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Type II restriction endonucleases cleave single-stranded DNAs in general

Koichi Nishigaki, Yoshio Kaneko*, Hidehiko Wakuda, Yuzuru Husimi and Toyosuke Tanaka

Department of Environmental Chemistry, Faculty of Engineering, Saitama University, 255 Shimo-Okubo, Urawa 338, Japan

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ABSTRACT

Restriction endonucleases (13 out of 18 species used for the test) were certified to cleave single-stranded(ss)DNA. Such enzymes as AvaII, HaeII, DdeI, AluI, Sau3AI, AccII, TthHB8I and HapII were newly reported to cleave ssDNA. A model to account for the cleavage of ssDNA by restriction enzymes was proposed with supportive data. The essential part of the model was that restriction enzymes preferentially cleave transiently formed secondary structures (called canonical structures) in ssDNA composed of two recognition sequences with two fold rotational symmetry. This means that a restriction enzyme can cleave ssDNAs in general so far as the DNAs have the sequences of restriction sites for the enzyme, and that the rate of cleavage depends on the stabilities of canonical structures.

INTRODUCTION

It is obvious that the use of type II restriction endonucleases is indispensable for DNA researches and genetic engineerings today. Type II restriction endonucleases are known to cleave double-stranded DNA (dsDNA) at or near the site of their recognition sequence which is usually of a two fold rotational symmetry. In rare cases such as HaeIII, HinfI, HhaI, SfaI, MboI, MboII, MspI, HpaII and BspRI, it is reported that these restriction endonucleases cleave single-stranded DNAs (ssDNAs) as substrates (1-5). The property has, however, been considered to be exceptional, attributed to a limited number of restriction endonucleases (5,6). There is a controversy over the mechanism of ssDNA cleavage of restriction endonucleases. Horiuchi and Zinder reported that a single-stranded fragment containing only a single recognition site was cleaved by restriction endonuclease HaeIII (1). In this context, Yoo and Agarwal demonstrated that a restriction endonuclease MspI could cleave synthetic oligonucleotides which were unable to form the double-stranded structure of a two fold symmetry (7). On the other hand, Blakesley and Wells presented that single-stranded DNAs were not cleaved by a restriction endonuclease HaeIII in denaturing conditions,

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suggesting that double-stranded structures of two fold rotational symmetry (termed as canonical structures) were absolutely required for the cleavage of ssDNAs by the enzyme (2,3). Hofer et al. supported the idea with the experiment that a restriction endonuclease HinfI predominantly cleaves the sites that can be intramolecularly formed in a canonical structure(8). Therefore, the conclusive mechanism of ssDNA cleavage of restriction endonucleases has remained to be elucidated.

In this paper, we first demonstrate experimentally that almost all restriction endonucleases can cleave ssDNAs. Secondly, a model explaining these facts and the experimental results so far presented is introduced with supportive evidences.

MATERIALS AND METHODS

Enzymes. Restriction endonucleases were obtained from Takara Shuzo Co. (Kyoto,Japan) with the exception of HpaII,MspI and DdeI which were purchased from P-L Biochemicals (Richmond,USA). Enzymatic activities were expressed to be such that one unit of an enzyme is just enough an amount to digest 1 μg Col EI DNA in a 50 μl standard reaction mixture in an hour at an optimal temperature. The buffer of reaction mixtures is HAE composed of 50 mM Tris-HCl(pH7.5), 60 mM NaCl, 5 mM $MgCl_2$ and 0.5 mM dithiothreitol. The reaction mixtures for enzymes BamHI,TthHB8I and Sau3AI were further adjusted to 100 mM NaCl. The reaction of ssDNA cleavage by the enzyme TthHB8I was done at 37°C or 45°C so as not to destroy the secondary structures of ssDNA, though the enzyme has an optimal temperature at 65°C.

DNAs. Double-stranded DNAs of bacteriophages M13, fd and fd107 (a kimeric phage produced by the combination of fd DNA and pBR322; see ref. 9) were prepared according to the method of Moses et al.(10). Each phage was propagated on its host E.coli(S26) and purified by successive polyethylene glycol (PEG) precipitations. Phages were recovered as a precipitate in 2 % PEG (11). ssDNAs were directly extracted with phenol from these phages. DNAs thus obtained were completely free from dsDNAs.

