

Insertion and Deletion Mutants of *FokI* Restriction Endonuclease*

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***FokI* restriction endonuclease recognizes the nonpalindromic pentadeoxyribonucleotide, 5'-GGATG-3':5'-CATCC-3' in duplex DNA and cleaves 9 and 13 nucleotides away from the recognition site. We have reported the presence of two distinct and separable protein domains within this enzyme: one for the sequence-specific recognition of DNA (the DNA binding domain) and the other for the endonucleases activity (the cleavage domain). Our studies have suggested that the two domains are connected by a linker region, which appears to be amenable for repositioning of the DNA-sequence recognition domain with respect to the catalytic domain. Here, we report the construction of several insertion (4-, 8-, 12-, 18-, 19-, or 23-amino acid residues) and deletion (4- or 7-amino acid residues) mutants of the linker region of *FokI* endonuclease. The mutant enzymes were purified, and their cleavage properties were characterized. The mutants have the same DNA sequence specificity as the wild-type enzyme. However, compared with the wild-type enzyme, the insertion mutants cleave predominantly one nucleotide further away from the recognition site on both strands of the DNA substrate. The four-codon deletion mutant shows relaxed specificity at the cut site while the seven-codon deletion appears to inactivate the enzyme. The DNA binding and cleavage domains of *FokI* appear to be linked by a relatively malleable linker. No simple linear relationship exists between the linker length and the distance of the cut site from the recognition site. Furthermore, the four-codon insertion mutants cleave DNA substrates containing hemi-methylated *FokI* sites; they do not cleave fully methylated substrates. These results are best explained as a consequence of protein-protein interactions between the domains.**

The *FokI* restriction endonuclease from *Flavobacterium okeanokoites* belongs to the type IIS class. It recognizes the asymmetric sequence 5'-GGATG-3' and cleaves double-stranded DNA at staggered sites 9 and 13 nucleotides away from the recognition site (1, 2). Several laboratories have independently cloned and sequenced the *FokI* restriction-modification system¹ (3, 4). Many laboratories have purified *FokI* endonuclease and characterized its properties (6–10). Our studies on proteolytic fragments of *FokI* endonuclease using trypsin have revealed an N-terminal DNA binding domain and a C-

terminal catalytic domain with nonspecific DNA cleavage activity (11, 12). Our studies have suggested that the two domains are connected by a linker region which is susceptible to cleavage by trypsin. We have also shown that insertion of four (or seven codons) between the recognition and cleavage domains of *FokI* can alter the cleavage distance of *FokI* within its substrate (13). Recently, Waugh and Sauer (14) have shown that single amino acid substitutions uncouple the DNA-binding and strand scission activities of *FokI* endonuclease. Furthermore, they have obtained a novel class of *FokI* restriction endonuclease mutants that cleave hemi-methylated DNA substrates (15). The modular structure of *FokI* suggested that it may be feasible to construct hybrid endonucleases with novel sequence-specificity by linking other DNA-binding proteins to the cleavage domain of *FokI* endonuclease. Recently, we reported the construction of the first "chimeric" restriction endonuclease by linking the *Ubx* homeo domain to the cleavage domain of *FokI* (16).

To further probe the linker region, we have constructed several insertion and deletion mutants of *FokI* endonuclease. Here we describe the properties of these mutants.

EXPERIMENTAL PROCEDURES

Escherichia coli RR1 strain was the host in all experiments. RR1 [pACYC*fokIM*] which carries a single copy of the *FokI* methyltransferase gene (*fokIM*) was used for the construction and expression of the insertion and deletion mutants of *FokI* endonuclease. The mutant genes were cloned into pRRS under the control of *lacUV5* promoter. The structure of pRRS*fokIR* and pACYC*fokIM* are described elsewhere (11). The pTZ19R plasmid DNA was used as the substrate to assay the activity of the mutant enzymes.

