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Functional domains in *Fok* I restriction endonuclease

(*Flavobacterium okeanokoites/Escherichia coli*/methyltransferase/restriction endonuclease/recognition and cleavage domains)

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ABSTRACT **The PCR was used to alter transcriptional and translational signals surrounding the *Flavobacterium okeanokoites* restriction endonuclease (*fokIR*) gene, so as to achieve high expression in *Escherichia coli*. By changing the ribosome-binding site sequence preceding the *fokIR* gene to match the consensus *E. coli* signal and by placing a positive retroregulator stem-loop sequence downstream of the gene, *Fok* I yield was increased to 5–8% of total cellular protein. *Fok* I was purified to homogeneity with phosphocellulose, DEAE-Sephadex, and gel chromatography, yielding 50 mg of pure *Fok* I endonuclease per liter of culture medium. The recognition and cleavage domains of *Fok* I were analyzed by trypsin digestion. *Fok* I in the absence of a DNA substrate cleaves into a 58-kDa carboxyl-terminal and 8-kDa amino-terminal fragment. The 58-kDa fragment does not bind the DNA substrate. *Fok* I in the presence of a DNA substrate cleaves into a 41-kDa amino-terminal fragment and a 25-kDa carboxyl-terminal fragment. On further digestion, the 41-kDa fragment degrades into 30-kDa amino-terminal and 11-kDa carboxyl-terminal fragments. The cleaved fragments both bind DNA substrates, as does the 41-kDa fragment. Gel-mobility-shift assays indicate that all the protein contacts necessary for the sequence-specific recognition of DNA substrates are encoded within the 41-kDa fragment. Thus, the 41-kDa amino-terminal fragment constitutes the *Fok* I recognition domain. The 25-kDa fragment, purified by using a DEAE-Sephadex column, cleaves nonspecifically both methylated (pACYCfokIM) and nonmethylated (pTZ19R) DNA substrates in the presence of $MgCl_2$. Thus, the 25-kDa carboxyl-terminal fragment constitutes the *Fok* I cleavage domain.**

We have undertaken a detailed study of the *Fok* I restriction modification system from *Flavobacterium okeanokoites*. *Fok* I is a member of type IIS class. Unlike other type II restriction enzymes, which have the cleavage site within or adjacent to their recognition sites, the type IIS enzymes cleave double-stranded DNA at precise distances from their recognition site. The *Fok* I endonuclease recognizes the nonpalindromic pentadeoxynucleotide 5′-GGATG-3′·5′-CATCC-3′ in duplex DNA and cleaves 9/13 nucleotides downstream of the recognition site (1). Because 10 base pairs (bp) are required for one turn of the DNA helix, this enzyme will probably interact with one face of DNA by binding at one point and cleave at another point on the next helical turn. This interaction implies the presence of two separate protein domains: one for the sequence-specific recognition of DNA and the other for the endonuclease activity. Once the DNA-binding domain is anchored at the recognition site, a signal is transmitted to the endonuclease domain, probably through allosteric interactions, and cleavage occurs. Indirect support for the presence of two domains comes from Szybalski *et al.* (2, 3). By combining *Fok* I with properly designed oligonucleotide adapters, they have cleverly devised a method that uses the separation between recognition site and endonuclease site to confer additional cleavage specificities (2, 4, 5).

Three laboratories including ours have independently cloned the *Fok* I restriction-modification system from *F. okeanokoites* (refs. 6–8; L.P.W. and S.C., unpublished work). Purification of *Fok* I endonuclease has been reported by other laboratories (9, 10).

In this paper we describe construction of an efficient overproducer clone of *Fok* I endonuclease using PCR and purification of large amounts of homogeneous enzyme. We also describe the separation and identification of functional recognition and cleavage domains of *Fok* I restriction endonuclease using trypsin digestion.

MATERIALS AND METHODS

Cloning Procedures and Growth of Cells. The *Fok* I restriction-modification system was cloned into pUC13 and pBR322 by selecting for the modification phenotype (L.P.W. and S.C., unpublished work). The nucleotide sequences of parts of this system were determined by using Sanger's sequencing method (11). The complete nucleotide sequence of the *Fok* I restriction-modification system has been published by other laboratories (7, 8). Experimental protocols for PCR are described elsewhere (12). Plasmid pUCfokIRM DNA linearized with *Bam*HI was used as the template.

