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The Type II restriction endonuclease MvaI has dual specificity

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ABSTRACT

The MvaI restriction endonuclease cuts 5′-CC↓AGG-3′/5′-CC↑TGG-3′ sites as indicated by the arrows. N4-methylation of the inner cytosines (C^{m4}CAGG/C^{m4}CTGG) protects the site against MvaI cleavage. Here, we show that MvaI nicks the G-strand of the related sequence (CCGGG/CCCGG, BcnI site) if the inner cytosines are C5-methylated: C^{m5}C↓GGG/CC^{m5}CGG. At M.SssI-methylated SmaI sites, where two oppositely oriented methylated BcnI sites partially overlap, double-nicking leads to double-strand cleavage (CC^{m5}C↓GGG/CC^{m5}C↑GGG) generating fragments with blunt ends. The double-strand cleavage rate and the stringency of substrate site recognition is lower at the methylation-dependent site than at the canonical target site. MvaI is the first restriction endonuclease shown to possess, besides the 'normal' activity on its unmethylated recognition site, also a methylation-directed activity on a different sequence.

INTRODUCTION

Type II restriction endonucleases (REases) are sequence-specific endonucleases that recognize short DNA sequences and cut the DNA at defined positions within or close to the recognition sequence. In the producer cell, the host DNA is protected by specific methylation of the recognition sequence. The specific methylation is established by DNA methyltransferases, which methylate a cytosine or an adenine in the recognition sequence to produce C5-methylcytosine, N4-methylcytosine or N6-methyladenine (1). The ability to cleave DNA at specific sites made Type II REases indispensable tools of molecular biology (2) as well as excellent model systems for the study of sequence-specific protein–DNA interactions (3,4). Since their discovery 40 years ago (5), the number of biochemically or genetically characterized Type II REases has risen to more than 3800 (6). This huge group of enzymes shows great diversity. Members are classified into subgroups according, among others, to the symmetry of the recognition sequence, the position of the cut site relative to the recognition sequence, the number of target sites the enzyme interacts with, etc. (1).

From the perspective of the present study, two subgroups of Type II REases are especially interesting. Enzymes in the Type IIM subgroup (methyl-directed REases) break the general rule of protection by DNA methylation; unlike most REases, they require methylated substrate site for activity (7). The other subtype that deserves special attention, are nicking REases, which cut only one strand of the substrate DNA. Such enzymes include natural nicking REases (8), isolated subunits of heterodimeric REases (9) and mutant REases engineered to cut only one strand of the substrate DNA (10).

The MvaI REase recognizes the sequence 5′-CC↓WGG-3′/5′-CC↑WGG-3′ (W stands for A or T) and cuts both strands as indicated generating one nucleotide 5′-overhangs (11). The cognate DNA-methyltransferase M.MvaI modifies the internal cytosines to produce N4-methylcytosine: (C^{m4}CWGG/C^{m4}CWGG) (11). C5-methylation of the same cytosines does not protect against MvaI cleavage (12). MvaI was shown to recognize its pseudosymmetric target site as a monomer (13). An interesting feature of the enzyme is its tolerance to a wide range of modifications within the recognition sequence (12). MvaI shares ~20% sequence identity and structural similarity with BcnI, an REase recognizing the related pseudopalindromic sequence CC/SGG (S stands for G or C) (13–15).

Here, we show that MvaI, in addition to its double-stranded cleavage activity on the canonical recognition sequence CCWGG, nicks BcnI sites (C$\underline{C}$↓GGG/CC$\underline{C}$GG) as indicated, if the underlined cytosines are C5-methylated (5-methylcytosine, ^{m5}C). This nicking activity results in double-strand scisions at CC^{m5}CGGG/CC^{m5}CGGG sites, where two methylated BcnI sites

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overlap. To our knowledge, MvaI is the first REase that has been shown to have such dual specificity: cleaving two different sequences, one of them in a methylation-dependent manner.

MATERIALS AND METHODS

Strains and growth conditions

The *Escherichia coli* strains DH10B F^- *endA1 recA1 galU galK deoR nupG rpsL ΔlacX74* φ80*lacZΔM15 araD139* Δ (*ara leu*) *7697 mcrA* Δ (*mrr- hsdRMS-mcrBC*) λ^- (16) and ER1821 F^- *glnV44, e14*$^-$ ($McrA^-$) *endA1 thi-1 Δ(mcrC-mrr)114::IS10* were used as cloning hosts.

Cells were grown in LB medium at 30°C or 37°C as indicated in the text. Ampicillin (Ap), kanamycin (Kn) and chloramphenicol (Cm) were used at 100, 50 and 25 μg/ml, respectively.

