A new methyl-directed site-specific DNA endonuclease MteI cleaves nonanucleotide sequence 5’-G(5mC)G(5mC)^NG(5mC)GC-3’/3’-CG(5mC)GN^(5mC)G(5mC)G-5’

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Translated from "Ovchinnikov bulletin of biotechnology and physical and chemical biology" V.8, No 1, pp 16-26, 2012

We have discovered and purified a new methyl-directed site-specific DNA endonuclease MteI from bacterial strain *Microbacterium testaceum* 17B. The enzyme recognizes methylated DNA sequence and doesn’t cleave unmethylated DNA. MteI is a first methyl-directed site-specific DNA endonuclease recognizing a prolonged DNA sequence and its activity depends on a number of 5-methycytosines and their positions in the recognition site. MteI cleaves DNA sequence 5’-G(5mC)G(5mC)↑NG(5mC)GC-3’/3’-CG(5mC)GN↓(5mC)G(5mC)G-5’ as indicated by arrows and this nonanucleotide is a minimal recognition site. The enzyme activity is significantly higher if 5’-GC-3’ dinucleotides in this site are replaced by 5’-G(5mC)-3’ dinucleotides and additional 5’-G(5mC)-3’ dinucleotides are present at 5’-ends in both DNA strands. Due to an ability to cleave

only prolonged methylated DNA sequences MteI may find a practical application in the molecular biology and epigenetics studies.

INTRODUCTION

Methyl-directed site-specific DNA endonucleases (MD endonucleases) recognize and cleave methylated DNA sequences, and don't cleave unmethylated DNA. MD endonucleases require only Mg^{2+} ions as a cofactor and their properties are similar to those of restriction endonucleases.

A few MD endonucleases are currently known which recognize and cleave DNA sequences containing 5-methylcytosine. In particular, MD endonuclease GlaI hydrolyzes the methylated DNA sequence 5'-R(5mC)GY-3 ', where R - purine, Y - pyrimidine [1].
Several MD endonucleases recognize the nucleotide sequences which differ in a number of 5-methylcytosines. The enzymes BlsI, BisI, PkrI and GluI recognize and cleave C5-methylated nucleotide sequence 5'-GCNGC-3 ', however, BlsI and BisI cut this sequence in the presence of two 5-methylcytosine, whereas PkrI and GluI are required 3 and 4 methylated bases, respectively, for an efficient DNA hydrolysis [2-5].
This paper describes a new MD endonuclease MteI, which recognizes methylated DNA sequence 5'-G(5mC)G(5mC)↑NG(5mC)GC-3'/3'-CG(5mC)GN↓(5mC)G(5mC)G-5' and cleaves it as indicated by arrows.

MATERIALS AND METHODS

The strain producer growth. The strain was grown in flasks containing LB liquid medium (1% Tryptone, 0.5% Yeast extract, 0.5% NaCl, pH 7.5) and cultured in a shaker at 30°C and stirring at 150 rev/min until a stationary phase of growth. The cells were precipitated by centrifugation at 5,000 rpm for 30 min at 4 °C in a centrifuge "Beckman" (USA), rotor JA-10. The cells pellet was stored at -20°C.

Enzyme isolation. Enzyme isolation was performed at 4°C using following buffer solutions:

Buffer A - 10 mM Tris-HCl pH 7.6; 0.1 mM EDTA and 7 mM 2-mercaptoethanol;
Buffer B - 10 mM K-phosphate, pH 7.2; 0.1 mM EDTA and 7 mM 2-mercaptoethanol.

Extraction. Frozen cell paste (20 g) was suspended in 60 ml of buffer A with 0.2 M NaCl, 1 mM Phenylmethylsulfonyl fluoride (PMSF), 0.1 mg/ml lysozyme. The cells were disrupted in the ultrasonic desintegrator Soniprep 150 ("MSE", UK). Cell debris was removed by centrifugation at 15,000 rpm for 30 min in a centrifuge J2-21 ("Beckman", USA), rotor JA-20.

Phosphocellulose chromatography. The supernatant was loaded to P-11 phosphocellulose column (30 ml) equilibrated with Buffer A with 0.2 M NaCl, and washed with two volumes of Buffer A with 0.2 M NaCl. Adsorbed material was eluted with 300 ml of NaCl linear gradient (0.2-1) in Buffer A. The fractions containing a target activity were collected.

Chemicals and substrates.

For experiments we have used DNA of bacteriophages λ, T7 and a variety of C5-methylated plasmids which were constructed before [2,4,6]. Besides, we have used restriction endonucleases, T4 DNA-ligase, T4 polynucleotidase, DNA- methyltrasferases HspAI and Fsp4HI and corresponding buffer solutions produced by Sibenzyme Ltd., Russia.

As a marker of the molecular weight of DNA fragments we have used 1 kb DNA Ladder (SIbenzyme, Russia.)

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Hydroxiapatite chtomatography.

The combined fractions were loaded to a hydroxiapatite column (4ml) equilibrated with buffer B and washed with 8 ml of it. The adsorbed material was eluted with 150 ml of a linear gradient 0,01M-0,2M of K-phosphate. 50 fractions were obtained and fractions 10-18 were collected.

Heparin-sepharose chromatography.

The combined fractions were loaded to a heparin-sepharose column (4 ml) equilibrated with Buffer A containing 0.1 M NaCl, and washed with 2 volumes of the same buffer. The adsorbed material was eluted with 150 ml of NaCl linear gradient (0.1-0.6 M) in Buffer A. 40 fractions were obtained, those of which, from 15^{th} to 23^{rd}, were collected.

Enzyme concentrating and storage.

The obtained fractions containing a target activity were dialyzed against 300 ml of Buffer A containing 55% Glycerol and 0.25 M NaCl during 20 h. The purified enzyme preparation was stored at -20°C.

Construction of the plasmids containing C5-methylated cytosines.

In order to determine MteI substrate specificity and to measure enzyme activity we have constructed a series of the plasmids carrying the genes of HspAI or Fsp4HI DNA-methyltransferases.

Construction of C5-methylated plasmids carrying the HspAI DNA-methyltransferase gene.

All the new plasmids containing gene of M.HspAI were constructed on the base of pHspAI plasmid [6] which was previously obtained by ligation of pUC19/EcoRI vector and a fragment of Haemophilus species AI genomic DNA carrying the HspAI DNA methyltransferase gene. The E. coli strain harboring this plasmid contains an active HspAI DNA methyltransferase which methylates first cytosine in C5 position of the sequence 5'-GCGC-3' forming a double-stranded methylated sequence 5'-G(5mC)GC-3'/3'-CG(5mC)G-5 '. In the present work we have inserted a variety of oligonucleotide duplexes into pHspAI DNA by ligation at PstI and Psp124BI restriction endonuclease sites. As a result four new plasmids carrying an additional methylated site formed by the modification of the inserted oligonucleotide duplexes with HspAI methylase were constructed.