Gel electrophoresis. Digested DNAs were ethanol precipitated and dissolved in a 1/10 x EP buffer (1 x EP: 40 mM Tris-acetic acid (pH 8.0), 20 mM sodium acetate, 2 mM EDTA). Gel electrophoresis was carried out with a 4 % polyacrylamide gel containing 8 M urea, whose top was attached with a stacking zone (2.5 % polyacrilamide gel containing 8 M urea),essentially following the method of Jeppesen(12). Agarose gel(1.4 %(W/V) agarose in EP buffer) was also used to separate the larger fragments.

Gel staining. Gels were stained with ethidium bromide (0.5 μg/ml) and/or with the silver staining method (13,14). The step of fixation of gels with the solution of 10 %(V/V) acetic acid and 45 %(V/V) methanol in water was particularly important for the quantitative silver staining.

Densitometry. Silver-stained gels were submitted to densitometric analysis using Densitomaster DM K-H (Atago,Tokyo) with a 430 nm filter. The proportionality between the amount of DNA and the corresponding band density was confirmed in the range between 0.5 ng/band and 20 ng/band also in our experimental system as reported by Beidler et al.(15). Peak areas in a densitometric chart were measured on a digitizer tablet TD 1000 (Watanabe Sokki,Tokyo) aided by microcomputer PC8001 (NEC,Tokyo) and calibrated to that of the reference band.

Computer analysis of local structure stability of DNA

A computer program "Maxmth" was worked out to estimate the stability of canonical structures formed by matching of recognition sequences. The contribution of distal parts of the sequence to the structural stability around the recognition site was neglected, and 8 to 12 nucleotides including the recognition sequence were considered to be matching partners. Hairpin loop effects are not considered. The algorithm of maximum matching by Needleman and Wunsch(16) was partly utilized to make up the program module to search possible structures. The thermodynamic parameters used were those by Salser (17).

RESULTS

Assay of ssDNA cleavage of various kinds of restriction enzymes

Cleavage of ssDNA by a restriction enzyme was assayed by gel electrophoresis. As shown in Fig.1, the restriction enzymes used here, MspI, HapII, AvaII, HaeII,DdeI,AluI,HpaII,Sau3AI,AccII,TthHB8I, HaeIII, HhaI, HinfI, EcoRI and AccI digested ssDNA to give specific bands. All bands produced by the above enzymes except for EcoRI and AccI could be assigned, according to their size, to be one of the whole fragments that were theoretically predicted including intermediate products. The case of HaeIII is shown in Fig. 2 as an example of assignment. In most cases enzymatic digestions were rather incomplete because the experimental conditions adopted here were fixed as a standard to be optimal ones for HaeIII except for a NaCl concentration which was chosen to be optimal for each enzyme. Restriction enzymes AccI and EcoRI cleaved fd107 ssDNA generating bands which were unable to assign. Since contaminating endonucleases were not