The PCR-generated DNA containing the *Fok* I modification gene (*fokIM*) was ligated into *Nco* I-cleaved and dephosphorylated pACYC184, and the recombinant DNA was transfected into *Escherichia coli* RB791 i^q or RR1 cells made competent as described by Maniatis *et al.* (13). After tetracycline selection, several clones were picked, and plasmid DNA was examined by restriction analysis for the *fokIM* gene fragment in the correct orientation with the chloramphenicol promoter. This construct expresses *Fok* I methylase constitutively. The plasmid DNA from these clones are resistant to *Fok* I digestion.

The PCR-generated DNA containing the *F. okeanokoites* I restriction endonuclease gene (*fokIR*) was ligated into *Bam*HI-cleaved and dephosphorylated high-expression vectors pRRS (12) or pCB (14). The recombinant DNA was transfected into competent *E. coli* RB791 i^q [pACYCfokIM] or RR1[pACYCfokIM] cells. After tetracycline and ampicillin antibiotic selection, several clones were picked, and plasmid DNA was examined by restriction analysis for *fokIR* gene fragment in the correct orientation relative to the vector promoters.

Purification of *Fok* I Endonuclease. RR1 cells [pACYCfokIM, pRRSfokIR] were grown in 6 liters of 2 × TY (1.6% tryptone/1% yeast extract/0.5% NaCl, pH 7.2) containing 20 μg of tetracycline per ml and 50 μg of ampicillin per ml at 37°C to an OD_{600} unit of 0.8 and then induced overnight with 1 mM

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Abbreviations: *fokIR*, gene coding for *Fok* I restriction endonuclease; *fokIM*, gene coding for *Fok* I methyltransferase.

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isopropyl β-D-thiogalactoside. The cells were harvested by centrifugation and then resuspended in 250 ml of buffer A [10 mM Tris phosphate, pH 8.0/7 mM 2-mercaptoethanol/1 mM EDTA/10% (vol/vol) glycerol] containing 50 mM NaCl. Cells were disrupted at maximum intensity on a Branson sonicator for 1 hr at 4°C. The sonicated cells were centrifuged at 12,000 × *g* for 2 hr at 4°C. The supernatant was then diluted to 1 liter with buffer A/50 mM NaCl. The supernatant was loaded onto a 10-ml phosphocellulose (Whatman) column preequilibrated with buffer A/50 mM NaCl. The column was washed with 50 ml of loading buffer, and the protein was eluted with a 80-ml total gradient of 0.05–0.5 M NaCl in buffer A. The fractions were monitored by OD_{280} absorption and analyzed by electrophoresis on SDS (0.1%)/polyacrylamide (12%) gels (15). Proteins were stained with Coomassie blue. Restriction endonuclease activity of the fractions was assayed by using pTZ19R as substrate. The fractions containing *Fok* I were pooled and fractionated with ammonium sulfate. The 50–70% ammonium sulfate fraction contained the *Fok* I endonuclease. The precipitate was resuspended in 50 ml of buffer A/25 mM NaCl and loaded onto a DEAE column. *Fok* I does not bind to DEAE, whereas many contaminating proteins do. The flow-through was concentrated on a phosphocellulose column. Further purification was achieved by using a gel filtration (AcA 44) column. The *Fok* I was purified to homogeneity by using this procedure.

Isolation of DNA-Binding Tryptic Fragments of *Fok* I Endonuclease with Oligo(dT)-Cellulose Affinity Column. *Fok* I endonuclease (160 μg) was incubated with 2.5 molar excess of annealed oligodeoxynucleotide substrate [5′-CCTCTGGATGCTCTC$(A)_{15}$-3′·5′-GAGAGCATCCAGAGG$(A)_{15}$-3′] in 10 mM Tris·HCl/50 mM NaCl/10% glycerol/10 mM $MgCl_2$ at room temperature for 1 hr in a final volume of 100 μl. Trypsin (10 μl, 0.2 mg/ml; Boehringer Mannheim, no. 109819) was added to initiate digestion; the ratio of trypsin to *Fok* I (by weight) was 1:80. Digestion continued for 10 min to obtain predominantly 41-kDa amino-terminal fragment and 25-kDa carboxyl-terminal fragments in the mixture. The reaction was quenched with a large excess of antipain (10 μg; Boehringer Mannheim, no. 1004646) and diluted in loading buffer (10 mM Tris·HCl, pH 8.0/1 mM EDTA/100 mM $MgCl_2$) to a final 400-μl vol. The solution was loaded onto an oligo(dT)-cellulose column (0.5 ml; Sigma, no. 0-7751) preequilibrated with loading buffer. The break-through volume was passed over the oligo(dT)-cellulose column six times. The column was washed with 5 ml of loading buffer and then eluted twice with 0.4 ml of 10 mM Tris·HCl, pH 8.0/1 mM EDTA. These fractions contain the tryptic fragments bound to the oligonucleotide DNA substrate. In a separate reaction, the trypsin digestion of *Fok* I–oligonucleotide complex was for 160 min to obtain predominantly the 30-kDa, 25-kDa, and 11-kDa fragments in the reaction mixture. The oligo(dT)-cellulose affinity column was used as described above to identify the tryptic fragments of *Fok* I that bound DNA.

