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Single amino acid substitutions uncouple the DNA binding and strand scission activities of *Fok* I endonuclease

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ABSTRACT **Single alanine substitution mutations at Asp-450 or Asp-467 of the type IIS restriction enzyme *Fok* I have no effect on the ability of the enzyme to bind strongly and selectively to its recognition site but completely eliminate its ability to cleave either strand of substrate DNA. Since wild-type *Fok* I shows no kinetic preference or required order of strand cleavage, these results indicate that *Fok* I, which evidently functions as a monomer, uses a single catalytic center to cleave both strands of DNA. In this respect, *Fok* I may resemble other monomeric enzymes that cleave double-stranded DNA.**

The *Fok* I endonuclease is a type IIS restriction enzyme (1) that recognizes the asymmetric sequence 5′-GGATG-3′ and makes staggered DNA cuts at sites that are 9 and 13 bases away as indicated in Fig. 1 (2). *Fok* I and other type IIS restriction enzymes appear to function as monomers (3). By contrast, the more familiar type II endonucleases, such as *Eco*RI and *Eco*RV, usually act as dimers to recognize and cleave palindromic DNA sequences. It is not known how both strands of DNA are cleaved by *Fok* I. A single molecule could contain two active sites, one to cleave each strand of DNA. Alternatively, a single set of active residues could be used to cleave both strands. Here we report the construction and characterization of two single amino acid substitutions in the *Fok* I endonuclease [Asp-450 to Ala and Asp-467 to Ala (D450A and D467A)] whose behavior indicates that *Fok* I uses a single catalytic center to cleave both strands of DNA. Specifically, we show that the mutants have normal DNA binding activity but are unable to cleave either strand of DNA.

MATERIALS AND METHODS

Site-Directed Mutagenesis. Single-nucleotide substitutions were incorporated at the desired locations in the cloned *Fok* I endonuclease gene (4, 5) by using a mutageneic oligonucleotide [5′-CCGCTATAAGCTTTAGTA(G/T)CCACGATCA-CACCGTAATCAATAGGGGATCCGACAGTATAAAT-TGCTCCG(G/T)CCGGTTT-3′] with mixed bases at positions corresponding to codons 450 and 467. A short segment of the cloned *Fok* I gene was amplified by PCR (6), using this primer in conjunction with another, nonmutagenic primer (5′-CTGGAGAAGGTTTGAAAGTACTGCGTCGAGC-3′). The resultant PCR fragment was cleaved with *Hin*dIII and *Kpn* I and inserted between these sites in the cloned *Fok* I gene. (The *Kpn* I site had previously been introduced into the cloned gene by an oligonucleotide-directed, single-base substitution in the Arg-286 codon.) Both mutations were identified by dideoxynucleotide sequencing (7) of plasmid DNAs isolated from independent transformants.

Activity Assay *in Vivo*. Activity assays *in vivo* (8) were performed essentially as described (9). Test plasmids carried either the wild-type or mutant *Fok* I endonuclease genes under the control of an isopropyl β-D-thiogalactopyranoside (IPTG)-inducible lacUV5 promoter. After transforming *Escherichia coli* strain JH140 (9) with plasmid DNA, the cells were spread on LB plates containing ampicillin (100 μg/ml) and 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) (35 μg/ml), both with and without 0.4 mM IPTG, and incubated at 37°C for 13 hr. In the absence of IPTG, cells expressing the wild-type *Fok* I endonuclease produced blue colonies on X-Gal plates. JH140 cells that already contained a compatible plasmid expressing the *Fok* I methyltransferase activity produced white colonies under the same conditions, indicating that induction of the SOS response is due to the action of *Fok* I endonuclease at its target site. No transformants were obtained when JH140 cells expressing the wild-type endonuclease were spread on plates containing IPTG, but white colonies were obtained under these conditions if the cells were also expressing the *Fok* I methyltransferase gene.

