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The Ser^{176} of T4 endonuclease IV is crucial for the restricted and polarized dC-specific cleavage of single-stranded DNA implicated in restriction of dC-containing DNA in host *Escherichia coli*

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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. Also the growth of dC-substituted T4 phage and host *Escherichia coli* cells is inhibited by *denB* expression presumably because of the inhibitory effect on replication of dC-containing DNA. Recently, we have demonstrated that an efficient cleavage by Endo IV occurs exclusively at the 5′-proximal dC (dC_1) within a hexameric or an extended sequence consisting of dC residues at the 5′-proximal and the 3′-proximal positions (dCs tract), in which a third dC residue within the tract affects the polarized cleavage and cleavage rate. Here we isolate and characterize two *denB* mutants, *denB*(*W88R*) and *denB*(*S176N*). Both mutant alleles have lost the detrimental effect on the host cell. Endo IV(W88R) shows no enzymatic activity (<0.4% of that of wild-type Endo IV). On the other hand, Endo IV(S176N) retains cleavage activity (17.5% of that of wild-type Endo IV), but has lost the polarized and restricted cleavage of a dCs tract, indicating that the Ser^{176} residue of Endo IV is implicated in the polarized cleavage of a dCs tract which brings about a detrimental effect on the replication of dC-containing DNA.

INTRODUCTION

Endonuclease IV (Endo IV) encoded by *denB* of bacteriophage T4 is implicated in restriction of deoxycytidine (dC)-containing DNA corresponding to the host *Escherichia coli* or its own genomic DNA in the host cells. Indeed, the synthesis of stable dC-substituted T4 (T4dC) genomic DNA requires a defective mutation of *denB* in addition to mutations of gene *42* (which encodes dCMP hydroxymethylase), gene *56* (dCTP/dUTPase) and either gene *46* (46/47 exonuclease) or *denA* (endonuclease II) (1). Under the *denB*$^-$, gene *42*$^-$, gene *56*$^-$, possibly *denA*$^-$ background, an additional mutation in *alc* (*unf*), whose product shuts off transcription of T4dC DNA (2), results in the generation of a plaque-forming T4 phage containing dC-substituted DNA (T4dC phage) (3). Furthermore, a deficiency in *denB* is indispensable for the synthesis of stable T4dC genomic DNA (1) and *denB* expression in *E. coli* cells is lethal (4), even though Endo IV has little effect on the degradation of T4dC (5) or host (6–8) genomic DNA after infection of T4 phage. These results suggest that Endo IV plays a crucial role in inhibition of the replication of T4dC or host genomic DNA rather than in its degradation.

Endo IV composed of 185 amino acid residues requires Mg^{2+} for activity, acts only on single-stranded (ss) DNA and generates 5′ termini containing exclusively dC (4,9–12, H. Ohshima, N. Hirano and H. Takahashi, submitted for publication). The enzyme does not cleave normal T4 genomic DNA-containing glucosylated deoxyhydroxymethylcytidine (gluc-dHMC) (4,9). Endo IV preferentially recognizes short nucleotide sequences that include 5′-dTdCdA-3′ (4,12) in a condition where a unique dC locates within a substrate oligonucleotide. Given that Endo IV is highly toxic to *E. coli* cells, we have synthesized the enzyme using a wheat germ cell-free protein synthesis system (13) and purified to homogeneity (4, H. Ohshima, N. Hirano and H. Takahashi, submitted for publication). Recently, we have demonstrated that a six-base sequence

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comprising dC residues at the first and sixth positions (refers as a dC_1–dC_6 tract or a 6 dCs tract), 5′-$dC_1dN_2dN_3dN_4dN_5dC_6$-3′ (where dN represents dA, dC, dG or dT), is crucial for the efficient cleavage by Endo IV, and a third dC at the $dN_2dN_3dN_4dN_5$ region of a dC_1–dC_6 tract restricts the cleavage activity at the 5′-proximal dC_1 site (restricted and polarized cleavage). A third dC residue placed within a dCs tract enhances the restricted and polarized cleavage. Furthermore, some of dCs tracts having sizes larger than six bases (extended dCs tract) also were shown to behave as a six dCs tract (H. Ohshima, N. Hirano and H. Takahashi, submitted for publication).

Replication of the *E. coli* chromosome occurs bidirectionally from a single locus known as *oriC* (14,15), whereas bacteriophage T4 makes use of two major replication mechanisms that are regulated to occur during certain phases of the infection cycle (16,17). At early period of T4 infection, origin-dependent replication proceeds from several origins and involves initiation from R-loops (18). As infection progresses, origin-dependent replication is repressed, and recombination-dependent replication predominates (19) and involves initiation from D-loops formed by strand invasion (20). Given that R-loops, D-loops and lagging strand of replication fork include ss regions of DNA, the regions of ssDNA in these replication-specific structures may become potential substrates for Endo IV *in vivo*. If this is the case, Endo IV might target certain sequences in the ssDNA regions of these replication-specific structures and thereby effectively inhibit the replication. In this regard, we previously identified that short nucleotide sequences that include 5′-dT/dCdAdCdTdTdC-3′ is the most susceptible to Endo IV cleavage *in vitro* (H. Ohshima, N. Hirano and H. Takahashi, submitted for publication), where the slash indicates the cleavage site by Endo IV. However, the mechanism of sequence recognition by Endo IV that is crucial for the inhibition of replication of dC-containing DNA remains unclear.