RESULTS AND DISCUSSION

Construction of an Efficient Overproducer Clone of *Fok* I Endonuclease Using PCR. We have used the PCR technique to alter transcriptional and translational signals surrounding the *fokIR* gene, so as to achieve high expression in *E. coli*. The ribosome-binding site preceding the *fokIR* and *fokIM* genes was altered to match the consensus *E. coli* signal. The oligonucleotide primers used to amplify the *fokIR* and *fokIM* genes are shown in Fig. 1*A*. Construction of the overproducer clone was done in two steps: (*i*) The PCR-generated *fokIM* gene was digested with *Nco* I and gel purified; it was then ligated into *Nco* I-cut, dephosphorylated pACYC184. Clones were obtained that carried the *fokIM* gene in the correct orientation for expression from the chloromphenicol promoter (Fig. 1*B*). This plasmid expresses *Fok* I methylase constitutively and protects the host from chromosomal cleavage, when *fokIR* gene is introduced into the host on a compatible plasmid. (*ii*) The PCR-generated *fokIR* fragment was ligated into the *Bam*HI site of two high expression vectors: pRRS, possessing a *lac* UV5 promoter (12) and pCB containing the strong *tac* promoter (14). In addition, these vectors contain the positive retroregulator stem-loop sequence (12) derived from the crystal protein-encoding gene of *Bacillus thuringiensis* downstream from the inserted *fokIR* gene. The recombinant plasmids were transfected into competent RR1[pACYCfokIM] or RB791I^q [pACYCfokIM] cells. Clones were obtained that carried the *fokIR* gene correctly oriented for efficient expression. These clones were examined for enzyme production. Both constructs (Fig. 1*B*) yield *Fok* I to a level of 5–8% of total cellular protein. The purification procedure used to obtain homogeneous *Fok* I

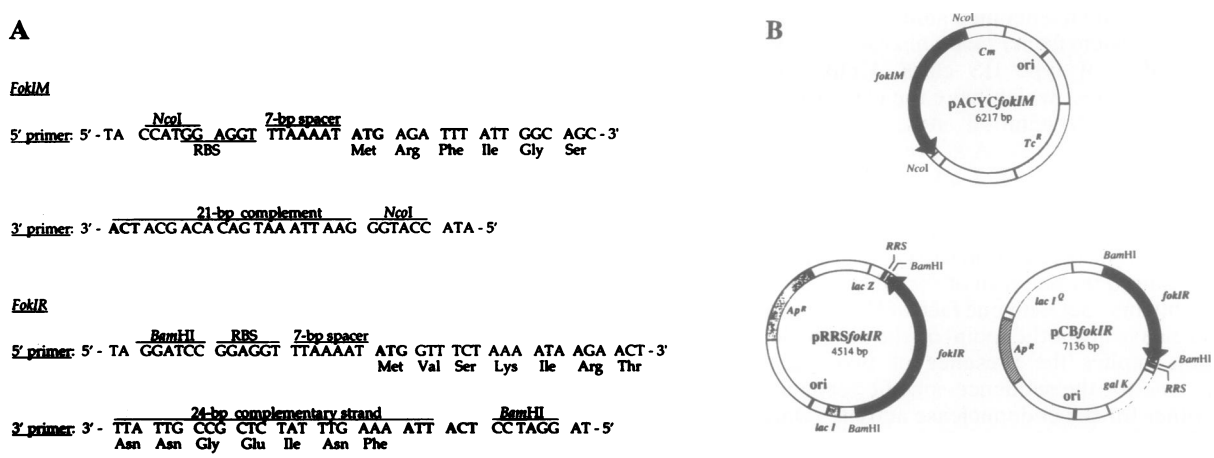

FIG. 1. Construction of high expression vectors of *Fok* I endonuclease. (*A*) Sequences of the 5′ and 3′ primers used to alter translation signals of *fokIM* and *fokIR* genes during PCR amplification. The consensus ribosome-binding site (RBS) for *E. coli* is separated from the ATG start codon by a 7-bp spacer. The *fokIM* primers are flanked by *Nco* I sites. The *fokIR* primers are flanked by *Bam*HI sites. Start and stop codons are shown in boldface letters. The 21-bp complement sequence is complementary to the sequence immediately following the stop codon of *fokIM*. (*B*) Structure of plasmids pACYCfokIM, pRRSfokIR, and pCBfokIR. The PCR-modified *fokIM* gene was inserted at the *Nco* I site of pACYC184 to form pACYCfokIM. The PCR-generated *fokIR* gene was inserted at the *Bam*HI sites of pRRS and pCB to form pRRSfokIR and pCBfokIR, respectively. pRRS possesses a *lac* UV5 promoter, and pCB contains a strong *tac* promoter. In addition these vectors contain the positive retroregulator sequence (*RRS*) downstream from the inserted *fokIR* gene.