1) pHspAI2 was obtained by insertion of a DNA fragment formed from the following synthetic oligonucleotides: Gl-1: 5'-c ggt agc gcg cgc tcc tct aga gtc gac ctg ca-3 ' Gl-2: 5'-ggtc gac tct aga gga gcg cgc gct acc gag ct-3 '

As a result a new methylated site appeared in the pHspAI2 plasmid:

5'-CGGTAG(5mC) G (5mC) G (5mC) G CTCCTCTAGAGTCGACCTGCA-3 '

3'-GCCATC G (5mC) G (5mC) G (5mC)GAGGAGATCTCAGCTGGACGT-5 '

2) pHspAI4 was obtained by insertion of a DNA fragment formed from the following synthetic oligonucleotides: Gl-3: 5'-c ggt agc gcag cgc tcc tct aga gtc gac ctg ca-3 ' Gl-4: 5'-ggtc gac tct aga gga gcg ctgc gct acc gag ct-3 '

As a result a new methylated site appeared in the pHspAI4:

5'-CGGTAG(5mC) G CAG(5mC) G CTCCTCTAGAGTCGACCTGCA-3 '

3'-GCCATC G (5mC)GTC G (5mC)GAGGAGATCTCAGCTGGACGT-5 '

3) pHspAI10 was obtained by insertion of a DNA fragment formed from the following synthetic oligonucleotides: Gl-9: 5'-t ggt agcgc gcag cgcgc tcc tct aga gtc gac ctg ca-3 ' Gl-10: 5'-ggtc gac tct aga gga gcgcg ctgc gcgct acc aag ct-3 '

As a result a new methylated site appeared in the pHspAI10:

5'-TGGTAG(5mC) G (5mC) G CAG(5mC) G (5mC) G CTCCTCTAGAGTCGACCTGCA-3 '

3'-ACCATC G (5mC) G (5mC)GTC G (5mC) G (5mC)GAGGAGATCTCAGCTGGACGT-5 '

4) pHspAI12 was obtained by insertion of a DNA fragment formed from the following synthetic oligonucleotides:Gl-11: 5'-t ggt agcgc gcag cgc tcc tct aga gtc gac ctg ca-3 ' Gl-12: 5'-ggtc gac tct aga gga gcg ctgc gcgct acc aag ct-3 '

As a result a new methylated site appeared in the pHspAI12 plasmid:

5'-TGGTAG(5mC) G (5mC) G CAG(5mC) G CTCCTCTAGAGTCGACCTGCA-3 '

3'-ACCATC G (5mC) G (5mC)GTC G (5mC)GAGGAGATCTCAGCTGGACGT-5 '.

Construction of C5-methylated plasmids carrying the Fsp4HI DNA-methyltransferase gene.

When constructing new substrates, we were also using pFsp4HI2 and pFsp4HI3 plasmids which contained the gene of Fsp4HI DNA-methyltransferase modifying the first cytosine in the sequence 5'-

GCNGC-3' to form the sequence 5'-G(5mC)NGC-3'/3'-CGN(5mC)G-5 '. In order to obtain new substrates we have inserted different oligonucleotide duplexes into pFsp4HI2 by ligation at the Bsp19I and HindIII restriction sites. As a result three new plasmids carrying an additional methylated site formed by the modification of the inserted oligonucleotide duplexes with Fsp4HI MTase were constructed.

1) pFsp4HI4 plasmid was obtained by the insertion of a DNA fragment formed from the following synthetic oligonucleotides:

Fsp3: 5'-cat gtg ccg ccg ccgctcgaa ttc taga-3'

Fsp4: 5'-agct tct aga att cga gcg gcg gcg gca-3'

As a result a new methylated site appeared in the pFsp4HI4 plasmid contained:

5'-CATGTG(5mC)C G (5mC)C G (5mC)C G CTCGAATTCTAGA-3'

3'-GTACAC G G(5mC) G G(5mC) G G(5mC)GAGCTTAAGATCT-5'

2) pFsp4HI6 plasmid was obtained by insertion of a DNA fragment formed from the following synthetic oligonucleotides: Fsp5: 5'-cat gtg ccg cag ccg ctcgaa ttc taga-3 ' Fsp6: 5'-agct tct aga att cga gcg gct gcg gca-3 '

As a result a new methylated site appeared in the pFsp4HI6 plasmid contained:

5'-CATGTG(5mC)C G (5mC)A G (5mC)C G CTCGAATTCTAGA-3'

3'-GTACAC G G(5mC) G T(5mC) G G(5mC)GAGCTTAAGATCT-5'

3) pFsp4HI8 plasmid was obtained by insertion of a DNA fragment formed from the following synthetic oligonucleotides: Fsp7: 5'-cat gtg ctg cag cag ctcgaa ttc taga-3' Fsp8: 5'-agct tct aga att cga gct gct gca gca-3'

As a result a new methylated site appeared in the pFsp4HI8 plasmid contained:

5'-CATGTG(5mC)T G (5mC)A G (5mC)A G CTCGAATTCTAGA-3'

3'-GTACAC G A(5mC) G T(5mC) G T(5mC)GAGCTTAAGATCT-5'

The site-specific DNA hydrolysis with MteI endonuclease was performed in the reaction mixture (20 ml) containing 1 mg of a DNA in SE-buffer “Y”(33 mM triacetate pH 7,9; 10 mM Mg-acetate; 66 mM K-acetate; 1 mM DTT) at 48°C. The reaction products obtained were separated by electrophoresis in 20% polyacrylamide gel (or in 1% agarose gel) containing TAE buffer and 7 M urea.

Enzyme activity determination.

An activity unit was a minimal amount of the enzyme that was necessary for complete digestion at a single recognition site of 1 mg of pHspAI10/DriI DNA additionally treated with M.Fsp4HI. The hydrolysis reaction was carried out in SE-buffer «Y» at 48°C for 1 hour in the total reaction volume of 20 mg.

To determine the primary structure of the 16S RNA gene fragment we carried out PCR with the following primers: 16S-direct 5'-AGAGTTTGATC (5mC) TGGCTCA-3' 16S-reverse 5'-TACGGYTACCTTGTTACGACTT-3'

The primary structure of the obtained PCR-product was determined on the ABI Prism 310 Genetic Analyzer (“Applied Biosystems”).

MteI recognition site and cleavage position determination on oligonucleotide duplexes.