To elucidate *in vivo* function of Endo IV, in this study we isolated two T4dC mutants with point mutations in *denB* gene that result in the replacement of Trp^{88} of Endo IV with Arg [*denB*(*W88R*)] or that of Ser^{176} with Asn [*denB*(*S176N*)]. We synthesized the Endo IV enzymes encoded by the mutant *denB* alleles and found that the enzymatic activities of Endo IV(W88R) and Endo IV(S176N) were <0.4 and 17.5% of that of wild-type Endo IV, respectively. Neither of the two mutant alleles had a detrimental effect on the synthesis of T4dC genomic DNA nor the growth of host cells. Furthermore, the sequence 5′-dCdTdT-3′ located at the $dN_2dN_3dN_4dN_5$ region of a dC_1–dC_6 tract was shown to enhance cleavage at the dC_1 site by wild-type Endo IV but not by Endo IV(S176N), indicating that the Ser^{176} residue is crucial for the restricted and polarized cleavage by Endo IV. These results indicate that the Ser^{176} residue of Endo IV implicated in the restricted and polarized cleavage at the dC_1 site of a dCdTdT-containing dC_1–dC_6 tract is responsible for the Endo IV-mediated restriction of dC-containing DNA in *E. coli* cells.

Table 1. Oligonucleotide substrates used and their abbreviated names

Oligonucleotide	Short name[a]
5′-$(dC)_{45}$-3′	$[(dC)_{45}]_{45/45}$
5′-$(dC)_{25}$-3′	$[(dC)_{25}]_{25/25}$
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′-$(dA)_9(dC)_6(dA)_{10}$-3′	$[dCdCdCdCdCdC]_{6/25}$
5′-$(dA)_{10}(dC)_5(dA)_{10}$-3′	$[dCdCdCdCdC]_{5/25}$
5′-$(dA)_{12}(dC)_1(dA)_{12}$-3′	$[dC]_{1/25}$
5′-$(dA)_9$dCdTdTdTdTdC$(dA)_{10}$-3′	$[dCdTdTdTdTdC]_{6/25}$
5′-$(dA)_9$dCdCdTdTdTdTdC$(dA)_{10}$-3′	$[dCdCdTdTdTdTdC]_{6/25}$
5′-$(dA)_9$dCdTdCdTdTdTdC$(dA)_{10}$-3′	$[dCdTdCdTdTdTdC]_{6/25}$
5′-$(dA)_9$dCdTdTdCdTdTdC$(dA)_{10}$-3′	$[dCdTdTdCdTdTdC]_{6/25}$
5′-$(dA)_9$dCdTdTdTdTdCdC$(dA)_{10}$-3′	$[dCdTdTdTdTdCdC]_{6/25}$

[a]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 [ ].

MATERIALS AND METHODS

Materials

Restriction and other enzymes for recombinant DNA technology were obtained from Takara Shuzo. T4 and T4dC genomic DNA were prepared as described previously (21). The plasmid pEUGFP was constructed as described previously (22) and pGEX-6p-1 was obtained from GE Healthcare. *Escherichia coli* strain KH5402-1 [*ilv*, *thyA*, *thr*(Am), *trpE9829*(Am), *metE*, *deo*, *supF6*(Ts)] (23) was provided by the National Institute of Genetics (Japan), and strains LE392 (*supF58*, *supE44*, *hsdR514*, *galK2*, *galT22*, *metB1*, *trpR55*, *lacY1*) and MC1061 (*sup*°, *hsdR*, *mcrB*, *araD139*, *araABC-leu7679*, *lacX74*, *galU*, *galK*, *rpsL*, *thi*) were described previously (24). λCI_{857} lysogen of LE392 and *E. coli* Bb were used as host strains for T4 phages (24). T4GT7 (3), T4dC(+) (24) and T4 *amC87* (gene *42*), *amE51* (gene *56*) and *unf39x6* (*alc-1*) were derived from our T4 phage stocks.

Oligonucleotide substrates used in this article were purchased from Oligo Texas Co. and are listed in Table 1. A 45-base oligonucleotide, 5′-dTdAdGdAdTdTdTdAd GdTdAdAdTdTdAdTdAdTdGdCdTdCdTdTdCdAdTd TdAdGdAdAdAdAdTdTdAdTdTdTdGdTdGdA-3′ (=Cy5-T4A), based on the sequence of T4 DNA and labeled at its 5′ end with Cy5, was commercially synthesized (Sigma-Aldrich, USA).

Genetic manipulation of T4 phage mutants and isolation of single point mutations in T4 *denB*

T4 phage cross and complementation test were performed as described in a previous paper (24). Isolation of *denB* mutations from *denB*-proficient multiple mutant strain of T4 was done as described previously (24). In brief, T4GT7, which contains mutations in gene *42* (*amC87*), gene *56* (*amE51*) and *alc* (*unf39*) in addition to the deletion NB5060 (Δ*rII*–Δ*denB*), was genetically crossed with an *alc* (*unf39*)-deficient mutant having *rII*- and *denB*-proficient genes. Genetic cross was performed on LE392 as host cells. LE392, LE392 (λCI_{857}), MC1061 and

Bb were used as host cells to identify the presence of respective mutation. After crossing on LE392, plaque formers on LE392 (λCI_{857}) at 30°C were screened for the presence of 'amber' mutation(s) by spotting on Bb and LE392. Then, phage clones giving no growth on Bb host were further examined by genetic complementation tests with *amC87* (gene *42*), *amE51* (gene *56*) on Bb cells. Thus, *rII*- and *denB*-proficient phage recombinants having mutations in gene *42* (*amC87*) and gene *56* (*amE51*) besides *alc* (*unf39*), were obtained. Two such T4 clones that give plaques on MC1061 at $\sim 10^{-4}$, but essentially no plaque on Bb host cells were used for further study. These clones may possibly have an additional mutation derived from the original GT7 that affects endonuclease II activity (25). However, this does not affect the results reported here and therefore we do not mention this mutation later.