Construction of Insertion and Deletion Mutants of *FokI* Endonuclease—Polymerase chain reaction technique was used to insert or delete amino acid residues within the proposed linker region of *FokI* endonuclease as described elsewhere (13). The polymerase chain reaction generated DNA containing the insertions or deletions were digested with *SpeI/SmaI* and gel-purified. The plasmid pRRS*fokIR* that contains a single *SpeI* site within the *fokIR* gene was cleaved with *SpeI/SmaI*, and the large 3.9-kilobase fragment was gel-purified and ligated to the polymerase chain reaction product. After selection on LB plates containing tetracycline (20 µg/ml) and ampicillin (60 µg/ml), the plasmid DNA from the surviving clones were screened for larger *SpeI/HindIII* inserts as compared to the wild-type fragment by agarose (1.5%) gel electrophoresis. The cultures of the positive clones were induced with isopropyl-β-D-thiogalactopyranoside. After sonication, the mutant enzymes were partially purified using a phosphocellulose column and then were assayed for restriction endonuclease activity. In addition, the recombinant plasmids from the active clones were isolated, and the presence of the insertions or deletions was confirmed by Sanger's dideoxy sequencing method (17). The construction of the (Gly₄Ser)₃ insertion mutant was done by synthesizing the appropriate complementary oligonucleotides. The oligonucleotides were annealed to yield the duplex shown below:

XbaI
5'-GGCTCTAGACGGCGGTGGAGGATCAGGGGAGGAGGTAGC-3' : 3'-CCCCC-
TCCTCCATCGCCGCTCCGCTAGTGTGATCAGG-5'
SpeI

It is flanked by *XbaI* and *SpeI* restriction sites. The annealed duplex was filled-in by using dNTPs and Klenow fragment to generate the *XbaI* and *SpeI* sites. It was then digested with *SpeI/XbaI*, purified using G-25

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¹ L. P. Wu and S. Chandrasegaran, unpublished results.

spun column and then ligated into *SpeI*-cleaved pRRS*fokIR* plasmid. The recombinants were screened for appropriate inserts using *SpeI*/*ScaI*/*HindIII* enzyme digestion. The presence of the insert in the right orientation within the gene was confirmed by using Sanger *et al.*'s (17) dideoxy sequencing method.

Overproduction and Purification of the Mutant Enzymes—The procedures for cell growth and purification of the mutant enzymes are similar to the one used for the wild-type *FokI* and the four (or seven) codon insertion mutants that are described elsewhere (13). The purity of the proteins were analyzed by 0.1% SDS-12% acrylamide gel electrophoresis (18), followed by staining the protein with Coomassie Blue (Fig. 1).

Preparation of DNA Substrates with a Single *FokI* Site—Substrate containing a single *FokI* site was constructed by using synthetic oligonucleotides: 5'-CTAGAGTCAGATAGCGAAGACTTCGGGGATGGGC-TTAATGGCCTTAGTTCACAA-3'; 3'-TCAGTCTATCGCTTCTGAAGCC-CCTACCCGAATTACCGGAATCAAGTGTTCGA-5'. The oligomers were phosphorylated and then annealed to form the duplex. It is flanked by *XbaI* and *HindIII* compatible ends. The fragment was ligated to *XbaI*/*HindIII*-cleaved pTZ19R plasmid. The recombinants were screened for the insert using *XbaI*/*HindIII* digestion.

A 300-bp fragment containing the insert was gel-purified after the digesting the recombinant plasmid with *PvuII* enzyme. The fragment was dephosphorylated by treatment with calf intestinal phosphatase and then was rephosphorylated using T4 polynucleotide kinase and [γ -³²P]ATP to obtain substrate that was labeled on both strands of the DNA. Digestion of the above labeled fragment with either *HinPI* or *XbaI*, followed by gel purification of the appropriate DNA fragment, yielded substrates that were labeled individually on each strand.