The MteI DNA hydrolysis position was determined by comparing DNA fragments lengths after cleavage of the [γ32P]-labeled synthetic oligonucleotide duplexes containing the MteI recognition site. The duplexes were formed with the following synthetic oligonucleotides:

Mte3: 5'-CCGTACTTTG(5mC)G(5mC)AG(5mC)G(5mC)TTGATTCCC-3'

Mte4: 5'-GGGAATCAAG(5mC)G(5mC)TG(5mC)G(5mC)AAAGTACGG-3'

Glu7: 5'-CCGTACTTTG(5mC)G(5mC)GG(5mC)G(5mC)TTGATTCCC-3'

Glu8: 5'-GGGAATCAAG(5mC)G(5mC)CG(5mC)G(5mC)AAAGTACGG-3'.

One of the duplex chains was labeled at the 5'-end with the radioactive phosphorus [32P] and an equimolar amount of the complementary oligonucleotide was added. Then the mixture was heated at 95°C for 5 minutes and was left to cool on a table. The reaction of hydrolysis was carried out in 10 mg of the reaction mixture containing SE-buffer «G» (10 mM Tris-HCl, pH 7.6; 10 mM MgCl2; 50 mM NaCl; 1 mM dithiothreitol) and the oligonucleotide duplex in a concentration of 74 nM at 55°C for 1 hour. The electrophoresis of digestion products was performed in 20% PAAG with 7 M Urea in Tris-borate buffer. The autoradiography of the gel was carried out with the Cyclone Storage System ("Packard Instrument Co." USA).

RESULTS AND DISCUSSION

Strain-producer description.

Cultural-morphological features.

While screening soil microorganisms, we found a bacterial strain-producer of the new DNA-endonuclease. On Luria-Bertrani agar medium (LB) the strain-producer forms a smooth, glossy, orange-pale colonies. The cells are rod-shaped with a diameter of 1-2 mm and 8 mm in length. Immobile. Gram positive.

Physiological and biochemical properties of strain-producer. Obligate aerobes. Catalase-positive. No oxidase detected. The cells grow at the temperatures from +10°C to +40°C, at a pH of 6 to 9. The nucleotide sequence of the 16S RNA gene fragment obtained:

1 actggcggcg tgcttacaca tgcaagtcga acggtgaagc agagcttgct ctgtggatca

61 gtggcgaacg ggtgagtaac acgtgagcaa cctgccctgg actctgggat aagcgctgga

121 aacggcgtct aatactggat atgagacgtg atcgcatggt caacgtttgg aaagatttttt

181 cggtctggga tgggctcgcg gcctatcagc ttgttggtga ggtaatggct caccaaggcg

241 tcgacgggta gccggcctga gagggtgacc ggccacactg ggactgagac acggcccaga

301 ctcctacggg aggcagcagt ggggaatatt gcacaatggg cgaaagcctg atgcagcaac

361 gccgcgtgag ggatgacggc cttcgggttg taaacctctt ttagcaggga agaagcgaaa

421 gtgacggtac ctgcagaaaa agcgccggct aactacgtgc cagcagccgc ggtaatacgt

481 agggcgcaag cgttatccgg aattattggg cgtaaagagc tcgtaggcgg tttgtcgcgt

541 ctgctgtgaa atcccgaggc tcaacctcgg gcctgcagtg ggtacgggca gactagagtg

601 cggtagggga gattggaatt cctggtgtag cggtggaatg cgcagatatc aggaggaaca

661 ccgatggcga aggcagatct ctgggccgta actgacgctg aggagcgaaa gggtggggag

721 caaacaggct tagataccct ggtagtccac cccgtaaacg ttgggaacta gttgtgggga

781 ccattccacg gtttccgtga cgcagctaac gcattaagtt ccccgcctgg ggagtacggc

841 cgcaaggcta aaactcaaag gaattgacgg ggacccgcac aagcggcgga gcatgcggat

901 taattcgatg caacgcgaag aaccttacca aggcttgaca tacaccagaa cgggccagaa

961 atggtcaact ctttggacac tggtgaacag gtggtgcatg gttgtcgtca gctcgtgtcg

1021 tgagatgttg ggttaagtcc cgcaacgagc gcaaccctcg ttctatgttg ccagcacgta

1081 atggtgggaa ctcatgggat actgccgggg tcaactcgga ggaaggtggg gatgacgtca

1141 aatcatcatg ccccttatgt cttgggcttc acgcatgcta caatggccgg tacaaagggc

Based on morphological and biochemical properties as well as the nucleotide sequence of the 16S ribosomal RNA gene, the strain was identified as Microbacterium testaceum 17B. The discovered DNA endonuclease was named MteI according to the nomenclature.

Enzyme purification.

The biomass yield was 5g/l. A 3.5 ml of the enzyme preparation was obtained from 20 g of biomass.

DNA substrates hydrolysis with MteI endonuclease.

The MteI substrate specificity preliminary analysis at unmethylated and different C5-methylated substrates.

As the substrates for the determination of MteI specificity at the first stage, we used λ and T7 phage DNA and pFsp4HI3, pHspAI2 and pHspAI10 plasmids DNA (see "Materials and Methods"). On the Fig.1 the results of treatment of the above mentioned substrates with MteI endonuclease are presented:

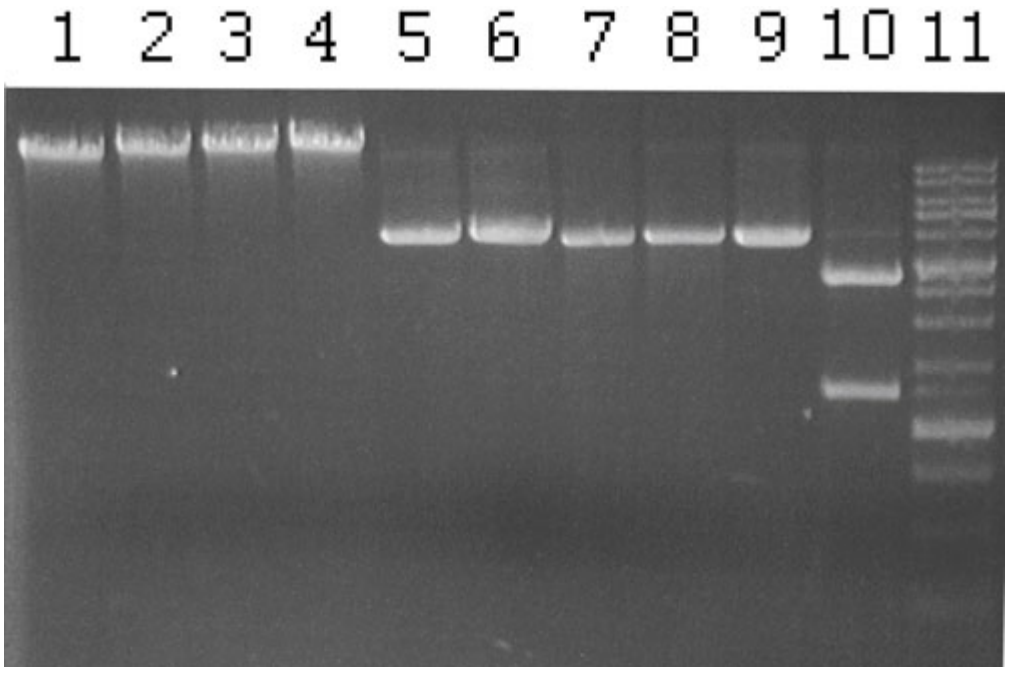


Fig. 1. MteI specificity on different DNA substrates. Reaction time is 1 hour. 1 ml of MteI preparation was added to a 20 ml of the reaction mixture. Lanes: 1 - λ DNA; 2 - λ DNA + MteI; 3 - T7 DNA; 4 - T7 DNA + MteI; 5 - pFsp4HI3/DriI; 6 - pFsp4HI3/DriI + MteI; 7 - pHspAI2/DriI; 8 - pHspAI2/DriI + MteI; 9 - pHspAI10/DriI; 10 - pHspAI10/DriI + MteI; 11 - 1 kb DNA marker.

As we can see from the Fig. 1, MteI does not cleave λ (lane 2), T7 phage DNA (lane 4); the DNA of pFsp4HI3 plasmids contains site 5'-G(5mC)GG(5mC)-3'/3'-(5mC)GC(5mC)G-5' (lane 6) and does not cleave pHspAI2 plasmid DNA, containing the 5'-G(5mC)G(5mC)G(5mC)GC-3 '/ 3'-CG(5mC)G(5mC)G(5mC)G-5' site (lane 8). But MteI cuts the linearized pHspAI10 plasmid (lane 10), which contains the 5'-G(5mC)G(5mC)GCAG(5mC)G(5mC)GC-3 '/ 3'-CG(5mC)G(5mC)GTCG(5mC)G(5mC)G-5 ' sequence.

MteI digestion of DNA substrates containing the gene of M.HspAI.

In addition to plasmid pHspAI10 which is the MteI substrate (Fig.1, lane 10), we have tested a possibility of the MteI endonuclease to digest pHspAI12 and pHspAI4 plasmid DNAs, which also included *hspAIM* gene, but differ in the pattern of methylation (see"Materials and Methods"). All these substrates contained several methylated variants of the 5'-GCGCAGCGC-3' sequence. In particular, pHspAI4 contained 5'-G(5mC)GCAG(5mC)GC-3'/3'-CG(5mC)GTCG(5mC)G-5' site, pHspAI12 contained 5'-G(5mC)G(5mC)GCAG(5mC)GC-3'/3'-CG (5mC)G(5mC)GTCG(5mC)G-5' site, whereas pHspAI10 contained 5'-G(5mC)G(5mC)GCAG(5mC)G(5mC)GC-3'/3'-CG(5mC)G(5mC)GTCG(5mC)G(5mC)G-5' site.

Moreover, for the analysis of the MteI substrate specificity we used the same three plasmids with additional modification with Fsp4HI methylase preparation [10]. As a result of the Fsp4HI MTase treatment a methylated cytosines appeared adjacent to the central nucleotide A or T in both chains of the 5'-GCGCAGCGC-3 '/ 3'-CGCGTCGCG-5' sequence.

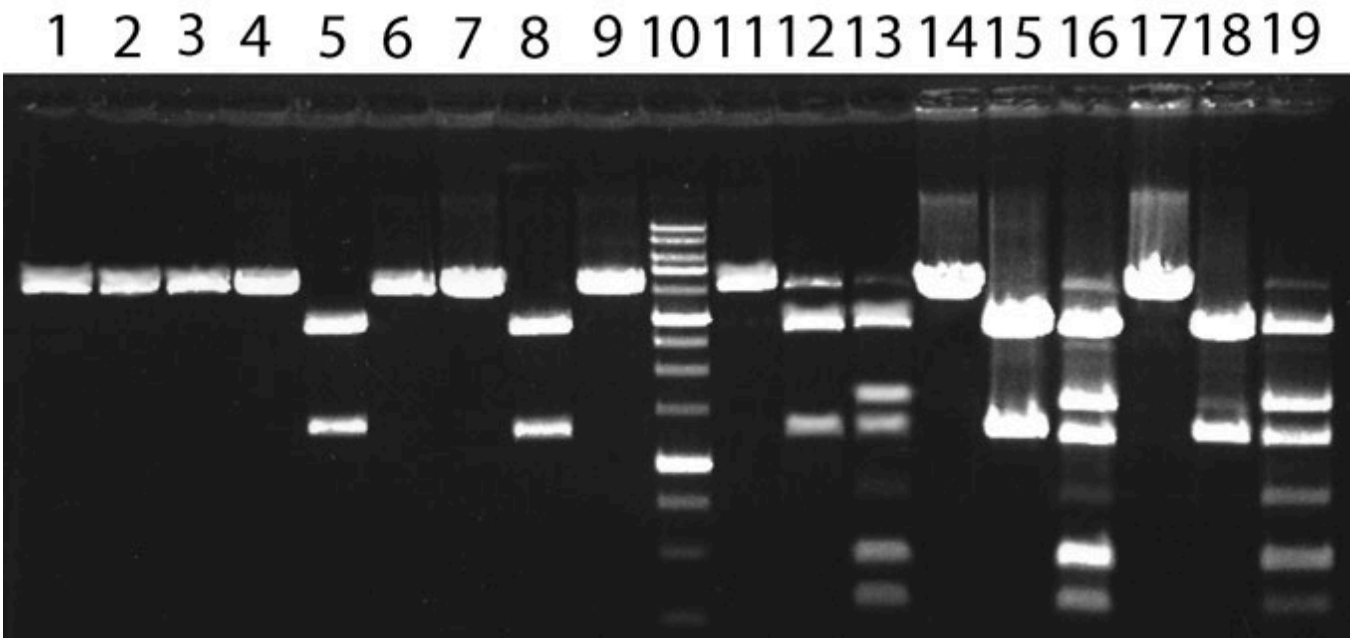
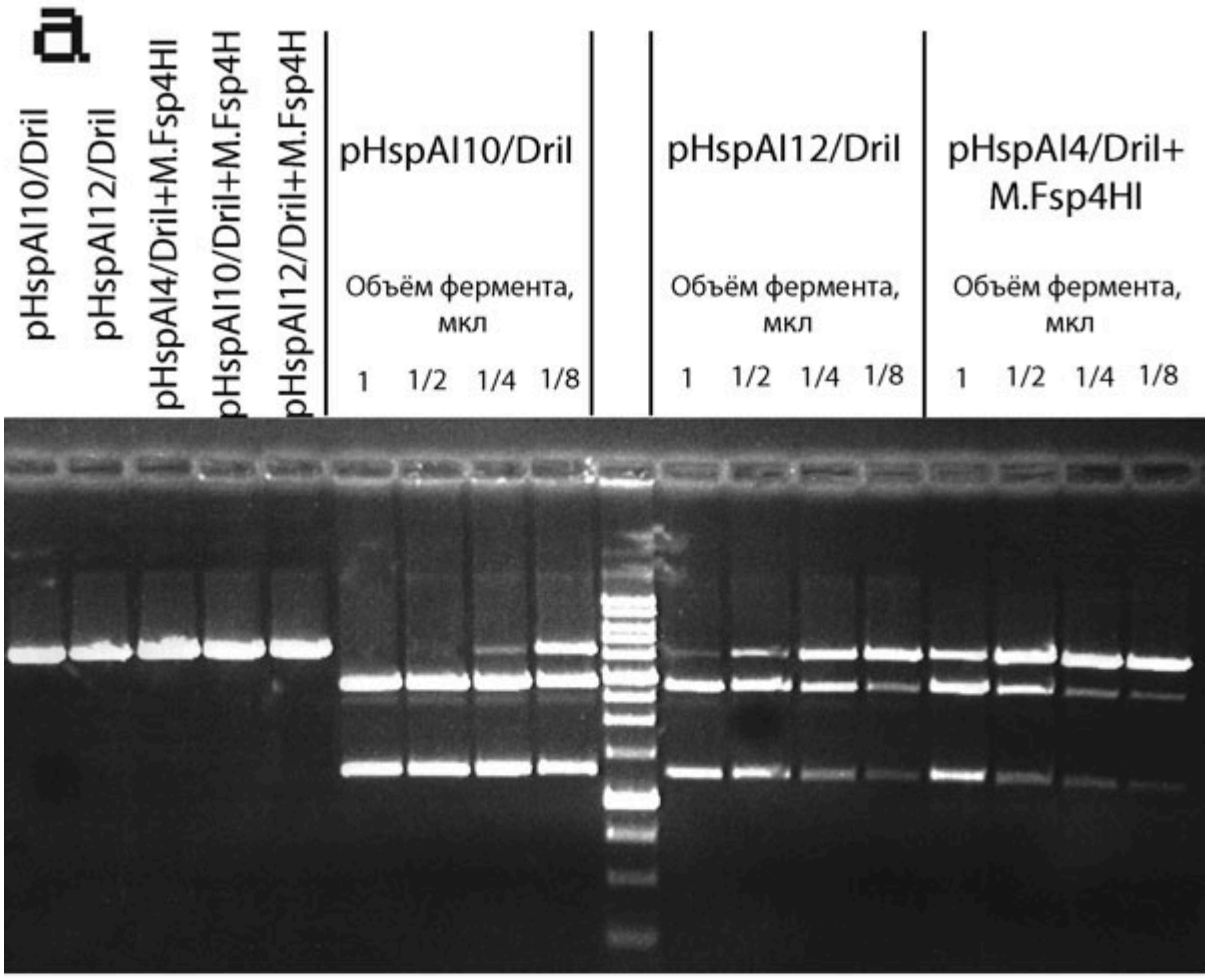


