Type II restriction endonuclease Mva1269I recognizes an asymmetric DNA sequence 5’-GAATGCN ↓-3’/5’-NG ↓ CATTC-3’ and cuts top and bottom DNA strands at positions, indicated by the “↓” symbol. Most restriction endonucleases require dimerization to cleave both strands of DNA. We found that Mva1269I is a monomer both in solution and upon binding of cognate DNA. Protein fold-recognition analysis revealed that Mva1269I comprises two “PD-(D/E)XK” domains. The N-terminal domain is related to the 5’-GAATTC-3’-specific restriction endonuclease EcoRI, whereas the C-terminal one resembles the nonspecific nuclease domain of restriction endonuclease FokI. Inactivation of the C-terminal catalytic site transformed Mva1269I into a very active bottom strand-nicking enzyme, whereas mutants in the N-terminal domain nicked the top strand, but only at elevated enzyme concentrations. We found that the cleavage of the bottom strand is a prerequisite for the cleavage of the top strand. We suggest that Mva1269I evolved the ability to recognize and to cleave its asymmetrical target by a fusion of an EcoRI-like domain, which incisves the bottom strand within the target, and a FokI-like domain that completes the cleavage within the nonspecific region outside the target sequence. Our results have implications for the molecular evolution of restriction endonucleases, as well as for perspectives of engineering new restriction and nicking enzymes with asymmetric target sites.

Type II restriction enzymes (REases) are one of the largest groups of endonucleases. They recognize short double-stranded DNA sequences and specifically cleave both strands of DNA at fixed positions within or near these sites (1), a property that has made them indispensable for DNA engineering. All structurally characterized REases have been found to share a common three-dimensional fold harboring a bipartite PD-(D/E)XK pattern of three moderately conserved charged catalytic/Mg2+ binding residues (2, 3). However, their amino acid sequences are strongly divergent. Typically, only enzymes that recognize identical or very similar DNA sequences may display similarity at the amino acid level (4). Recent bioinformatics analyses suggested that REases belong also to at least three other folds, GIY-YIG, HNH, and PLD (5-7). Therefore, identification of the catalytic and DNA recognition residues of REases is extremely challenging.

Most of type II REases (type IIA) recognize palindromic DNA sequences and cleave both DNA strands within the target at symmetrical positions. They usually act as homodimers, in which each of two identical subunits interacts symmetrically with the same part of the DNA sequence (3). Type II REases that recognize asymmetric targets are classified as type IIA (8). Of these, there are nine prototypes that cleave both DNA strands within the recognition sequence. Two of them, BbvCI and Bpu10I, have been characterized (9, 10). Both enzymes are composed of two mutually homologous, but non-identical subunits. This feature suggests that Bpu10I and BbvCI recognize and cleave DNA in an “almost symmetrical” manner, reminiscent of type I IF REases.

Type IIS REases are those type IIA enzymes that cleave at least one strand of the DNA duplex outside of the recognition sequence (1). This group of enzymes can be further subdivided based on the distance between the recognized sequence and the site of cleavage. One subgroup, represented by an archetypal REase, FokI, includes those type IIS enzymes that cleave both DNA strands distant from the target site. FokI comprises two physically and functionally distinct domains, one for DNA binding and another for catalysis, which are connected by a linker (11). Initially, FokI binds to DNA as a monomer via the DNA binding domain. In the presence of divalent metal ions, the catalytic domains of two FokI monomers transiently dimerize and then cleave the DNA at a fixed position near one of the recognition sites (12-14). FokI dimerization is necessary for efficient double-stranded DNA cleavage, because mutants defective in dimerization exhibit impaired DNA cleavage (12). It is likely that other REases of this subgroup could act in a similar manner (15, 16), although some of them can efficiently cleave one strand of cognate DNA. Their dimerization is only required to carry out the cleavage of the second DNA strand (17, 18).

The other subgroup of type IIS enzymes, “short-distance” cutters, includes REases that cleave both DNA strands very close to the recognition site. Due to spatial constraints provided by the proximity of the DNA target sequence and the position where the cleavage occurs, it seems doubtful that the FokI-like DNA recognition and cleavage mechanism is applicable to these short-distance REases. Indeed, kinetic studies by Bath et al. (15) and experiments aimed at developing nicking enzymes from representatives of this group of enzymes (19, 20) suggested that short-distance REases may possess two active sites. However, this possibility was never studied in detail.

The type IIS REase from Micrococcus varians RFL1269, Mva1269I, is a short-distance cutter that recognizes an asymmetric DNA sequence 5’-GAATGCN ↓-3’/5’-NG ↓ CATTC-3’ and cuts top and bottom
DNA strands at positions indicated by arrows. Reaction products have 3′-overhangs that are two nucleotides long. In this work, we investigated the DNA cleavage mechanism and sequence-structure-function relationships of Mva1269I using bioinformatics and biochemical analysis. Our experiments indicate that Mva1269I is a mononuclear enzyme possessing two active sites responsible for the sequential cleavage of each DNA strand, which has evolved by fusion of a sequence-specific nuclease domain, similar to EcoRI, to a nonspecific nuclease domain, similar to FokI.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Reagents—**Micrococcus varians RFL1269 was obtained from the Fermentas UAB collection. Escherichia coli strain ER2267 (New England Biolabs) was used as a host in cloning experiments. ER2566 (New England Biolabs) and PET-21b(+) (Novagen) were used for overexpression of Mva1269I. All strains were grown in LB medium at 37 °C according to standard protocols (21). Transformations of *E. coli* cells were carried out either by using the CaCl2-heat shock method or by electroporation (21). All enzymes, kits, standard sequencing primers, molecular mass standards, 3′ and 5′ end-labeled M13/pUC sequencing primers, molecular mass standards, were from Fermentas UAB. To construct the Mva1269I target-deficient plasmid pUC57Mva, pUC57 DNA was linearized with Mva1269I, and cohesive DNA ends were blunt-ended by T4 DNA polymerase and ligated. The substrate with two Mva1269I sites, pUC57-2, was constructed as follows. The DNA of pUC57 was linearized with AatII, cohesive ends blunt-ended, and the resulting fragment ligated with a synthetic DNA duplex identical in sequence to the multiple cloning site of pUC57 (nucleotide coordinates 401–436) and containing the Mva1269I target in the middle. The insertion was verified by sequencing. The plasmids used in DNA cleavage studies were purified by centrifugation in CsCl-ethidium bromide.