Overproduction and Purification of Wild-Type and Mutant *Fok* I Endonucleases. The wild-type and mutant *Fok* I genes were cloned into the pET11d expression vector (10), using the unique *Nco* I site to join the *Fok* I coding sequences to the bacteriophage T7 φ10 promoter and gene 10 ribosome binding site. The expression vectors were transformed into BL21/DE3 cells containing pLysS and pDW179 (a compatible plasmid that expresses the *Fok* I methyltransferase gene) and grown to midlogarithmic phase (A_{600} = 0.5) in LB broth (11) at 37°C. Expression was induced by adding 2 mM IPTG to the medium. After 4 hr, the cells were chilled on ice and collected by centrifugation at 4000 × *g*. The pellet was washed with 200 ml of ice-cold lysis buffer [100 mM Tris·HCl, pH 8.1 (25°C)/200 mM NaCl/1 mM EDTA/14 mM 2-mercaptoethanol/5% (vol/vol) glycerol], and the cells were collected again by centrifugation. The pellet was resuspended in 10 ml of lysis buffer per g of cells, and the cells were disrupted by sonication. Polyethyleneimine was then added to a final concentration of 0.25%, and the mixture was centrifuged at 10,000 × *g* to remove the nucleic acids and cellular debris. Four volumes of ice-cold, ammonium sulfate-saturated lysis buffer were mixed with the polyethyleneimine supernatant, and after 30 min on ice the protein was collected by centrifugation at 10,000 × *g*. The pellet was resuspended in 10 ml of ice-cold low salt buffer [10 mM Tris·HCl, pH 8.1 (25°C)/0.1 mM EDTA/1.4 mM 2-mercaptoethanol/5% glycerol] per g of cells and dialyzed exhaustively against this buffer. The dialysate was applied to a DEAE-Sephacel column (Pharmacia) that had been equilibrated with low salt buffer. *Fok* I was recovered in the flow-through fraction. The fractions that contained *Fok* I (by SDS/PAGE) were pooled and applied directly to a Biorex 70 column (Bio-Rad) equilibrated with the same buffer. *Fok* I bound to the column and was subsequently eluted with a linear gradient from 0 to 1 M KCl. The

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Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; DMS, dimethyl sulfate.

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activity eluted at ≈0.2 M KCl under these conditions, and the preparation was nearly homogeneous at this point as judged by SDS/PAGE (12). The peak fractions from the Biorex 70 column were pooled and purified further by gel filtration (Superose 12; Pharmacia) with an FPLC apparatus.

Dimethyl Sulfate (DMS) and DNase Protection Experiments. DMS and DNase I protection experiments were performed essentially as described (13). ^{32}P-labeled substrates were prepared by annealing synthetic oligodeoxyribonucleotides (Fig. 1) in TES (11) after the 5′ end of one strand was phosphorylated with polynucleotide kinase and [γ-^{32}P]ATP. The double-stranded substrates were purified by gel electrophoresis. About 2×10^4 Cherenkov cpm of DNA was mixed with various amounts of protein in 200 μl of DMS buffer [50 mM sodium cacodylate, pH 7.5/10 mM $MgCl_2$/0.1 mM EDTA/150 mM KCl/1 mM dithiothreitol (DTT)/0.25 mg of bovine serum albumin (BSA) per ml/5 μg of sonicated salmon sperm DNA per ml] or 100 μl of DNase I buffer (10 mM Tris·HCl, pH 7.5/10 mM $MgCl_2$/1.5 mM $CaCl_2$/0.1 mM EDTA/50 mM KCl/1 mM DTT/25 μg of sonicated salmon sperm DNA per ml/0.1 mg of BSA per ml) and allowed to equilibrate at room temperature for 5–10 min; $MgCl_2$ was omitted from some of the DMS reactions, as indicated in Fig. 3. DMS reactions were initiated by adding 1 μl of DMS and were terminated after 3 min by ethanol precipitation. The DNA was recovered by centrifugation, and then the pellets were resuspended in 90 μl of 0.5 M piperidine/5 mM EDTA and incubated at 90°C for 20 min. The DNA was recovered by evaporating the piperidine/EDTA solution in a Speed-Vac, and the pellets were twice resuspended in 50 μl of H_2O and evaporated to dryness to remove all traces of piperidine. Finally, the pellets were resuspended in 10 μl of 95% formamide/20 mM EDTA, and then the reaction products were resolved by electrophoresis through a 12% acrylamide (19:1) slab gel containing 8 M urea and TBE buffer (11). After electrophoresis, the gel was frozen and then exposed to x-ray film. For DNase reactions, 5 μl of DNase I (0.05 μg/ml) was added to the protein/DNA mixture. After 15 min at room temperature, the reactions were stopped by ethanol precipitation, and the DNA was recovered by centrifugation. The pellets were resuspended in 10 μl of 95% formamide/20 mM EDTA and incubated at 90°C for 4 min; then the reaction products were resolved by electrophoresis as described above.