Fig. 2. Cleavage of plasmid DNAs by MteI and PkrI endonucleases. Reaction time - 8 hours. 1 µl of the enzyme preparation was added to a 20 µl of reaction mixture. Lanes: 1 - pHspAI4/DriI; 2 - pHspAI4/DriI + MteI; 3 - pHspAI4/DriI + PkrI; 4 - pHspAI10/DriI; 5 - pHspAI10/DriI + MteI; 6 - pHspAI10/DriI + PkrI; 7 - pHspAI12/DriI; 8 - pHspAI12/DriI + MteI; 9 - pHspAI12/DriI + PkrI; 10 - 1 kb DNA Ladder; 11 - pHspAI4/DriI + M.Fsp4HI; 12 - pHspAI4/DriI + M.Fsp4HI + MteI; 13 - pHspAI4 / DriI + M.Fsp4HI + PkrI; 14 - pHspAI10/DriI + M.Fsp4HI; 15 - pHspAI10/DriI + M.Fsp4HI + MteI; 16 - pHspAI10/DriI + M.Fsp4HI + PkrI; 17 - pHspAI12/DriI + M. Fsp4HI; 18 - pHspAI12/DriI + M.Fsp4HI + MteI; 19 - pHspAI12/DriI + M.Fsp4HI + PkrI.

Fig. 2 shows the results of the MteI digestion of the linearized pHspAI4, pHspAI10 and pHspAI12 DNA plus MteI digestion of the same substrates modified with M.Fsp4HI. As a comparison, we used the DNA endonuclease PkrI, which recognizes and cleaves the 5'-GCNGC-3 ' sequence if there are at least three 5-methylcytosines in both DNA chains of the recognition site [5].

Figure 2 shows that MteI doesn't cut pHspAI4 (lane 2), but it cleaves pHspAI10 and pHspAI12 (lanes 5 and 8, respectively), as well as it digests all these plasmids modified with M.Fsp4HI (lanes 12, 15 and 18, respectively). In this case, a complete MteI DNA digestion is not observed only on the 12th lane, which allows us to consider the sequence 5'-G(5mC)G(5mC)AG(5mC)GC-3'/3'-CG(5mC)GT(5mC)G(5mC)G-5' to be a minimal recognition site, while the site 5'-G(5mC)GCAG(5mC)GC-3'/3'-CG (5mC)GTCG(5mC)G-5' (lane 2) is not a substrate for MteI. Noteworthy, DNA endonuclease PkrI doesn't cleave modified 5'-GCGCAGCGC-3' sequence in two cases (lanes 3 and 9), when the central pentanucleotide sequence contains only two methylated cytosines, that corresponds to the previously described PkrI substrate specificity [5]. But PkrI cleaves pHspAI12 and pHspAI10 DNA plasmids previously modified with M.Fsp4HI at 5'-GCNGC-3' sites, containing four 5-methylcytosines. Thus, DNA endonuclease MteI recognizes the extended C5-methylated sequence of 5'-G(5mC)G(5mC)AG(5mC)GC-3'/3'-CG(5mC)GT(5mC)G(5mC)G-5'and significantly differs in substrate specificity from the previously described DNA endonucleases BisI, BlsI, GluI and PkrI which recognize the modified sequence 5'-GCNGC-3'/3'-CGNCG-5' with the presence of 2-4 5-methylcytosines.

Fig. 3 shows the results of a more detailed definition of MteI specific activity on the pHspAI10 and pHspAI12 linearized plasmids, and on the same plasmids, modified with Fsp4HI MTase.