**DNA Manipulations—**Recombinant plasmid construction, restriction mapping, construction of nested deletions, agarose gel electrophoresis, and purification of DNA were carried out using standard techniques (21). The nucleotide sequence was determined using the Cycle Reader DNA Sequencing Kit and standard 5′-end-labeled M13/pUC sequencing primers. Genomic DNA from *Micrococcus varians* RFL1269 was extracted and purified as described previously (21). Details on the construction of the *M. varians* RFL1269 gene library, isolation of clones carrying the cloned gene for Mva1269I methyltransferase, and subsequent cloning of the full-length gene for Mva1269I REase are available upon request.

**Expression and Purification of wt Mva1269I and Its Mutant Variants—**To protect the DNA of ER2566 in vivo from Mva1269I REase, the gene for Mva1269I methyltransferase was subcloned into pACYC184 (22). The resulting plasmid rendered genomic DNA resistant to Mva1269I cleavage. Then, the gene for Mva1269I REase was inserted into PET-21b(+) under the control of the isopropyl 1-thio-β-D-galactopyranose-inducible phase T7 promoter, and introduced into methylation-proficient ER2566 cells. Site-directed mutagenesis of Mva1269I was performed by the “megaprimer” method (23). The presence of the desired mutations, as well as the absence of additional mutations, was confirmed by DNA sequencing. Details on the construction of all aforementioned clones are available upon request.

To purify wt Mva1269I and its mutant variants, cultures were grown to 0.5 A (λ = 550 nm) and induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside. After 3 h at 37 °C, the cells were harvested by centrifugation and suspended in 10 mM Tris-HCl (pH 8.5), 7 mM mercaptoethanol, 1 mM Na2EDTA, 0.05% Triton X-100, 10% glycerol, 0.1 mM NaCl. The cell suspension was sonicated, and then cell debris was removed by centrifugation. The extract was subjected to column chromatography (successively, heparin-Sepharose, DEAE-52 cellulose, Bordo-Sepharose, and P11-phosphocellulose sorbents) using NaCl gradient. Fractions with Mva1269I activity were dialyzed against storage buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM diithiothreitol, 0.1 mM Na2EDTA, 50% glycerol, 0.025% Triton X-100), and stored at −20 °C. Mva1269I mutants lacking the double-stranded cleavage activity were purified using the same scheme. However, in this case, samples of individual fractions were analyzed by SDS-PAGE electrophoresis, and those fractions that contained the abundant protein band corresponding to that of the wild-type enzyme were pooled. The final preparations of all proteins were analyzed by SDS-PAGE, stained with Coomassie Brilliant Blue R-250, scanned, and quantified using 1Dwiniv software to verify their purity. Concentrations of purified proteins were estimated using the Bradford assay (24).

**Oligodeoxynucleotides—**The oligodeoxynucleotide sequences designed for the top strand of double-stranded DNA substrates for Mva1269I were: S1, 5′-GGCGATCAATGCTTGAAGGTT-3′ (Mva1269I target underlined); PTO1, 5′-GGCGATCAATGCTTGAAGGTT-3′ (s denotes the position of phosphorothioate substitution); and N1, 5′-GGCGATCAATGCTTGAAGGTT-3′ (no Mva1269I target). The sequences of the oligodeoxynucleotides used for the bottom strand were: S2, 5′-CCCTTCTAGGATCGATCG-3′; PTO2, 5′-CCCTTCTAGGATCGATCG-3′; and N2, 5′-CCCTTCTAGGATCGATCG-3′.

All purified proteins, salt-free oligodeoxynucleotides were purchased from MWG Biotech. Phosphorothioate-substituted oligonucleotides were supplied as a racemic mixture of R and S diastereomers. They were used without further purification. Concentrations of oligonucleotides were evaluated spectrophotometrically. For the DNA–binding experiments and cleavage assays, either top or the bottom strands of the duplexes were 5′-labeled with [γ-32P]ATP (Amersham Biosciences) by using T4 polynucleotide kinase. To ensure that all labeled oligonucleotides are hybridized to the duplex, the unlabeled complementary oligonucleotides were used in slight excess (with a ratio of 1:1.2). Complementary oligonucleotides were annealed by heating the solution to 95 °C in a water bath and then allowed to cool slowly. Non-denaturing polyacrylamide gel electrophoresis, followed by radioactivity detection, showed no detectable traces of unhybridized radiolabeled single-stranded oligonucleotides.

**DNA Cleavage Assay I: Cleavage of One-site (pUC57) and Two-site (pUC57-2) Substrates by Mva1269I—**Cleavage reactions were performed at 25 °C with 5 μM supercoiled plasmid DNA in Buffer R (Fermentas UAB, 10 mM Tris-HCl, pH 8.5, 10 mM MgCl2, 100 mM KCl, 1.5 μM BSA) and 0.4 μM Mva1269I. Aliquots were removed at timed intervals, and the cleavage reactions were stopped by adding 0.3 volume of gel loading buffer (60 mM EDTA, 0.3% SDS, 60% glycerol, 0.012% bromophenol blue). The reaction products were fractionated by agarose gel electrophoresis and visualized after staining with ethidium bromide. The stained gels were scanned, and DNA bands were quantified using 1Dwiniv software.

**DNA Cleavage Assay II: Cleavage of One-site Substrate pUC57 by Mva1269I Mutants—**Cleavage reactions were performed at 37 °C with 2.5 μM supercoiled plasmid DNA in Buffer R and Mva1269I variants at the concentrations indicated. Reactions were stopped by adding 0.3 volume of gel loading buffer (see above). The reaction products were analyzed by agarose gel electrophoresis.