The plaque formers on MC1061 cells are expected to have mutation in *denB* in addition to the mutations in gene *42* (*amC87*), gene *56* (*amE51*) and *alc* (*unf39*). Plaques appeared on MC1061 cells were purified and the DNAs were subjected to the polymerase chain reaction (PCR) with a 5′ primer and a 3′ primer (4) to amplify the *denB* region. The sequence of this region was determined by the dideoxy chain termination method to identify individual points mutations of *denB*.

Construction of *denB* expression plasmids

The plasmids pBR*denB*^{am}, pBR*denB*(*W88R*)^{am} and pBR*denB*(*S176N*)^{am} were constructed as described previously (4). The *denB*^{am}, *denB*(*W88R*)^{am} and *denB*(*S176N*)^{am} genes, in which the TAC codon for Tyr^{38} is replaced with a TAG nonsense codon, were constructed by PCR with a 5′ primer containing a BamHI site and a 3′ primer containing a SalI site as well as with 5′ and 3′ mutagenic primers (4). The PCR products were introduced into the BamHI–SalI sites of pBR322 (Takara Shuzo) to yield pBR*denB*^{am}, pBR*denB*(*W88R*)^{am} and pBR*denB*(*S176N*)^{am}, and the resulting plasmids were cloned in non-amber-suppressing host cells. The nucleotide sequences of the plasmid inserts were confirmed by the dideoxy chain termination method. All PCR primers were synthesized by Sigma-Aldrich. PCR was performed for 25 cycles of incubation at 98°C for 10 s, 55°C for 10 s and 72°C for 30 s with Prime STAR HS DNA polymerase (Takara Shuzo) in a Takara PCR Thermal Cycler MP.

The effect of *denB* alleles on *E. coli* growth was examined by growth curve analysis of *E. coli* KH5402-1 cells (23) harboring pBR*denB*^{am}, pBR*denB*(*W88R*)^{am} or pBR*denB*(*S176N*)^{am}. The cells were grown overnight at 42°C in LB liquid broth containing thymine and ampicillin at 2 and 100 μg/ml, respectively (LB-Thy-Amp). The resulting cells were used to inoculate (1:100 dilution) fresh LB-Thy-Amp liquid broth and were incubated with shaking ($\sim 140\,min^{-1}$) at 42 or 30°C. Samples were removed from the cultures at various times to measure the optical density at 600 nm (OD_{600}). Given that *E. coli* KH5402-1 cells harbor a temperature-sensitive (Ts) allele of the *supF* suppressor gene, the cells transformed with pBR*denB*^{am}, pBR*denB*(*W88R*)^{am} or pBR*denB*(*S176N*)^{am} would be expected to produce the encoded Endo IV proteins when grown at 30°C but not at 42°C.

Production and purification of GST-Endo IV fusion proteins

A DNA fragment encoding a glutathione S-transferase (GST) fusion protein of Endo IV was constructed and subjected to *in vitro* transcription as described previously (4). 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 (4). 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 (4). 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. The Endo IV mutant proteins were obtained as described for the wild-type protein.

Kinetic analysis of Endo IV activity

Hydrolysis of oligonucleotide substrates (10 μM) was performed as described previously (4, H. Ohshima, N. Hirano and H. Takahashi, submitted for publication). 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 nucleotides 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 analysis of an oligonucleotide corresponding to a T4 DNA sequence by Endo IV

Hydrolysis of a 45-base oligonucleotide (Cy5–T4A, see Materials section) based on the sequence of T4 DNA and labeled at its 5′ end with Cy5 (Sigma-Aldrich) was

performed as described above for the Endo IV assay at a substrate concentration of 10 μM and enzyme concentrations of either 0.5–4 μg/ml for wild-type Endo IV or 1–8 μg/ml for Endo IV(S176N). 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

Screening for single point mutations in *denB*

To clarify the biological significance and function of *denB* and the gene product, Endo IV, we screened and characterized the *denB* mutations and their gene products. The isolation of *denB* mutations was originally described in a previous paper (24). T4 mutant phages having multiple mutations in gene *42* (*amC87*), gene *56* (*amE51*) and *alc* (*unf39*) were plated on MC1061 strain as the host and plaque formers were further characterized in details (see Materials and Methods section). Thus, we identified two types of T4 phage mutants with a single point mutation in *denB* besides the multiple mutations, which resulted in plaque-formable T4 phages with dC-containing DNA on a restriction-minus and suppressor-negative host, such as MC1061. By sequence analysis, the first type of *denB* mutation revealed to have a transition mutation (U to C) in the codon Trp^{88} (UGG) changed to codon Arg (CGG). This mutation coincided with the *denB-s19* mutation of T4dC(+) phage (24) and was named as *denB*(*W88R*). The second type of *denB* mutation had also a transition mutation (G to A) which resulted in a change of codon from Ser^{176} (AGC) to Asn (AAC), and was named as *denB*(*S176N*).