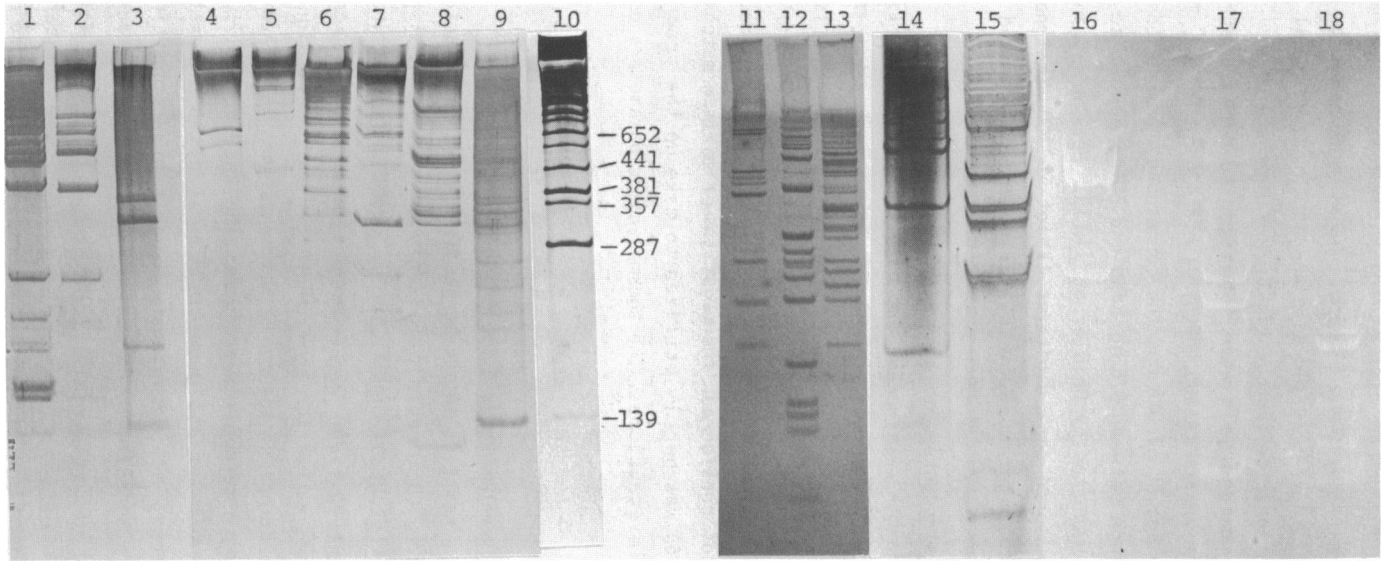


Figure 1. Cleavage of ssDNAs by restriction endonucleases. Single-stranded DNA fragments produced by digestion with restriction enzymes (1)MspI, (2)HapII,(4)AvaII,(5)HaeII,(6)DdeI, (7)AluI, (8)Sau3AI, (9)AccII, (10)TthHB8I, (11)HaeIII,(12)HhaI, (13)HinfI, (14)HpaII, (16)BamHI, (17)EcoRI and (18)AccI for each lane were migrated in denaturing gel electrophoresis using 4 % polyacrylamide gel containing 8 M urea at 60°C (1-15) or in 1.4 % agarose gel without denaturant (16-18) at room temperature. DNA used as a substrate is fd ssDNA in 1-2, and 9-14 or fd107 ssDNA in 4-8 and 16-18. Bands were silver-stained with the methods described in the text. Lanes (3) and (15) are for double-stranded fragments digested with HaeIII as references.

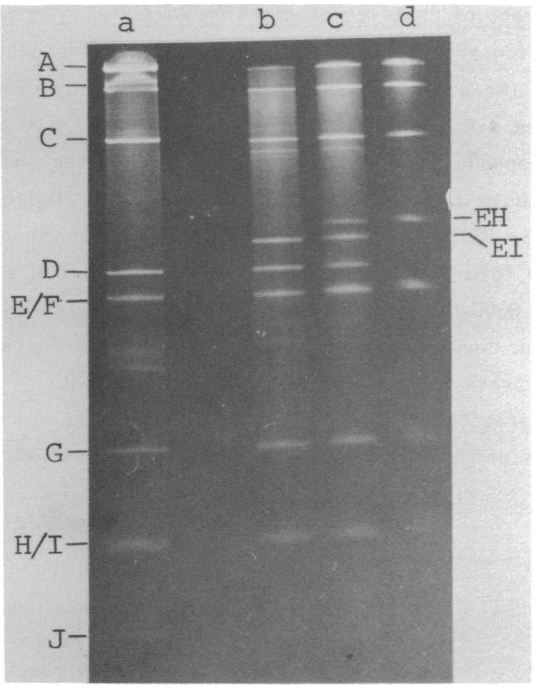


Figure 2. Assignment of digestion products of fd ssDNA by restriction endonucleases HaeIII. Digestion products were dissolved in formamide and subjected directly to denaturing gel electrophoresis as described in Fig.1. The linear relationship between the logarithm of the degree of polymerization and the logarithm of the mobility of a polynucleotide was used for assignment of each band with an aid of knowledge about the species of DNA fragments which were expected from fd DNA sequence. Completely digested fragments with restriction enzyme HaeIII were named with a capital letter from A to J in the order of the length of the fragment. Gel electrophoresis of HaeIII digestion products; a) fd RF(double-stranded)DNA, b) fd ssDNA, c) fd ss + M13 ss DNAs, and d) M13 ssDNA.

detected from the result after vigourous digestion of dsDNAs with these enzymes (40 units/μg DNA),the results obtained here may imply the unknown action of these enzymes such as relaxation phenomena ("star activity" (5)). Among all restriction enzymes tested,BamHI and FokI could not cleave ssDNA. As BamHI is known to be very unstable and FokI is peculiar in its recognition and restriction site as a type II restriction enzyme, the experimental conditions adopted here may be insufficient for these enzymes to cleave ssDNAs.