The cleavage assays with plasmid substrates were performed by adding pTZ19R (1.5 μ g) and the enzyme (40 nM) in a total reaction volume of 15 μ l containing 10 mM Tris-HCl (pH 7.5 at 37 °C), 50 mM NaCl, 10 mM MgCl₂, and 1 mM 1,4-dithioerythritol. The reactions were incubated at 37 °C for 4 h. The digests were analyzed by agarose gel (1.2%) electrophoresis (Fig. 2). For determining the cleavage distance from the recognition site within the DNA substrate, ³²P-labeled substrates (~10,000 cpm) were mixed with 0.5 μ g of pTZ19R and 45 nM of enzymes in a total volume of 10 μ l containing 10 mM Tris-HCl (pH 7.5 at 37 °C), 50 mM NaCl, 10 mM MgCl₂, and 1 mM 1,4-dithioerythritol. The reaction mixture was incubated at 37 °C for 2 h. The samples were electrophoresed on 7% PAGE² containing 7 M urea in 1 × TBE, the gel dried, and then exposed to x-ray film (see Fig. 3).

Cleavage of Hemi-methylated DNA Substrates—The four oligonucleotides used in these experiments were kindly provided by Dr. Waugh (Hoffmann-La Roche). These are the same oligonucleotides that were used by Waugh and Sauer (15) to show that two of their eight *FokI* endonuclease missense mutants cleave hemi-methylated DNA substrates. Each of the four oligomers were phosphorylated using T4 polynucleotide kinase and [γ -³²P]ATP. By annealing various combinations of the synthetic substrates, it was possible to construct an unmethylated substrate, two hemi-methylated substrates and a fully methylated substrate. 0.02 pmol of each labeled substrate and about 70 nM sites (pTZ19R) were mixed with 70 nM of *FokI* or insertion mutants (TAEI or KSEL) in 15 μ l of reaction buffer described above and digested at 37 °C for 1 h. The samples were analyzed on 9% PAGE containing 7 M urea, and the gel was dried and then exposed to an x-ray film (see Fig. 4).

Filter Binding Assays—The filter binding assays were performed in duplicate using *FokI* or mutant enzymes and the four different synthetic substrates. Nine different concentrations for each of the enzymes were made by serial dilutions (5000, 2000, 667, 222, 74.1, 24.7, 8.23, 2.74, and 0.914 nM). The enzymes were mixed with 40 nM of each labeled substrate along with 50 μ g/ml poly(dI-dC) and 50 μ g/ml bovine serum albumin in 50 μ l of buffer A (10 mM Tris phosphate (pH 8.0), 7 mM 2-mercaptoethanol, 1 mM EDTA, 50 mM NaCl, and 10% (v/v) glycerol). The samples were incubated at 22 °C for 1 h and then loaded on to nitrocellulose filters. The filters were washed twice with 0.5 ml of buffer A, dried, and counted using the scintillation counter.

RESULTS AND DISCUSSION

Construction of Insertion and Deletion Mutants of *FokI* Endonuclease—Previously, we have shown that introduction of additional amino acid residues (4 or 7 residues) between the recognition and cleavage domains of *FokI* can alter the spacing between the recognition site and the cleavage site within the

DNA substrate (13). Secondary structure prediction of *FokI* endonuclease based on its primary amino acid sequence revealed a long stretch of α -helix region at the junction of the recognition and cleavage domains. If the helix constituted the linker that connects the two domains of the enzyme, we reasoned that the cleavage distance of *FokI* from the recognition site could be altered by changing the length of this spacer. Insertion of either four codons or seven codons into the linker region of *FokI* was expected to shift the cleavage distance 1 and 2 base pairs, respectively, away from the recognition site. Close examination of the amino acid sequence of the linker region revealed the presence of two KSEL repeats separated by amino acids EEK. Therefore, KSEL and KSELEEK were inserted within the linker region of *FokI*. Both mutants cleaved DNA in a similar way and not necessarily in a distance-dependent way (13). Thus, we could not establish a clear relationship between the length of the connector region of *FokI* and the cleavage distance from the recognition site within its DNA substrate.

