substrate, $(dT)_8$, a good substrate for the exonuclease activity of nuclease T1, was examined on endonuclease activity. As shown in Fig. 7, the inhibition on cleavage of ϕ X174 RFI DNA increased in proportion to the amount of the oligonucleotide. The endonuclease activity was completely inhibited by adding 0.42 A_{260} unit of oligomer to 0.01 A_{260} unit of the form I DNA; this inhibited activity was gradually recovered by increasing the amount of form I DNA or by increasing the incubation time. These results suggest that $(dT)_8$ is a competitive inhibitor for the endonuclease activity and both endo- and exo-nuclease activities of nuclease T1 have a common active site.

DISCUSSION

We have shown that nuclease T1 has an endonuclease activity preferential to circular DNAs in addition to the exonuclease activity toward double- and single-stranded DNA. The properties of the endonuclease activity toward form I DNA are summarized in Table II and were compared with those of the known exonuclease activity (1,2). Both of these activities are similarly inactivated by heat treatment. The pH optimum range of the endonuclease is similar

Fig. 6 Electrophoretic mobility of exo- and endo-nuclease activities of nuclease T1. (a) Nuclease T1 (300 units, A_{280} 0.13 unit) was electrophoresed on two 7% polyacrylamide gels (17 x 0.6 cm) at pH 8.9 at 5 mA/tube for 200 min. One gel was stained with Coomassie brilliant blue for protein. The other was frozen and cut into 0.5 mm slices so as to extract the enzyme with 20 mM Tris-HCl buffer, pH 7.5, containing 10 mM $MgCl_2$, 1 M NaCl and 20% glycerol (0.5 ml/mm slices). (b) Exonuclease activity was measured with the standard assay mixture by 20 μ l of the extract toward calf thymus heat-denatured DNA for 3 h at 60°C. (c) Endonuclease activity was assayed under the reaction conditions described in "METHODS" with ϕ X174 RFI DNA and 10 μ l of the extract for 30 min at 60°C. Arrows indicate the position of the slices.

Fig. 7 Effect of $(dT)_8$ on cleavage of ϕ X174 RFI DNA by nuclease T1. (a) The reaction mixture (45 μ l) consisting of ca. 0.01 A_{260} unit of ϕ X174 RFI DNA, 10 μ l of 1 M glycine-NaCl-NaOH, pH 9.1, 10 μ l of 0.2 M $MgCl_2$, 0-0.42 A_{260} unit of $(dT)_8$ and nuclease T1 (0.25 unit) was incubated for 30 min at 60°C. The reaction was stopped by adding 15 μ l of B.J. solution and each sample was electrophoresed on 1.2% agarose gels. A_{260} unit of $(dT)_8$: (1) 0.021, (2) 0.042, (3) 0.1, (4) 0.21, (5) 0.42, (6) 0, (7) 0, without nuclease T1. (b) Constant amount (0.21 A_{260} unit) of $(dT)_8$ was incubated with various amount of ϕ X174 RFI DNA and nuclease T1 (0.05 unit). ϕ X174 RFI DNA (A_{260} unit): (1) 0.01, (2) 0.02, (3) 0.04, (4) 0.01, without nuclease T1. The other reaction conditions are the same as those described in (a). (c) Constants amount of $(dT)_8$ (0.21 A_{260} unit) and the substrate (0.01 A_{260} unit) were incubated with nuclease T1 (0.05 unit) for longer time. Incubation time (h): (1) 0.5, (2) 1, (3) 1.5, (4) 2. The other reaction conditions are the same as described in (a).

to that of the exonuclease activity toward native DNA and the temperature optimum is identical. In a certain report, it was indicated that the melting temperature of form I DNA exceeded 104°C in standard saline citrate and that form II DNA melted at 89°C (11,12). Our experiment on agarose gel electrophoresis also confirmed that the pattern of forms I, II and III was not affected at 85°C, but that forms II and III underwent denaturation at 92°C (data not shown). However, the effect of temperature on the detailed structure of circular DNAs is still not clear. Mg^{2+} is required for both activities; the dependence of endonuclease activity on Mg^{2+} concentration is similar to that of exonuclease activity and EDTA inactivates both activities. Nuclease T1 is a multimolecular type enzyme and shows three main protein bands on polyacrylamide gels (Fig. 6). Both endo- and exo-nuclease activities always comigrate with these proteins. These results prove that nuclease T1 has an intrinsic endonuclease activity.

TABLE II Properties of Endo- and Exo-nuclease Activities of Nuclease TT1

Property	Endonuclease activity ^{a)}	Exonuclease activity ^{b)}
pH optimum:	pH 7.5-9.1	pH 8.5-9.3 (dsDNA) pH 9.4-9.5 (ssDNA) pH 8.5-9.0 (RNA)
Temperature optimum:	85°C~	85°C
Effect of Mg ²⁺ :	Activates (2.5-125 mM)	Activates (1-100 mM)
Effect of EDTA:	Inactivates	Inactivates
Percent inactivation by heat 80°C for 10 min:	Partially inactivated	10%
90°C for 10 min:	Mostly inactivated	>80%

a) ϕ X174 or PM2 Form I DNA was used as a substrate.

b) Calf thymus heat-denatured DNA was used as a substrate (1,2).