Plasmids, oligonucleotides and DNA techniques

Plasmid pUP41 (Ap^R) carries a Kn^S allele of the kanamycin resistance gene, which can revert to Kn^R phenotype by a C to T mutation (17). Plasmid pSTB–MSssI (Cm^R) carries the gene of the SssI DNA methyltransferase under the control of the arabinose P_{BAD} promoter and the AraC protein. It was constructed by transferring an NsiI–PstI fragment carrying the s*ssIM* and *araC* genes from the pBAD24-based (18) expression plasmid pB–MSssI (to be published later) into the PstI site of the ColE1-compatible plasmid vector pST76-C (19). Transcription of the s*ssIM* gene in pSTB–MSssI can be induced by arabinose and repressed by glucose. Plasmid pACYC184-M.PspGI (Cm^R), which encodes the PspGI methyltransferase, was a gift of Shuang-yong Xu (New England Biolabs). To introduce a SmaI site, the partially self-complementary oligonucleotide AK244 (Table 1) was ligated into the unique XbaI site of pACYC184-M.PspGI to yield pACYC184-M.PspGI(S).

Oligonucleotides (Table 1) were synthesized in the BRC (Szeged) or were purchased from Integrated DNA Technologies. The oligonucleotides used as endonuclease substrates (AK252 through 255) were gel-purified preparations. Double-stranded oligonucleotides were labeled by a filling-in reaction (see below). AK252 and AK253 are complementary to AK254 and AK255. Double-stranded oligonucleotides were prepared by heating the complementary strands to 80°C, then incubating the mixture in 50 mM Tris–HCl pH 7.5, 0.9 M NaCl for 1 h at 53°C. AK254 and AK255 contain a 5′-TTT extension allowing labeling of the annealed complementary strands (AK252 or AK253) by a filling-in reaction using Klenow polymerase and [α-^{32}P]dATP. The AK252/254 duplex contains an unmodified BcnI site, whereas in AK253/254 and AK252/255 the BcnI site is C5-methylated in one strand as shown in Table 1 and Figure 5. In duplex A253/255 both strands are methylated. The 441 bp AvaII–SspI fragment of pUC18 was radioactively labeled by filling in the AvaII end using [α-^{32}P]dCTP.

Recombinant DNA techniques followed standard protocols (20). DNA sequence was determined by an automated sequencer (ABI). Cleavage of radioactively labeled DNA fragments and oligonucleotides was analyzed by electrophoresis in 10% or 6% polyacrylamide gels containing 7 M urea (20). After electrophoresis, the digestion products were detected by conventional autoradiography or by a phosphor image analyzer. MvaI was purchased from Fermentas (conventional and FastDigest enzyme preparations) and Sigma. MvaI digestions were routinely performed using the conventional Fermentas enzyme in Fermentas R buffer (10 mM Tris–HCl pH 8.5, 10 mM $MgCl_2$, 100 mM KCl and 0.1 mg/ml BSA) at 37°C as recommended by the manufacturer. All other restriction enzymes, DNA polymerase I Klenow fragment, and T4 DNA ligase were from Fermentas or from New England Biolabs. Deoxyadenosine- and deoxycytidine 5′-[α-^{32}P]triphosphate were purchased from Izotóp Intézet Kft. (Budapest).

Preparation of methylated DNA

C-terminal His-tagged M.SssI DNA methyltransferase was purified from the *E. coli* strain ER1821(pBHNS–MSssI) using a slightly modified version of the procedure described previously (21). Plasmid DNA and gel-purified DNA fragments were methylated in 50 μl reactions containing 1 to 5 μg DNA, 50 mM Tris–HCl pH 8.5, 50 mM

Table 1. Deoxyoligonucleotides used in this work

Name	Sequence	Properties	Use
AK240	GAATGAACTGCAGGACGAGG	Positions 171–190 of pUP41	Primers for sequencing through the $SmaI_{279}$ site
AK241	AGTACGTGCTCGCTCGATGC	Positions 438–419, complementary strand of pUP41	
AK244	CTAGATCTG**CCCGGG**CAGAT	Partially self-complementary, contains an SmaI site	Introducing a SmaI site
AK245	CATGTAACTCGCCTTGATCG	Positions 2275–2294 of pUP41	Primers for sequencing through the $BcnI_{2411}$ site
AK246	ACGCTCACCGGCTCCAGATT	Positions 2523–2504, complementary strand of pUP41	
AK252	TAATTGTTGCCGGGAGGCCAGAGTAAG	Forms duplex with AK254 and AK255	Testing cleavage of the BcnI site
AK253	TAATTGTTGC^{m5}CGGGAGGCCAGAGTAAG		
AK254	TTTCTTACTCTGGCCTCCCGGCAACAATTA	Forms duplex with AK252 and AK253	
AK255	TTTCTTACTCTGGCCTCC^{m5}CGGCAACAATTA		

Sequences are written in 5′–3′ direction. In AK244 the SmaI site (CCCGGG) is shown in bold. In AK252–AK255, the BcnI site (CCSGG) is underlined.