Limited Proteolysis. Purified *Fok* I and the D450A and D467A mutants were incubated at 37°C with thermolysin (14) or at room temperature with trypsin (15) for various times as described. The reaction products were resolved by SDS/PAGE (6% stacking gel, 15% running gel) (12).

Substrates and Activity Assays *in Vitro*. Six synthetic oligonucleotides (see Fig. 4, A–F) were used to construct substrates for *Fok* I. Two of these (C and D) were phosphorylated enzymatically with polynucleotide kinase, so that when they were combined with other oligonucleotides to create "nicked" duplexes, the nicks would have 5′ phosphate and 3′ hydroxyl termini. Each full-length oligonucleotide (A and F) was labeled at its 5′ end with polynucleotide kinase and [γ-^{32}P]ATP in a separate reaction. Nicked duplexes then were formed by annealing each full-length ^{32}P-labeled oligonucleotide (A or F) with a 5-fold molar excess of the complementary fragments (D + E or B + C, respectively) in reaction buffer (see below). Control substrates were prepared by annealing each full-length ^{32}P-labeled oligonucleotide (A or F) with a 5-fold molar excess of its unlabeled, full-length complement (F or A). Reactions were also carried out with each full-length, ^{32}P-labeled oligonucleotide by itself. Activity assays were performed by combining the substrates (≈20 nM) with the wild-type or the mutant endonucleases (≈2 nM) in 50-μl reaction mixtures that also contained 10 mM Tris·HCl (pH 8.0 at 25°C), 60 mM KCl, 10 mM $MgCl_2$, and 5 mM 2-mercaptoethanol. The reaction mixtures were incubated at 37°C for 20 min, and the reactions were stopped by adding an equal volume of 95% formamide/20 mM EDTA/0.2% SDS. The samples were incubated at 95°C for 4 min immediately before they were loaded onto 15% acrylamide (19:1) slab gels containing 8 M urea and 1× TBE (11). After electrophoresis, the gels were frozen at −80°C and exposed to x-ray film.

Phosphorthioate-Substituted Deoxyribonucleotides. Synthetic oligodeoxyribonucleotides with phosphorthioate linkages at unique locations were prepared on a model 392 automated DNA synthesizer (Applied Biosystems), using a synthesis cycle written by Applied Biosystems (ABI User Bulletin no. 58, February 1991) and modified by D.S.W. for use with the two-column instrument (details available on request). The sulfurizing reagent was tetraethylthiuram disulfide.

FIG. 1. Synthetic oligodeoxyribonucleotide substrate for *Fok* I endonuclease. The recognition sequence (5′-GGATG-3′) is boxed, and the cleavage sites are indicated by arrows. Guanines that are protected by *Fok* I from DMS modification, or that manifest enhanced reactivity with DMS in the presence of *Fok* I, are indicated by solid and open circles, respectively. Solid lines indicate the area on each strand of the substrate that is strongly protected from DNase I digestion by *Fok* I; dashed lines signify areas of partial protection and/or enhanced reactivity. Examples of DMS and DNase I protection experiments are shown in Fig. 3.

RESULTS AND DISCUSSION

Comparison of the crystal structures of the *Eco*RV and *Eco*RI endonucleases bound to their recognition sequences revealed a common sequence motif (Pro-Asp-Xaa$_{15-19}$-Glu/Asp-Xaa-Lys), which lies in close proximity to the scissile phosphodiester bonds in the protein–DNA complexes (16, 17). The acidic residues in this motif are thought to chelate a magnesium ion that forms part of the enzyme active site, and mutations at these positions in *Eco*RV and *Eco*RI inactivate the enzymes without affecting their ability to bind to their recognition sites (17). As shown in Fig. 2, the *Fok* I sequence from residues 447–469 contains a reasonable match to the active site sequences of *Eco*RI and *Eco*RV (18); the similarity between the *Fok* I and *Eco*RI sequences is especially strong. To test whether this *Fok* I sequence was important for activity, we changed each of the two conserved acidic residues (Asp-450 and Asp-467) to alanine by site-directed mutagenesis.