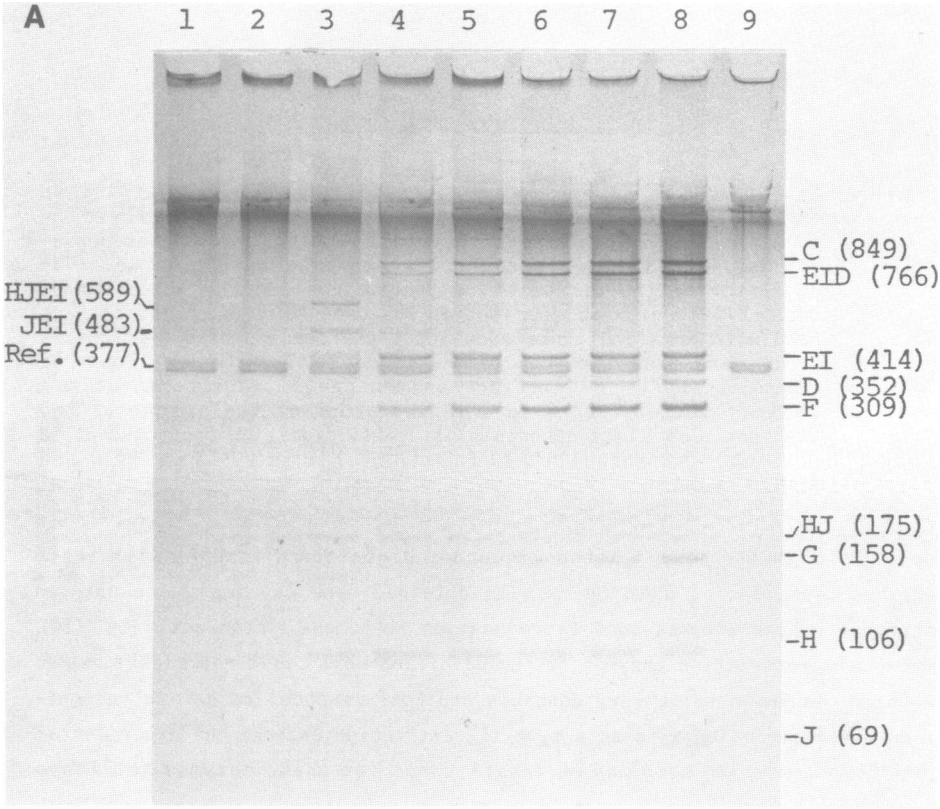
Preferential cleavage of restriction sites by HaeIII

Each restriction site of ssDNA is cleaved in a different reaction rate as shown in Fig.3. (As a certain fragment is generated by cleaving both of the two restriction sites that bound the fragment, the rate of producing a certain fragment depends on the rate of cleaving both the ends.)

The result shows that the 8th restriction site of HaeIII in the fd sequence (called as "GGCC-8") is not restricted with a vigorous digestion such as that of 40 fold excess amount of enzyme and 20 fold excess time. On the contrary, the GGCC-6 in HJ fragment is eventually cleaved, giving two fragments H and J. Some fragments called HJ, J and F are produced in prominently higher rates than those of fragments G and D.