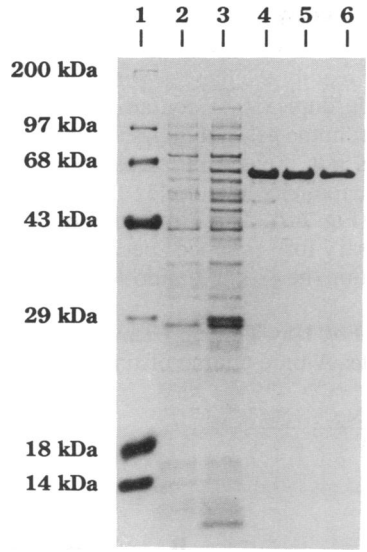

FIG. 2. SDS/PAGE profiles at each step in the purification of *Fok* I endonuclease. Lanes: 1, protein standards; 2, crude extract from uninduced cells; 3, crude extract from cells induced with 1 mM isopropyl β-D-thiogalactoside; 4, phosphocellulose pool; 5, 50–70% $(NH_4)_2SO_4$ fractionation pool; and 6, DEAE pool.

endonuclease is described earlier. SDS/PAGE profiles of protein species at each stage of purification are shown in Fig. 2. The sequence of the first 10 amino acids of the purified enzyme was determined by protein sequencing; the determined sequence was the same as that predicted from the nucleotide sequence.

Analysis of *Fok* I Endonuclease by Trypsin Cleavage in the Presence of a DNA Substrate. Trypsin is a serine protease, which cleaves at the carboxyl-terminal side of lysine and arginine residues. This is a very useful enzyme to study the domain structure of proteins and enzymes. Fig. 3*B* shows a time course of trypsin digestion of *Fok* I endonuclease with a 2.5 molar excess of oligonucleotide substrate and 10 mM $MgCl_2$. At the 2.5-min time point only two major fragments, other than intact *Fok* I, are present—a 41-kDa and a 25-kDa fragment. Upon further digestion, the 41-kDa fragment degrades into a 30-kDa and 11-kDa fragment. The 25-kDa fragment appears resistant to any further trypsin digestion. This fragment appears less stable when the trypsin digestion of *Fok* I–oligonucleotide complex is done without $MgCl_2$. Only three major fragments (30 kDa, 25 kDa, and 11 kDa) appear at the 160-min time point. Each of these fragments (41 kDa, 30 kDa, 25 kDa, and 11 kDa) were purified by reversed-phase HPLC, and their amino-terminal amino acid sequences were determined (Table 1). By comparing these amino-terminal sequences with the predicted sequence of *Fok* I, the 41-kDa and 25-kDa fragments are identified as amino-terminal and carboxyl-terminal fragments, respectively. In addition the 30-kDa fragment is amino-terminal.

The DNA-binding properties of these fragments were analyzed by using an oligo(dT)-cellulose column (Fig. 4). Trypsin digestion of *Fok* I endonuclease in a 2.5 molar excess of oligodeoxynucleotide substrate [5′-CCTCTGGATGCTC-$TC(A)_{15}$-3′·5′-GAGAGCATCCAGAGG$(A)_{15}$-3′] and 10 mM $MgCl_2$ for 10 min yields the 41-kDa amino-terminal fragment and 25-kDa carboxyl-terminal fragments as the predominant species in the reaction mixture (Fig. 4, lane 3). When this mixture is passed over an oligo(dT)-cellulose column, only the 41-kDa amino-terminal fragment is retained by the column, suggesting that the DNA-binding property of *Fok* I endonuclease is in the amino-terminal two-thirds of the enzyme. The 25-kDa fragment is not retained by the oligo(dT)-cellulose column. Trypsin digestion of *Fok* I–oligonucleotide complex for 160 min yields predominantly the 30-kDa, 25-kDa, and 11-kDa fragments (Fig. 4, lane 5). When this reaction mixture is passed over an oligo(dT)-cellulose column, only the 30-kDa and 11-kDa fragments are retained. It appears that these species bind DNA, and they arise from further degradation of 41-kDa amino-terminal fragment. The 25-kDa fragment is not retained by an oligo(dT)-cellulose column. This fragment also does not bind to DEAE-Sephadex and, thus, could be purified by passage through a DEAE column and being recovered in the break-through volume (Fig. 5*A*). This purified fragment cleaves nonspecifically both unmethylated DNA substrate (pTZ19R; Fig. 5*B*) and methylated DNA substrate (pACYCfokIM; data not