We first assayed the D450A and D467A mutants in strain JH140 by using a sensitive test in which endonuclease activity results in SOS induction, which in turn is detected by increased synthesis of β-galactosidase (8, 9). The wild-type and mutant *Fok* I genes were placed under the control of a synthetic, IPTG-inducible lacUV5 promoter on a high-copy-number plasmid. In this context, the fully repressed wild-type *Fok* I gene can be maintained in *E. coli* in the absence of *Fok*

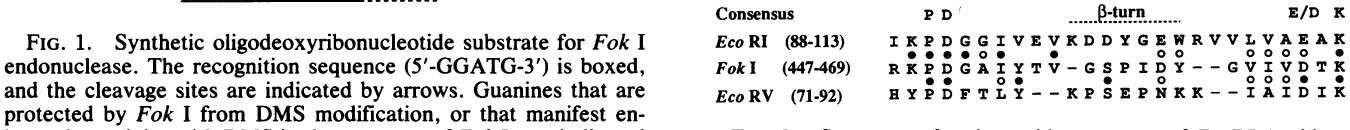

FIG. 2. Segments of amino acid sequences of *Eco*RI (residues 88–113), *Eco*RV (residues 71–92), and *Fok* I (residues 447–469) endonucleases are aligned for comparison. Identical residues are indicated by solid circles; conservative substitutions are identified by open circles. Gaps are indicated by dashes. The consensus sequence of the proposed active site motif (16) is shown on the top line.