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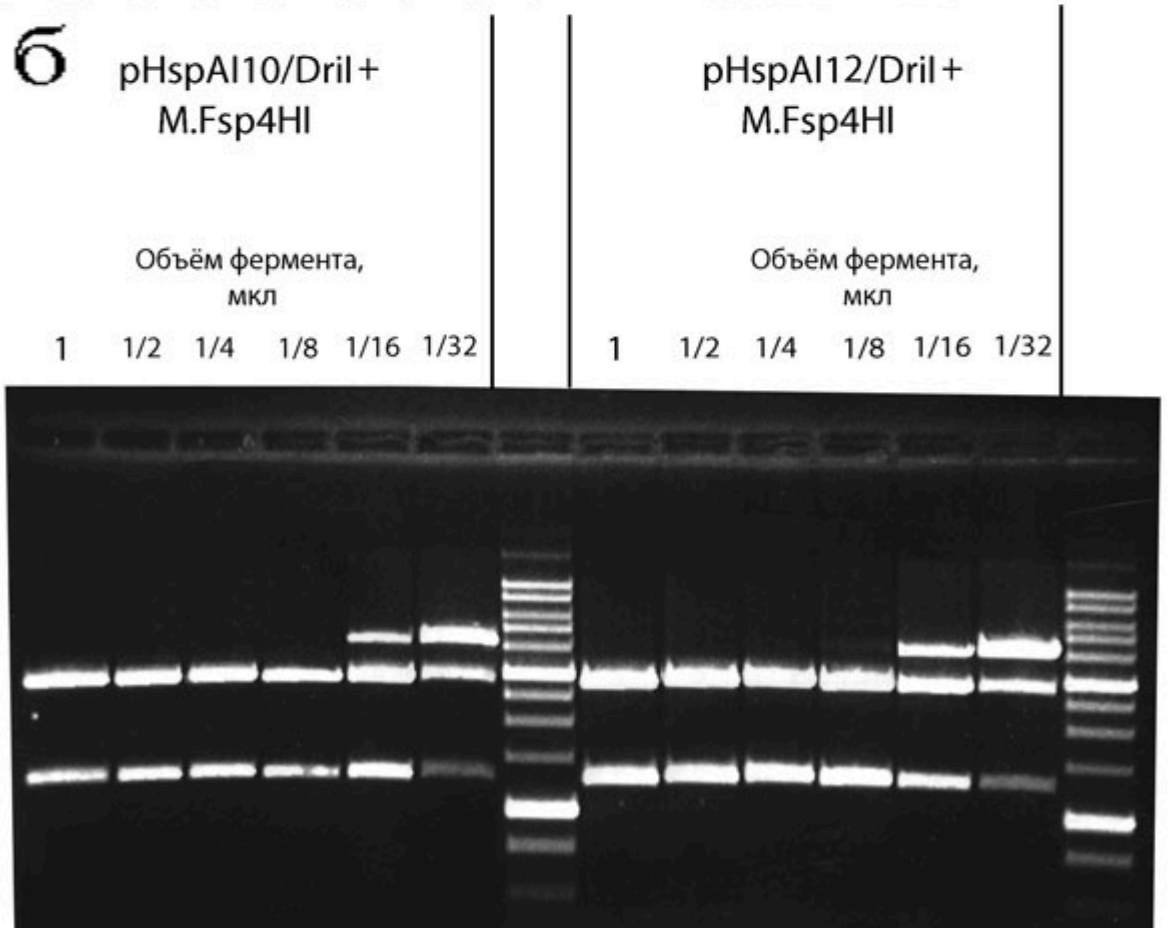

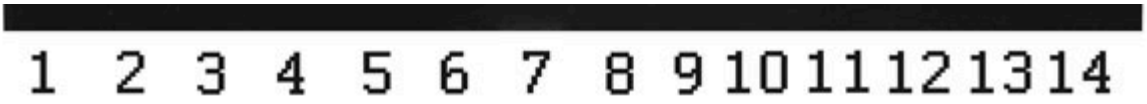

Fig. 3. Comparison of MteI activity in hydrolysis of different plasmid DNAs. Determination of the enzyme activity was carried out by the DNA digestion in multiple dilutions of the enzyme preparation: the reaction mixture with 1 μl of MteI was diluted subsequently in two, four or eight times with the reaction mixture without the enzyme. Reaction time - 1 hour. Lanes: (a) 1 - pHspAI10/DriI; 2 - pHspAI12/DriI; 3 - pHspAI4/DriI + M.Fsp4HI; 4 - pHspAI10/DriI + M.Fsp4HI; 5 - pHspAI12/DriI + M.Fsp4HI; 6 - pHspAI10/DriI + 1 μl MteI; 7 - pHspAI10/DriI + 1/2 μl MteI; 8 - pHspAI10/DriI + 1/4 μl MteI; 9 - pHspAI10/DriI + 1/8 μl MteI; 10 - 1 kb DNA Ladder, 11 - pHspAI12 / DriI + 1 μl MteI; 12 - pHspAI12/DriI + 1/2 μl MteI; 13 - pHspAI12/DriI + 1/4 μl MteI; 14 - pHspAI12/DriI + 1/8 μl MteI; 15 - pHspAI4/DriI + M . Fsp4HI + 1 μl MteI; 16 - pHspAI4/DriI + M.Fsp4HI + 1/2 μl MteI; 17 - pHspAI4/DriI + M.Fsp4HI + 1/4 μl MteI; 18 - pHspAI4/DriI + M.Fsp4HI + 1/8 μl MteI; (b) 1 - pHspAI10/DriI + M.Fsp4HI + 1 μl MteI; 2 - pHspAI10/DriI + M.Fsp4HI + 1/2 μl MteI; 3 - pHspAI10/DriI + M.Fsp4HI + 1/4 μl MteI; 4 - pHspAI10/DriI + M.Fsp4HI + 1/8 μl MteI; 5 - pHspAI10/DriI + M.Fsp4HI + 1/16 μl MteI; 6 - pHspAI10/DriI + M.Fsp4HI + 1/32 μl MteI; 7 - 1 kb DNA Ladder, 8 - pHspAI12/DriI + M.Fsp4HI + 1 μl MteI; 9 - pHspAI12/DriI + M.Fsp4HI + 1/2 μl MteI; 10 - pHspAI12/DriI + M.Fsp4HI + 1/4 μl MteI; 11 - pHspAI12/DriI + M.Fsp4HI + 1/8 μl MteI; 12 - pHspAI12/DriI + M.Fsp4HI + 1/16 μl MteI; 13 - pHspAI12/DriI + M.Fsp4HI + 1/32 μl MteI; 14 - 1 kb DNA Ladder.

