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A hexanucleotide sequence (dC_1–dC_6 tract) restricts the dC-specific cleavage of single-stranded DNA by endonuclease IV of bacteriophage T4

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ABSTRACT

Endonuclease (Endo) IV encoded by *denB* of bacteriophage T4 is an enzyme that cleaves single-stranded (ss) DNA in a dC-specific manner. Previously we have demonstrated that a dTdCdA is most preferable for Endo IV when an oligonucleotide substrate having a single dC residue is used. Here we demonstrate that Endo IV cleaves ssDNAs exclusively at the 5′-proximal dC where a sequence comprises dC residues both at the 5′ proximal and 3′ proximal positions (a dCs tract-dependent cleavage). The dCs tract-dependent cleavage is efficient and occurs when a dCs tract has at least 6 bases. Some dCs tracts larger than 6 bases behave as that of 6 bases (an extended dCs tract), while some others do not. One decameric dCs tract was shown to be cleavable in a dCs tract-dependent manner, but that with 13 dCs was not. The dCs tract-dependent cleavage is enhanced by the presence of a third dC residue at least for a 6 or 7 dCs tract. In contrast to the dCs tract-dependent cleavage, a dCs tract-independent one is generally inefficient and if two modes are possible for a substrate DNA, a dCs tract-dependent mode prevails. A model for the dCs tract-dependent cleavage is proposed.

INTRODUCTION

Endonuclease (Endo) IV encoded by *denB* of bacteriophage T4 is a unique enzyme that cleaves single-stranded (ss) DNA in a dC-specific manner (1–5). Only limited information on the mechanism of substrate recognition by Endo IV has been available (1–4) and the small number of Endo IV-related proteins in the genome sequence databases have limited the amount of insight provided by such proteins into mechanisms of Endo IV action. Given that Endo IV is highly toxic to *Escherichia coli* cells, we have synthesized the enzyme with the use of a wheat germ cell-free protein synthesis system (6) and purified it to homogeneity (5). Endo IV requires Mg^{2+} for activity, acts on ssDNA, generates 5′ termini containing exclusively dC (1,3,5) and does not cleave normal T4 genomic DNA containing glucosylated deoxyhydroxymethylcytidine (1,5).

In addition, the enzyme processed both phiX174 circular ssDNA and heat-denatured T4dC genomic double-stranded (ds) DNA substrates to oligonucleotides comprising several hundred bases (5), suggesting that the digestion of dC sites was highly restricted in each substrate and that the sequence surrounding dC residues or the size of dC-containing oligonucleotides might affect the cleavage efficiency. To characterize the mode of endonucleolytic cleavage by Endo IV, we used two methods; an acid-solubility assay of oligonucleotide substrates and a cleavage pattern analysis of Cy5-labeled substrates. The acid-solubility assay is based on the activity of Endo IV to generate acid-soluble products from an acid-insoluble oligonucleotide substrate. We synthesized oligonucleotide substrates that consist of two dA-copolymers adjacently placed to the 5′- and 3′-ends of a central target sequence. The substrates used were so designed as to become acid-soluble when cleaved in the target sequence by Endo IV. The enzymatic activity of Endo IV was defined as the ability to generate acid-soluble products from an acid-insoluble substrate. The rationale of this method was described in detail (5). We successfully quantified Endo IV activity using oligonucleotide substrates with a single dC residue and found a marked preference of Endo IV for the sequence 5′-dTdCdA-3′ (5). The cleavage pattern analysis of Cy5-labeled substrates gives us information as to the actual sites as well as cleavage efficiencies (5).

Here we used several Cy5-labeled oligonucleotide substrates based on T4 genomic sequence and phiX174 ssDNA sequence in addition to the acid-solubility assay, and we found that the cleavage events occur at the limited dC sites in which a sequence tract comprising dC residues at both the 5′- and 3′-proximal positions. The feature

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of cleavage by Endo IV deduced from the results on the basis of the acid-solubility assay and cleavage analysis of Cy5-labeled oligonucleotides are as follows: Endo IV recognizes a hexanucleotide consisting of two dC residues at the first and sixth positions, named a 6 dCs tract (dC_1–dC_6 tract), in which the cleavage event occurs exclusively at the dC_1 site. A third dC residue placed within a dC_1–dC_6 tract improves affinity between the substrate and Endo IV. Furthermore, some dCs tracts larger than 6 bases (an extended dCs tract) have been shown to behave as a cleavable dCs tract while some others do not. A decameric dCs tract works as an extended dCs tract cleavable by Endo IV. A model for the dCs tract-dependent cleavage by Endo IV is presented.

MATERIALS AND METHODS

Materials

Restriction and other enzymes for recombinant DNA technology were obtained from Takara Shuzo. The plasmid pEUGFP was constructed as described previously (7) and pGEX-6p-1 was obtained from GE Healthcare. Oligonucleotides used for the acid-solubility assay in this article were purchased from OligoTexas Co. and are listed in Table 1. Five Cy5-labeled oligonucleotides, Cy5-T4A, Cy5-T4B, Cy5-T4BG, Cy5-T4Be1 and Cy5-T4Be2 are based on the sequence of T4 DNA. Two Cy5-labeled oligonucleotides, Cy5-phiX174-6 and Cy5-phiX174-6b, are based on the sequence of phiX174 ssDNA. Cy5-phiC174-6 is the same as used in the cleavage experiment of Figure 3 in the previous paper (5). All of these oligonucleotides labeled at their 5′-end with Cy5 were commercially synthesized (Sigma-Aldrich, Japan). Cy5-T4A, 5′-dTdAdGdAdT dTdTdAdGdTdAdAdTdTdAdTdAdTdGdCdTdCdTdTd CdAdTdTdAdGdAdAdAdAdTdTdAdTdTdTdGdTd GdA-3′; Cy5-T4B, 5′-dAdTdTdGdGdAdAdAdCdTdTdA dAdTdGdTdAdAdTdTdCdAdCdTdTdCdAdTdTdGdCd GdAdAdTdGdCdTdTdAdGdCdCdAdG-3′; Cy5-T4BG, 5′-dAdTdTdGdGdAdAdAdCdTdTdAdAdTdGdTdAdA dTdTdCdAdCdTdTdCdAdTdTdGdGdGdAdAdTdGdC dTdTdAdGdCdCdAdG-3′; Cy5-T4Be1, 5′-dAdTdTdGd GdAdAdAdCdTdTdAdAdTdGdTdAdAdTdTdCdAdCd GdTdTdCdAdTdTdGdCdGdAdAdTdGdCdTdTdAdGd CdCdAdG-3′; Cy5-T4Be2, 5′-dAdTdTdGdGdAdAdAdC dTdTdAdAdTdGdTdAdAdTdTdCdAdGdGdTdTdCdA dTdTdGdCdGdAdAdTdGdCdTdTdAdGdCdCdAdG-3′; Cy5-phiX174-6, 5′-dTdTdGdGdAdTdGdAdGdGdAdGd AdAdGdTdGdGdCdTdTdAdAdTdAdTdGdCdTdTdGd GdCdAdCdGdTdTdCdGdTdCdAdAdG-3′; Cy5-phiX174 -6b, 5′-dTdTdGdGdAdTdGdAdGdGdAdGdAdAdGdTdGdGdCd TdTdAdAdTdAdTdGdGdTdTdGdGdCdAdCdGdTdTdCdGd TdCdAdAdG-3′.