Detrimental effect of wild-type and mutant-type *denB* alleles on *E. coli* growth

As described in a previous paper (4), attempts to clone the wild-type *denB* gene in several different expression vectors, including pET21a under strict control of the T7 promoter, were unsuccessful, presumably because of a highly toxic effect of the *denB* product (Endo IV) on the host *E. coli* cells. We therefore examined the effect of *denB* expression on *E. coli* by using KH5402-1 (*supF6*Ts) cells. The *E. coli* strain has a temperature-sensitive allele of the *supF* suppressor gene (23) with a plasmid containing an amber mutant allele of *denB*, in which the TAC codon for Tyr^{38} is mutated to a TAG nonsense codon. The *E. coli* KH5402-1 cells were used to examine the effects of T4 phage-encoded *denB* (4) and *ndd* (26) genes on the growth of host cells, and a significant growth defect was observed when KH5402-1 cells transformed with a plasmid containing an amber mutant allele of *denB* (4) or *ndd* (26) gene were cultured at 30°C.

To know whether the *denB*(*W88R*) and *denB*(*S176N*) alleles allow the growth of *E. coli* cells, we transformed KH5402-1 cells with a plasmid-containing *denB* mutant alleles in which an amber mutation had been introduced at the codon for Tyr^{38} (TAC). Cells cultured at 30°C would thus be expected to produce the full-length Endo IV mutant enzymes, whereas those cultured at 42°C would not. We previously showed that culture at 30°C of KH5402-1 cells transformed with pBR*denB*^{am} reduced colony-forming ability compared with that apparent at 42°C, and most (99%) of the original colonies that formed at 30°C did not give rise to colonies in the presence of the selection drug (ampicillin) at 42°C (4). These results indicated that most colonies that formed at 30°C consisted of cells lacking pBR*denB*^{am} and that most cells that expressed wild-type Endo IV were not viable. Here we examined the effects of *denB* expression on the growth of KH5402-1 cells transformed with pBR*denB*^{am}. KH5402-1 cells transformed with pBR*denB*^{am} revealed essentially no growth at 30°C, but normal growth at 42°C, and KH5402-1 cells transformed with pBR322 grow normally both at 30 and 42°C (Figure 1A). In contrast, the growth at 42 or 30°C of KH5402-1 cells transformed with pBR322 containing amber mutant forms of *denB*(*W88R*) or *denB*(*S176N*) alleles did not differ substantially from that of those transformed with pBR322 alone (Figure 1B). These results suggested that *E. coli* cells expressing the intact Endo IV mutant enzymes, Endo IV(W88R) and Endo IV(S176N), were viable and that, in contrast

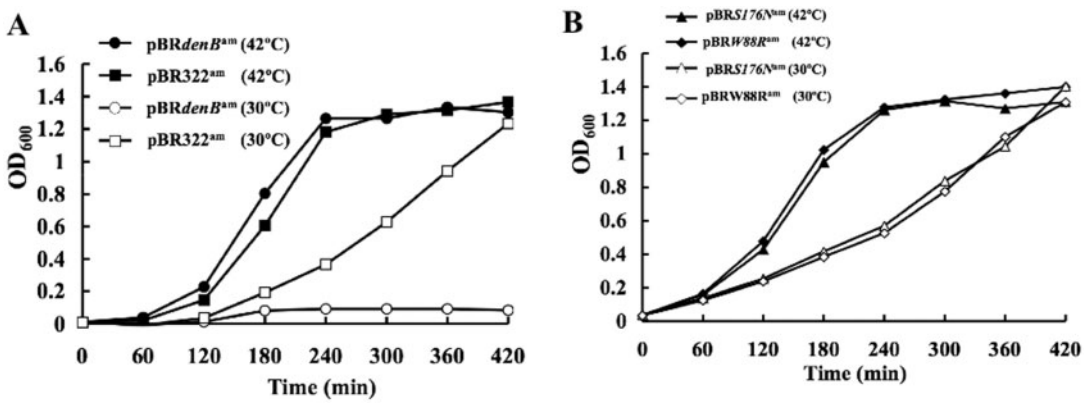

Figure 1. Effect of *denB*(*W88R*) and *denB*(*S176N*) alleles on *E. coli* growth. Cultures of KH5402-1 cells harboring either pBR322 or pBR*denB*^{am} (**A**) or pBR*W88R*^{am} or pBR*S176N*^{am} (**B**) were grown overnight in LB-Thy-Amp liquid broth at 42°C, and the cells were then used to inoculate fresh LB-Thy-Amp liquid broth and incubated at 42°C or 30°C. Samples were removed at the indicated times for determination of OD_{600}. Data are means of values from two independent experiments.

to wild-type Endo IV, these mutant enzymes do not show detrimental effect on the host cells.

Enzymatic activities of Endo IV(W88R) and Endo IV(S176N)

To examine the Endo IV activity encoded by these *denB* mutant alleles, we synthesized wild-type and mutant Endo IV as GST fusion proteins by *in vitro* translation with a wheat germ cell-free protein synthesis system (4). The amounts of purified Endo IV(W88R) and Endo IV(S176N) obtained were similar to that of wild-type Endo IV. We previously developed the acid-solubility assay for Endo IV activity (4). The acid-solubility assay is based on the endonucleolytic activity of Endo IV to generate acid-soluble products from an acid-insoluble oligonucleotide substrate. All of the oligonucleotide substrates were so designed as to be acid-insoluble before Endo IV cleavage and to generate acid-soluble products after the cleavage. The acid-solubility assay was successfully applied to the kinetic analysis of Endo IV with oligonucleotide substrates consisting of two stretches of appropriate length of oligo(dA) attached to both the 5′ and 3′ termini of varied dC-containing sequence (4, H. Ohshima, N. Hirano and H. Takahashi, submitted for publication). All of the oligonucleotide substrates used in this study were identical to those used in the previous study (H. Ohshima, N. Hirano and H. Takahashi, submitted for publication).