A direct relationship has been shown between the length of the protein connector regions of *EcoR124* and *EcoR124/3* (belonging to the type I class) and their related but different recognition site 5'-GAA(N₆)RTGG-3' and 5'-GAA(N₇)RTGG-3', respectively, where R = G or A and N = G, A, T, or C. The recognition sites differ only in the length of the nonspecific spacer (shown in bold type). This difference nevertheless places the two specific domains of the *EcoR124/3* sequence 3.4 Å further apart and rotates them 36° with respect to those of *EcoR124*, which implies major structural differences in the proteins recognizing the sequences (19). This is accommodated in the protein structure by altering the number of amino acid repeats (Thr-Ala-Glu-Leu)₂ and (Thr-Ala-Glu-Leu)₃, respectively, within the connector region (19).

To further probe the structure of the linker region between the recognition and cleavage domains of *FokI* endonuclease, we have constructed a series of mutants with various number of amino acid residue deletions or insertions ranging from -7 to +23 residues (Table I). The amino acid segments KSEL, TAEI, and KSELEEK were used as basic units of insertion or deletion. As indicated above, the TAEI segment was observed in the protein connector regions of *EcoR124* and *EcoR124/3* (type I) enzymes. Multiples of the basic units and a combination thereof were inserted between the recognition and catalytic domains of *FokI* to form the mutants. The method used to construct the insertion and deletion mutants are the same as the one described elsewhere (13). In addition, we have also constructed a 18-amino acid residue insertion mutant that includes the glycine linker, (Gly,Ser)₃. The clones of each mutant were induced with 1 mM isopropyl- β -D-thiogalactopyranoside for 4–5 h for optimal expression of the enzymes. The mutant enzymes were purified using a similar procedure described elsewhere (11, 13). SDS-PAGE profiles of the mutant enzymes are shown in Fig. 1.

Analysis of Sequence Specificity and the Cleavage Distances from the Recognition Site of the Mutant Enzymes—The agarose gel electrophoretic profile of the products of pTZ19R substrate cleavage by *FokI* and the deletion and insertion mutants are shown in Fig. 2. The profiles are very similar, suggesting that deletion and insertions ranging from -4 to +23 residues do not disrupt the sequence-specificity of the enzymes. Several clones of seven codon deletion mutants were identified; however, none of these clones showed any enzymatic activity indicating that seven residue (KSELEEK) deletion probably inactivates the enzyme (data not shown). All digestions were done at similar protein concentrations. Larger insertion mutants show partial digests. These reactions proceed to completion either by increasing the enzyme concentration or by digesting for longer time periods.

² The abbreviation used is: PAGE, polyacrylamide gel electrophoresis.

TABLE I
Insertion/deletion mutants of FokI restriction endonuclease

Amino acid sequence at the insertion/deletion site ^a	No. of amino acid insertion	Activity ^b
....QLVKSELRHKLK....	-7	-
....QLVEEKKSELRHKLK....	-4	+
....QLVKSELEKKSELRHKLK....	wt	+
....QLVTAEKSELEKKSELRHKLK....	+4	+
....QLVTAEKSELEKKSELRHKLK....	+8	+
....QLVTAEKSELEKKSELRHKLK....	+12	+
....QLVTAEKSELEKKSELRHKLK....	+19	+
....QLVTAEKSELEKKSELRHKLK....	+23	+
....QLRGGGGGGGGGGGGQLVKSELEKKSELRHKLK....	+18 ^c	+

^a The inserted aa residues are shown in bold type.

^b Activity based on the cleavage of pTZ19R DNA substrate. The cleavage pattern of the substrate by these mutants (as determined by the agarose gel electrophoresis) were similar to the wild-type (wt) FokI.

^c The inserted aa residues contain (G-G-G-S)₃ linker.