NaCl, 10 mM DTT, 250 μg/ml BSA, 0.16 mM S-adenosylmethionine (NEB) and 0.85 μM M.SssI. After incubation at 30°C for 30 min, the DNA was purified by phenol–chlorophorm extraction and ethanol precipitation.

For methylation of pUP41 CG sites *in vivo*, *E. coli* DH10B was co-transformed with pUP41 and pSTB–MSssI. Ap^R Cm^R double-transformants were grown at 30°C, and M.SssI expression was induced at OD_{550} ~0.5 with 0.1% arabinose. Methylation status of the purified plasmid DNA was tested by Hin6I digestion. Hin6I cannot digest GCGC sites when the underlined cytosine is methylated.

RESULTS

M.SssI-specific methylation creates new MvaI cleavage sites

The plasmid pUP41 constructed to detect C to U or ^{m5}C to T deaminations contains a mutant allele of the kanamycin resistance gene (17). An artificially created mutation in the plasmid results in Leu94Pro replacement leading to kanamycin sensitive phenotype. A single C to T mutation can revert this mutation to yield the wild-type Leu94 and Kn^R phenotype. The C to T mutation results in the disappearance of one of the two SmaI sites (CCCG GG) and the appearance of a new MvaI (CCWGG) site (17). In the course of our work with the CG-specific DNA (cytosine-5) methyltransferase M.SssI (22), we noticed that M.SssI methylation of pUP41 *in vivo* or *in vitro* led to the appearance of two MvaI fragments (~1250 and 450 bp), which were not present in the digest of the unmethylated plasmid (Figure 1). Disappearance of the 1482-bp fragment and the concomitant appearance of a ~1250-bp fragment resembled the revertant state, but this change in the restriction pattern was not accompanied by reversion to Kn^R phenotype, indicating that the mutation yielding the change to WT L94 did not occur.

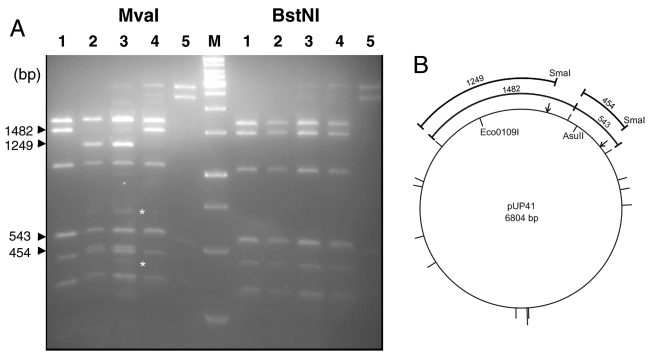

Figure 1. (**A**) Digestion of SssI-methylated pUP41 DNA with MvaI and BstNI. Lanes 1, unmethylated pUP41; lanes 2, pUP41 methylated by M.SssI *in vitro*; lanes 3, pUP41 purified from cells expressing M.SssI; lanes 4, pUP41 purified from cells in which M.SssI production was repressed by glucose; lanes 5, pSTB–MSssI (from uninduced cells). Sizes of fragments that differ between the unmethylated and M.SssI-methylated DNAs are shown in base pairs. The band corresponding to the 543-bp fragment remains visible in the methylated sample because it also contains a comigrating 540-bp fragment. The extra fragments that appear in sub-stoichiometric amounts are indicated by asterisk. M, 1 kb DNA Ladder (Fermentas). (**B**) Restriction map of the plasmid pUP41. MvaI (spikes) and SmaI (arrows) sites are indicated on the perimeter.

To test the connection between the new MvaI sites and M.SssI-specific methylation, DH10B cells were co-transformed with pUP41 and the plasmid pSTB–MSssI, which carries the gene of the SssI methyltransferase. After a 4 h growth in the presence of arabinose to induce M.SssI expression, part of the culture was harvested for plasmid isolation. Cells from the rest of the culture were sedimented by centrifugation, resuspended in fresh LB/Ap/Cm medium containing 0.2% glucose and grown overnight for plasmid isolation. Comparison of the digestion patterns showed that the new cleavage sites, which were detectable in the plasmid prepared from the arabinose-induced culture, disappeared upon glucose repression, and the MvaI pattern corresponding to the known pUP41 sequence was restored (Figure 1). Reversibility of the change in the digestion pattern ruled out the possibility that the new cleavage sites were created by mutations. The observed changes in the digestion pattern were specific for MvaI, they were not detectable for the isoschizomer BstNI (Figure 1).