The *denB* product of T4dC phages would not be expected to possess enzymatic activity, because Endo IV catalyzes endonucleolytic cleavage of dC-containing ssDNA. To confirm this expectation, we examined the enzymatic activities of Endo IV(W88R), Endo IV(S176N) and wild-type Endo IV with 45-base 5′-$(dC)_{45}$-3′ ($[(dC)_{45}]_{45/45}$), 25-base 5′-$(dC)_{25}$-3′ ($[(dC)_{25}]_{25/25}$) and various oligonucleotides as substrates listed in Table 1. The activity of wild-type Endo IV decreased as the length of dC tract decreased from 15 to 5, and its enzymatic activity with $[dC]_{1/25}$ did not differ from that with $[dCdCdCdCdC]_{5/25}$ (Figure 2). As expected, Endo IV(W88R) showed essentially no Endo IV activity, which was <0.4% of that of wild-type Endo IV with $[(dC)_{25}]_{25/25}$. In contrast, Endo IV(S176N) showed a substantial level of Endo IV activity, which was 17.5% of that of wild-type Endo IV with $[(dC)_{25}]_{25/25}$ and equal to that of wild-type Endo IV with $[dCdCdCdCdC]_{5/25}$, even though the *denB*(*S176N*) allele allowed the synthesis of T4dC genomic DNA and the growth of host cells. In addition, the activity of wild-type Endo IV increased markedly (by a factor of 20) as the length of dC tract increased from five to six, whereas that of Endo IV(S176N) did not (by a factor of 2). These results indicated that the S176N mutation might affect the sequence preference of Endo IV.

Sequence preferences of Endo IV and Endo IV(S176N)

To examine a difference in the enzymatic activity with $[dCdCdCdCdCdC]_{6/25}$ between wild-type Endo IV and Endo IV(S176N) in more detail, we compared their kinetic parameters for the hydrolysis of this substrate (Figure 3). The V_{max} of Endo IV(S176N) with $[dCdCdCdCdCdC]_{6/25}$ was reduced by a factor of 1.9 and its K_m was increased by a factor of 1.6 compared with the corresponding values of wild-type Endo IV, indicating that the *denB*(*S176N*) mutation resulted in both a lower hydrolysis rate and a lower substrate affinity.

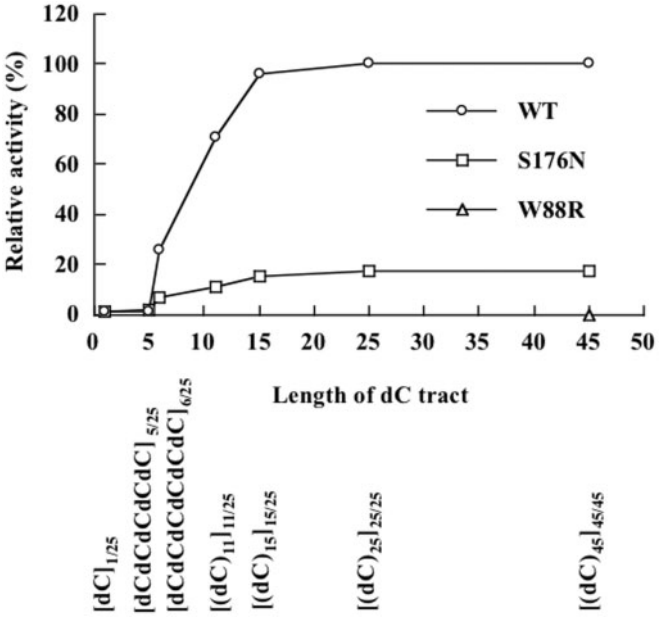

Figure 2. Effect of dC tract length on enzymatic activities of wild-type and mutant (S176N or W88R) forms of Endo IV. Enzymatic activity was determined by measurement of the amount of acid-soluble nucleotides released from the substrate (10 μM). All substrates with the exception of $[(dC)_{45}]_{45/45}$ contained 25 nt. The specific activity of the wild-type (WT) enzyme with the $[(dC)_{25}]_{25/25}$ substrate was ~8.0 U/mg. Relative activity was calculated by dividing the enzymatic activity of each Endo IV enzyme observed with each substrate by that apparent with the wild-type enzyme and $[(dC)_{25}]_{25/25}$ as substrate. Data are means of values from two independent experiments.

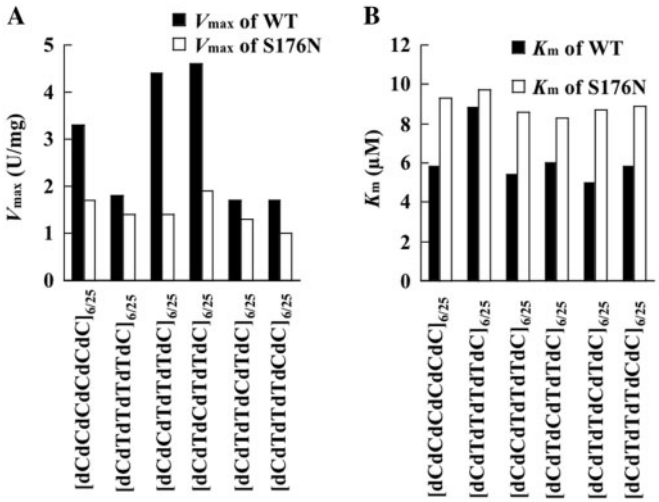

Figure 3. Analysis and comparison of the sequence preferences of wild-type and S176N mutant forms of Endo IV. The V_{max} (**A**) and K_m (**B**) of the wild-type (WT) and S176N mutant forms of Endo IV were determined with series of 25-base oligonucleotides $[dCdYdYdYdC]_{6/25}$ (where dY represents dT or dC) and shown by black columns (WT) and white columns (S176N) in the figure. Data are means of values from two independent experiments.