The effect of temperature on preferential cleavage

The digestion of ssDNA by HaeIII was carried out in various tempera-



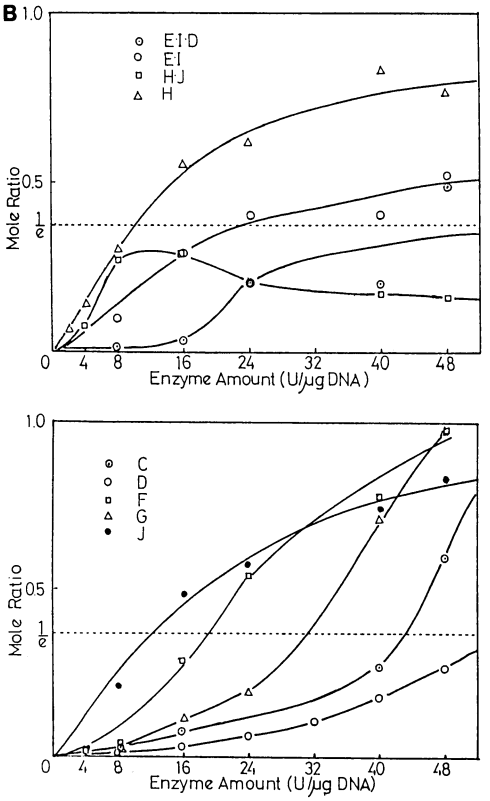


Figure 3. The rate of production of the each restriction fragment by HaeIII analysed by gel electrophoresis.
(A) Electrophoretic patterns : fd ssDNAs were treated at 37°C for 5 h with various amounts of HaeIII; (a) 0 U,(b) 4 U,(c)8 U, (d)16 U, (e)24 U, (f)32 U, (g)40 U , (h)48 U ,and internalreference band of 377 nucleotides only. Electrophoresis was carried out under denaturing conditions at 60°C using 4 % polyacrylamide gel containing 8 M urea which was equipped with a stacking zone of 2.5 %(V/V) polyacrylamide. Silver staining was adopted.
(B) Graphic presentations : The amount of fragments were quantitated by densitometry and were normalized from the recovery of the internal reference. Mole ratio is expressed to be the mole of a fragment recovered over the mole of ssDNA used as a substrate.

tures. Figure 4 shows the temperature dependence of HaeIII restriction site cleavage monitored by the amount of fragments produced. The optimal temperature to be generated is named T_{max}, which is characteristic of each fragment. If the enzyme activity is responsible to the temperature dependence, all fragments should have the same T_{max}. But this was not the case. Together with the fact that HaeIII holds its activity up to

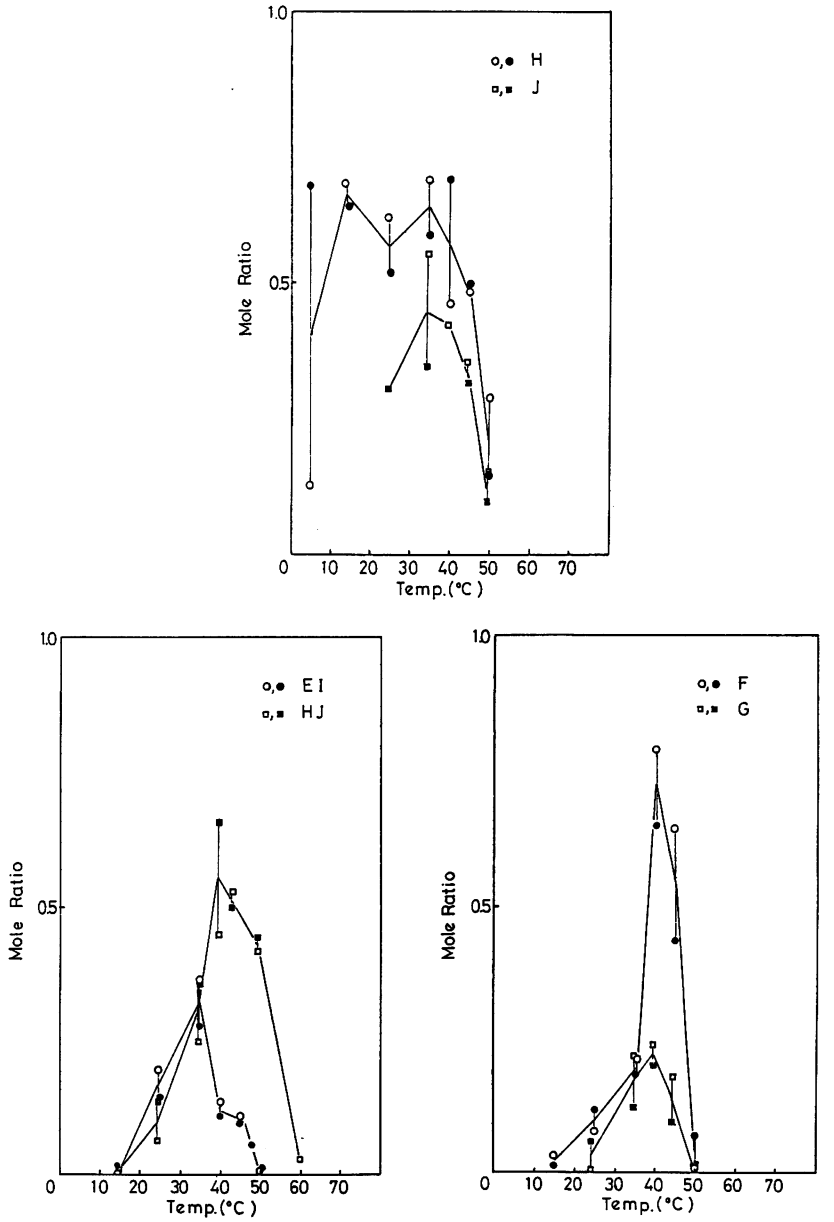


Figure 4. The profiles of temperature dependence of the amount of fragments produced by HaeIII.
The results of two separate examinations are plotted and the mean values of them are connected with a line. Mole ratio is the same as shown in Fig.3.