Table 1. Oligonucleotide substrates used and their abbreviated names

Oligonucleotide	Short name
5′-$(dA)_5(dC)_{15}(dA)_5$-3′	$[(dC)_{15}]_{15/25}$
5′-$(dA)_7(dC)_{11}(dA)_7$-3′	$[(dC)_{11}]_{11/25}$
5′-$(dA)_9(dC)_7(dA)_9$-3′	$[dCdCdCdCdCdCdC]_{7/25}$
5′-$(dA)_9(dC)_6(dA)_{10}$-3′	$[dCdCdCdCdCdC]_{6/25}$
5′-$(dA)_{10}(dC)_5(dA)_{10}$-3′	$[dCdCdCdCdC]_{5/25}$
5′-$(dA)_{12}(dC)$ $(dA)_{12}$-3′	$[dC]_{1/25}$
5′-$(dA)_9$dCdTdTdTdTdC$(dA)_{10}$-3′	$[dCdTdTdTdTdC]_{6/25}$
5′-$(dA)_9$dCdCdTdTdTdC$(dA)_{10}$-3′	$[dCdCdTdTdTdC]_{6/25}$
5′-$(dA)_9$dCdTdCdTdTdC$(dA)_{10}$-3′	$[dCdTdCdTdTdC]_{6/25}$
5′-$(dA)_9$dCdTdTdCdTdC$(dA)_{10}$-3′	$[dCdTdTdCdTdC]_{6/25}$
5′-$(dA)_9$dCdTdTdTdCdC$(dA)_{10}$-3′	$[dCdTdTdTdCdC]_{6/25}$
5′-$(dA)_9$dCdAdAdAdAdC$(dA)_{10}$-3′	$[dCdAdAdAdAdC]_{6/25}$
5′-$(dA)_9$dCdAdCdAdAdC$(dA)_{10}$-3′	$[dCdAdCdAdAdC]_{6/25}$
5′-$(dA)_9$dCdGdGdGdGdC$(dA)_{10}$-3′	$[dCdGdGdGdGdC]_{6/25}$
5′-$(dA)_9$dCdGdCdGdGdC$(dA)_{10}$-3′	$[dCdGdCdGdGdC]_{6/25}$
5′-$(dA)_9$dCdCdAdTdTdC$(dA)_{10}$-3′	$[dCdCdAdTdTdC]_{6/25}$
5′-$(dA)_9$dCdCdTdAdTdC$(dA)_{10}$-3′	$[dCdCdTdAdTdC]_{6/25}$
5′-$(dA)_9$dCdCdTdTdAdC$(dA)_{10}$-3′	$[dCdCdTdTdAdC]_{6/25}$
5′-$(dA)_{10}$dCdTdTdTdC$(dA)_{10}$-3′	$[dCdTdTdTdC]_{5/25}$
5′-$(dA)_9$dCdTdTdTdTdTdC$(dA)_9$-3′	$[dCdTdTdTdTdTdC]_{7/25}$

Suffix numbers indicate the nucleotide length of the target sequence (referred as a six-base tract) and total length of oligonucleotides, respectively. Suffix number is often omitted in the text. A dC_1–dC_6 tract is defined as a hexanucleotide comprising two dC residues at the first and sixth positions and the sequences are shown in the parentheses [ ]. All of the oligonucleotides in the table were acid-insoluble as their intact form and the cleavage products became acid-soluble by extensive treatment with Endo IV.

Production and purification of GST-Endo IV fusion protein

A DNA fragment encoding a GST fusion protein of Endo IV was constructed and subjected to *in vitro* transcription as described previously (5). The resulting mRNA was then translated in a wheat germ cell-free protein synthesis system with the use of a dialysis cup (molecular size cutoff of 12 000 Da; Daiichi Pure Chemicals) as described previously (5). The dialysis unit containing the reaction mixture was incubated for 96 h at 26°C, with the original amount of substrate mRNA being supplemented and the external solution changed every 24 h. The GST-Endo IV fusion protein was purified as described previously (5). The fusion protein was cleaved by incubation with PreScission protease (10 U/ml; GE Healthcare) for 4 h at 4°C in a glutathione-Sepharose 4B MicroSpin column (GE Healthcare). The flow-through fraction contained a protein of the predicted size for Endo IV (21.1 kDa), as revealed by SDS–polyacrylamide gel electrophoresis on a 12% gel and staining with Coomassie brilliant blue. The protein concentration of this fraction was estimated by densitometric analysis with NIH Image software and the use of trypsin inhibitor (20.1 kDa) as a standard.

Kinetic analysis of Endo IV activity

Hydrolysis of oligonucleotide substrates (10 μM) was performed by the acid-solubility assay as described previously (5). Enzyme and substrate were incubated for 30 min at 37°C in a reaction mixture (20 μl) containing 10 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM dithiothreitol and bovine serum albumin (0.1 mg/ml). The reaction was stopped by the addition of 30 μl of 25 mM EDTA (pH 8.0) and 50 μl of 10% trichloroacetic acid. The resulting mixture (100 μl) was maintained on ice for 15 min and then centrifuged at 5000*g* for 15 min at 4°C. The amount of acid-soluble nucleotide in the supernatant fraction was quantified by measurement of absorbance at 260 nm, with molar absorption coefficients of 15 200,

7050, 12 010 and 8400 $M^{-1}cm^{-1}$ for dA, dC, dG and dT, respectively. One unit was defined as the amount of enzyme producing 1 μmol of acid-soluble nucleotides per minute, and specific activity was defined as the enzymatic activity per milligram of enzyme. The amount of enzyme was varied such that the amount of product increased in proportion to that of the enzyme. The concentration of the substrate was also varied from 3 to 30 μM such that it spanned the K_m. Oligonucleotide substrates were synthesized by Texas Genomics, Japan. Kinetic parameters were determined by a least-squares fit of the data in Lineweaver–Burk plots.

Cleavage pattern analysis of Cy5-labeled oligonucleotides

Hydrolysis of Cy5-labeled oligonucleotides was performed as described above for the Endo IV assay at a substrate concentration of 10 μM. Two kinds of cleavage conditions were adopted for detecting cleavage bands. For a low Cy5-specific activity cleavage, Cy5-labeled oligonucleotides (10 μM) were diluted with an equal volume of non-Cy5-labeled oligonucleotides having an identical sequence and concentration. For a high Cy5-specific activity cleavage, Cy5-labeled oligonucleotides (10 μM) commercially supplied were used without diluting by non-labeled oligonucleotides. Cleavages of oligonucleotides with a low Cy5-specific activity were performed with varied enzyme concentration (0.5–4 μg/ml). Cleavages of oligonucleotides with a high Cy5-specific activity were done at an enzyme concentration of 0.66 μg/ml. The reaction products were separated by electrophoresis on a 10% polyacrylamide gel containing 7 M urea and were visualized with a Variable Image Analyzer Typhoon 8600 (GE Healthcare).