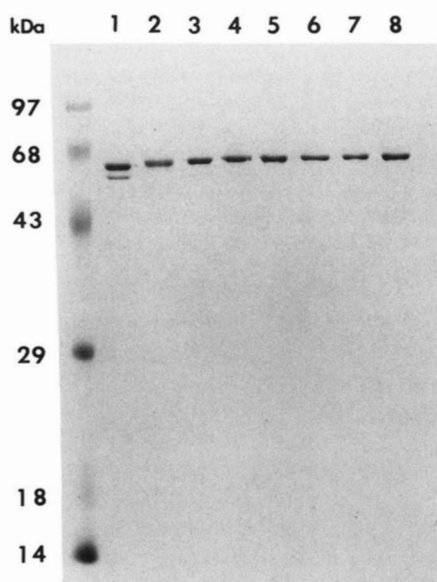


FIG. 1. SDS-PAGE profiles of purified deletion and insertion mutants of FokI endonuclease. Lanes: 1, KSEL deletion; 2, wild-type FokI; 3, TAEI insertion; 4, TAEIΔTAEI insertion; 5, TAEIΔTAEIΔTAEI insertion; 6, 19-amino acid residue insertion; 7, 23-amino acid residue insertion; and 8, glycine linker insertion.

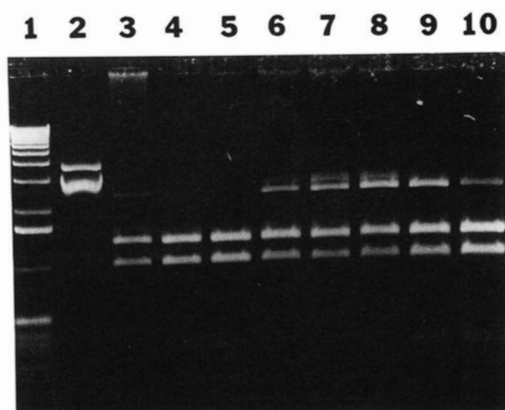


FIG. 2. Agarose gel electrophoretic profile of pTZ19R substrate cleavage by deletion and insertion mutants of FokI endonuclease. Lanes: 1, 1-kilobase ladder; 2, no enzyme; 3, KSEL deletion; 4, wild-type FokI; 5, TAEI insertion; 6, TAEIΔTAEI insertion; 7, TAEIΔTAEIΔTAEI insertion; 8, 19-amino acid residue insertion; 9, 23-amino acid residue insertion; and 10, glycine linker insertion.

To determine the distance of cleavage by the insertion and deletion mutants from the recognition site, the cleavage products of the ³²P-labeled DNA substrates containing a single FokI site were analyzed by PAGE (Fig. 3). The digestion products