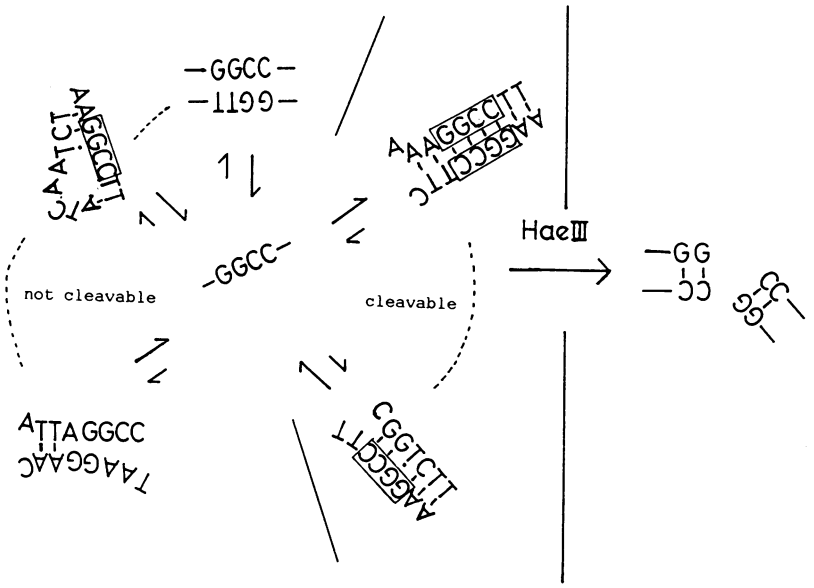


Figure 5. Schematic diagram of dynamic equilibrium of local secondary structures of ssDNA.
A certain recognition site transfers from a structure to another in a life-time defined from the structural stability. The 'cleavable' structures mean that they can be substrates for the restriction enzyme concerned. As described in the text, the majority of the 'cleavable' structures is thought to be canonical ones.

70°C (3), the result obtained here expresses the effect of the structural change of ssDNA caused by the elevation of temperature.

A model for cleavage of ssDNA by restriction endonucleases

The phenomena described above can be explained by the following model. Single-stranded DNAs are assumed to be in an equilibrium between varieties of conformations. The local conformation of ssDNA changes continuously from one to another as shown in Fig.5. Some of the local structures transiently formed are effective as substrates of a certain enzyme. Therefore the stability of the effective structure is reflected on the rate of the enzymatic reaction, since the more stable the structure, the more abundant the existance. According to this model, the preferential cleavage or the difference in the rate of cleavage among restriction sites can be interpreted to be an expression of the difference in the stabilities of the effective structures which involve those restriction sites. The temperature

Table 1. The most stable canonical structures for restriction sites of HaeIII and their Gibbs free energies.

Fragment name	ΔG (kcal/M)	Secondary structure	Fragment name	ΔG (kcal/M)	Secondary structure
HJ	-22.9	TAAGGCCGAT ******** CCTCCGGCTA	EI	-16.85	ATCGGCCTCC ** **** TATCCGGCTC
J	-23.75	GGTGGCCTCA ** **** * CCTCCGG-CTA	G	-12.85	GTT-GGCCAGA * **** * T-AGCCGGAAT
F	-19.65	CAAGGCC-AAT ******* * GTTCCGGCCT	D	-14.25	CTCGGCC-TAT **** * TTTCCGGCCT
C	-16.85	AGCGGCCTTT * ****** TAACCGGAAC	H	-15.3	TAAGGCCGAT * ***** * ACTCCGG-TGG

The base pair of G-T was not taken into consideration. The local stabilities are calculated about 10 bases around the restriction sequence.

dependence shown above is another expression of the stabilities of those effective structures. The majority of the effective structures as substrates for a restriction enzyme can be a priori supposed to be the canonical structures which are essentially identical to restriction sites in double-stranded DNA.