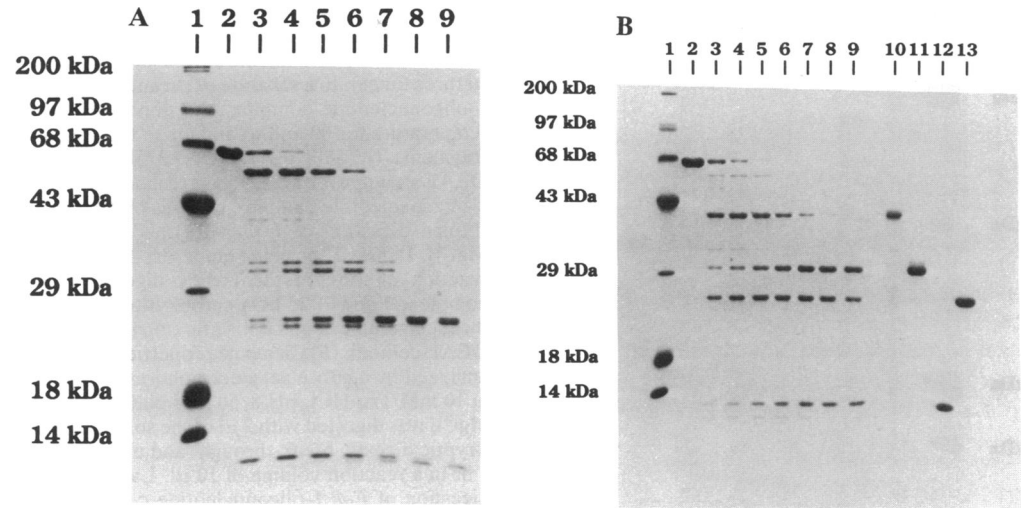

FIG. 3. SDS/PAGE profiles of tryptic fragments at various time points of trypsin digestion of *Fok* I endonuclease. (*A*) The enzyme (200 μg) in a final volume of 200 μl containing 10 mM Tris·HCl/50 mM NaCl/10 mM $MgCl_2$ was digested with trypsin at room temperature. The trypsin/*Fok* I ratio was 1:50 by weight. (*B*) Trypsin digestion of *Fok* I endonuclease in the presence of the oligodeoxynucleotide substrate, 5′-CCTCTGGATGCTCTC-3′·5′-GAGAGCATCCAGAGG-3′. The molar ratio of *Fok* I to oligonucleotide duplex was 1:2.5. *Fok* I (200 μg) was preincubated with the oligonucleotide duplex in a 180-μl volume containing 10 mM Tris·HCl/50 mM NaCl/10% glycerol/10 mM $MgCl_2$ at room temperature for 1 hr before adding trypsin (20 μl, 0.2 mg/ml). Aliquots (28 μl) from the reaction mixtures were removed at different time intervals and quenched with excess antipain. Lanes (*A* and *B*): 1, protein standards; 2, *Fok* I endonuclease; 3, 2.5 min (of trypsin digestion); 4, 5.0 min; 5, 10 min; 6, 20 min; 7, 40 min; 8, 80 min; and 9, 160 min. Lanes 10–13 in *B*: HPLC-purified tryptic fragments of 41 kDa (lane 10), 30 kDa (lane 11), 11 kDa (lane 12), and 25 kDa (lane 13).

Table 1. Amino-terminal sequences of *Fok* I fragments from trypsin digestion

Fragment	Amino-terminus sequence	DNA substrate
8 kDa	VSKIRTFG*VQNPGKFENLKRVVQVFDRNS	−
58 kDa	SEAPCDAIIQ	−
25 kDa	QLVKSELEEK	+
41 kDa	VSKIRTFGWV	+
30 kDa	VSKIRTFGWV	+
11 kDa	FTRVPKRVY	+

*, Unidentified amino acid.

shown) in the presence of $MgCl_2$. These products are small, indicating that the endonuclease domain is relatively nonspecific in cleavage. The products were dephosphorylated with calf intestinal phosphatase and rephosphorylated using polynucleotide kinase and [γ-^{32}P]ATP. The ^{32}P-labeled products were digested to mononucleotides with DNase I and snake venom phosphodiesterase. Analysis of the mononucleotides by polyethylenimine-cellulose chromatography indicates that the 25-kDa fragment cleaves preferentially phosphodiester bonds 5′ to guanine > adenine >> thymine ~ cytosine. The 25 kDa carboxyl-terminal fragment thus constitutes the cleavage domain of *Fok* I endonuclease.