RESULTS

A hexanucleotide (dC_1–dC_6 tract) is a basic framework for an efficient cleavage by Endo IV

A dC-homopolymer, $(dC)_{45}$, was efficiently cleaved by Endo IV while three other homopolymers ($(dA)_{45}$, $(dG)_{45}$ and $(dT)_{45}$) were not (5), being consistent with the notion that Endo IV cleaves ssDNA by an exclusively dC-specific manner (1,3,5). To know the basic feature of dC-specific cleavage by Endo IV, we examined the cleavage activity using various oligonucleotides as the substrates (Table 1). The activity of Endo IV reduced gradually as the number of dC residues in the 25-base oligonucleotide substrate decreased from 25 to 6 and then markedly declined as the number of dC residues decreased from six to five (Table 2). The V_{max} value (3.3 U/mg) of Endo IV with a substrate ($[dCdCdCdCdCdC]_{6/25}$) containing six consecutive dC residues within a target site was comparable to that with $[dCdCdCdCdCdCdC]_{7/25}$. On the other hand, the V_{max} value with a substrate containing five consecutive dC residues ($[dCdCdCdCdC]_{5/25}$) was significantly low (0.38 U/mg) and the same level to that apparent with $[dC]_{1/25}$. In addition, the affinity of oligonucleotide substrate (K_m value) to Endo IV became weaker by the size of dC residues in the target site of the substrate decreasing from six to five. Although the acid-solubility assay does not give us information of the cleavage site in these oligonucleotide substrates by Endo IV, these results strongly suggest that the size of dC-containing target in an oligonucleotide substrate used must be longer than 6 bases for an efficient cleavage by Endo IV. Although we have not examined extensively, an oligonucleotide substrate, $[dCdTdTdTdTdTdC]_{7/25}$ gives V_{max} and K_m values comparable to those of dC_1–dC_6 tracts (Table 3), at least some of 7 dCs tract (dC_1–dC_7 tract) behave like a 6 dCs tract. For dCs tracts larger than 6 bases (referred as extended dCs tracts) will be described below.

Furthermore, since the kinetic parameters of Endo IV with $[dCdCdCdCdCdC]_{6/25}$ were significantly more favorable than those with $[dCdCdCdCdC]_{5/25}$, we assumed that at least two dC residues at the first and sixth positions of a 6-base tract (dC_1–dC_6 tract) in the oligonucleotide substrate might be crucial for an efficient cleavage by Endo IV. To test this assumption, we examined the kinetic parameters of Endo IV with oligonucleotide substrates in which the internal positions ($dN_2dN_3dN_4dN_5$) of a dC_1–dC_6 tract were replaced with dA, dG or dT (Table 2). The V_{max} values with $[dCdTdTdTdTdC]_{6/25}$, $[dCdAdAdA dAdC]_{6/25}$ and $[dCdGdGdGdGdC]_{6/25}$ were 1.8, 2.6 and

Table 2. Kinetic parameters of Endo IV with oligonucleotide substrates containing a target sequence

Oligonucleotide	V_{max} (U/mg)	K_m (μM)
$[(dC)_{15}]_{15/25}$	14.0 (4.24)	6.0 (1.03)
$[(dC)_{11}]_{11/25}$	6.1 (1.85)	5.1 (0.88)
$[dCdCdCdCdCdCdC]_{7/25}$	3.2 (0.97)	6.3 (1.09)
$[dCdCdCdCdCdC]_{6/25}$	3.3 (1.00)	5.8 (1.00)
$[dCdCdCdCdC]_{5/25}$	0.38 (0.11)	12.6 (2.17)
$[dC]_{1/25}$	0.40 (0.12)	14.1 (2.43)
$[dCdTdTdTdTdC]_{6/25}$	1.8 (0.55)	8.8 (1.52)
$[dCdAdAdAdAdC]_{6/25}$	2.6 (0.79)	8.8 (1.52)
$[dCdGdGdGdGdC]_{6/25}$	2.7 (0.82)	9.3 (1.60)

The V_{max} and K_m values of Endo IV were determined with the indicated oligonucleotides shown by short names (Table 1). The relative values were calculated by dividing the values for each substrate by those for $[dCdCdCdCdCdC]_{6/25}$ and shown in the parentheses ( ). Data are means of two independent experiments.

Table 3. Kinetic parameters of Endo IV with various dC_1–dC_6 tracts and the effects of third dC residue in the tracts

Oligonucleotide	V_{max} (U/mg)	K_m (μM)
$[dCdCdCdCdCdC]_{6/25}$	3.3 (1.00)	5.8 (1.00)
$[dCdTdTdTdTdC]_{6/25}$	1.8 (0.55)	8.8 (1.52)
$[dCdCdTdTdTdC]_{6/25}$	4.4 (1.33)	5.4 (0.93)
$[dCdTdCdTdTdC]_{6/25}$	4.6 (1.39)	6.0 (1.03)
$[dCdTdTdCdTdC]_{6/25}$	1.7 (0.51)	5.0 (0.86)
$[dCdTdTdTdCdC]_{6/25}$	1.7 (0.51)	5.8 (1.00)
$[dCdAdCdAdAdC]_{6/25}$	2.6 (0.79)	5.4 (0.93)
$[dCdGdCdGdGdC]_{6/25}$	2.8 (0.85)	6.8 (1.17)
$[dCdCdAdTdTdC]_{6/25}$	3.0 (0.91)	5.2 (0.90)
$[dCdCdTdAdTdC]_{6/25}$	2.1 (0.64)	4.9 (0.84)
$[dCdCdTdTdAdC]_{6/25}$	4.3 (1.30)	5.4 (0.93)
$[dTdCdAdCdTdTdC]_{7/25}$	7.1 (2.15)	4.8 (0.83)
$[dCdTdTdTdTdTdC]_{7/25}$	2.3 (0.70)	12.6 (2.20)
$[dCdCdCdCdCdCdC]_{7/25}$	3.2 (0.97)	6.3 (1.09)

The V_{max} and K_m values of Endo IV were determined with the indicated oligonucleotides shown by short names (Table 1). Others are the same as described in Table 2.

2.7 U/mg, respectively, being comparable to 3.3 U/mg with $[dCdCdCdCdC]_{6/25}$, but significantly higher than that with $[dCdCdCdCdC]_{5/25}$, supporting the notion that two dC residues in the first and sixth positions of a dC_1–dC_6 tract are crucial for an efficient cleavage by Endo IV. We therefore adopted $[dCdTdTdTdTdC]_{6/25}$ as the basic configuration of the substrate to scan for dC residues that affect the kinetic parameters of Endo IV.

Effects of a third dC residue within a dC_1–dC_6 tract on the kinetic parameters of Endo IV

The K_m values with $[dCdTdTdTdTdC]_{6/25}$, $[dCdAdAdA\ dAdC]_{6/25}$ and $[dCdGdGdGdGdC]_{6/25}$ were consistently higher than that with $[dCdCdCdCdCdC]_{6/25}$ (Table 2). Therefore, we assumed that an additional dC residue in the dC_1–dC_6 tract might affect the enzyme-substrate affinity (K_m value). To know the effect of an additional (third) dC residue within the dC_1–dC_6 tract, we determined the kinetic parameters for hydrolysis of oligonucleotide substrates, in which one of the dT residues of $[dCdTdTdTdTdC]_{6/25}$ was replaced with a dC residue. The K_m values with $[dCdCdTdTdTdC]_{6/25}$, $[dCdTdCd\ TdTdC]_{6/25}$, $[dCdTdTdCdTdC]_{6/25}$ and $[dCdTdTdTd\ CdC]_{6/25}$ oligonucleotides were reduced compared to that with $[dCdTdTdTdTdC]_{6/25}$, indicating that an additional dC residue in a dC_1–dC_6 tract improves the enzyme-substrate affinity to the level of that with $[dCdCdCdCd\ CdC]_{6/25}$. This is also the case with derivatives of $[dCdAd\ AdAdAdC]_{6/25}$ and $[dCdGdGdGdGdC]_{6/25}$, in which one of the internal dA or dG was replaced with a dC residue (Table 3).