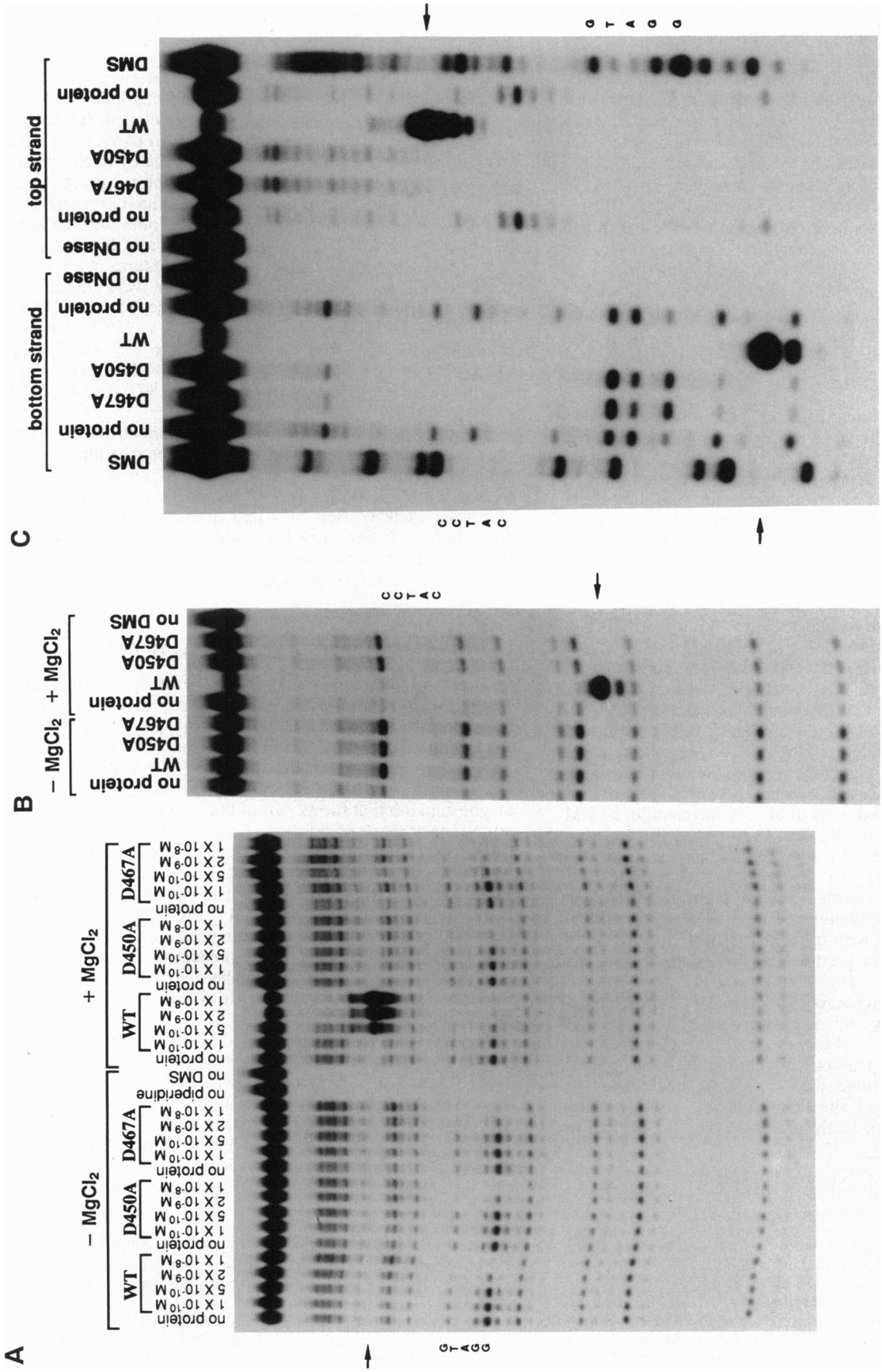

FIG. 3. (*A*) DMS protection experiment with various concentrations of wild-type (WT) and mutant (D450A, D467A) *Fok* I endonucleases as indicated. The substrate used in this experiment is shown in Fig. 1; the top strand was labeled at its 5′ end with ^{32}P. Reactions were carried out in the presence or absence of magnesium as indicated. Positions of recognition sequence (5′-GGATG-3′) and cleavage sites are also indicated. The faint, secondary bands that appear on the autoradiogram are caused by contamination of the synthetic oligonucleotides with a small amount of the $n - 1$ and $n - 2$ forms. (*B*) DMS protection experiment with a fixed concentration (10 nM) of wild-type and mutant *Fok* I endonucleases. Reactions were carried out in the presence or absence of magnesium, with either the top strand or the bottom strand of the substrate (Fig. 1) labeled at its 5′ end with ^{32}P as indicated. Positions of recognition sequence and cleavage sites are also indicated. (*C*) DNase I protection experiment with a fixed concentration (10 nM) of wild-type and mutant *Fok* I endonucleases. The substrate for these reactions was the same one used in the DMS protection experiments. DMS reactions were run alongside the DNase I reactions to provide markers on the gel. Approximate position of the recognition sequence and the cleavage site on each strand is indicated.

I methyltransferase activity; however, transformants of strain JH140 are blue on X-Gal plates, indicating that the SOS response is induced. In contrast, both mutants produced white colonies on X-Gal plates (i.e., they do not induce the SOS response) even when expression was stimulated by the addition of IPTG. Hence, both the D450A and D467A mutants appear to be inactive *in vivo*.

We overproduced and purified wild-type *Fok* I and the D450A and D467A mutants and assayed their enzymatic and DNA binding properties *in vitro*. DMS protection experiments (19) were used to probe potential major groove interactions between substrate DNA and wild-type *Fok* I, the D450A mutant, or the D467A mutant. The results of these studies are shown in Fig. 3 *A* and *B* and are summarized in Fig. 1. Each of the proteins protected the three guanines in the recognition sequence (5′-GGATG-3′) and enhanced DMS reactivity at two guanines adjacent to the recognition sequence. None of the proteins, however, protected or enhanced the reactivity of any other guanines on either strand of the DNA. In particular, the failure to protect the guanines that are immediately adjacent to the scissile bonds suggests that *Fok* I is not intimately associated with the major groove in this vicinity. This is not surprising, since there are no requirements for particular bases at or near the sites of cleavage. Dissociation constants for the wild-type and mutant enzymes were estimated from the concentration dependence of the DMS protection patterns (Fig. 3*A*; data not shown). These values ($K_d \approx 1$ nM) are essentially the same for the mutant and wild-type endonucleases and are similar in the presence or absence of magnesium. Cleavage of the substrate DNA (Fig. 3 *A* and *B*, arrows) is observed only for the wild-type enzyme in the presence of magnesium; neither mutant is capable of cleaving either strand of the DNA.