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DNA Cleavage Assay III: Cleavage of Oligoduplexes by Mva1269I—Cleavage reactions with unmodified (S1/S2) and phosphorothioate-modified (S1/PTO2, PTO1/S2, or PTO1/PTO2) oligoduplexes (5 nM, 5'-32P-labeled on either of the strands) and Mva1269I (20 nM) were performed in Buffer R at 25 °C. Aliquots were removed at timed intervals, and the cleavage reactions were stopped by adding 0.5 volume of stop solution (95% formamide, 0.1% bromphenol blue, 0.1% xylene cyanol FF, 20 mM EDTA, pH 8.0). Before loading, reaction samples were denatured for 5 min at 96 °C and cooled in ice. The reaction products were analyzed by denaturing gel electrophoresis (20% acrylamide, 1× TBE (8.9 mM Tris, 8.9 mM boric acid, 0.2 mM EDTA), 7 M urea). Radio-labeled DNA was detected and quantified by using Cyclone Storage Phosphor System and OptiQuant 3.0 software (PerkinElmer Life Sciences).

DNA Binding Assays—DNA binding by wt Mva1269I and by mutant proteins was analyzed by the electrophoretic mobility-shift assay, using the 24-bp specific (S1’/S2’) or non-specific (N1’/N2’) oligoduplexes (the asterisk denotes the 5'-32P-labeled oligonucleotide). To investigate the effect of Ca2+ ions on the DNA-binding properties of the wt enzyme, the duplexes (1 nM) were incubated for 10 min at room temperature with varying amounts of the wt enzyme in 20 µl of binding buffer (10 mM Tris-HCl, pH 8.5, 100 mM KCl, 3 µM BSA, 10% (v/v) glycerol) supplemented either with 10 mM CaCl2 or with 0.1 mM EDTA. Samples were loaded onto 8% (w/v) polyacrylamide gels (29.1 (w/w) acrylamide/bisacrylamide) and run at 6 V/cm in 20 mM Tris acetate (pH 8.5) buffer, which was supplemented with 10 mM calcium acetate in those cases when calcium ions were used in the DNA-binding reaction. After electrophoresis, the gels were dried and analyzed using a Cyclone Storage Phosphor System with OptiQuant 3.0 software. DNA-binding properties of mutant enzymes were examined in the presence of Ca2+ ions.

In DNA displacement experiments, the radiolabeled S1’/S2’ duplex (1 nM) was mixed with Mva1269I (2 nM) in binding buffer supplemented with 10 mM CaCl2 and incubated for 10 min at room temperature. Then, varying amounts of an unlabeled 174-bp DNA fragment, containing a single site for Mva1269I, were added. The mixtures were analyzed by non-denaturing PAGE as above. The 174-bp DNA fragment was generated by PCR using pUC57 as a template, standard M13/pUC direct and reverse primers, followed by gel purification.

Determination of Strand Specificity—pUC57 DNA was nicked with the appropriate Mva1269I mutant at 37 °C. The nicked form was then gel-purified and used as a template for run-off sequencing. The standard M13/pUC primers and BigDye® Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) were used in sequencing reactions.

Sucrose Gradient Velocity Centrifugation—Mva1269I (485 nM), either alone or together with marker proteins, in a total volume of 250 µl, was layered on a 12-m1.5 to 20% (w/w) linear sucrose concentration gradient in 10 mM Tris-HCl, pH 8.5, 100 mM KCl, and 5 mM CaCl2. The test tubes were then centrifuged at 20 °C in an SW 50Ti rotor using a Beckman L8—70 ultracentrifuge at 40,000 rpm for 19 h. After centrifugation, 300-µl fractions were taken starting from the top and analyzed by SDS-PAGE. The REase activity was detected by adding cofactor Mg2+ ions and λ DNA as a substrate. The following marker proteins were used: chymotrypsinogen (M, 25,000), ovalbumin (M, 43,000), bovine serum albumin (M, 66,000), and aldolase (M, 158,000). Centrifugation experiments of Mva1269I-DNA complexes were performed essentially as above, except that 485 nM of protein was combined either with a DNA mixture containing 4 nM of radioactively labeled and 960 nM of unlabelled oligoduplex S1/S2 (protein:DNA ratio, 1:2), or with a mixture of DNA containing 4 nM labeled and 1920 nM unlabeled oligoduplex S1/S2 (protein:DNA ratio, 1:4). To investigate the sedimentation of free DNA, the same volume (250 µl) of a DNA mixture containing 4 nM labeled and 1920 nM unlabeled oligoduplex S1/S2 was centrifuged.

To detect radioactively labeled DNA, aliquots of fractions were spotted onto a Hybond N+ membrane (Amersham Biosciences), and dried samples were analyzed using Cyclone Storage Phosphor System and OptiQuant 3.0 software.

Protein Structure Prediction—Secondary structure prediction and tertiary fold-recognition (FR, recognition of similarity to known protein structures without significant sequence identity) was carried out via the GeneSilico meta-server at genesilico.pl/meta/ (25). Because the FR procedure is designed to identify similarities at the level of individual protein domains, we carried out the initial analysis by dividing the Mva1269I sequence into a set of overlapping fragments corresponding to the size of typical domains (~250 amino acids). FR alignments to the structures of selected templates were used as a starting point for homology modeling using the “FRankensteins Monster” approach (26), comprising cycles of model building, evaluation, realignment in poorly scored regions, and merging of best scoring fragments. Regions, which could not be modeled because of the lack of the appropriate template structure, were added “de novo” using the fragment insertion method ROSETTA (27). This procedure has led to successful predictions of numerous protein structures (validated by experimental analyses; e.g., see the results of the CASP6 evaluation available at predictioncenter.lnl.gov/casp6/), including those of REases and other nucleases (e.g., 28–31). The reader is referred to the abovementioned articles for a detailed description of the modeling methodology.

RESULTS

Mva1269I Restriction-modification System Consists of Two Genes—Cloning and sequencing of the M. varians RFL1269 DNA region coding for the Mva1269I REase and methyltransferase revealed that the Mva1269I restriction-modification system consists of two convergently transcribed genes, which are separated by 125 bp (GenBank™ accession number DQ074451). The mva1269IR gene codes for the Mva1269I REase of 685 amino acid residues (calculated M, 78,400). Mva1269I shares 31% amino acid sequence identity with its isoschizomer BsmI (GenBank™ accession number AY079085). The mva1269IM gene encodes the M.vma1269I methyltransferase of 626 amino acid residues (calculated M, 72,597). Sequence analysis revealed that M.Mva1269I is composed of two modules exhibiting significant sequence similarity to DNA methyltransferases that generate either m4Co rm6A (32).