The V_{max} values with $[dCdCdTdTdTdC]_{6/25}$ and $[dCdT\ dCdTdTdC]_{6/25}$ were 2.4- and 2.6-fold higher than that with $[dCdTdTdTdTdC]_{6/25}$, while those with $[dCdTdTdC\ dTdC]_{6/25}$ and $[dCdTdTdTdCdC]_{6/25}$ oligonucleotides were almost the same level as that with $[dCdTdTd\ TdTdC]_{6/25}$ (Table 3). To analyze why $[dCdCdTdTdT\ dC]_{6/25}$ and $[dCdTdCdTdTdC]_{6/25}$ were more susceptible to cleavage by Endo IV than others, we first determined the kinetic parameters for the hydrolysis of 5′-$(dA)_9$dCd RdRdRdRdC$(dA)_{10}$-3′ (= $[dCdRdRdRdRdC]_{6/25}$, where dR represents dA or dG) and of 5′-$(dA)_9$dCdRdCdRdR dC$(dA)_{10}$-3′ (= $[dCdRdCdRdRdC]_{6/25}$) series of oligonucleotides. The V_{max} values with $[dCdRdCdRdRdC]_{6/25}$ were not markedly increased compared with those with $[dCdRdRdRdRdC]_{6/25}$ (Table 3), indicating that the hydrolysis rate of Endo IV with $[dCdDdCdDdDdC]_{6/25}$ is greater when dD represents dT than when it represents dA or dG.

To identify the dT residues of $[dCdCdTdTdTdC]_{6/25}$ and $[dCdTdCdTdTdC]_{6/25}$, which affect the susceptibility to Endo IV cleavage, we determined the kinetic parameters for hydrolysis of $[dCdCdAdTdTdC]_{6/25}$, $[dCdCd\ TdAdTdC]_{6/25}$ and $[dCdCdTdTdAdC]_{6/25}$ oligonucleotides. The V_{max} value with the sequence $[dCdCdTdT\ dAdC]_{6/25}$ containing the trinucleotide, 5′-dCdTdT-3′, was 2.4-fold greater than that apparent with $[dCdTdTdT\ dTdC]_{6/25}$ (Table 3). Given that, in addition to $[dCdCdTd\ TdAdC]_{6/25}$, both $[dCdCdTdTdTdC]_{6/25}$ and $[dCdTdCd\ TdTdC]_{6/25}$ contain the 5′-dCdTdT-3′ sequence, this trinucleotide within a dC_1–dC_6 tract enhances the cleavage activity as well as the enzyme-substrate affinity. These results indicate that a third dC residue within a dC_1–dC_6 tract may enhance the cleavage efficiency by Endo IV.

Cleavage of a dC_1–dC_6 tract by Endo IV occurs at dC_1 site (polarized cleavage)

To know the dC residue whose 5′ phosphodiester bond is cleaved by Endo IV in the dC_1–dC_6 tract, we examined the cleavage pattern of a Cy5-labeled 45-base oligonucleotide based on a T4 DNA sequence which contains a 5′-dCd TdCdTdTdC-3′ hexameric tract in the middle portion of the oligonucleotide (Cy5-T4A). This oligonucleotide is comparable to $[dCdTdCdTdTdC]_{6/25}$ used for the acid-solubility assay in the previous section. The oligonucleotide $[dCdTdCdTdTdC]_{6/25}$ is one of the optimal substrates for Endo IV having a hexameric dCs tract with a third dC (Table 3), we are able to know the exact cleavage position (s) in the 6 dCs tract using Cy5-T4A. As a result, cleavages occurred efficiently at dC_{20}, but very slightly if any at dC_{22} and dC_{25} (Figure 1). It is remarkable that an efficient cleavage occurred exclusively at the first dC (dC_{20}) in spite of three possible cleavage sites within the 6 dCs tract (a dC_{20}–dT_{21}–dC_{22}–dT_{23}–dT_{24}–dC_{25}). The efficient cleavage of the 6 dCs tract within Cy5-T4A is consistent with the result obtained by the acid-solubility assay (Table 3), and strongly suggests that an efficient cleavage within a 6 dCs occurs at the 5′-proximal end (dC_1 position) even in the oligonucleotide $[dCdTdCdTdTdC]_{6/25}$ used for the acid-solubility assay. Although the acid-solubility assay used in the kinetic analysis by Endo IV does not provide the cleavage site of substrates with a dCs tract, it is reasonable to assume that cleavage events occur at the first dC (dC_1) position within the target tract of oligonucleotide substrates used.

To confirm the assumption that Endo IV cleaves ssDNAs at the dC_1 position in a dC_1–dC_6 tract-depending manner (6 dCs tract-dependent polarized cleavage), we performed a series of cleavage experiments using Cy5-labeled oligonucleotides. Cy5-T4B is a 45mer Cy5-labeled oligonucleotide based on the T4 sequence, having two consecutive hexameric dCs tracts, $dC_{21}dA_{22}dC_{23}dT_{24}\ dT_{25}dC_{26}$, and $dC_{26}dA_{27}dT_{28}dT_{29}dG_{30}dC_{31}$ in the middle portion of the oligonucleotide (Figure 1). Cy5-T4B has 8 dC residues in the 45 nt and other dCs tracts with varied sizes will be described in the later section. Results of cleavage analysis of Cy5-T4B by Endo IV are shown in Figure 2. Two cleavage bands, marked b and d, correspond to the cleavage products occurred at dC_{21} and dC_{26}. Since the b and d bands correspond to the cleavage product occurred at the dC_1 position of 6 dCs tracts, $dC_{21}dA_{22}dC_{23}dT_{24}dT_{25}dC_{26}$ and $dC_{26}dA_{27}dT_{28}dT_{29}d\ G_{30}dC_{31}$, respectively, these results are consistent with and supportive of the 6 dCs tract-dependent polarized cleavage model. An efficient cleavage at dC_{21} (marked b) in a $dC_{21}dA_{22}dC_{23}dT_{24}dT_{25}dC_{26}$ tract is comparable to the 6 dCs tract with a third dC mentioned in the previous section. Another efficient cleavage at dC_{26} is presumably due to the presence of dC_{31} that constitutes a 6 dCs tract, $dC_{26}dA_{27}dT_{28}dT_{29}dG_{30}dC_{31}$. To confirm the 6 dCs

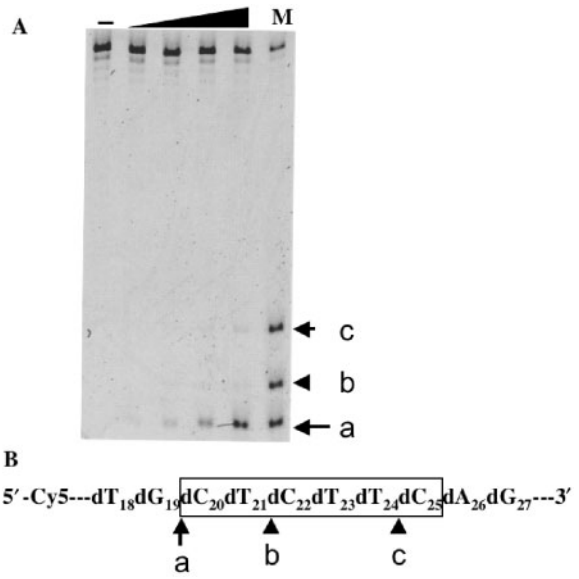

Figure 1. Cleavage pattern analysis of a T4-based oligonucleotide Cy5-T4A by Endo-IV. (**A**) A 45-base oligonucleotide (Cy5-T4A) based on the T4 DNA and labeled at its 5′ end with Cy5 was used as the substrate at a low Cy5-specific activity (10 μM) for assay of the activity of varied amount of Endo IV (0.5, 1, 2 or 4 μg/ml). The reaction products were separated by electrophoresis on a 10% polyacrylamide gel containing 7 M urea and were visualized with an image analyzer. Lane (–) represents a reaction mixture incubated in the absence of enzyme. Lane M represnts a mixture of oligonucleotides labeled at their 5′ ends with Cy5 and with sequences identical to those of residues 1–19 (a), 1–21 (b) and 1–24 (c) of the substrate. Cleavage bands are shown by horizontal leftward arrows. (**B**) A part of Cy5-T4A sequence (from dT_{18} to dG_{27}) is shown. Box shows a dC_1–dC_6 tract with upward arrows (a, b and c) corresponding to the cleavage sites shown in (A).