MvaI cuts M.SssI-methylated SmaI sites

Restriction mapping revealed that the methylation-dependent cleavage sites overlapped with the two SmaI sites in pUP41. Further evidence to support that M.SssI-specific methylation sensitized the SmaI sites to MvaI cleavage, came from digestions of pACYC184–M.PspGI(S). This plasmid carries the gene of the PspGI methyltransferase (23), and the MvaI sites of the plasmid are protected against MvaI digestion by PspGI-specific methylation (Shuang-yong Xu, personal communication). When pACYC184–M.PspGI(S) containing a single SmaI site was methylated with M.SssI *in vitro,* then digested with MvaI, the plasmid was linearized, whereas the unmethylated plasmid was not digested (data not shown).

To determine the exact position of the cleavage, the 1243 bp EcoO109I–AsuII fragment containing the $SmaI_{279}$ site (Figure 1B) was methylated with M.SssI *in vitro*, then digested with MvaI. The digested fragments were used as templates to sequence towards the SmaI site from both directions. The uncleaved fragment served as control. The sudden drop of sequencing signal intensity in the run-off reactions indicated that the cleavage occurred in both strands between the third (methylated) C and the G (Figure 2). (In the run-off reactions the polymerization products carried an extra A at the 3′-end, which is a non-templated addition by Taq polymerase (24).) These results confirmed that the methyl-directed cleavage occured at the SmaI sites and showed that it produced blunt ends (Figure 2).

In the MvaI digests of M.SssI-methylated pUP41, in addition to the strong ~1250 and 450 bp bands, also 2–3 very faint extra bands were detectable (Figure 1), which became stronger upon prolonged digestion (Supplementary Figure S1). Restriction mapping suggested that these fragments, which were partial digestion products in sub-stoichiometric amounts, were created by scissions at $CC^{m5}CGGT$ sequences. However, because

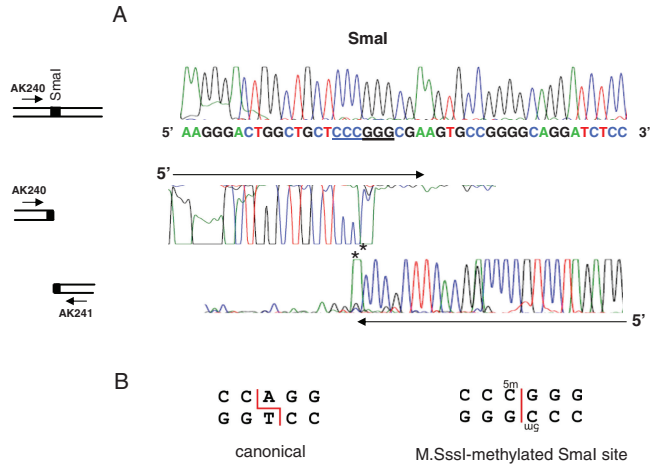

Figure 2. MvaI cleavage at M.SssI-methylated SmaI sites. (**A**) Sequencing through the methylated $SmaI_{279}$ site of pUP41 using intact or MvaI-cleaved templates as indicated by the scheme on the left. The terminal adenines, denoted by asterisk, are template-independent additions by Taq polymerase. (**B**) MvaI cleavage at the canonical recognition sequence and at the M.SssI-methylated SmaI site.

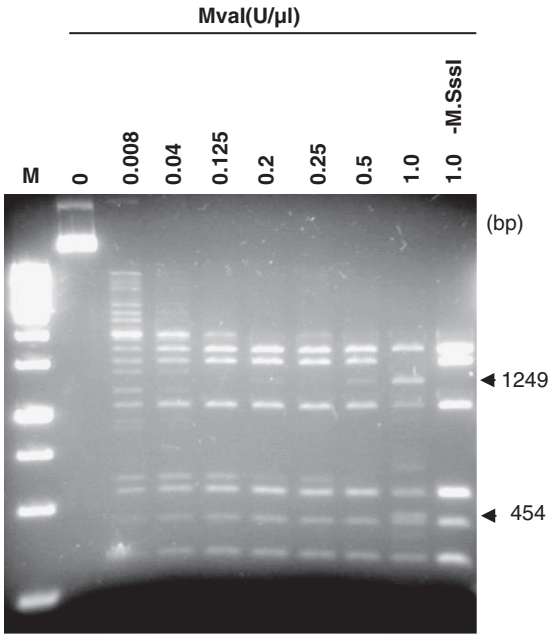

Figure 3. Comparison of MvaI cleavage rates on the canonical CCWGG/CCWGG and on the M.SssI-methylated SmaI site ($CC^{m5}CGGG/CC^{m5}CGGG$). Digestion of pUP41 plasmid DNA (~0.5 µg) methylated *in vitro* by M.SssI. MvaI concentrations are shown above the lanes. M, 1 kb DNA Ladder (Fermentas); -M.SssI, unmethylated plasmid. Appearance of the 1249 and 454 bp fragments indicates cleavage of the two methylated SmaI sites present in pUP41.

complete digestion could not be reached, cleavage of these sites was not further analyzed. Appearance of these extra bands indicates that the methyl-directed activity of MvaI is less specific than the canonical activity (Supplemenatary Figure S1).