The 41-kDa amino-terminal fragment–oligonucleotide complex was purified by agarose gel electrophoresis. Trace amounts of ^{32}P-labeled oligonucleotide duplex were incorporated into the complex to monitor it during gel electrophoresis. The band corresponding to the complex was excised and recovered by electroelution in a dialysis bag. Analysis of the complex by SDS/PAGE revealed the 41-kDa amino-terminal fragment as the major component. The 30-kDa amino-terminal fragment and the 11-kDa carboxyl-terminal fragment are present as minor components. The latter two

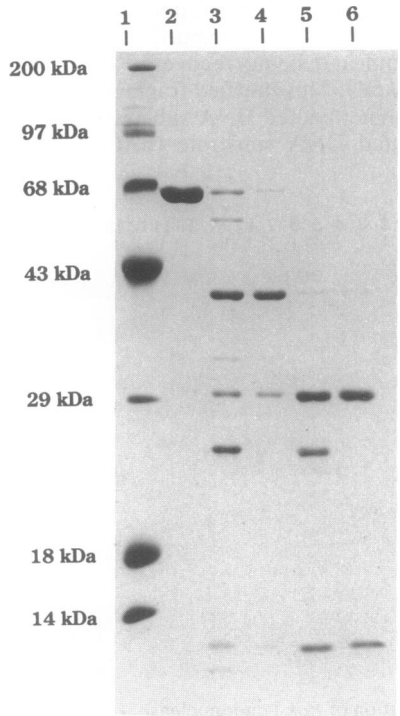

FIG. 4. SDS/PAGE profiles of the tryptic fragments of *Fok* I endonuclease that bound to the oligo(dT)-cellulose column. Lanes: 1, protein standards; 2, *Fok* I endonuclease; 3, 10-min trypsin digestion mixture of *Fok* I–oligonucleotide complex; 4, tryptic fragments that bound to the oligo(dT)-cellulose column; 5, 160-min trypsin digestion mixture of *Fok* I–oligonucleotide complex; 6, tryptic fragments that bound to the oligo(dT)-cellulose column.

fragments appear to bind DNA jointly and comigrate with the 41-kDa amino-terminal fragment–oligonucleotide complex. The complex readily exchanges with a ^{32}P-labeled specific oligonucleotide duplex that contains the *Fok* I recognition site, as seen from the gel-mobility-shift assays (Fig. 6*A*). The complex does not, however, exchange with a ^{32}P-labeled nonspecific oligonucleotide duplex that lacks the *Fok* I recognition site (Fig. 6*B*). These results indicate that all information necessary for sequence-specific recognition of DNA is encoded within the 41-kDa amino-terminal fragment of *Fok* I.

Analysis of *Fok* I by Trypsin Cleavage in the Absence of a DNA Substrate. A time course of trypsin digestion of *Fok* I