Since the activity of Endo IV(S176N) did not markedly increase compared with that of wild-type Endo IV as the length of dC tract increased from five to six (Figure 2), the *denB*(*S176N*) mutation might affect recognition of 5′-$dC_1dN_2dN_3dN_4dN_5dC_6$-3′ (dC_1–dC_6) tract by Endo IV (H. Ohshima, N. Hirano and H. Takahashi, submitted for publication). To examine this possibility, we compared the kinetic parameters of wild-type Endo IV and Endo IV(S176N) for the hydrolysis of $[dCdTdTdTdTdC]_{6/25}$, $[dCdCdTdTdTdC]_{6/25}$, $[dCdTdCdTdTdC]_{6/25}$, $[dCdTdTdCdTdTdC]_{6/25}$ and $[dCdTdTdTdCdC]_{6/25}$ oligonucleotides (Figure 3). The K_m and V_{max} of Endo IV(S176N) with $[dCdTdTdTdTdC]_{6/25}$ were similar to those of wild-type Endo IV with this substrate (Figure 3), indicating that the *denB*(*S176N*) mutation does not affect recognition of the dC_1 and dC_6 residues of a dC_1–dC_6 tract by Endo IV. In contrast, the V_{max} values of wild-type Endo IV with $[dCdCdTdTdTdC]_{6/25}$ and $[dCdTdCdTdTdC]_{6/25}$, both of which contain 5′-dCdTdT-3′ at the $dN_2dN_3dN_4dN_5$ region, were ~2.5-fold greater than its V_{max} with $[dCdTdTdTdTdC]_{6/25}$ (Figure 3A), whereas the V_{max} values of Endo IV(S176N) with all the substrates tested were similar to each other (Figure 3A), indicating that the *denB*(*S176N*) mutation disrupts recognition of a 5′-dCdTdT-3′ sequence located at the $dN_2dN_3dN_4dN_5$ region of a dC_1–dC_6 tract by Endo IV. In addition, the K_m values of wild-type Endo IV with $[dCdCdTdTdTdC]_{6/25}$, $[dCdTdCdTdTdC]_{6/25}$, $[dCdTdTdCdTdC]_{6/25}$ and $[dCdTdTdTdCdC]_{6/25}$, all of which contain a dC residue at the $dN_2dN_3dN_4dN_5$ region, were ~1.6-fold lower than its K_m with $[dCdTdTdTdTdC]_{6/25}$ (Figure 3B), whereas the K_m values of Endo IV(S176N) with all the substrates tested were substantially identical (Figure 3B), indicating that Endo IV(S176N) markedly reduces the affinity to the third dC residue within a dC_1–dC_6 tract. Thus, the mutant enzyme is unable to recognize a 5′-dCdTdT-3′ sequence within a dC_1–dC_6 tract and did not show enhanced cleavage at the same site observed by wild-type Endo IV.

Endo IV(S176N) is not able to cleave at the 5′ proximal dC (dC_1) in a dC_1–dC_6 tract

We have previously demonstrated that a 5′-dCdTdT-3′ sequence placed at the $dN_2dN_3dN_4dN_5$ region of a dC_1–dC_6 tract directs the cleavage activity of Endo IV at the dC_1 site (H. Ohshima, N. Hirano and H. Takahashi, submitted for publication). Since Endo IV(S176N) did not recognize 5′-dCdTdT-3′ element within a dC_1–dC_6 tract, the *denB*(*S176N*) mutation might affect the restricted and polarized cleavage at the dC_1 site by Endo IV. To examine this possibility, we compared the cleavage patterns of a Cy5-labeled 45-base oligonucleotide corresponding to a T4 DNA sequence containing a 5′-$dC_1dT_2dC_3dT_4dT_5dC_6$-3′ tract (Cy5–T4A) by wild-type Endo IV and Endo IV(S176N) (Figure 4). The maximal concentrations of wild-type Endo IV and Endo IV(S176N) used in these assays were 4 and 8 μg/ml, respectively, and the cleavage products were separated by electrophoresis on a polyacrylamide gel containing 7 M urea. The substrate contains three dC residues at positions 20, 22 and 25, which correspond to the dC_1, dC_3 and dC_6 positions,