To compare the cleavage rates at the canonical and at the methylation-dependent sites, pUP41 DNA was methylated *in vitro* by M.SssI, then digested with MvaI using different enzyme concentrations. Under the conditions of the experiment, ~10-fold higher concentration of MvaI was needed to reach complete digestion at the methylated SmaI sites, than at the canonical sites (Figure 3). Similar or somewhat bigger differences were observed for other plasmids in which the methylated SmaI site was in different sequence contexts (data not shown). Digestion of a plasmid, in which the SmaI site partially overlapped with a BspRI (GGCC) site revealed that overlapping BspRI-specific methylation ($CCCGGG^{m5}CC/GG^{m5}CCCGGG$) blocks cleavage of CG-methylated SmaI sites by MvaI (data not shown).

To exclude that the detected new activity was due to a contaminating enzyme in the Fermentas preparation, it was tested whether MvaI purchased from another commercial source shows the same phenomenon. MvaI obtained from Sigma gave similar digestion pattern (data not shown).

MvaI nicks M.SssI-methylated BcnI sites

The experiments described above determined that MvaI can cut, besides the canonical CCWGG, also the $CC^{m5}CGGG$ site. Although the two sequences showed some similarity, the ability of the enzyme to act on both substrates was puzzling. The SmaI site is 1 bp longer than the CCWGG site, and it is a perfect palindrom, whereas the canonical site has a quasi-palindromic sequence. The different ways of cleaving the two targets, i.e. staggered cut for the canonical site and blunt cut for the SmaI site (Figure 2), were even harder to accept because the cleavage mode is a tightly determined feature of Type II REases (13). Inspection of the SmaI site suggested an alternative interpretation. The SmaI site contains two partially overlapping and oppositely oriented BcnI sites (CCSGG), which differ from MvaI sites only in the central base pair. We hypothesized that the real second target site of MvaI is the methylated BcnI site, which is nicked by the enzyme, and the double-strand cleavage observed at the SmaI site was the result of double-nicking at the overlapping BcnI sites. One of the reasons why this idea seemed attractive was the monomeric nature of MvaI, which made nicking activity seem plausible. In principle, double-nicking at the SmaI site can produce blunt ends by two mechanisms: nicking the BcnI site in the G-strand (i.e. in the strand with G in the central position) or in the complementary C-strand (Figure 4A). To distinguish between these alternatives, pUP41 plasmid DNA was methylated with M.SssI *in vitro*, digested to completion with MvaI, and used as template to sequence through one of the BcnI sites from both directions. When the G-strand was used as template, intensity of the sequencing signal dropped suddenly at the BcnI site, whereas it stayed constant with the C-strand template (Figure 4B), indicating that MvaI nicks the G-strand.

These experiments determined that M.SssI methylation makes BcnI sites sensitive to nicking by MvaI, but it was not clear whether methylation of both strands was required for cleavage to occur. To address this question, double-stranded oligonucleotides containing unmethylated (AK252/254), hemimethylated (AK253/254, AK252/255)

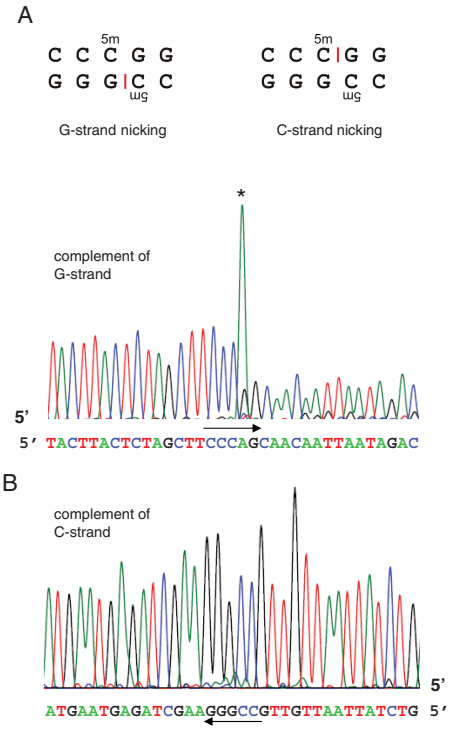

Figure 4. Strand-specific nicking of M.SssI-methylated BcnI sites by MvaI. (**A**) Possible nicking mechanisms at M.SssI-methylated BcnI sites. (**B**) Sequencing through the M.SssI-methylated and MvaI-digested $BcnI_{2411}$ site of pUP41. Asterisk, template-independent addition by the Taq polymerase.