The relative amount of nuclease TT1 to cleave various kinds of DNA are summarized in Table III. The rate of degradation of single-stranded circular DNA to 5'-mononucleotides is almost equivalent of that of the same amount of double-stranded linear DNA. In case of PM2 DNA, the nicking rate of form I to form II is 20 times faster than the exonucleolytic degradation of double-stranded DNA. However, the endonucleolytic conversion to form III from form I and form II DNA is 10-20 times slower than that of exonucleolytic degradation. Although the nicking rate of form I' is slower than that of form I and form II, nuclease TT1 also cleaves form I' DNA. The relative rate of nicking for form I and form II to the degradation of linear DNA by nuclease TT1 is similar to that of venom phosphodiesterase. But the nicking activity for form I' DNA by nuclease TT1 is much higher than that of venom phosphodiester-

TABLE III The Amount of Nuclease TT1 Required for Cleaving of Various Substrates

DNA substrate	Products	Nuclease TT1 required ^{a)} (units/A ₂₆₀ DNA)
ss Calf thymus	5'-mononucleotides	2.3
ds Calf thymus	5'-mononucleotides	5.2
ϕ X174 ss Circular	5'-mononucleotides	5.3
ϕ X174 RFI	Form II & Form III	28
ϕ X174 RFI & Form II	Form III	56
PM2 Form I	Form II & Form III	0.25
PM2 Form I & Form II	Form III	50
PM2 Form I'	Form III	125-250
ColE1 Form I & Form II	Form III	85
PTF62 Form II	Form III	100

a) Amount of nuclease TT1 (unit) required for cleaving of 1.0 A₂₆₀ unit of various DNA for 30 min at 60°C.

ase (3). The reason for this may be the difference in preference for single-stranded DNA between the two enzymes. That is, venom phosphodiesterase has an endonuclease activity on single-stranded linear DNA (3), but nuclease T1 does not (1). The relative rate of digestion of single-stranded DNA compared to double-stranded DNA by venom phosphodiesterase is higher than that of nuclease T1 (1,3). On the other hand, extracellular nuclease of *Alteromonas espejiana* BAL31 is highly specific for single-stranded DNA and gives a single-chain scission of superhelical PM2 DNA, but it fails to attack form I' DNA (13-15). It is also known that single-strand specific nuclease S1 converts superhelical DNA, containing unpaired or weakly hydrogen bonded regions, into unit length DNA, but form I' DNA, as well as linear duplex molecules, are relatively resistant to attack by this enzyme (16-18).

With regard to the location of cleavage sites, it has been reported that these regions are readily denaturable in topologically constrained superhelical molecules in the case of nuclease S1 (16). On venom phosphodiesterase, it is known that four of five the enzyme-susceptible sites of PM2 DNA are at or near early denaturation regions, but one of these sites was not (4) and some of the (A+T)-rich regions on ϕ X174 DNA were definitely cleaved (5). In the case of nuclease T1, the cleavage sites on ϕ X174 RFI DNA were also located in several definite regions. However data were not sufficient for speculating on the enzymatic function in consideration of the cleavage points in relation to the substrate sequence.

The mechanism of complete degradation of form I DNA by nuclease T1 is summarized as follows. Form I DNA undergoes a single strand scission to produce form II DNA, which is subsequently nicked at or near the opposite site to produce unit length linear DNA, form III. Endonuclease activity of nuclease T1 is essential for these steps. Linear double-stranded DNA is then completely degraded to 5'-mononucleotides from either terminus by the exonuclease activity of nuclease T1. At this step, no evidence for the endonuclease activity could be found, with the exception of ColE1 DNA, which sometimes showed several discrete bands on agarose gels following digestion of nuclease T1 (data not shown). The detailed mode of attack of the exonuclease is not yet known, as to whether one of the strands of a duplex DNA molecule is predominantly degraded or not. Although the rate of conversion decreased, form I' DNA was also degraded as well as form I. As mentioned above, other nucleases which cleave superhelical covalently closed circular DNA (form I) at a single site are single-strand specific and nonsuperhelical covalently closed circular DNA (form I') is resistant to attack by these

enzymes. On this point nuclease TTI can be distinguished from these enzymes. In conclusion, nuclease TTI which cleaves superhelical covalently closed circular DNA at a single site degrades both double- and single-stranded DNA exonucleolytically, and nonsuperhelical covalently closed circular DNA also undergoes attack.

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** The following abbreviations are used: form I, superhelical covalently closed circular DNA; form I', nonsuperhelical covalently closed circular duplex DNA; form II, circular duplex DNA with one nick; form III, linear duplex DNA; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

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