Tentative estimate of stabilities of canonical structures.

The stabilities of canonical structures composed of two recognition sequences in ssDNA were estimated as follows. The stability of a canonical structure in ssDNA is assumed to be determined by the local secondary structure surrounding the restriction site. The most stable structures and the Gibbs free energies for them were calculated using a computer program "Maxmth" in which the free energy of the formation of the most stable comlex between two sequences is obtained under the condition that the recognition sequences such as GGCC of HaeIII should complement each other. As shown in Table 1, the stabilities thus obtained have good correlations with the experimental results (Fig.3 and Fig.4). Canonical structures from which fragments HJ, J and F are produced are more stable than those for fragments G and D, which are quite consistent to the result obtained in Fig.3. Similar relations were obtained when the extent of the sequence

Table 2. The list of restriction enzymes examined for cleavage of ssDNA.

	Enzyme Name	Restriction Site	Number of Fragments fdss Obs./Th.	Number of Fragments fd107ss Obs./Th.	Result	References
1	AccI	GTBHAC		9/3	±	
2	AccII	CG/CG	23/16		++	
3	AvaII	G/GDCC		3/8	+	
4	AluI	AG/CT	16/16	14/32	++	4)N.
5	BamHI	G/GATCC		0-1/3	-	
6	BbeI	GGCGC/C		4/1	+	
7	DdeI	C/TNAG	24/25	25/33	++	
8	EcoRI	G/AATTC		9/1	±	
9	FokI	GGATG(N)$_9$/		0/16	-	
10	HaeII	PuGCGC/Py	4/1	7/15	+	2)N.
11	HaeIII	GG/CC	9/10		++	1),2),4)Y.
12	HapII	C/CGG	13/15		++	
13	HpaII	C/CGG	11/15		++	2)N./4)Y.
14	HhaI	GCG/C	23/23		++	2),4)Y.
15	HinfI	G/ANTC	22/24		++	3),4),8)Y.
16	MspI	C/CGG	23/15		++	7)Y.
17	Sau3AI	/GATC		24/26	++	
18	TthHB8I	T/CGA	21/10		++	
The other enzymes reported to be ss-cleavable						
e1	SfaI	GG/CC				2)Y.
e2	MboI	/GATC				3)Y.

B=A/C, H=G/T, D=A/T, -; not cleavable, ±; not determined, +; cleavable, ++; cleavable and results well-assigned. N.;(reported to be)not cleavable, Y.; cleavable. Obs.; observed band number in this experiment, Th.; theoretically expected band number.

which affects the stability of a canonical structure is set to be 8 or 12, though setting to be more than 14 gave no correlation. These support our model and suggest that it is sufficient to consider the local structure composed of around 10 nucleotides for an interaction with a restriction enzyme.

DISCUSSION

In this paper it was demonstrated that restriction enzymes cleave ssDNAs not as an exceptional behavior but as a general one. As summarized in Table 2, 16 out of 18 restriction enzymes used in our experiment gave specific bands and 13 of them were proved to give genuine products of their actions. On the whole 8 enzymes were newly added to those 9 species which cleaved ssDNAs. As commented in RESULTS, restriction enzymes tested in our experiment were chosen only under the criterion to have restriction sites in fd (or fd107) ssDNA and were examined only in a sigle standard buffer under standard conditions. All other restriction enzymes not examined here