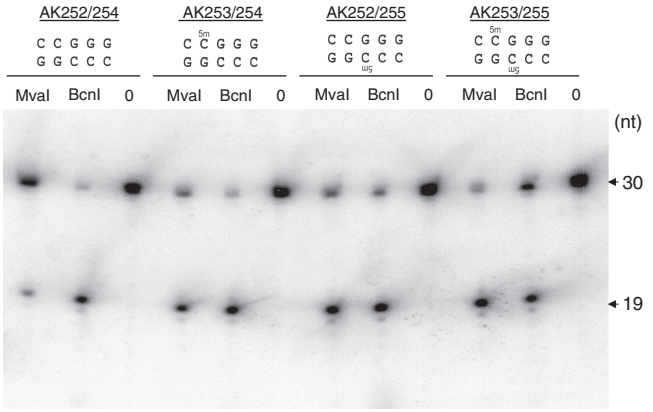

Figure 5. MvaI digestion of oligonucleotides containing unmethylated, hemimethylated or fully methylated BcnI sites. Electrophoresis of cleavage products in a 10% denaturing polyacrylamide gel. The 30-mer oligonucleotide duplexes contained unmethylated (AK252/254), hemimethylated (AK253/254 and AK252/255) or fully methylated (AK253/255) BcnI sites as shown above the gel. In all duplexes, the G-strand (the strand with G in the central position) was radioactively labeled at the 3′-end. Appearance of a 19-nt fragment indicates cleavage of the G-strand.

or fully methylated (AK253/255) BcnI sites were prepared and ^{32}P-labeled as described in 'Materials and Methods' section. The hemimethylated duplexes differed in that in AK253/254 the G-strand (C^{m5}CGGG/CCCGG), whereas in AK252/255 the C-strand (CCGGG/CC^{m5}CGG) was methylated (see also Figure 5). In all four duplexes the G-strand (AK252 or AK253) was radioactively labeled at the 3′-end. Digestion mixtures contained ~0.5 µg pUC18 plasmid DNA, and completeness of digestion was checked by agarose gel electrophoresis of an aliquot of the reaction. Digestion products of the 30-bp oligonucleotides were analyzed by electrophoresis in denaturing polyacrylamide gels (Figure 5). Nicking of the G-strand or double-strand cut at the BcnI site was indicated by the appearance of a 19-nt fragment. The unmethylated duplex was highly but not completely resistant to MvaI digestion. We considered that the small amount of cleaved product obtained with the unmethylated susbstrate was the result of cutting oligonucleotides that had remained single-stranded after the annealing reaction. However, in control reactions, the 5′-labeled, single-stranded AK252 oligonucleotide was not cleaved by MvaI (data not shown). Thus, the weak digestion detected with the AK252/254 duplex indicated that even the unmethylated BcnI site was nicked at low frequency by MvaI (see more on this below). Majority of the hemimethylated and fully methylated duplexes was cut by MvaI, showing that CG-specific methylation of either strand sensitizes the BcnI site for nicking. The fully methylated AK253/255 appeared to be a better substrate than the hemimethylated duplexes.

Although the main goal of digesting the duplexes with BcnI was to obtain an exact size marker, which co-migrates with the oligonucleotide produced by MvaI digestion, these experiments yielded new information on the methylation sensitivity of BcnI: ^{m5}C-hemimethylation at the indicated positions (C<u>C</u>GGG/CCCGG and CCGG G/CC<u>C</u>GG) does not protect against BcnI digestion (Figure 5). The ~50% digestion of the target site methylated on both strands (C<u>C</u>GGG/CC<u>C</u>GG) (Figure 5) is in agreement with previous observations (cited in the REBASE database (6)).