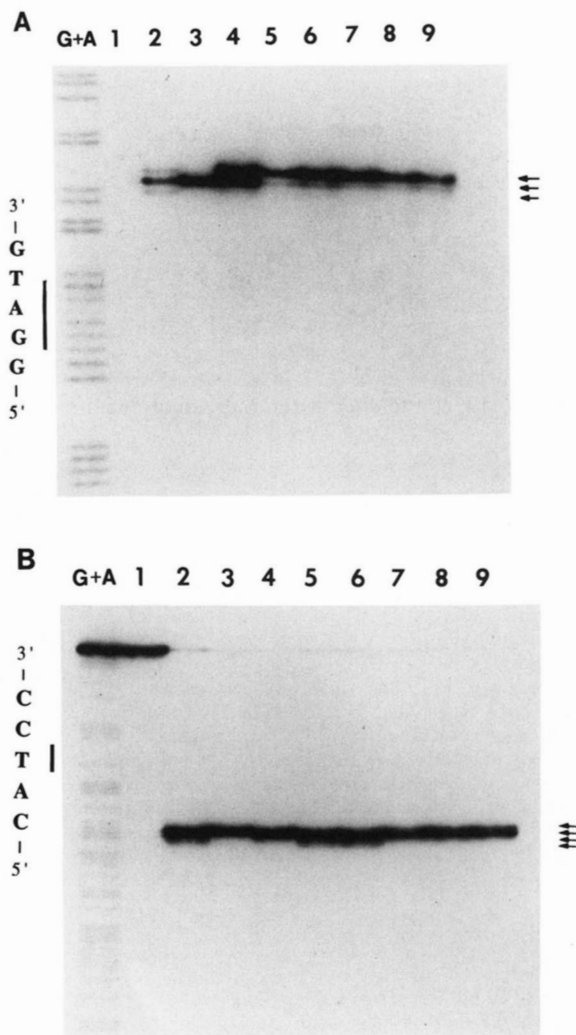


FIG. 3. PAGE (denaturing gel) profiles of the digestion products of the DNA substrate containing a single FokI site by the deletion and insertion mutants of FokI endonuclease (A) corresponds to the 5'-GGATG-3' strand and (B) corresponds to the complementary strand, 5'-CATCC-3'. Lanes: G+A, Maxam-Gilbert (G + A) sequencing reactions using the substrate; 1, no enzyme; 2, KSEL deletion; 3, wild-type FokI; 4, TAEI insertion; 5, TAEIΔTAEI insertion; 6, TAEIΔTAEIΔTAEI insertion; 7, 19-amino acid residue insertion; 8, 23-amino acid residue insertion; and 9, glycine linker insertion.

were analyzed alongside the Maxam-Gilbert (G + A) sequencing reactions (20) of the substrates. The cut sites of the insertion mutants are all shifted 1 base pair away from the recognition site on both strands of the DNA substrates as compared to the wild-type enzyme. A small amount of cleavage similar to that of

wild-type enzyme is also observed. It is more pronounced with the four codon insertion (TAEL) mutant. Relaxation of specificity at the cut site is much more prevalent on the 5'-CATCC-3' strand than the 5'-GGATG-3' strand in the case of the insertion mutants. A similar relaxation of specificity at the cut site was observed with the "chimeric" restriction endonuclease produced by linking *Ubx* homeo domain to the cleavage domain of *FokI* (16). The four-codon deletion (KSEL) mutant shows only relaxation of specificity at the cut site. The cleavage occurs predominantly at the site similar to the wild-type enzyme. The cut site is not shifted one bp closer to the recognition site as expected.

There appears to be no simple relationship between the length of the protein connector region of *FokI* and the cleavage distance from the recognition site within its DNA substrate. The recognition and cleavage domains of *FokI* are likely held together by a non-structured loop. There is probably some association between the recognition and nuclease domains of *FokI*. This domain-domain interaction is likely to be weak since mixing of the purified *FokI* recognition domain with the nuclease domain does not reconstitute *FokI* endonuclease.³ No sequence-specific cleavage of the substrate is observed with such a mixture; only nonspecific nuclease activity is observed. Furthermore, the 18-amino acid residue insertion mutant that includes the glycine linker, (Gly₄Ser)₃, also shows the same sequence-specificity as the wild-type enzyme (Fig. 2, lane 10); it also cleaves predominantly one nucleotide further away from the recognition site on both strands of the DNA substrate (Fig. 3, lane 9). The glycine linker should neither exhibit a propensity for ordered secondary structure (5) nor show any tendency to interfere with the folding of the individual domains of the mutant enzyme. It is also unlikely to interfere with the domain-domain interaction that occurs due to the association of the two domains. This protein-protein interaction between the domains probably leads to the cleavage of the substrate at a precise distance from the recognition site by the mutant enzymes. This may also explain the absence of a linear relationship between the length of the linker region of *FokI* and cleavage distance from the recognition site within the DNA substrate.