DNA Cleavage by Mva1269I—Studies of DNA cleavage by BsmI, an isoschizomer of Mva1269I, demonstrated that the enzyme cleaved substrates with one and two recognition sites at similar rates and cleaved the two-site DNA by acting independently at each site (15). Based on these observations, the authors suggested that BsmI does not need to interact with two target sites and that it probably is a monomer with two active sites, one for the cleavage of each DNA strand. To test if Mva1269I performs like BsmI, the cloned mva1269IR gene was overexpressed and Mva1269I purified. The enzyme was found to be >90% pure based on SDS-PAGE (Fig. 1).

DNA cleavage experiments were carried out by using the supercoiled (SC) form of plasmids with one site (pUC57) and two sites (pUC57-2) as substrates. Like BsmI, Mva1269I linearized (Fig. 2A, see the curve L) the SC form of pUC57 without the accumulation of a nicked intermediate (Fig. 2A, see the curve OC). Hence, the cleavage of both DNA strands is highly concerted and Mva1269I does not dissociate from the target during the reaction. In the course of the incubation of Mva1269I with the two-site plasmid the initial accumulation of the full-length linear DNA form (Fig. 2B, the curve L) was followed by cleavage of the remain-
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Mva1269I cleavage properties are very similar to those of BsmI, BsmBI, Bsal, and SapI, all of which are short-distance cutters (15). Thus, we carried out experiments aimed to understand how Mva1269I and, by indication, other short-distance REases recognize specific DNA and perform double-stranded DNA cleavage.

Mva1269I Is a Monomer in Solution—The quaternary structure of free and DNA-bound Mva1269I was examined by sucrose gradient centrifugation. Centrifugation of Mva1269I in the absence of specific DNA showed that Mva1269I sediments as a protein of ~82,000 (Fig. 3A). The calculated $M_r$ of Mva1269I is 78,400. Our results, therefore, suggest that Mva1269I is a monomer in the absence of specific DNA, as found for other type IIS REases (33–35).

Mva1269I Monomer Forms a Complex with One DNA Molecule—It is well known that some REases require divalent metal ions for specific DNA binding, and form stable specific protein-DNA complexes in the presence of non-catalytic Ca$^{2+}$ ions (36). Likewise, our initial DNA binding experiments revealed that Mva1269I did not discriminate between specific and nonspecific DNA in the absence of the catalytic cofactor Mg$^{2+}$, and that specific binding took place in the presence of Ca$^{2+}$ ions (see Fig. 8A). Therefore, Ca$^{2+}$ ions were used in all sucrose gradient centrifugation experiments. To study Mva1269I oligomerization in the presence of specific DNA, the enzyme was mixed either with a 2- or 4-fold molar excess of radioactively labeled specific DNA oligoduplex and subjected to sucrose gradient centrifugation. After centrifugation, individual fractions were tested for Mva1269I activity and for their radioactivity. Two peaks of radioactivity were observed (Fig. 3B). One of them represented the free DNA that, relative to the marker proteins, yielded an apparent molecular weight of ~21,000 (the calculated $M_r$ is ~14,600). The increased estimated molecular weight of free DNA can be explained by its cylindrical shape, resulting in increased hydrodynamic friction compared with that experienced by spherically shaped marker proteins. The second radioactivity peak overlapped with the peak of Mva1269I activity (data not shown), suggesting that Mva1269I and specific DNA form a complex with apparent $M_r$ of ~124,000 (Fig. 3C). The apparent molecular weight of the complex equaled the sum of molecular weights of one protein molecule and two DNA molecules ($82,000 + 21,000 \times 2 = 124,000$). However, the radioactivity counting of free and bound DNA fractions revealed that only a half of the DNA was bound when it was mixed with Mva1269I in a 2-fold molar excess, and one-fourth of the DNA was bound when DNA was added in a 4-fold excess. These results suggested that a monomer of Mva1269I forms a 1:1 complex with DNA. Identical centrifugation results were obtained when regular specific oligoduplex was replaced by a modified one, where the scissile phosphates were replaced by phosphorothioates, and catalytic Mg$^{2+}$ ions were used instead of Ca$^{2+}$ ions (data not shown). This modification rendered the oligoduplex resistant to Mva1269I cleavage (see Fig. 9D).

To clarify whether one or two DNA molecules interact with the Mva1269I monomer, a DNA displacement experiment was performed. The specific complex was first generated by incubating Mva1269I with the radioactively labeled specific 24-bp oligoduplex. Then, the 174-bp DNA fragment with a single Mva1269I recognition site was added in increasing amounts to the specific complex. If a monomer of Mva1269I interacts with two DNA molecules, an additional complex, containing a single protein molecule and two DNA fragments of different length should be formed, because the non-labeled 174-bp fragment gradually replaces the 24-bp oligoduplex. In our gel shift assay, however, no new site (Fig. 2B, the curve $L_{1/2}$). Therefore, it appears that the two cleavage reactions are separate and sequential. Also, the initial rates of supercoiled DNA cleavage were similar irrespective of the number of Mva1269I targets (0.63 nM/min and 0.66 nM/min for one- and two-site substrates, respectively). This is in sharp contrast with the cleavage mode of the best studied type IIS representative, FokI, and of several other type IIS enzymes, most of which require two sites for optimal performance (15).

FIGURE 1. Analysis of purified wild-type Mva1269I restriction endonuclease and its mutants by SDS-10% PAGE followed by Coomassie Brilliant Blue staining. 1-μg samples of each purified protein were analyzed, except for E112A. In the latter case a 0.5-μg sample was loaded due to low concentration. Lane M, PageRuler Prestained Protein Ladder (Fermentas UAB).