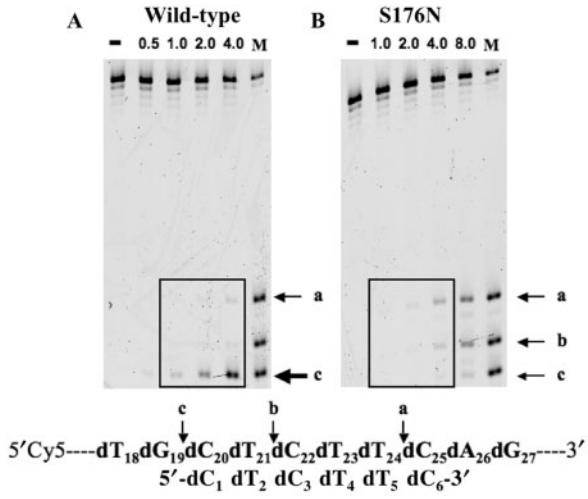

Figure 4. Comparison of cleavage patterns of oligonucleotide containing the 5′-dCdTdCdTdTdC-3′ corresponding to a T4 DNA sequence by wild-type and S176N mutant forms of Endo IV. A 45-base oligonucleotide (Cy5–T4A) based on the sequence of T4 DNA and labeled at its 5′ end with Cy5 was used as the substrate (10 μM) for assay of the activity of wild-type (0.5, 1.0, 2.0 or 4.0 μg/ml) (**A**) or S176N mutant (1.0, 2.0, 4.0 or 8.0 μg/ml) (**B**) forms of Endo IV. 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 represents a mixture of oligonucleotides labeled at their 5′ ends with Cy5 and with sequences identical to those of residues 1 to 19, 1 to 21 or 1 to 24 of the substrate. Cleavage sites of the substrate are indicated by arrows, and dC sites in dG_{19}–dC_{20}–dT_{21}, dT_{21}–dC_{22}–dT_{23} and dT_{24}–dC_{25}–dA_{26} are indicated by a, b and c, which correspond to the dC_1, dC_3 and dC_6 sites of the 5′-$dC_1dT_2dC_3dT_4dT_5dC_6$-3′ tract, respectively.

respectively, of the 5′-$dC_1dT_2dC_3dT_4dT_5dC_6$-3′ tract. Wild-type Endo IV (Figure 4A) cleaved exclusively at the dC_1 site in dG_{19}–dC_{20}–dT_{21} (marked c) while Endo IV(S176N) (Figure 4B) cleaved most efficiently at the dC_6 site in dT_{24}–dC_{25}–dA_{26} (marked a) and less efficiently at the dC_3 site in dT_{21}–dC_{22}–dT_{23} (marked b), indicating that the *denB*(*S176N*) mutation disrupts the restricted and polarized cleavage at the dC_1 site (marked c) of the 5′-$dC_1dT_2dC_3dT_4dT_5dC_6$-3′ tract by Endo IV. These results suggest that Ser^{176} of Endo IV recognizing the third dC residues included 5′-dCdTdT-3′ within a dC_1–dC_6 tract is crucial for the restricted and polarized cleavage at the dC_1 site by Endo IV. In addition, although Endo IV(S176N) has lost the ability to restrict the cleavage site to the dC_1 site, it retains an ability to cleave dC-specific manner and a 5′-dTdCdA-3′ preference that is characteristic of wild-type Endo IV (4).

DISCUSSION

Role of Ser^{176} in the sequence preference of Endo IV

Only limited information on the sequence preference of Endo IV has been available (4,12), and the small number of Endo IV-related proteins in the genome sequence databases has restricted the amount of insight provided by such proteins into the mechanism of sequence recognition by Endo IV. Recently, we have found that

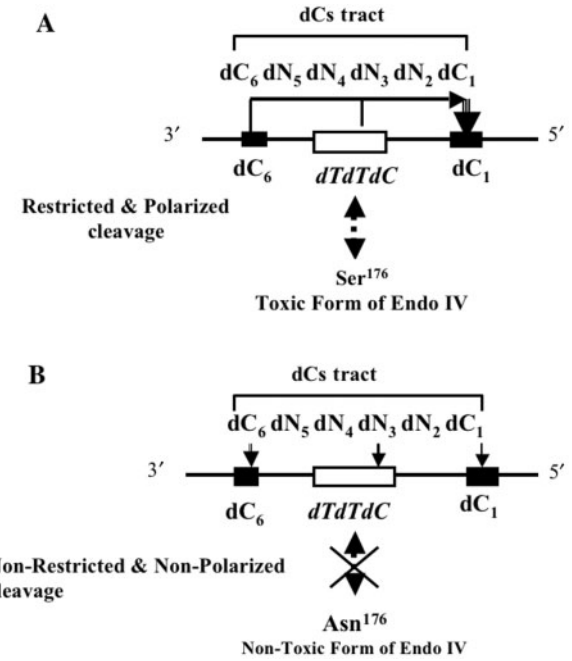

Figure 5. Schematic representation of the dCs tract model for restricted dC-specific cleavage by Endo IV and the role of Ser^{176}. **(A)** A dCs tract is shown as a horizontal line with two closed boxes (dC_6 and dC_1) from the 3′ to 5′ direction. A downward arrow indicates the point where an endonucleolytic cleavage by Endo IV occurs and a rightward arrow indicates the element enhancing and restricting the enzymatic activity (V_{max} and K_m) of Endo IV. Endo IV binds to both dC_1 and dC_6 residues, and especially the Ser^{176} residue of the enzyme contributes to recognition of a dC residue located at the $dN_2dN_3dN_4dN_5$ region of a dCs tract and leads the enzyme to exhibit cytotoxicity and recognize the 5′-dCdTdT-3′ trinucleotide element enhancing the cleavage activity at the dC_1 site. This interaction is required for the restricted and polarized cleavage at the dC_1 site by Endo IV. (**B**) Replacement of Ser^{176} with Asn disrupts the interaction between Endo IV and a dC residue located at the $dN_2dN_3dN_4dN_5$ region, resulting in the losses of restricted and polarized cleavage at the dC_1 site and cytotoxicity.

a 5′-$dC_1dN_2dN_3dN_4dN_5dC_6$-3′ (dC_1–dC_6) tract is crucial for the efficient cleavage by wild-type Endo IV (Figure 5A) (H. Ohshima, N. Hirano and H. Takahashi, submitted for publication). A third dC residue located at the $dN_2dN_3dN_4dN_5$ region of a dC_1–dC_6 tract improves the affinity of Endo IV to substrates, and a 5′-dCdTdT-3′ sequence at the $dN_2dN_3dN_4dN_5$ region restricts and enhances the Endo IV cleavage at the dC_1 site (Figures 4A and 5A) (H. Ohshima, N. Hirano and H. Takahashi, submitted for publication).