Cleavage of Hemi-methylated DNA Substrates by the Four-codon Insertion Mutants—Recently, Waugh and Sauer (15) have identified a novel class of *FokI* restriction endonuclease mutants that cleave hemi-methylated substrates. To test if the deletion and insertion mutants of *FokI* cleave hemi-methylated DNA sites, the same four oligonucleotides described by Waugh and Sauer were used as substrates. These synthetic oligonucleotides were kindly provided by Waugh (Hoffmann-La Roche). By annealing various combination of the ³²P-labeled oligonucleotides, we obtained an unmethylated substrate, two hemi-methylated substrates, and the fully methylated substrate. Cleavage assays with the hemi-methylated substrates were performed with the deletion and insertion mutants of *FokI* endonuclease. Of these only the four codon insertion (TAEL and KSEL) mutants cleave hemi-methylated substrates (Fig. 4). Both the wild-type and the four codon insertion mutants cleave unmethylated substrate to yield two fragments, and none of the enzymes cleave the doubly methylated substrate. In addition, the two mutant enzymes and not the wild-type enzyme cleave both forms of hemi-methylated DNA. Although the cleavage of hemi-methylated substrates is not as efficient as the unmethylated substrate, it proceeds reasonably well. The TAEL insertion mutant appears to cleave the hemi-methylated substrates better than the KSEL insertion mutant.

Why does the four amino acid residue insertions enable the mutant enzymes to cleave hemi-methylated DNA and not the larger inserts? The four codon insertions could increase the

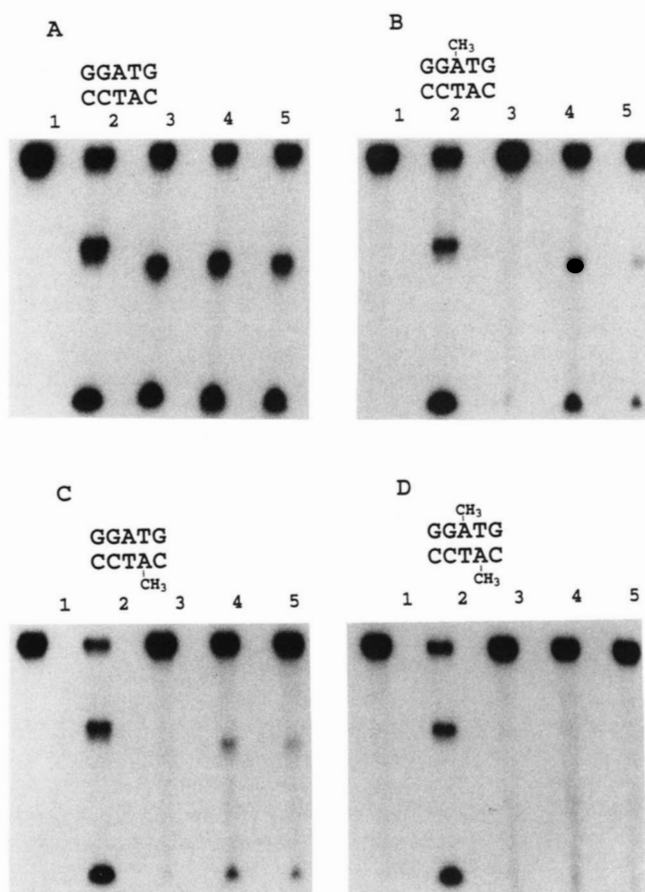


FIG. 4. Cleavage of unmethylated, hemi-methylated and fully methylated DNA substrates by wild-type *FokI* and the four codon (TAEL and KSEL) insertion mutants. Panels: A, unmethylated substrate; B, hemi-methylated (5'-GGATG-3') substrate; C, hemi-methylated (5'-CATCC-3') substrate; and D, fully methylated substrate. Lanes: 1, uncleaved substrates; 2, *PvuII* enzyme; 3, wild-type *FokI*; 4, TAEL insertion mutant; and 5, KSEL insertion mutant. The sequence of the substrate is 5'-*CTAGAGTCAGAATTCGAAGACTTGCCGGATGATCTGCAGGCCAGCTGTGGCGTCTAAATTGA-3' : 3'-TCAGTCTTAAGCTTCTGAACGGCCTACTAGACGTCCGGTCGACACCGCAGATTTAACTTCGA*-5'. The methylation sites are indicated by *. *PvuII* cleavage produces oligonucleotides 22- and 44-mer as products.