FIGURE 2. Mva1269I performance on plasmids with one (A) or two (B) recognition sites. Graphs illustrate the decline or accumulation of various forms of plasmid DNA (SC, supercoiled; OC, open-circle; L, linear; $L_{1/2}$, two linear products). Reactions were performed by using 0.4 nM Mva1269I and 5 nM plasmid, in Buffer R, at 25 °C.
bands corresponding to potential additional complexes were observed (Fig. 3D). Once displaced, the 24-bp oligoduplexes moved as a free DNA. Therefore, it seems that in the presence of Ca\(^{2+}\) ions, one mole- cule of Mva1269I binds one DNA molecule to form a specific complex.

Taken together, these results raise the question of how monomeric Mva1269I can perform a double-stranded DNA cleavage. Two possibilities must be considered: (i) Mva1269I has two active sites per monomer, one for each DNA strand, or (ii) Mva1269I has only one active site, which cleaves one DNA strand and then, after appropriate conformational changes in the complex, cleaves the second DNA strand. If two active sites exist, mutational inactivation of any of them should convert Mva1269I into a nicking enzyme. Conversely, a totally inactive mutant enzyme should appear if only one active site serves to cleave both DNA strands. A search for putative active sites and their mutational analysis was carried out to distinguish between these two possibilities.

Fold-recognition Analysis of Mva1269I.—Because of notorious difficulties in the identification of functionally important residues in REases sequences, we used the protein fold-recognition (FR) approach to identify suitable structural templates for modeling of Mva1269I. The N-terminal region (aa 1–212) was found to be related to the REase EcoRI (top rank according to the mGenThreader method, intermediate ranks according to three other FR methods, and with the ultimate top rank assigned to the EcoRI structure by the consensus server Pcons). The C-terminal region (aa 481–687) was found to be related to the catalytic domain of FokI (top rank according to SAM-T02 and 3DPSSM, second rank according to mGenThreader, and with the ultimate top rank assigned by Pcons). The central region of Mva1269I (aa 213–480) did not show any significant similarity to known protein structures.

The sequence alignments reported by different FR methods exhibited differences. To optimize the sequence to structure fit, we used the FRan- kenstein’s Monster approach (26). It uses protein modeling to evaluate differences in the resulting models and sequence alignments revealed that the C-terminal domain contains a putative active site comprising a \(\beta\)-hairpin motif serving as a scaffold for an "orthodox" catalytic motif Pro-Asp\(^{537}\)-\(\chi\)\(^{18}\)Glu\(^{554}\)-Ser-Lys\(^{556}\) (conserved in the sequence alignment in Fig. 4). The N-terminal domain exhibits a similar structural scaffold. However, its putative catalytic motif (Ala-Asp\(^{595}\)-\(\chi\)\(^{18}\)Glu\(^{112}\)-Phe-Ser) is non-orthodox and lacks the Lys residue. There are, however, known examples in the PD-(D/E)\(_2\)K superfamily of REases that lack some of the common catalytic residues and yet are potent nucleases (37). Thus, we used the preliminary structural models to direct and interpret mutagenesis experiments.

The Catalytic Motif Pro-Asp\(^{537}\)-\(\chi\)\(^{18}\)Glu\(^{554}\)-Ser-Lys\(^{556}\) in the C-terminal FokI-like domain of Mva1269I Is Responsible for Cleavage of the Top Strand.—To test whether amino acid residues Asp\(^{537}\), Glu\(^{554}\), and Lys\(^{556}\) from the orthodox catalytic motif in the C-terminal domain are involved in DNA cleavage by Mva1269I, single substitution mutants D537A, E554A and K556A, as well as a double mutant E554A/K556A, were constructed by site-directed mutagenesis, and the corresponding proteins were purified to apparent homogeneity (Fig. 1). Mutant proteins D537A and E554A/K556A did not cleave the DNA substrate (Fig. 5, lanes 5 and 6, respectively), whereas the E554A and K556A mutants exhibited low, but detectable, double-stranded DNA cleavage activity (Fig. 5, lanes 1–2 and 3–4, respectively). Surprisingly, a clearly visible DNA fragment of 6 kb appeared in reaction mixtures preincu- bated with D537A and E554A/K556A, after their heating for 10 min at 70 °C (Fig. 5, lanes 7 and 8, respectively). This observation suggested that a short double-stranded DNA region, located about ~6 kb from the end of the linear \(\lambda\) DNA molecule and separating neighboring nicks on opposite DNA strands, was melted during the heating. Indeed, there are two adjacent inverted Mva1269I targets out of the 46 cleavage sites found in \(\lambda\) DNA. These are located ~6 kb from the right end of \(\lambda\) DNA (nucleotide positions 42475 and 42493). Therefore, the appearance of the fragment after the heating could be due to the strand-specific nicking of Mva1269I targets by mutant proteins, resulting in melting of the DNA fragment between the nicks. Restriction mapping confirmed this suggestion: Ehel, which has a unique target at the position 45679 of \(\lambda\) DNA.
DNA (Fig. 5, lane 11), cleaved the fragment of ∼6 kb into two pieces of the expected size (Fig. 5, lanes 9 and 10).

The properties of Mva1269I mutants were further investigated using both the pUC57 DNA, which bears a single Mva1269I target sequence, and its target-free derivative pUC57ΔMva (Fig. 6). The supercoiled form (SC) of pUC57 was completely converted into the open-circle form (OC) by mutants D537A and E554A/K556A at 1.25 nM (or higher) protein concentrations after a 1-h incubation. Conversely, the target-free plasmid remained intact after incubation with 15 nM of both mutants (Fig. 6, compare lane K3 with lane K1). This finding suggests that the nicking of pUC57 was site-specific. In addition, no traces of linear form (L) of pUC57, the product of double-stranded DNA cleavage, were observed at 15 nM (Fig. 6) or even higher protein concentrations (up to 100 nM; data not shown), indicating that the cleavage of the second strand was abrogated by the mutations. In contrast, the E554A and K556A mutants exhibited some double-stranded DNA cleavage activity at elevated concentrations, resulting in linearization of pUC57 (Fig. 6, appearance of the L form). All these results matched perfectly those obtained with the phage λ DNA as a substrate (Fig. 5). It is noteworthy that ∼20 times lower concentration of the wild-type enzyme (0.0625 nM) was enough to completely convert the SC-pUC57 into the linear form (Fig. 6). Therefore, it appears that the specific DNA nicking activity of mutants D537A and E554A/K556A is ∼20 times lower compared with the specific double-stranded endonucleolytic activity of wt Mva1269I.