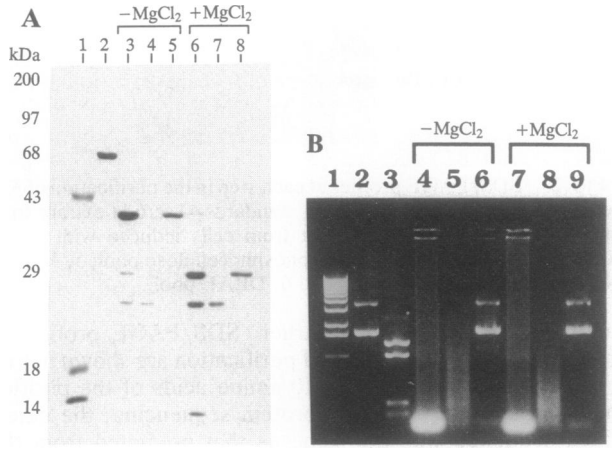

FIG. 5. Analysis of cleavage properties of the tryptic fragments of *Fok* I endonuclease. *Fok* I (390 μg) was incubated with a 2.5 molar excess of oligodeoxynucleotide duplex [5′-CCTCTGGATGCTCTC-3′·5′-GAGAGCATCCAGAGG-3′] in a total volume of 170 μl containing 10 mM Tris·HCl, pH 8/50 mM NaCl/10% glycerol at room temperature for 1 hr. Digestion with trypsin (30 μl; 0.2 mg/ml) without $MgCl_2$ was for 10 min at room temperature to maximize yield of the 41-kDa amino-terminal fragment; the reaction was quenched with excess antipain. The tryptic digest was passed through a DEAE column. The 25-kDa carboxyl-terminal fragment, which does not bind to DEAE-Sephadex, was recovered in the break-through volume. This purified 25-kDa fragment cleaves pTZ19R only with $MgCl_2$. All other tryptic fragments (41 kDa, 30 kDa, and 11 kDa) are retained by the column and were eluted with 0.5 M NaCl buffer (200 μl three times). In a separate experiment, the trypsin digestion of *Fok* I–oligonucleotide complex was done with 10 mM $MgCl_2$ at room temperature for 60 min to maximize the yield of 30-kDa and 11-kDa fragments. (*A*) SDS (0.1%)/12% PAGE profiles of fragments from the DEAE column. Lanes 3–5 correspond to trypsin digestion of *Fok* I–oligonucleotide complex without $MgCl_2$; lanes 6–8 correspond to trypsin digestion of *Fok* I–oligonucleotide complex with 10 mM $MgCl_2$. Lanes: 1, protein standards; 2, *Fok* I endonuclease; 3 and 6, reaction mixture of the tryptic digests of *Fok* I–oligonucleotide complex; 4 and 7, 25-kDa carboxyl-terminal fragment in the break-through volume; 5 and 8, tryptic fragments of *Fok* I that bound to the DEAE column. (*B*) Cleavage properties of the tryptic fragments were analyzed by agarose gel electrophoresis. One microgram of pTZ19R in 10 mM Tris·HCl, pH 8/50 mM NaCl/1 mM dithiothreitol/10 mM $MgCl_2$ was digested with 2 μl of the solution containing the fragments (tryptic digests, break-through, and eluate, respectively) at 37°C for 1 hr in a reaction volume of 10 μl. Lanes 4–6 correspond to trypsin digestion of *Fok* I–oligonucleotide complex without $MgCl_2$. Lanes 7–9 correspond to trypsin digestion of *Fok* I–oligonucleotide complex with 10 mM $MgCl_2$. Lanes: 1, 1-kilobase (kb) ladder; 2, pTZ19R; 3, pTZ19R digested with *Fok* I endonuclease; 4 and 7, reaction mixture of the tryptic digests of *Fok* I–oligonucleotide complex; 5 and 8, 25-kDa carboxyl-terminal fragment in the break-through volume; 6 and 9, tryptic fragments of *Fok* I that bound to the DEAE column. The intense bands at gel bottom correspond to excess oligonucleotides. Controls containing either trypsin or antipain without *Fok* I failed to degrade the plasmid substrates, indicating the lack of contaminating nonspecific nuclease activity in these preparations.

endonuclease in the absence of the DNA substrate is shown in Fig. 3*A*. Initially, *Fok* I cleaves into a 58-kDa fragment and an 8-kDa fragment. The 58-kDa fragment does not bind DNA substrates and is not retained by the oligo(dT)-cellulose column. On further digestion, the 58-kDa fragment degrades into several intermediate tryptic fragments. However, the complete trypsin digestion yields only 25-kDa fragments as major components (appears as two overlapping bands). Each of these species (58 kDa, 25 kDa, and 8 kDa) was purified by reversed-phase HPLC, and their amino-terminal amino acid sequences were determined (Table 1). Comparison of the amino-terminal sequences to the predicted *Fok* I sequence revealed the 8-kDa fragment to be amino-terminal and the 58-kDa fragment to be carboxyl-terminal. This result further supports our conclusion that the amino terminus of *Fok* I is responsible for the recognition domain. Sequencing the amino terminus of the 25-kDa fragments revealed more than a single component. A time course of trypsin digestion of *Fok* I endonuclease with a nonspecific DNA substrate yields a profile similar to that obtained when trypsin digestion of *Fok* I occurred without any DNA substrate (data not shown).

Functional Domains in *Fok* I Restriction Endonuclease. Analysis of functional domains of *Fok* I (with and without substrates) by using trypsin is summarized in Fig. 7. Binding of DNA substrate by *Fok* I is accompanied by alteration in