affinity of the mutants for the hemi-methylated DNA. We have compared the binding affinities of wild-type enzyme and both four codon insertion mutants by filter binding assays using the synthetic oligonucleotide substrates (Fig. 5). The binding affinities were measured in the presence of nonspecific DNA, poly(dI-dC). The results suggest wild-type *FokI* can bind to hemi-methylated and even fully methylated sites, although ~100 fold less efficiently than to unmethylated sites (8). The TAEL and KSEL insertion mutants show similar results suggesting that insertions do not affect the binding step. Furthermore, this model does not account for the inability of the larger insertion mutants to cleave hemi-methylated *FokI* sites.

In a more plausible model, the rate-limiting step of *FokI* cleavage reaction could involve the dissociation of the nuclease domain from the DNA-recognition domain. The methyl groups may inhibit the dissociation of the nuclease domain from the DNA recognition domain through hydrophobic interactions or even steric effects. The four amino acid residue insertions may have partly uncoupled the nuclease domain from the recognition domain resulting in the cleavage of the hemi-methylated substrates. Due to the added flexibility associated with large insertions, these may not uncouple the *FokI* nuclease domain from the DNA binding domain. The model is consistent with the observation that chimeric restriction endonucleases obtained

³ Y.-G. Kim, L. Li, and S. Chandrasegaran, unpublished results.

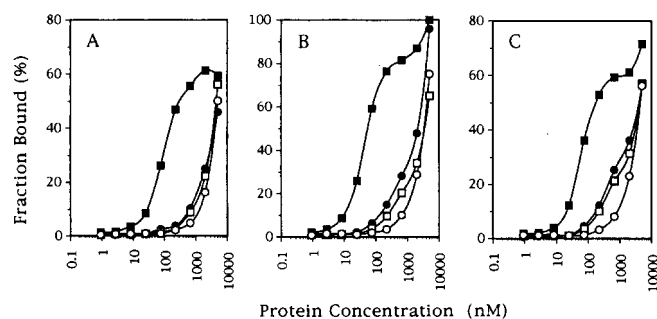


FIG. 5. Affinity of wild-type *FokI* and four codon (TAEI and KSEL) insertion mutants for unmethylated, hemi-methylated and fully methylated DNA substrates determined using filter-binding assays in presence of nonspecific DNA, poly(dI-dC). A, B, and C correspond to wild-type *FokI*, TAEI, and KSEL insertion mutants respectively. ■, unmethylated substrate; ●, hemi-methylated substrate where 5'-GGATG-3' is methylated; □, hemi-methylated substrate where the complementary strand 5'-CATCC-3' is methylated and ○ corresponds to the fully methylated substrate.

by linking other DNA-binding proteins to the nuclease domain of *FokI* exhibit not only sequence-specific cleavage that is determined by the DNA-binding protein but also nonspecific nuclease activity which can be controlled by lowering the concentration of $MgCl_2$ (16). The dissociation of the enzyme from the cleaved product as a rate-limiting step in the *FokI* cleavage reaction could not be ruled out at this time.

In summary, our results suggest that large insertions between the DNA-recognition domain and the catalytic domain of *FokI* do not disrupt the activity of the enzyme. Internal deletions of seven or more codons of the linker region appears to result in the inactivation of the enzyme. These findings are of importance for future engineering of chimeric restriction enzymes especially when one encounters protein-folding problems with the fusions. Several laboratories are in the process of determining the crystal structures of *FokI* and *FokI*-DNA complexes. These studies will provide detailed information about

the mechanism of the *FokI* cleavage reaction and the domain-domain interactions within the protein-DNA complex at atomic resolution.

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