To determine whether the DNA nicking by E554A/K556A was strand-specific, the nicked form of pUC57ΔMva (Fig. 6) was used as a substrate. The DNA sequencing reaction was terminated when the DNA strand that carried the bottom strand of the Mva1269I target (5'-GCATTC-3') was used as a primer.
sequencing template. Termination occurred at the position, which coincided with the position of Mva1269I cleavage on the bottom strand. In contrast, no DNA sequencing termination was observed when the top strand was used as a template. Identical results were obtained when pUC57, nicked by mutant proteins D537A, E554A, or K556A, was purified and sequenced. Thus, the mutants D537A and E554A/K556A are strand-specific nicking enzymes that cut only the bottom strand of the Mva1269I target. Hence, we conclude that the motif Pro-Asp537-Glu554-Ser-Lys556 represents an active site involved in the cleavage of the top strand.

Residues Asp93 and Glu112 Belong to the Second Active Site in the N-terminal EcoRI-like Domain of Mva1269I Involved in Cleavage of the Bottom Strand—To test the prediction of the putative active site in the EcoRI-like domain, alanine mutants of Asp93 and Glu112 were constructed by site-directed mutagenesis and the corresponding proteins purified (Fig. 1). Both mutant proteins demonstrated nearly identical biochemical properties. Therefore, only experiments with the D93A mutant are described below. The D93A mutant exhibited practically undetectable cleavage activity on λ DNA at very high protein concentration (400 nM) after 1-h or even after 17 h of incubation (Fig. 7, lanes 1 and 2, respectively). Nevertheless, a 6-kb fragment appeared after the heating of the reaction mixture, which had been incubated for 17 h (Fig. 7A, lane 4) as in the previous experiments where nicking mutants of the C-terminal domain were analyzed (see Fig. 5). Furthermore, incubation of the D93A mutant protein with the supercoiled (SC) pUC57 DNA has led to its conversion mostly into the nicked (OC) form (Fig. 7B). However, a much higher concentration of the D93A mutant protein (>2500 nM) was required to nick >90% of the SC substrate in 1 h, compared with the concentrations of the mutatn proteins of the C-terminal active site required for complete nicking (1.25 nM). In addition, a small amount of linear DNA accumulated during incubation with the D93A mutant protein (Fig. 7B, appearance of the L form). In the control reaction, the DNA of target-free pUC57ΔMva remained unaltered after the incubation with the 2500 nM mutant enzyme (Fig. 7B, compare lane K2 with lane K1). Therefore, the D93A mutant appears to be a nicking endonuclease with a residual double-stranded DNA cleavage activity.

We reasoned that mutants in the N- and C-terminal domain of Mva1269I may be responsible for independent nicking of either strand of the target. Thus, if the D93A mutant specifically nicks the top strand target, it should be able to linearize the substrate in which the bottom strand is already nicked, whereas the substrate nicked by the D93A mutant protein should be linearized by the addition of bottom-strand nicking mutants.

We observed that the nicked (OC) form of pUC57, generated by E554A/K556A (Fig. 7C, lane 1), was converted into the linear form (L) by both the wt enzyme and by the D93A mutant (Fig. 7C, lanes 2 and 3, respectively). Similarly, pUC57 nicked by D93A (Fig. 7C, lane 4), was linearized by both the wt enzyme and by E554A/K556A (Fig. 7C, lanes 5 and 6, respectively). Furthermore, run-off sequencing of pUC57 previously nicked by D93A confirmed that this mutant cleaved the top strand of cognate DNA preferentially. The sequencing was terminated only when the DNA strand containing the top strand of the Mva1269I target served as a sequencing template. Termination occurred at the position coinciding with the top strand cleavage of the Mva1269I target, whereas the bottom strand of the target was sequenced without termination. Therefore, we conclude that Asp93 (and Glu112) belongs to the N-terminal active site, which is responsible for the cleavage of the bottom DNA strand, and that mutants of that active site cleave the top strand of DNA preferentially. However, the cleavage of the bottom strand is not completely inactivated by single mutations D93A or E112A.

The DNA-Binding Properties of Mva1269I and the Mutants—To test whether mutations of the predicted catalytic amino acid residues alter the DNA-binding properties of mutant proteins, DNA binding studies were carried out using the electrophoretic mobility shift assay. Two synthetic DNA oligoduplexes of 24 bp were used as substrates. One of them, hereafter referred to as “specific DNA,” contained a single Mva1269I target in the very middle of the sequence. The oligoduplex referred to as “nonspecific,” contained three substitutions in the Mva1269I target site. We observed that the wild-type enzyme formed a single specific complex with DNA and discriminated efficiently against the nonspecific DNA, in the presence of Ca²⁺ ions (Fig. 8A). It should be
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DNA cleavage experiments demonstrated that >2000 times higher concentration of the REase with the mutation in the N-terminal active site (D93A; see Fig. 7B) was required to nick >90% of the target DNA, when compared with mutants of the C-terminal active site (Fig. 6). This observation indicated that the specific nicking activity of former mutants was decreased more than 2000-fold. Such a decrease could be explained by a greatly lowered affinity for specific DNA. However, DNA binding assays showed that DNA-binding properties of D93A are nearly identical to those for the wild-type enzyme (Fig. 8B). Therefore, it seems that there must be other reasons of this sharp decrease in the specific nicking activity.

Mva1269I Cleaves Two DNA Strands Successively —DNA binding and cleavage studies revealed that mutants of the C-terminal active site are much more active nicking enzymes than mutants of the N-terminal active site. Such a different effect of mutations could be easily explained by the DNA cleavage mechanism, in which the cleavage of the bottom strand by the N-terminal active site is a prerequisite for the cleavage of the top strand by the C-terminal active site.