As it is shown in the Fig. 3, the minimal MteI activity was observed with pHspAI4 DNA substrate modified with Fsp4HI MTase (Fig.3, lanes 15-18). This result is consistent with the above made conclusion that the sequence 5'-G(5mC)G(5mC)AG(5mC)GC-3'/3'-CG(5mC)GT(5mC)G(5mC)G-5' is the minimal site, and it cleaves with MteI worse than the others. pHspAI12 plasmids containing the 5'-G(5mC)G(5mC)GCAG(5mC)GC-3'/3'-CG(5mC)G(5mC)GTCG(5mC)G-5' sequence (Fig. 3a, lanes 11-14) is a better substrate. MteI partially cleaves a linear form of these DNA plasmids (Fig. 3a, lanes 11 and 15) and, thus, its activity is less than 1 unit/μl. A significantly greater activity (2 u/ μl in terms of the considered substrate) was observed when digesting the 5'-G(5mC)G(5mC)GCAG(5mC)G(5mC)GC-3'/3'-CG(5mC)G(5mC)GTCG(5mC)G(5mC)G-5' sequence in pHspAI10 plasmid (Fig. 3, lanes 6-9). Finally, MteI activity is at 8 u/μl when the substrate contains the site 5'-G(5mC)G(5mC)G(5mC)AG(5mC)GC-3'/3'-CG(5mC)G(5mC)GT(5mC)G(5mC)G-5'and 5'-G(5mC)G(5mC)G(5mC)AG(5mC)G(5mC)GC-3'/3'-CG(5mC)G(5mC)GT(5mC)G(5mC)G(5mC)G-5' (pHspAI12 and pHspAI10 plasmids, respectively, modified with M.Fsp4HI). The results are summarized in Table 1.

Methylated sequence	Plasmid with MteI recognition site	The number of 5-methylcytozynes in methylated sequence	MteI activity in plasmid DNA hydrolysis
5'-G(5mC)G CAG(5mC)G C-3' **3'-C G(5mC)GTC G(5mC)G-5'**	pHspAI4	4	-
5'-G(5mC)G(5mC)G CAG(5mC)G C-3' **3'-C G(5mC)G(5mC)GTC G(5mC)G-5'**	pHspAI12	6	< 1 u/μl
5'-G(5mC)G(5mC)A G(5mC)G C-3' **3'-C G(5mC) G T(5mC)G(5mC)G-5'**	pHspAI4/M.Fsp4HI	6	< 1 u/μl
5'-G(5mC)G(5mC)G CAG(5mC)G(5mC)G C-3' **3'-C G(5mC)G(5mC)GTC G(5mC)G(5mC)G-5'**	pHspAI10	8	2 u/μl
5'-G(5mC)G(5mC)G(5mC)A G(5mC)G C-3' **3'-C G(5mC)G(5mC)G T(5mC)G(5mC)G-5'**	pHspAI12/M.Fsp4HI	8	8 u/μl
5'-G(5mC)G(5mC)G(5mC)A G(5mC)G(5mC)G C-3' **3'-C G(5mC)G(5mC)G T(5mC)G(5mC)G(5mC)G-5'**	pHspAI10/M.Fsp4H	10	8 u/μl

Table 1. MteI activity in hydrolysis of different DNA substrates containing the *hspAIM* gene.

Based on the data obtained we have chosen the pHspAI10 DNA, linearized with the restriction enzyme DriI and modified with M.Fsp4HI as a canonical substrate for MteI. Thus, for one unit of MteI activity we have taken a minimal amount of the enzyme that was necessary for a complete hydrolysis of 1 μg of pHspAI10/DriI DNA + M.Fsp4HI in 20 μl reaction mixture containing SE-buffer «Y» at 48°C for 60 minutes. The MteI activity on canonical substrate is 8 u/μl.

Hydrolysis of DNA substrate containing the *fsp4HIM* gene.

As a possible substrate for MteI we tested a number of plasmids containing the gene of Fsp4HI MTase (see "Materials and Methods"). Fig. 4 shows the results of MteI digestion of such substrates and pHspAI10 as a control.

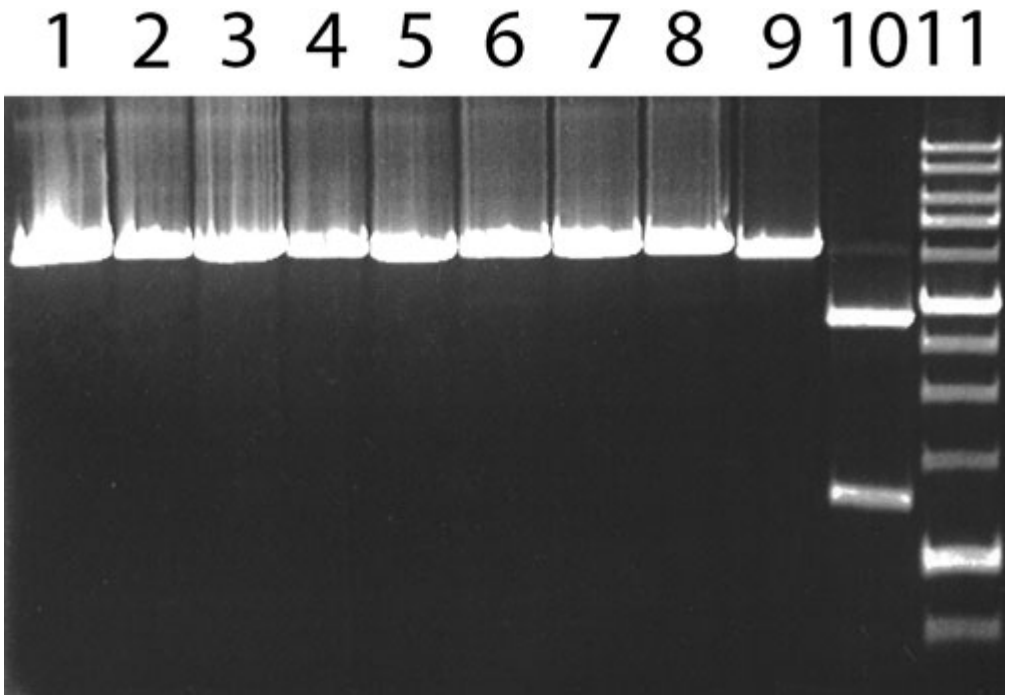

Fig. 4. MteI hydrolysis of the DNA substrates containing the gene of Fsp4HI MTase. Lanes: 1 - pFsp4HI3/DriI; 2 - pFsp4HI3/DriI + MteI; 3 - pFsp4HI4/DriI; 4 - pFsp4HI4/DriI + MteI; 5 - pFsp4HI6/DriI; 6 - pFsp4HI6/DriI + MteI; 7 - pFsp4HI8/DriI; 8 - pFsp4HI8/DriI + MteI; 9 - pHspAI10/DriI; 10 - pHspAI10/DriI + MteI; 11 - 1 kb DNA ladder.