The results of DNase I protection experiments are shown in Fig. 3*C* and are summarized in Fig. 1. The mutants show a clear pattern of protection that is centered around the recognition sequence. The DNA between the recognition sequence and the cleavage sites is also protected from DNase I digestion, but the strongly protected region does not completely encompass the cleavage sites themselves. Some positions in the vicinity of the cleavage sites are partially protected from the action of DNase I, while others show enhanced reactivity. It is possible that the mutant proteins are only weakly or transiently associated with this region of the DNA. It is more difficult to discern the DNase I footprint of wild-type *Fok* I because a significant fraction of the substrate DNA is cleaved. Nevertheless, the faint protection that can be seen appears similar to that observed for the D450A and D467A mutants.

The results presented so far show that the D450A and D467A mutants have no detectable endonuclease activity but interact with the *Fok* I target site in a manner that is qualitatively and quantitatively similar to the wild-type enzyme. Hence, both mutations have the properties expected for active site residues; they uncouple the sequence-specific DNA binding and strand scission activities of the enzyme. If

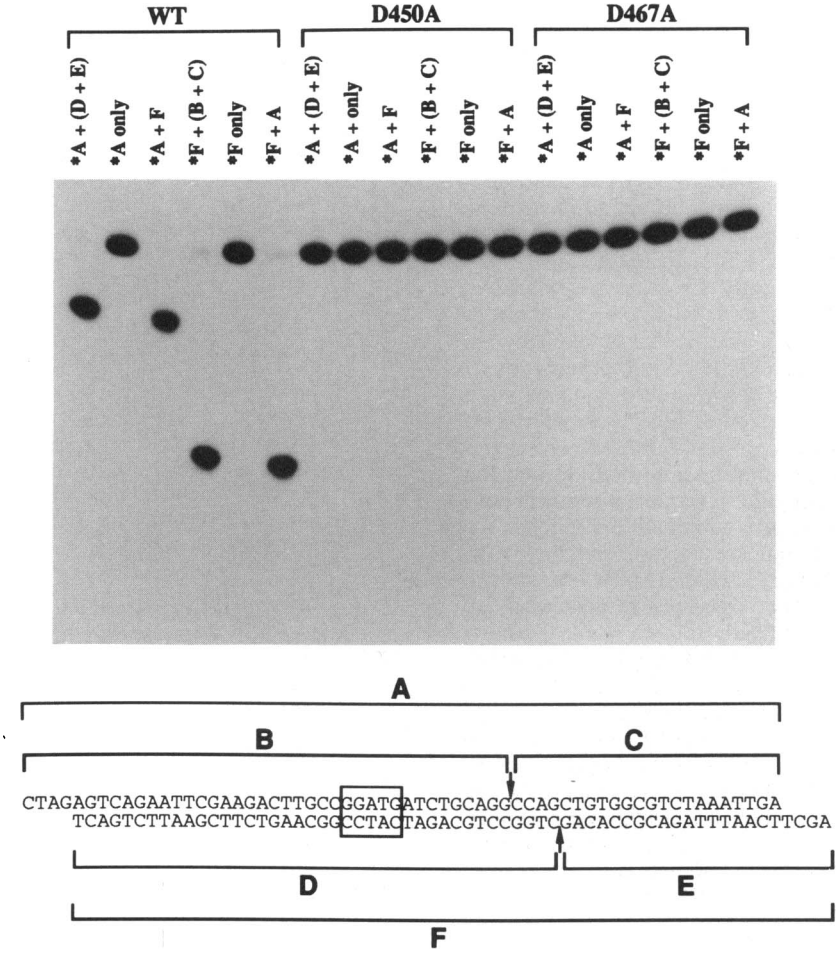

FIG. 4. Activity assays of wild-type (WT) and mutant (D450A, D467A) *Fok* I endonucleases. (*Lower*) Full-length substrate and fragments used in this experiment (A–F) are shown. *Fok* I recognition sequence is boxed, and cleavage sites are indicated by arrows. (*Upper*) Autoradiogram of a denaturing 15% acrylamide gel showing the reaction products obtained when various combinations of fragments A–F are incubated with the enzymes. Asterisk indicates which oligonucleotide was labeled at its 5′ end with ^{32}P in each reaction.