Substitution of the non-bridging oxygen on the phosphate backbone by sulfur at the site of cleavage in either DNA strand typically decreases or abolishes cleavage by REases (40, 41). If both catalytic sites of Mva1269I cleave their appropriate DNA strands independently from each other, the presence of phosphorothioate in one DNA strand should not interfere with the cleavage of the other strand. However, if the cleavage of the top strand is strictly followed by the cleavage of the bottom strand, inhibition of the first cleavage reaction should reduce the cleavage of both strands to the same extent. Accordingly, to test the mechanism of sequential cleavage, we produced four 24-bp oligoduplexes comprising the Mva1269I target: (i) with no phosphorothioate substitutions, (ii) with a single substitution located at the position of cleavage of the top strand, (iii) with a single substitution located at the position of cleavage of the bottom strand, or (iv) with two substitutions that are located at positions of cleavage of the top and bottom strands. It should be noted that the phosphorothioate-substituted oligonucleotides used in this study were a racemic mixture of R, S, and R,S diastereomers, which probably confer different levels of resistance against cleavage by Mva1269I. Fig. 9 shows the kinetics of cleavage of all four oligoduplexes by wt Mva1269I. In the absence of modification, both strands were cleaved with nearly identical rates (Fig. 9A). This observation suggests that cleavage of both strands is highly concerted, and confirms the results of cleavage of one-site substrates where no nicked intermediates were detected (see Fig. 2). Substitution of the non-bridging oxygen by sulfur at the site of cleavage in the top strand greatly decreased the rate of cleavage of this strand (Fig. 9B, marked by open circles). However, the bottom strand was cleaved almost as efficiently as in DNA substrate having no modifications (Fig. 9, B and A, respectively, marked by filled circles). Small changes could be seen if initial rates of bottom-strand cleavage in unmodified and top-strand modified substrates were compared. Therefore, it seems that the cleavage of the bottom strand is virtually independent from the cleavage of the top strand. In contrast, the phosphorothioate modification in the bottom strand of DNA sharply decreased the cleavage rates of both strands (Fig. 9C), suggesting that cleavage of the top (non-modified) strand depends on the cleavage of the bottom (modified) DNA strand. These results support a sequential mechanism of double-stranded DNA cleavage by Mva1269I and explain the different nicking activities resulting from mutations in the predicted N- and C-terminal active sites.
The results for cleavage of the substrate with modifications in both DNA strands, were unexpected (Fig. 9D). Fig. 9C shows that Mva1269I cleaved the modified bottom DNA strand (marked by filled circles) relatively slowly in duplexes carrying the unmodified top strand. On the other hand, the top strand modification had only a weak effect on the rate of cleavage of the bottom (unmodified) strand (Fig. 9B). Therefore, one might expect that Mva1269I would cleave the modified bottom strand in duplexes carrying the double modification, with a rate comparable to that in Fig. 9C. Surprisingly, double modification nearly completely blocked the cleavage of both DNA strands (Fig. 9D). Therefore, it seems that the non-bridging oxygens at the position of the top strand cleavage somehow affects cleavage of the bottom strand. This conclusion is also in agreement with the earlier observation that the bottom strand of the substrate, when carrying the modification in the top strand, was cleaved slightly more slowly than the unmodified substrate. However, more detailed studies are required to shed light on the impact of non-bridging oxygens located at the position of cleavage in one strand on the cleavage of the complementary strand.

**DISCUSSION**

To date, two strategies for double-stranded DNA cleavage have been described for type II REases and related nucleases. In one of them, two identical active sites are brought together by dimerization of the respective catalytic domains. The DNA target is bound in a symmetric manner. The symmetry may be broken at the atomic level, but in general the interactions of both nuclease domains with the DNA and with its partner are highly similar. This strategy is common for the orthodox type IIP REases such as EcoRI, the type IIE, IIF enzymes (3), and for many homing endonucleases from the HNH and LAGLIDADG superfamilies (e.g. I-CreI) (42) that cut within their symmetric (or nearly symmetric) target sites. The same strategy is used by the type IIS REases such as EcoRV, which recognizes an asymmetric sequence 5'-GAATTC-3' and uses two nuclease domains to cleave both strands sequentially. Computational fold-recognition analyses reveal that the N-terminal domain of Mva1269I is remotely, but statistically significantly related to EcoRI, a type IIP enzyme, which recognizes a symmetric sequence 5'-G↓AATTC-3' and cuts it as indicated by the "↓" symbol. On the other hand, the C-terminal domain of Mva1269I seems to be related to the nonspecific nuclease domain of the FokI enzyme. It occurred to us that the pattern of recognition and cleavage of the bottom strand by Mva1269I (5'-G↓CATTC-3') is strikingly similar to that of EcoRI (only 1 bp difference), whereas the cleavage of the top strand occurs in a nonspecific region outside the recognition sequence (5'-GAATTCN↓N-3'), similarly to FokI. Site-directed mutagenesis of the amino acid residues predicted to form the active sites revealed that the EcoRI-like domain is responsible for the bottom-strand cleavage, whereas the FokI-like domain is responsible for the top-strand cleavage. Amino acid substitutions in either active site generated site-specific nicking enzymes with very interesting properties. The DNA binding and cleavage analysis of the wt enzyme and the mutants revealed that the cleavage of the bottom strand preceded the cleavage of the top strand. Thus, Mva1269I possesses two active sites, like enzymes with two identical or closely related nuclease domains, but uses them to achieve sequential cleavage of both strands, as found with enzymes with only one active site.
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It is noteworthy that the pattern of cleavage by Mva1269I (3’ extensions 2 bp long) and the patterns of cleavage of its relatives, i.e. EcoRI and FokI (5’ extensions 4 bp long), are very different, which suggests that the mutual orientation of the two active sites in Mva1269I is likely to be novel. Examples of evolutionarily related REases, which have changed their pattern of cleavage by modification of the domain-domain interface, have been well documented in the literature (31, 47, 48). Here, we describe the first case of a REase, which has evolved a novel cleavage pattern by recruiting domains from two different enzymes. It remains to be determined, whether the two nuclease domains in Mva1269I are rigidly connected and remain in approximately the same orientation with and without the DNA or whether the linker is flexible and both domains may move around. A preliminary model (Fig. 10) suggests that both nuclease domains of Mva1269I could simultaneously bind to the target sequence without steric clashes or extensive mutual interactions (which can be mediated by the central part of the protein, not included in the current model). The data collected so far strongly suggest that it is the N-terminal EcoRI-like domain, which governs the action of Mva1269I, because it is required to make the first incision within the specific target sequence, which only then allows the FokI-like domain to complete the reaction by cleaving the other strand outside the recognition sequence.