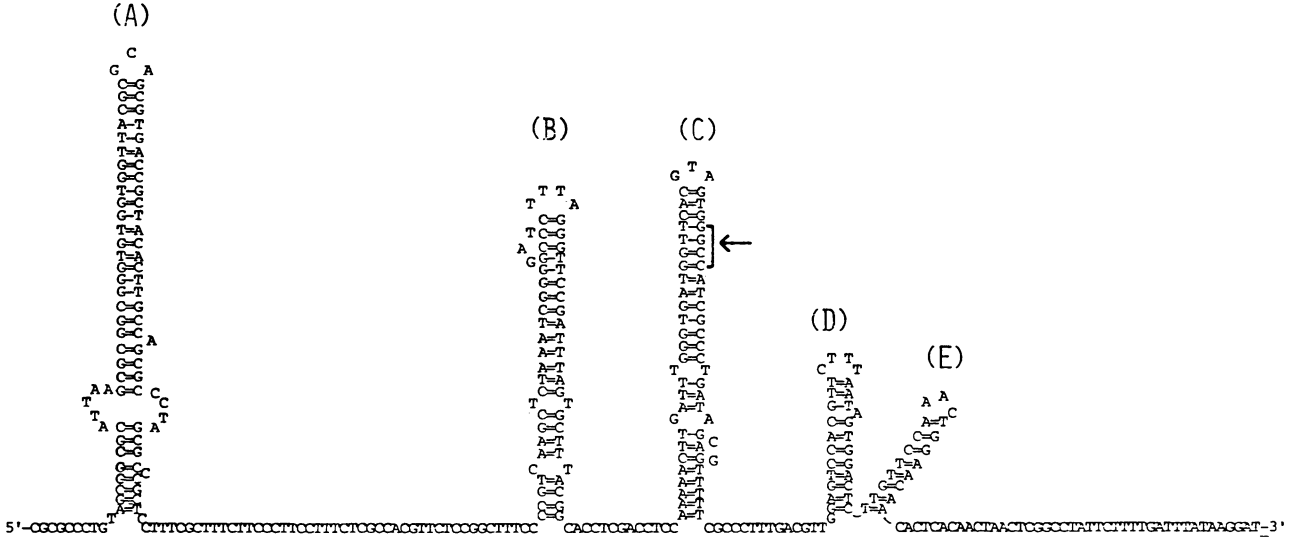


Figure 6. Schematic drawing of the secondary structure around the 8th HaeIII restriction site (GGCC-8) in fd ssDNA. This structure reported by Beck and Zink(18) was essentially supported by our experiment using the computer program for secondary structure "SEW" made by Suyama et al.(19).

seem to have as equal a possibility to cleave ssDNA as those tested here. This possibility is reinforced by the plausible model presented here for the mechanism of ssDNA cleavage by restriction enzymes. According to the model, some restriction enzymes poorly cleave a certain ssDNA due to the unstabilities of the canonical structures included in the DNA. Not restriction enzymes but ssDNAs are rather responsible for the apparent uncleavage of ssDNA. This ,however,does not exclude the possibility of characteristic behaviors of those enzymes with ssDNAs. As fd ssDNA was cleaved by HaeIII without leaving intermediates except a fragment EI, it means that the intermediates such as a fragment HJ were indeed digested to final products in one of the following three cases; (1) cleavage of intermolecularly formed canonical structures,(2) cleavage of relaxed structures which resemble but differ from canonical structures or (3) cleavage of bona fide single-stranded restriction sites.

Though at present it is not clear whether a bona fide single-stranded restriction site was cleaved or not, the rate of cleavage, if possible, seems to be far lower than that of the cleavage of canonical structures. Consequently it bears the same result whether double-stranded structures (called as canonical structures here) are absolutely required or not absolutely required to be cleaved by restriction enzymes. The possibility of cleavage of single-stranded restriction sites should be clarified using a simple and well-defined experimental system utilizing synthetic DNA oligomers. In such an experiment the effect of inter-molecularly forming the canonical structures must be considered.

One of the most significant points elucidated in this study is that the canonical structures to be cleaved are, in general, relatively unstable ones in the secondary structures of ssDNA. There are a number of much more stable secondary structures not canonical and competitive. A typical example is the structure around "GGCC-8" shown in Fig.6. As GGCC-8 is buried in a stable but not canonical structure,which is supposed to exist by Schaller et al.(20) and Shishido and Ikeda (21), it is not cleaved by HaeIII. The fact that cleavage of ssDNA by restriction enzyme requires an excess amount of enzyme is explained by the effect of unstabilities of canonical structures. Further, the temperature dependence of the rate of cleavage is deduced from the model. The amount and stability of competitive structures to canonical ones is specific to each restriction site. This seems to affect the temperature dependence of cleavage of each canonical structure. T_{max} designated in this paper probably reflects the

temperature at which a canonical structure melts. Our finding that most of restriction enzymes can cleave ssDNAs will promote the applications of restriction enzymes to a wide range of researches aimed at structure analysis of ssDNA as done here. Restriction enzyme can be a specific "probe" of higher structure of DNA. Another important application in genetic engineering is to directly obtain ss DNA fragments.

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*Present address: Institute of Biotechnology, Seibu Chemical Industry Ltd., 1120 Motoaho Kamikawa-mura, Kodama-gun, Saitama Prefecture 367-02, Japan.

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