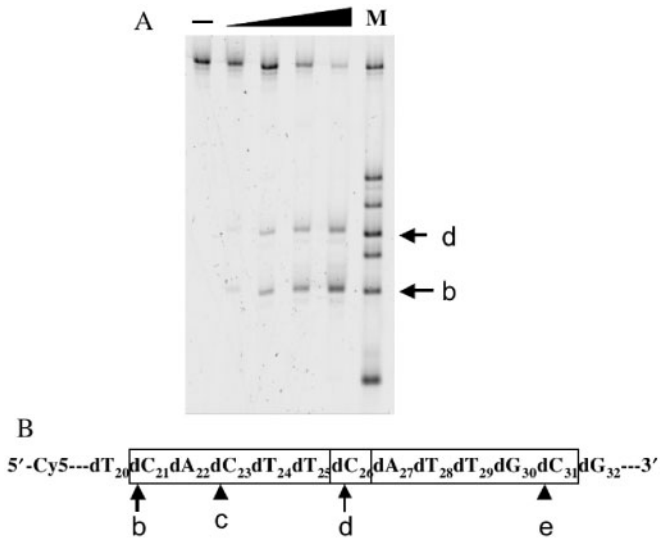

Figure 2. Cleavage pattern analysis of a T4-based oligonucleotide Cy5-T4B by Endo IV. (**A**) A 45-base oligonucleotide (Cy5-T4B) based on the sequence of T4 DNA and labeled with its 5′ end with Cy5 was used as the substrate. Lane (–) represents a reaction mixture without enzyme. Lane M represents a mixture of oligonucleotides labeled at their 5′ ends with Cy5. Marks b and d indicate bands corresponding to the cleavage products shown in (B). Others are the same as described in the legend to Figure 1. (**B**) A relevant portion of Cy5-T4B sequence is shown. Two 6 base dCs tracts ($dC_{21}dA_{22}dC_{23}dT_{24}dT_{25}dC_{26}$ and $dC_{26}dA_{27}dT_{28}dT_{29}dG_{30}dC_{31}$) were boxed. Four dC residues within the sequence which Endo IV might cleave, are shown by arrows (b, c, d and e).

tract-dependent cleavage at dC_{26} (marked d in Figure 2), a derivative of Cy5-T4B, in which the dC_{31} was replaced by dG (Cy5-T4BG), was used. If the cleavage event at dC_{26} of Cy5-T4B is dependent on the presence of dC_{31}, Cy5-T4BG does not give a cleavage band marked d in Figure 2. As shown in Figure 3, a band corresponding to band d was not detected in Endo IV-treated Cy5-T4BG, indicating that a dC residue at 31 position (dC_{31}) is crucial for the cleavage event at dC_{26}, supporting the 6 dCs tract-dependent polarized cleavage. These results are consistent with the results obtained by the acid-solubility assay for dCs tracts and indicating that an efficient cleavage occurs at dC_1 residue depending on the presence of dC_6.

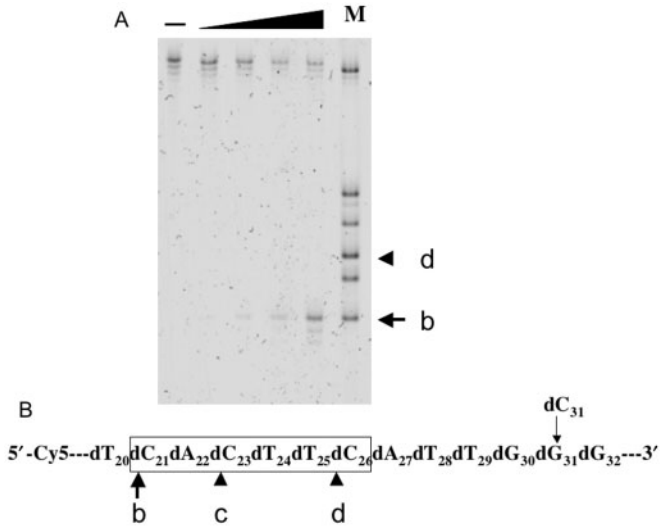

Figure 3. Cleavage pattern analysis of a T4-based oligonucleotide Cy5-T4BG by Endo IV. (**A**) A 45-base oligonucleotide (Cy5-T4BG) that the dC31 of Cy5-T4B was substituted with dG, was used as the substrate. Marks b and d with a horizontal arrow indicate the band positions in the gel corresponding to the cleavage sites (b, c and d) shown in (**B**). Others are the same described in Figure 1.

Some dCs tracts larger than 6 bases behave as a dCs tract cleavable by Endo IV

In addition to the 6 dCs tracts analyzed in the previous section, Cy5-T4B has a 7 dCs tract having a $dC_{31}dG_{32}dA_{33}dA_{34}dT_{35}dG_{36}dC_{37}$, which might be recognized and cleaved as a dCs tract that is larger than 6 bases. If this is the case, a dCs tract having 7 or more bases will be recognized by Endo IV and behaves as an extended dCs tract.

A dCs tract-dependent polarized cleavage predicts that cleavages of a substrate having consecutive dCs tracts cleavable by Endo IV occur in a non-consecutive manner. That is, a substrate molecule with 2 dCs tracts consecutively, once cleaved at the 5′ proximal dC residue within 1 dCs tract should destruct a dC tract adjacent to the cleaved dCs tract. However, if 2 dCs tracts recognizable by Endo IV occur separately (non-consecutively) in a substrate molecule, once-cleaved molecules will possibly