From both the structural and evolutionary point of view, Mva1269I represents an exceptionally interesting case of domain fusion. The key properties of the presumed ancestral EcoRI-like and FokI-like domains have been maintained, but within the single Mva1269I polypeptide they are oriented with respect to each other in a novel way to produce 2-nucleotide rather than 4-nucleotide staggered ends. Interestingly, our sequence analyses revealed that many other type IIS enzymes that cleave both DNA strands very close to their recognition sequence such as BsmBI, Bsml, Bsal, and SapI (19, 20) also contain a pair of strongly divergent active sites. The DNA cleavage mechanisms of these REases have been studied previously (15). All of these enzymes, including the Mva1269I isoschizomer Bsml, which shares 31% amino acid sequence identity (Fig. 4), cleave plasmids containing a single site or two sites with a similar rate. They do not require two copies of their targets and thus resemble orthodox type IIP REases (15). Several putative reaction mechanisms have been put forward to explain how these type IIS enzymes could perform double-stranded cuts. One such mechanism was based on the presence of two active sites per monomeric protein, and this proposal was later indirectly supported by the isolation of several SapI and Bsal mutants able to selectively nick either top or bottom DNA strands (19, 20). However, it appears that the reaction mechanism of SapI differs from that of Mva1269I. SapI introduces the first cut in nearly random fashion (respectively to the DNA strand) (19), whereas the order of cleavage by Mva1269I is strictly sequential. It remains unknown if the Mva1269I-like DNA cleavage mechanism is used by some other short-distance REases. However, the similarity of Mva1269I and Bsml as well as the conservation of amino acid residues involved in the formation of both Mva1269I active sites allows us to predict that the equivalent mutations should convert Bsml into a nicking enzyme.

The modular structure of Mva1269I and other short-distance type IIS REases suggests that enzymes of this group could be excellent targets for protein engineering, not only because they can be used to generate site-specific nickases (as demonstrated in this article), but because the individual domains could be selectively targeted by mutagenesis aiming at changing (or extending) the sequence specificity, without disturbing the functionality of the other domain. Our results also suggest that it may be possible to generate novel REases by recombination of the nuclease domains of existing enzymes. It has been shown that a combination of genetic recombination and computational protein design can lead to the engineering of hybrid homing nucleases with new specificities (49). Our current structural model of Mva1269I (Fig. 10) is probably of too low resolution to unambiguously guide atomic-level protein design, but it will serve as a useful platform for further experiments to study (and modify) protein-DNA and protein-protein interactions, until such time as a high resolution crystal structure of this interesting enzyme is obtained.

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REFERENCES
1. Roberts, R. J., Belfort, M., Bestor, T., Bhagwat, A. S., Bickle, T. A., Bitinaite, J., Blumenthal, R. M., Degtyarev, S. Kh., Dryden, D. T. F., Dyvig, K., Firman, K., Gromova, E. S., Gumport, R. I., Halldor, S. E., Hattman, S., Heitman, J., Hornby, D. P., Janulaitis, A., Jeltsch, A., Josephsen, J. Kiss, A., Klaenhammer, T. R., Kobayashi, I., Kong, H., Kruger, D. H., Lacks, S., Marinus, M. G., Miyahara, M., Morgan, R. D., Murray, N. E., Nagaraja, V., Piekarowicz, A., Pingoud, A., Raleigh, E., Rado, D. N., Reich, N., Repin, V. E., Selker, E. U., Shaw, P. C., Stein, D. C., Stoddard, B. L., Szybalski, W., Trautstein, T. A., Van Etten, J. L., Vitor, J. M., Wilson, G. G., and Xu, S. Y. (2003) Nucleic Acids Res. 31, 1805–1812
2. Bujnicki, J. M. (2003) Curr. Protein Pept. Sci. 4, 327–337
3. Pingoud, A., Fuxreiter, M., Pingoud, V., and Wende, W. (2005) Cell Mol. Life Sci. 62, 685–707
4. Pingoud, V., Sudina, A., Geyer, H., Bujnicki, J. M., Lurz, R., Luder, G., Morgan, R., Kubareva, E., and Pingoud, A. (2005) J. Biol. Chem. 280, 4289–4298
5. Aravind, L., Makarova, K. S., and Koonin, E. V. (2000) Nucleic Acids Res. 28, 3417–3432
6. Bujnicki, J. M., Radlinska, M., and Rychlewski, L. (2001) Trends Biochem. Sci. 26, 9–11
Restriction Endonuclease Mva1269I Has Two Centers

1. Sasnauskas, R., Sasnauskas, G., Lagunavičius, A., Vilkaitytė, G., Lubys, A., and Siksnys, V. (2000) J. Biol. Chem. 275, 30878–30885
2. Roberts, R. J., Vincze, T., Posfai, J., and Macelis, D. (2003) Nucleic Acids Res. 31, 418–420
3. Simons, K. T., Ho, C. S., and Baker, D. (2005) J. Mol. Biol. 348, 631–640
4. Stankevicius, K., Lubys, A., Timinskas, A., Vaitkevicius, D., and Janulaitis, A. (1998) Nucleic Acids Res. 26, 1084–1091
5. Wah, D. A., Hirsch, J. A., Dorner, L. F., Schildkraut, I., and Aggarwal, A. K. (1997) J. Biol. Chem. 272, 248–254
6. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
7. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
8. Chang, A. C., and Cohen, S. N. (1978) J. Mol. Biol. 129, 1084–1091
9. Vincze, T., Posfai, J., and Macelis, D. (2003) Nucleic Acids Res. 31, 418–420
10. Sasnauskas, R., Sasnauskas, G., Lagunavičius, A., Vilkaitytė, G., Lubys, A., and Siksnys, V. (2000) J. Biol. Chem. 275, 30878–30885
11. Fujita, T., and Takagi, T. (1995) J. Mol. Biol. 253, 1084–1091