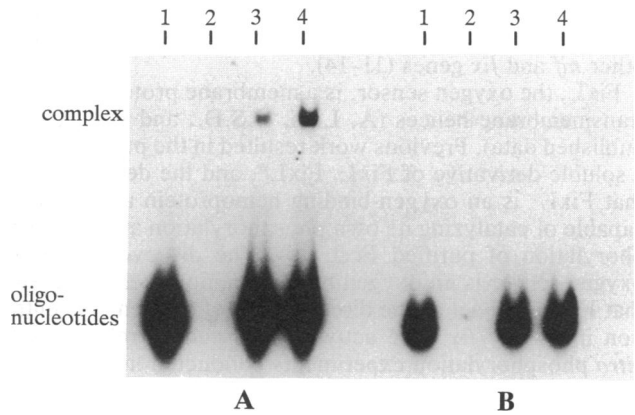

FIG. 6. Analysis of sequence-specific binding of DNA by 41-kDa amino-terminal fragment by using gel-mobility-shift assays. *Fok* I endonuclease (200 μg) was incubated with a 2.5 molar excess of oligodeoxynucleotide duplex (5′-CCTCTGGATGCTCTC-3′·5′-GAGAGCATCCAGAGG-3′) in a volume of 180 μl containing 10 mM Tris·HCl, pH 8.0/50 mM NaCl/10% glycerol at room temperature for 1 hr. Trace amounts of ^{32}P-labeled oligonucleotide duplex were incorporated into the complex to monitor it during gel electrophoresis. Digestion with trypsin (20 μl; 0.2 mg/ml) was for 12 min at room temperature to maximize yield of the 41-kDa amino-terminal fragment; the reaction was quenched with excess antipain. The 41-kDa amino-terminal fragment–oligonucleotide complex was purified by agarose gel electrophoresis. The band corresponding to the complex was excised and recovered by electroelution in a dialysis bag (≈600 μl). For the exchange reaction, the complex (10 μl) was incubated with 1 μl of ^{32}P-labeled specific (or nonspecific) oligonucleotide duplex in a volume of 20 μl containing 10 mM Tris·HCl/50 mM NaCl/10 mM $MgCl_2$ at 37°C for 30 and 120 min, respectively. One microliter of the specific probe contained 12 pmol of 5-CCTC-TGGATGCTCTC-3′·5′-GAGAGCATCCAGAGG-3′ and ≈50 × 10^3 cpm. One microliter of the nonspecific probe contained 12 pmol of 5′-TAATTGATTCTTAA-3′·5′-ATTAAGAATCAATT-3′ and ≈2.5 × 10^3 cpm. The reaction mixtures were analyzed on an 8% nondenaturing polyacrylamide gel. (*A*) Lanes: 1, specific oligonucleotide duplex; 2, 41-kDa amino-terminal fragment–oligonucleotide complex; 3 and 4, specific probe incubated with complex for 30 and 120 min, respectively. (*B*) Lanes: 1, nonspecific oligonucleotide duplex; 2, 41-kDa amino-terminal fragment–oligonucleotide complex; 3 and 4, nonspecific probe incubated with complex for 30 and 120 min, respectively.

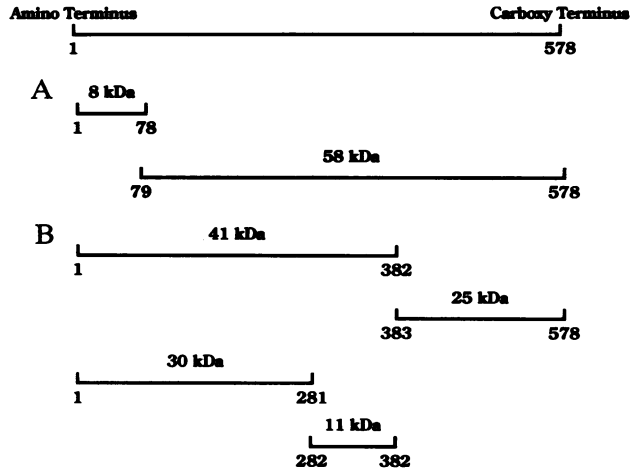

FIG. 7. Tryptic map of *Fok* I endonuclease. *Fok* I endonuclease fragmentation pattern without (*A*) and with (*B*) oligonucleotide substrate.

enzyme structure. Our study supports the presence of two separate protein domains within this enzyme: one for the sequence-specific recognition and the other for the endonuclease activity. Our results indicate that the recognition domain is at the amino terminus of the *Fok* I endonuclease, whereas the cleavage domain is probably in the carboxyl-terminal third of the molecule. Mutational analysis of the enzyme can precisely define the domain structure—i.e., the recognition and cleavage domains within *Fok* I endonuclease. The modular structure of the enzyme suggests that it may be feasible to construct chimeric endonucleases of different sequence specificity by linking other DNA-binding proteins (e.g., zinc finger motifs, homeo domain motifs, and DNA-binding domains of *lambda*, *lac* repressors, *cro*, etc.) to the cleavage domain of *Fok* I endonuclease.

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