Digestion of double-stranded oligonucleotides showed that even unmethylated BcnI sites were nicked by MvaI at low rate (Figure 5). However, due to the high sensitivity of phosphor imaging simple visual evaluation of band intensity can overestimate the relative amount of material in faint bands. To obtain more reliable quantitative data, the phosphor image was analyzed by the ImageQuant software. For these experiments, to exclude the possibility of any artifact that might arise from the chemical synthesis or annealing of the oligonucleotides, a gel-purified DNA fragment was used. The ^{32}P-labeled 441 bp AvaII-SspI fragment of pUC18 containing a single BcnI site but no canonical MvaI site was extensively digested with MvaI and the cleavage products were analyzed by denaturing gel electrophoresis (Figure 6). With this substrate nicking of the BcnI site in the G-strand gives rise to a 175-nt long ^{32}P-labeled single-stranded fragment. pUC18 plasmid DNA added to some of the reactions served as internal control to monitor digestion. Completeness of digestion was tested by agarose gel electrophoresis of parts of the digestion mixtures (Figure 6C). Some nicking did occur in the MvaI-digested samples but, as densitometric analysis indicated, its amount was negligible (Figure 6B). This is in agreement with the lack of observable double-strand

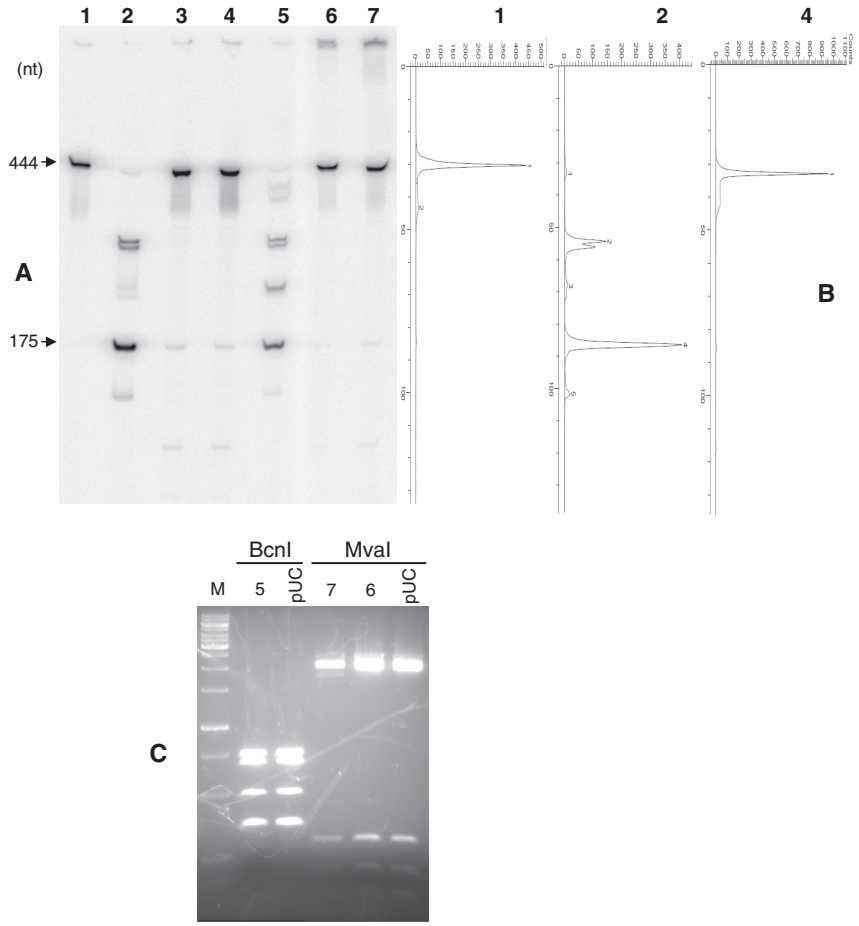

Figure 6. MvaI digestion of the ^{32}P-labeled 444 bp pUC18 DNA fragment containing an unmethylated BcnI site. (**A**) Electrophoresis in a 6% denaturing polyacrylamide gel. Lane 1, undigested; lanes 2 and 5, digested with BcnI; lanes 3 and 6, digested with 0.5 U/µl MvaI; lanes 4 and 7, digested with 1.0 U/µl MvaI. Samples in lanes 5, 6 and 7 also contained pUC18 DNA as internal control to test completeness of digestion. Nicking of the G-strand or double-strand cleavage at the single BcnI site produces a 175-nt single-stranded labeled fragment. (**B**) ImageQuant line graph of lanes 1, 2 and 4. (**C**) Agarose gel electrophoresis of aliquots of the samples in lanes 5, 6 and 7. pUC, pUC18 completely digested with BcnI or MvaI. M, 1 kb DNA Ladder (Fermentas).

cleavage at unmethylated SmaI sites in pUP41 (Supplementary Figure S1).