Fok I relies on a different set of active residues to cleave each strand of DNA, then disabling one catalytic center by mutation might be expected to give rise to an enzyme with strand-specific "nicking" activity. Since this is not observed, it seems likely that *Fok* I has a single active site. There are, however, two caveats. (*i*) Single mutations could disable two active sites through global conformational changes, but several observations suggest that neither mutation dramatically alters the structure of the enzyme. First, both mutants were stably expressed in *E. coli* and behaved exactly like the wild-type enzyme during purification. Second, when the wild-type endonuclease and mutants were digested with thermolysin or trypsin *in vitro*, all three proteins showed the same pattern of metastable intermediates in essentially the same proportions (data not shown). (*ii*) It is possible that *Fok* I has two sets of active residues but that there is an obligatory order of strand scission. According to this model, the D450A and D467A mutations inactivate only one of two catalytic centers, but neither strand can be cleaved because the first event is blocked by the mutation. To test this possibility, we prepared different DNA substrates in which one strand of the duplex or the other was nicked at the appropriate location and assayed cleavage by wild-type *Fok* I and the mutants. As shown in Fig. 4, the wild-type enzyme cleaves both nicked substrates but neither mutant can cleave either nicked DNA. We also prepared different DNA substrates in which a phosphate oxygen atom in the scissile linkage on one or the other strand of the substrate was replaced by sulfur. The thio substitution inhibited cleavage of that strand by wild-type *Fok* I by at least 100-fold but had no influence on the ability of the enzyme to cleave the other strand (data not shown). The mutant enzymes were unable to cleave either thio-substituted substrate. Taken together, these experiments indicate that there is neither an obligatory order of strand scission nor a discernable kinetic preference for either strand in the cleavage reaction.

How might *Fok* I cleave both strands of DNA by using only a single catalytic center? This could be accomplished by cleavage of one strand, movement to the second site, and cleavage of the second strand. Although the scissile phosphodiester bonds are almost directly across the major groove from each other in B-form DNA, this distance is still about 18 Å. The movement could involve a conformational change in the protein or the DNA or dissociation and reassociation of the complex. Alternatively, the reaction may proceed via a complex that includes two molecules of *Fok* I, with each molecule cleaving one strand of the DNA. There is no evidence for *Fok* I oligomerization in solution (20), and the asymmetric nature of the recognition sequence seems inconsistent with the use of a symmetric dimer. Still, evidence for higher-order complexes of some restriction enzymes with DNA is beginning to emerge (21, 22), and so this possibility cannot be ignored. Many other enzymes evidently act as monomers but must cleave double-stranded DNA. Perhaps these enzymes and enzymes like *Fok* I use a similar mechanism of action.

The report by Theilking *et al.* (16), noting the similarity between the active site sequences of *Eco*RI and *Eco*RV, led us to investigate the role of the homologous residues, Asp-450 and Asp-467, in *Fok* I. The phenotypes we observe for the D450A and D467A mutants are consistent with the proposed active site function of this motif and demonstrate its predictive value. However, when we examined the amino acid sequences of the other cloned type IIS enzymes, *Hga* I and *Mbo* II (23), we were unable to find strong matches to the motif as it is currently defined. Both sequences contain Pro-Asp sequences and Glu/Asp-Xaa-Lys sequences but the distance between these elements is either much larger or much smaller than the 15–19 amino acids seen in *Eco*RI, *Eco*RV, and *Fok* I. Thus, the motif may be more degenerate than currently imagined, or there may be several different evolutionary lineages for restriction enzymes.

Recently, Li *et al.* (15) reported that *Fok* I endonuclease can be divided into two domains by limited digestion with trypsin. The N-terminal domain lacks endonuclease activity but retains the ability to bind specifically to the recognition site. The other domain, a 25-kDa C-terminal fragment, manifests nonspecific endonuclease activity. Our results are consistent with these findings, since the D450A and D467A mutations would be included in the C-terminal nuclease domain of *Fok* I.

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