In this report, we have shown that the affinity of Endo IV(S176N) to the third dC residue located at the $dN_2dN_3dN_4dN_5$ region is markedly reduced and therefore a 5′-dCdTdT-3′ sequence at the $dN_2dN_3dN_4dN_5$ region does not either restrict or enhance the cleavage activity of Endo IV(S176N) at the dC_1 site (Figures 4B and 5B), whereas Endo IV(S176N) retains an ability to cleave dC-specific manner and a 5′-dTdCdA-3′ preference (Figure 4B). These results suggest that the Ser^{176} residue that contributes to recognition of the third dC residue located at the $dN_2dN_3dN_4dN_5$ region of a dC_1–dC_6 tract is crucial for enhancement of the cleavage activity of Endo IV with a dCdTdT-containing dC_1–dC_6 tract and the restricted and polarized cleavage at the dC_1 site by Endo IV. In addition, because the Ser^{176} residue markedly increases the hydrolysis rate of Endo IV with a dCdTdT-containing dC_1–dC_6 tract, Ser^{176} may contribute to accommodation of the cleavage site (dC_1 site) to the active site of Endo IV enzyme, resulting in the efficient cleavage at the dC_1 site by Endo IV.

Possible mechanism of Endo IV action *in vivo*

A deficiency in *denB* suppresses the lack or arrest of T4dC genomic DNA synthesis in a gene *56*$^-$ (1,27,28) or gene *42*$^-$, gene *46*$^-$, gene *56*$^-$ (1) background, respectively, and T4dC phage mutants with a *denB*$^+$ background cease synthesizing their genomic DNA within 30 min after infection (1). In addition, several *denB* point mutations that allow stable T4dC genomic DNA synthesis in the gene *56*$^-$ background result in a complete loss of Endo IV activity (confirmed *in vitro*) (29). Moreover, *denB* expression in *E. coli* cells is lethal (4). Together, these various observations so far suggest that a lack of Endo IV activity is indispensable for the replication of dC-containing DNA.

We have now identified the *denB*(*W88R*) and *denB*(*S176N*) alleles that allowed plaque-forming T4dC phage in the gene *42*$^-$, gene *56*$^-$, *alc*$^-$, possibly *denA*$^-$ background and did not exhibit the detrimental effect on host cell growth, suggesting that these *denB* alleles do not inhibit the replication of both T4dC and host genomic DNAs. Indeed, Endo IV(W88R) exhibited essentially no Endo IV activity (<0.4% of that of wild-type Endo IV), consistent with the suggested indispensability of a deficiency of *denB* for the replication of dC-containing DNA. However, Endo IV(S176N) possessed a substantial level of Endo IV activity (17.5% of that of wild-type Endo IV) and a 5′-dTdCdA-3′ preference. The comparison of the enzymatic activities of wild-type Endo IV and Endo IV(S176N) indicated that the *denB*(*S176N*) mutation did not enhance the cleavage activity of Endo IV with a dCdTdT-containing dC_1–dC_6 tract and lost the restricted and polarized cleavage at the dC_1 site, which might be responsible for the loss of the ability of Endo IV to restrict dC-containing DNA in the host cells.

Replication of *E. coli* genome initiates from a single origin, *oriC* (14,15), whereas bacteriophage T4 makes use of two major mechanisms of replication initiation, which involve R-loops at several origins, *oriA*, *oriE*, *oriF* and *oriG*, and D-loops in the process of recombination-dependent replication (16–20). However, because dCdTdT-containing dC_1–dC_6 tracts are not found abundantly in the proximal regions of any of T4 and *E. coli* origins, Endo IV may not specifically target the initiation-specific structures of replication at these origins but target various ssDNA regions of replicating DNA, such as those involved in R-loops, D-loops and lagging strand of replication fork. In fact, no sequence consisting of more than six consecutive dC residues crucial for the efficient cleavage by Endo IV is found in T4 genomic DNA (168 903 bp), whereas dCdTdT-containing dC_1–dC_6 tracts being the enhanced target sequence by Endo IV are found

frequently in T4 genomic DNA with an average frequency of 1 per 938 bp. Thus, recognition of the sequences that include a dCdTdT-containing dC_1–dC_6 hexanucleotide tract in T4 genomic DNA by Endo IV may be crucial for the effective inhibition of T4dC genomic DNA synthesis.

The mechanism of restriction of dC-containing DNA in the host cells by Endo IV remains elusive. The *denB*(*S176N*) mutation reduced the enzymatic activity with the oligo(dC) substrates and the V_{max}/K_m values with the substrates containing a dCdTdT-containing dC_1–dC_6 tract only by a factor of ~5 (Figures 2 and 3), raising a question why the *denB*(*S176N*) allele allows both T4 and *E. coli* replication-apparatuses to replicate their dC-containing genomic DNAs. A five-fold reduction of the Endo IV activity *in vivo* may simply make both T4 and *E. coli* replication-apparatuses possible to replicate dC-containing DNA due to contribution of the endogenous repair systems. Alternatively, if the cleavage at the dC_1 site of a dCdTdT-containing dC_1–dC_6 tract is critical for the inhibition of both replications by T4 and *E. coli* replication-apparatuses, a loss of the restricted cleavage at the dC_1 site of the tract may greatly reduce the accumulation of damage to both replicating DNAs *in vivo*. *In vivo* studies of the effects of *denB* mutant alleles on *E. coli* growth, which retain the restricted cleavage at the dC_1 site of the tract but reduce the enzymatic activity, may provide insight into the mechanism of Endo IV-mediated restriction of dC-containing DNA in *E. coli* cells.

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Conflict of interest statement. None declared.

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