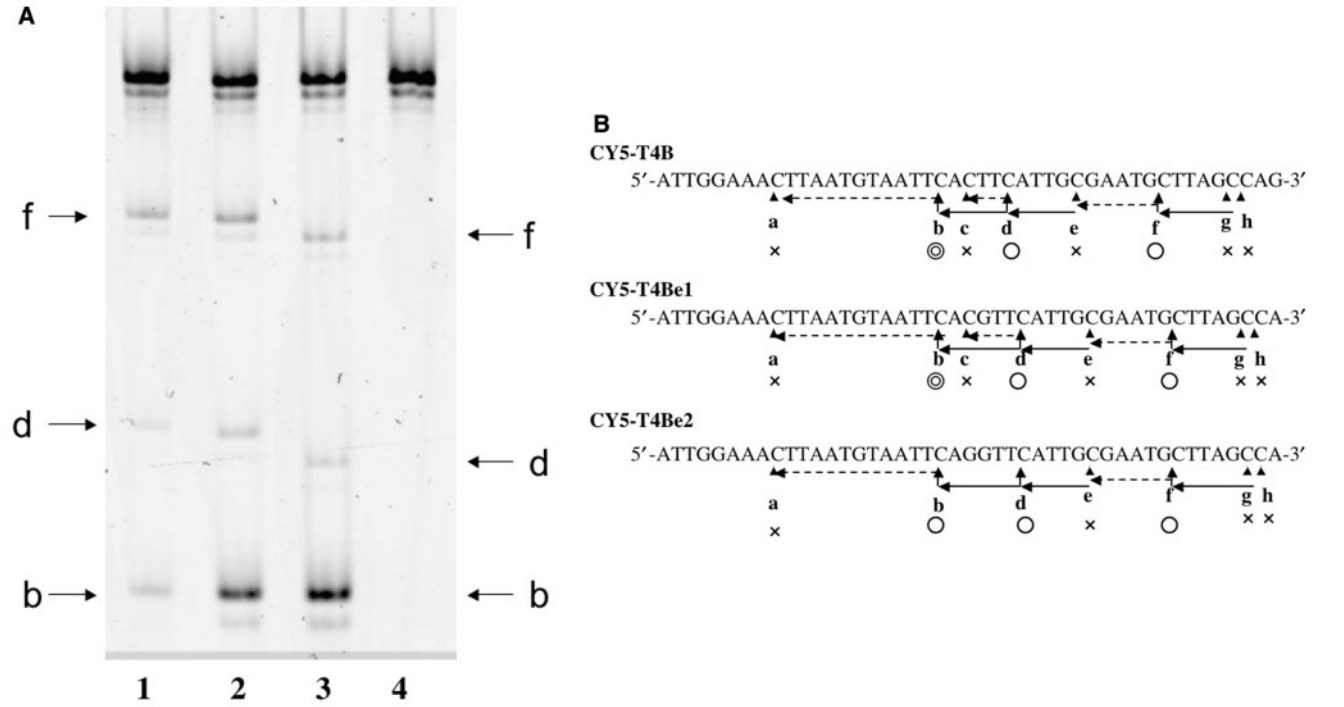

Figure 4. Cleavage pattern analysis of Cy5-T4B derivatives by Endo IV. (**A**) Three 45-base oligonucleotides (Cy5-T4B, Cy5-T4Be1 and Cy5-T4Be2) were used as the substrate. Cleavages were done at a high Cy5-specific activity (10 μM/ml) and a low enzyme concentration (0.66 μg/ml). Lanes 1 and 2 show cleavage products of Cy5-T4Be2 and Cy5-T4Be1, respectively, corresponding to marks with an arrow b, d and f at the left-hand side. Lane 3 shows cleavage products of Cy5-T4B by marks with an arrow b, d and f at the right-hand side. Lane 4 represents a reaction mixture incubated without enzyme. (**B**) Lower cases (a∼h) below the sequences of Cy5-T4B, Cy5-T4Be1 and Cy5-T4Be2 represent possible cleavage positions (dC sites) in each oligonucleotide. Marks with a vertical arrow (b, d and f) correspond to the cleavage products shown in (A). Horizontal arrows with solid line represent dCs tract cleavable by Endo IV and those with dotted line represent dCs tract non-cleavable by Endo IV.

be cleaved again at a 5′-proximal position depending a condition used for the cleavage. Since Cy5-labeled oligonucleotides we used are 5′ end-labeled molecules, we may miss the cleavage products occurred at the 3′-proximal region of a substrate by the secondary cleavage at a 5′ proximal dCs tract-dependent cleavage event.

To detect 3′-proximal cleavage products, we used an Endo IV-cleavage condition in which a high specific Cy5 activity of the oligonucleotides and a lower amount of enzyme were used (see Materials and Methods section). As a result, a cleavage band corresponding to the arrow f (cleaved at dC_{37}) was detected in addition to bands b and d (Lanes 1, 2 and 3 in Figure 4). In contrast, a cleavage band corresponding to an arrow e (cleaved at dC_{31}) was not detected, which would be expected if the 7 dCs tract ($dC_{31}dG_{32}dA_{33}dA_{34}dT_{35}dG_{36}dC_{37}$) was cleaved at the 5′-proximal dC residue. These results reveal that an appreciable cleavage event did not occur at dC_{31} in a 7 dCs tract, $dC_{31}dG_{32}dA_{33}dA_{34}dT_{35}dG_{36}dC_{37}$ tract-dependent manner. A dCs tract-dependent and polarized cleavage at dC_{37} by a 6 dCs tract ($dC_{37}dT_{38}dT_{39}dA_{40}dG_{41}dC_{42}$) is apparent in the same cleavage products.

In a previous paper (5), we used a Cy5-labeled oligonucleotide based on phiX174 ssDNA (referred as Cy5-phiX174-6 in this article) and observed a strong cleavage band corresponding to 32-nt long (5′-dT_1-dG_{32}-3′, band marked c in Figure 5). This cleavage product should

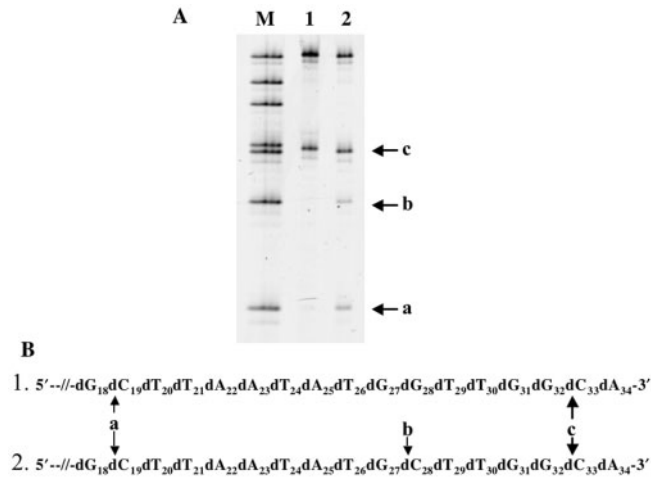

Figure 5. Cleavage analysis of Cy5-phiX174-6 and Cy5-phiX174-6b by Endo IV. (**A**) 45 base Cy5-labeled oligonucleotides based on the sequence of phiX174 ssDNA [Cy5-phiX174-6 (used in Ref. 5) and its derivative (Cy5-phiX174-6b) were cleaved by Endo IV]. Cleavages were done as described in the legend of Figure 4. Lane M represents a size marker mixture of oligonucleotides labeled at the 5′ end with Cy5. Lanes 1 and 2 represent cleavage products of Cy5-phiX174-6b and Cy5-phiX174-6, respectively. Bands a, b and c with arrows alongside the gel correspond to the cleavage sites shown in (**B**). (B) Nucleotide sequences of relevant portions in the substrate oligonucleotides Cy5-phiX174-6b (1) and Cy5-phiX174-6 (2) were shown with possible cleavage sites (lower cases and vertical arrows) in each sequence.