DISCUSSION

An accidental observation with M.SssI-methylated DNA led to the discovery that the Type IIP REase MvaI has two specificities. We have shown that, in addition to cutting its well-known recognition site (CC↓AGG/CC↑TGG), MvaI can nick BcnI sites if the underlined cytosines are C5-methylated (C$\underline{C}$↓GGG/CC$\underline{C}$GG). The single-strand scission occurs in the G-strand as indicated. Because at SmaI sites the nicking activity manifests in double-stranded cuts, it was straightforward to compare the cleavage rates between the two recognition sites. The methyl-directed double-stranded cleavage of the SmaI site is substantially slower than cleavage of the canonical MvaI sites, but it is still a relatively robust activity, not an obscure side-reaction. The methylation-dependent activity was detected in two different commercial MvaI preparations. One of these enzymes (Fermentas) was prepared from an overexpressing *E. coli* strain, whereas the Sigma enzyme was purified from the native host *Micrococcus varians* Rfl 19. Thus, it can be concluded that the new activity was not due to a contaminating enzyme in the commercial preparations, it is an inherent property of MvaI.

MvaI formally combines features of 'typical' Type II REases cutting unmethylated sequences with those of methyl-directed Type II REases, which require methylated substrate sites. To our knowledge, MvaI is the first REase, for which such dual specificity has been shown. The new, methylation-dependent activity represents nicking and double-stranded cleavage specificities ($C^{m5}C$↓GGG/CC^{m5}GG and CC^{m5}C↓GGG/CC^{m5}C↑GGG) that were not known before. It must be noted, however, that the methyl-directed activity is less specific than the canonical activity, recognition of the substrate sequence is less tightly determined than for the CCWGG site.

When we try to interpret the new MvaI activity in the light of previous results, the following data can be considered. There are four Type IIP REases (MspI (25), HinPI (26), MvaI (13) and BcnI (27)), which were shown by X-ray crystallography to act as monomers.

All four enzymes contact their palindromic or pseudopalindromic substrate sites asymmetrically, contacting both strands of the recognition sequence. The asymmetry of the recognition complexes suggest that these enzymes act as nicking enzymes and cut the double-stranded substrate in two sequential nicking reactions. The cleavage mechanism has so far been tested for MvaI and HinPI. MvaI was shown to preferentially cleave the A-strand of the target site (30) and HinPI displayed nicking activity on supercoiled DNA (28). Further support for the nicking mechanism comes from observations with MvaI, BcnI and the monomeric DNA mismatch repair protein MutH. There is structural similarity between the three proteins (15), and MutH was shown to be a nicking enzyme, making single-strand scissions on the unmethylated strand of hemimethylated Gm6ATC sites (29). Against this background, the nicking activity of MvaI detected in this study is not surprising. Nicking the strand with the purine base in the center (the G-strand) may relate to the preferential cleavage of the A-strand observed for the canonical site (30).

The canonical recognition site of MvaI is characterized by A/T ambiguity: the enzyme accepts A : T or T : A (W), but exludes G : C and C : G (S) base pairs in the center of the target sequence. PspGI, another REase recognizing CCWGG flips the central adenine and thymine out of the helix and uses this mechanism to discriminate between W and S (31). In the MvaI recognition complex, no flipping of the central base pairs was observed (13). In an intact helix, because of the geometry of the base pairs and the position of the groups that can act as hydrogen bond donors or acceptors, differentiation between the W and S base pairs has to rely mainly on interactions in the minor groove (32). There are observations to suggest that this mechanism is less perfect than recognition of single base pairs mediated predominantly by interactions in the major groove. For example, the SinI DNA methyltransferase, whose normal target sequence is GGWCC, methylates, albeit at much lower rate, also GGSCC sites. Moreover, relaxed specificity M.SinI mutants with impaired capacity to discriminate between the two sites were relatively easy to isolate, suggesting that the W versus S discrimination is less tightly determined than recognition of well-defined unique base pairs (33,34). From the crystal structure of the MvaI–DNA complex, it is not entirely clear how recognition of the W base pairs is accomplished, especially as in the crystal only one of the two possible binding modes was represented (13). The results presented here demonstrate that adding a C5-methyl group to the indicated cytosines (C$\underline{C}$GGG/CC$\underline{C}$GG) in either strand seriously impairs the $\underline{a}$bility of $\underline{M}$vaI to discriminate between W and S in the center of the target sequence. The effects seem to be additive, as can be concluded from the more complete cleavage of the duplex modified on both strands (Figure 5). One of the modified cytosines is in the center of the BcnI site, at the position occupied by the A : T base pair in the canonical recogniton sequence. It is tempting to speculate that the 5-methylcytosine mimics the thymine in the major groove, and this is a major factor in the recognition of the methylated BcnI site. However, in such a model, binding to the C-strand would involve cleavage of the C-strand as can be inferred from the crystal structure (13), which would be inconsistent with the experimental observation of G-strand nicking (Figure 4). It needs structural studies to determine how C5-methylation of the cytosines leads to the observed change of sequence specificity.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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