be generated by a cleavage event at dC_{33} depending on a 7 dCs tract ($dC_{33}dA_{34}dC_{35}dG_{36}dT_{37}dT_{38}dC_{39}$), if the 7 dCs tract (an extended dCs tract) with a third dC (dC_{35}) works as a dCs tract cleavable by Endo IV. To know whether an extended 7 dCs tract-dependent cleavage occurs or not, two derivatives of Cy5-T4B, named Cy5-T4Be1 and Cy5-T4Be2, were synthesized and used for the cleavage analysis by Endo IV. Cy5-T4Be1 is identical to Cy5-T4B except for an addition of dG between dC_{23} and dT_{24} of Cy5-T4B. Thus, Cy5-T4Be1 has an extended heptamer dCs tract with a third dC residue, $dC_{21}dA_{22}dC_{23}dG_{24}dT_{25}dT_{26}dC_{27}$, being identical to the 7 dCs tract of phiX174-6 mentioned above. Cy5-T4Be2 is also a derivative of Cy5-T4Be1 in which a third dC (dC_{23}) was substituted with a dG residue. Accordingly, Cy5-T4Be2 has an extended 7 dCs without a third dC residue. Results of cleavage analysis using Cy5-T4Be1 and Cy5-T4Be2 were shown in Figure 4. An appreciable cleavage event (marked b in lane 1, Figure 4) was observed at dC_{21} of Cy5-T4Be2 without a third dC in an extended 7 dCs tract. A cleavage event at dC_{21} of Cy5-T4Be1 with a third dC (dC_{23}) in an extended 7 dCs tract, was obviously more efficient than that of Cy5-T4Be2 without a third dC (marked b in lane 2, Figure 4), indicating that a third dC within an extended 7 dCs tract has an enhancing effect on the polarized cleavage at dC1 as suggested by the acid-solubility assay. This is comparable to the same effect mentioned previously by a third dC residue in a basal 6 dCs tract. These results indicate that a 7 dCs tract having sequence, dCdAdGdGdTdTdC, is cleavable by Endo IV. Thus, at least one 7 dCs tract having dCdGdAdAdTdGdC is not cleavable by Endo IV while the other 7 dCs tract having dCdAdGdGdTdTdC is cleavable.

Although we do not examine extensively whether dCs tracts having a longer size than 7 dCs work as a cleavable target by Endo IV, a 10 dCs tract having a decameric nucleotide, $dC_{19}dT_{20}dT_{21}dA_{22}dA_{23}dT_{24}dA_{25}dT_{26}dG_{27}dC_{28}$ (see Figure 3 of Ref. 5) appears to be cleaved in an extended dCs tract-dependent manner. To clarify whether this is true, we did a cleavage analysis using a Cy5-phiX174-6b, in which the 10th dC from the cleavage point (dC_{19}) has been substituted with dG. Results are shown in Figure 5. When the Cy5-phiX174-6b was cleaved by Endo IV, two bands a and b disappeared (Lane 1, Figure 5A) and band c remained unaffected. In contrast, the original Cy5-phiX174-6 cleaved by Endo IV gave three cleavage bands, a, b and c (Lane 2, Figure 5). These results indicate that the cleavage event at dC_{19} occurred in a 10 dCs tract-dependent manner. Although we have not examined the size limit of dCs tracts cleavable by Endo IV, a tridecameric nucleotide, dCdTdTdAdAdTdGdTdAdA dTdTdC, is not cleavable in an apparent dCs tract-dependent manner (absence of a band in Figure 2, marked a in Figure 4B). Details of the features of extended dCs tracts including the size limit and sequence feature that works as a cleavable tract by Endo IV are under way and will be published elsewhere.

DISCUSSION

Restricted cleavage of ssDNAs by Endo IV

We have previously demonstrated that highly purified Endo IV catalyses endonucleolytic cleavage of ssDNA specifically at the 5′ phosphodiester bond of dC (5), consistent with the previous observations (1,3), and that the efficiency of such cleavage is highly dependent on the identity of the nucleotides surrounding the dC residue (5). We also observed that the digestion of phiX174 ssDNA, heat-denatured T4dC genomic dsDNA and a phiX174-based oligonucleotide by Endo IV was limited (5). Indeed, the cleavage products of phiX174 ssDNA generated by Endo IV were largely acid-insoluble under our assay conditions (5), whereas subsequent treatment of these products with *E. coli* exonuclease I rendered them acid-soluble, as demonstrated for cleavage by partially purified Endo IV of fd ssDNA (1,3). These results indicate that Endo IV does not cleave dC-containing ssDNAs solely in a dC-specific manner, but does in a highly restricted manner.

The basic feature of target sequence by Endo IV

The cleavage rate and substrate affinity of Endo IV with $[dCdCdCdCdCdC]_{6/25}$ were significantly higher than those with $[dCdCdCdCdC]_{5/25}$, suggesting that the length of dC-containing sequence is important for an efficient cleavage by Endo IV. Therefore, we assumed that a 6-base tract having two dC residues at the first and sixth positions (dC_1–dC_6 tract) should be crucial for the efficient cleavage by Endo IV (Figure 6). This was supported by data of the acid-solubility assay with oligonucleotide substrate that have the internal positions (dN_2~dN_5) of a dC_1–dC_6 tract ($dC_1dN_2dN_3dN_4dN_5dC_6$) replaced with dA, dG or dT (Tables 2 and 3). It turned out that the V_{max} values with $[dCdTdTdTdTdC]_{6/25}$, $[dCdAdAdAdAdC]_{6/25}$ and $[dCdG dGdGdGdC]_{6/25}$ were comparable to that with $[dCdCd CdCdCdC]_{6/25}$, but significantly higher than that with $[dCdCdCdCdC]_{5/25}$. Because two dC residues (dC_1 and dC_6) in the dC_1–dC_6 tract largely increase the hydrolysis rate of Endo IV, it is likely that these two dC residues contribute to accommodation of the cleavage site to the active site of Endo IV enzyme, which makes the basic configuration to be highly susceptible to Endo IV cleavage. Since the V_{max} value with $[dCdTdTdTdTd TdC]_{7/25}$ was also comparable to those with $[dCdCdCdC dCdC]_{6/25}$ and $[dCdCdCdCdCdCdC]_{7/25}$ (Table 3), it is likely that a 7-base (dC_1–dC_7) tract behaves likewise as a dC_1–dC_6 tract does. All of the dCs tracts having 5 dCs or shorter examined were not cleavable in a dCs tract-dependent manner. In contrast, all of the 6 dCs tracts tested are cleavable by Endo IV while at least 1 dCs tract having heptamer sequence.

A dCs tract-dependent cleavage occurs exclusively at the first dC residue (dC_1) within the dCs tract

The acid-solubility assay revealed that an efficient cleavage event by Endo IV occurs in a hexameric dCs tract-dependent manner. To identify the dC residue whose 5′ phosphodiester bond is cleaved by Endo IV in the

dC_1–dC_6 tract, first we performed cleavage pattern analysis using a Cy5-labeled oligonucleotide based on a T4 DNA sequence (Cy5-T4A) containing a 5′-dCdTd CdTdTdC-3′ hexameric dCs tract that is identical to the target hexamer of $[dCdTdCdTdTdC]_{6/25}$ used for the acid-solubility assay in the previous section (Tables 2 and 3). The cleavage pattern analysis of Cy5-T4A has revealed that an efficient cleavage occurs almost exclusively at the first dC (dC_1) position within the 6 dCs tract. Although the acid-solubility assay of an oligonucleotide does not provide information as to sites in which the actual cleavage event occurred, the result of cleavage pattern analysis strongly suggests that cleavage event in the $[dCdTdCdTdTdC]_{6/25}$ occurred at the first dC residue in the target tract. It is remarkable that an efficient dC-specific cleavage occurs exclusively at the dC1 position within the 6 dCs tract in spite of 3 dC residues possibly cleavable by Endo IV. A 6 dCs tract-dependent cleavage at the dC_1 position was also confirmed using a set of Cy5-labeled oligonucleotides, Cy5-T4B and Cy5-T4BG. Cy5-T4B has two consecutive 6 dCs tracts, $dC_{21}dA_{22}dC_{23}dT_{24}dT_{25}dC_{26}$ and $dC_{26}dA_{27}dT_{28}dT_{29}dG_{30}dC_{31}$, which is cleaved at dC_{21} and dC_{26} corresponding to the dC1 position of respective 6 dCs tract giving two cleavage bands, b and d (Figure 2). When Cy5-T4BG—i.e. the dC_{31} residue of Cy5-T4B was replaced with dG—was used, a cleavage band corresponding to d (see Figure 3) disappeared. These results indicate that a cleavage event at the dC_{26} of Cy5-T4B is depending on the dC_{31}.

Some extended dCs tracts larger than 6 bases function as a target dCs tract for the polarized cleavage by Endo IV, but some others do not

We have shown that a 6 dCs (dC_1–dC_6) tract is basal and minimal for the dCs tract-depending polarized cleavage by Endo IV. Moreover, some dCs tracts larger than 6 bases ((dC_1–dC_x) tract in which x is bigger than 6) behave as a 6 dCs tract does. Also we have demonstrated that one 7 dCs tract having a sequence dCdAdGdGdTdTdC, functions as a target dCs tract for polarized cleavage by Endo IV and that the other 7 dCs tract having a sequence dCdGdAdAdTdGdC does not function as a cleavable dCs tract. Another 7 dCs tract having a sequence dCdAdC dGdTdTdC was efficiently cleaved at the dC_1 site. So at least some of 7 dCs tracts are cleavable as an extended dCs tract by Endo IV but some others are not. Also a 10 dCs tract having a sequence, dCdTdTdAdAdTdAdTdGdC is cleavable at the 5′-proximal dC position (dC_1) (Figure 3 in Ref. 5). When a dC residue at the 3′-proximal position (dC_{10}) is replaced by dG, a cleavage event at the 5′-proximal position (dC_1) does not occur, indicating that the cleavage event at the dC_1 position in the 10 dCs tract occurred in a dCs tract-depending manner and the further extended dCs tract, having 13 dCs tract, does not.

Two modes of dC-specific cleavage by Endo IV: a dCs tract-independent inefficient cleavage and a dCs tract-dependent efficient cleavage

We have previously shown that a dTdCdA is the most preferable sequence cleaved by Endo IV in a condition in which an oligonucleotide with a single dC residue is used as a substrate (5). In this article, we have revealed that an efficient cleavage by Endo IV occurs exclusively at the 5′-proximal position (dC_1) within a dCs tract having a minimal size of 6 bases (a dCs tract-dependent polarized cleavage). As we discuss in the next section, it is likely that a dCs tract-dependent cleavage of substrate ssDNA starts by recognizing a dC residue ('recruiting dC') at the 3′-proximal position and then scans 'cut-site dC' to the 5′-direction on the substrate. If the 'cut-site dC' is 6 bases from the 'recruiting dC', a cleavage event occurs. Since a dCs tract-dependent cleavage by Endo IV occurs more efficiently than a dCs tract-independent cleavage does, it is reasonable to assume that the dCs tract-dependent cleavage prevails for natural ssDNA substrates. Actually, when a 45-nt long Cy5-T4A with a single 6 dCs tract was used, a cleavage band (marked c in Figure 1) presumably due to a dCs tract-independent cleavage at dTdCdA was detected, although it was very slight. However, when Cy5-T4BG with multiple dCs tracts in addition to the relevant dCs tract (dCdAdCdTdTdC) was used, no cleavage band (marked d) was detected (Figure 3). These results imply that a dCs tract-independent cleavage occurs for a substrate where dC residues are very rare. Accordingly, a dCs tract-dependent cleavage is prevailing for a substrate DNA having multiple dC residues.

A 'dCs Tract Model' for polarized cleavage by Endo IV

In this article, we have demonstrated that Endo IV cleaves ssDNAs in a restricted manner in which an efficient cleavage event occurs exclusively at the dC1 position in a dCs tract-dependent manner. The minimal size of the dCs tract recognized by Endo IV is a hexamer (6 dCs tract). Both the acid-solubility assay and cleavage analysis of Cy5-labeled oligonucleotides indicated that all of the 6 dCs tracts are cleavable by Endo IV, but less than 6 dCs tracts are not. Furthermore, at least some dCs tracts larger than 6 bases and up to at least 10 bases are cleavable in a dCs tract-dependent manner as extended dCs tracts. A model explaining the dCs tract-dependent and polarized cleavage by Endo IV is presented in Figure 6. First of all, a dC residue in a substrate ssDNA must be recognized by Endo IV molecule, since cleavage events occur in a dCs tract-dependent manner. Cleavage events within the dCs tracts occurring exclusively at the 5′-proximal dC (dC_1) and the 3′-proximal dC are variable in distance from the cleavage site dC. Accordingly it is rational to assume that Endo IV first recognizes a dC residue at the 3′-proximal position (dC_6 or dC_x) and that scans a dC residue to the 5′-direction on the substrate. After an Endo IV molecule binds to the 3′-proximal dC as a 'recruiting dC', the enzyme molecule scans the substrate DNA to the 5′-direction to find a dC residue to be cleaved. If the 5′-proximal dC residue locates exactly 6 nt apart from the recruiting dC (dC_6 or dC_x), a cleavage event occurs at the dC_1 residue ('cut-site dC'). If a 5′-proximal dC residue locates at less than 6 bases, no cleavage event occurs. In contrast, if the 5′-proximal dC locates apart more than 6 nt (extended dCs tracts) from the 3′-proximal dC residue, cleavages occur depending presumably on the

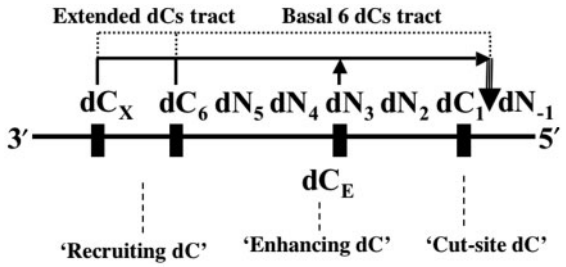

Figure 6. Schematic representation of 'dCs Tract Model' for polarized cleavage by Endo IV. A basal dC_1–dC_6 tract (6 dCs tract) is shown as a horizontal line with two filled boxes (dC_6 and dC_1) from 3′ to 5′ direction. Also, an extended dCs tract is shown by two filled boxes (dC_x and dC_1) in which dC_x locates at the extended position from dC_1 (apart more than 6 bases from dC_1). Endo IV scans ssDNA from 3′ to 5′ direction and recognizes dC residue ('Recruiting dC') at the 3′ proximal position of dC_1 ('Cut-site dC'). A cleavage event occurs at the dC_1 residue shown by a vertical arrow when Endo IV first binds to the recruiting dC residue at the dC_6 or extended dC position. A dC residue (dC_E) that strengthens dCs tract-dependent cleavage reaction at dC_1 residue is also shown.

sequences composing the extended tract. At least 1 dCs tract having 10 bases works as a dCs tract cleavable by Endo IV. A third dC residue within a cleavable dCs tract may enhance the enzyme-substrate binding or the cleavage efficiency ('Enhancing dC') at the 'cut-site dC' locating further to the 5′-direction. Recently, we have isolated a *denB* mutation whose gene product appears to have altered the activity of the dCs tract-dependent polarized cleavage (8). Analysis of the mutant Endo IV may shed more light on the biological and biochemical significance of the enzyme.

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Conflict of interest statement. None declared.

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