As it is shown in the Fig.4, MteI doesn't cleave pFsp4HI4 (lane 4), pFsp4HI6 (lane 6) and pFsp4HI8 DNA (lane 8). Thus, the 5'-G(5mC)CG(5mC)AG(5mC)CGC-3'/3'-CGG(5mC)GT(5mC)GG(5mC)G-5' sequence, which is present in pFsp4HI6, is not a MteI substrate (lane 6), although its underlined central part coincides with the central part of pHspAI4, methylated with Fsp4HI. The obtained results and the Table 1 analysis have shown that the enzyme is able to cleave the 5'-G(5mC)AG(5mC)-3'/3'-(5mC)GT(5mC)G-5' sequence only with an additional G(5mC)-dinucleotide at the 5'-end of the upper and/or the bottom strand, therefore leading to the formula of the minimal site 5'-G(5mC)G(5mC)AG(5mC)GC-3'/3'-CG(5mC)G(5mC)G(5mC)G-5', which is the minimal substrate for the new enzyme. **At the same time MteI shows a significantly greater activity when replacing the 5'-GC-3' dinucleotides on the 5'-G(5mC)-3' ones occurs in theie recognition sites or an additional dinucleotides 5'-G(5mC)-3 'appear at the 5'-end in both DNA strands.** It should be noted that methylation of cytosines adjacent to the central nucleotide of the recognition sequence (A or T) with Fsp4HI MTase also leads to a significant increase of the MteI activity.

MteI hydolysis of λ DNA, methylated with HspAI and Fsp4HI methylases. The further definition of MteI substrate specificity was carried out in a digestion of λ DNA methylated with M.HspAI and M.Fsp4HI. The results of this analysis are shown in Fig. 5.

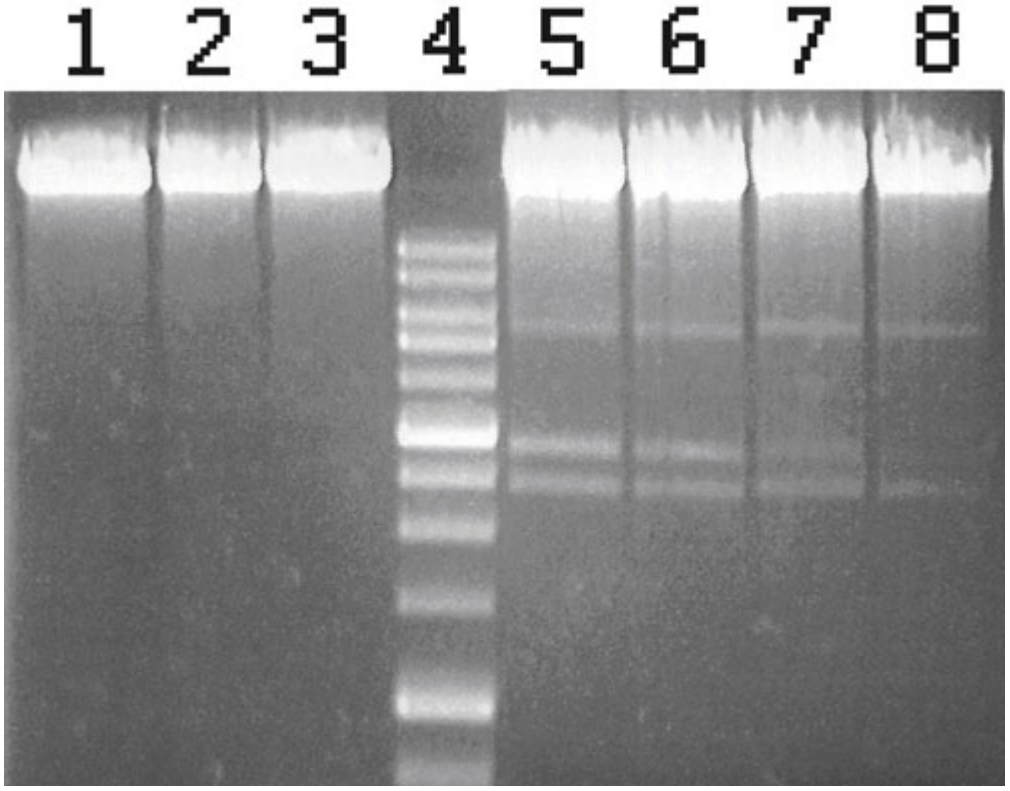

Fig. 5. MteI digestion of λ DNA methylated with HspAI and Fsp4HI MTases. Reaction time - 30 min. The reaction volume - 20 μl. Lanes: 1 - λ DNA + M.HspAI + M.Fsp4HI; 2 - λ DNA + M.HspAI + M.Fsp4HI + R.HspAI; 3 - λ DNA + M.HspAI + M.Fsp4HI + R.Fsp4HI; 4 – 1 kb DNA Ladder, 5 - λ DNA + M.HspAI + M.Fsp4HI + 4 u of MteI; 6 - λ DNA + M.HspAI + M.Fsp4HI + 2 u of MteI; 7 - λ DNA + M.HspAI + M.Fsp4HI + 1 u of MteI; 8 - λ DNA + M.HspAI + M.Fsp4HI + 0,5 u of MteI.

The complete λ DNA methylation with HspAI and Fsp4HI MTases was tested by HspAI (lane 2) and Fsp4HI (lane 3) restriction endonucleases digestion. From Fig. 5 it is clear that when 4, 2, 1, or 0.5 units of MteI (lanes 4, 5, 6, 7, respectively) were added to the reaction mixture, the appearance of three bands was observed. The analysis of the DNA structure has shown that 5'-GCGCTGCGC-3' sequence at the 2498-2506 position and the 5'-GCGCGGCGC-3' sequence at the position of 5437-5445 are present in λ DNA. After λ DNA treatment with HspAI and Fsp4HI MTases these sites are transformed to 5'-G(5mC)G(5mC)AG(5mC)GC-3'/3'-CG(5mC)GT(5mC)G(5mC)G-5’ and to 5'-G(5mC)G(5mC)GG(5mC)GC-3'/3'-CG(5mC)GC(5mC)G(5mC)G-5' respectively. These methylated sequences correspond to the minimal MteI site indicated above. As it is seen from the Fig. 5, the electrophoretic mobility of the formed DNA fragments (~ 2500 and ~ 2940 bp) corresponded to the products of the DNA digestion at the positions of 2498 and 5437. The observed less bright third band of ~ 5500 bp, was probably formed from the first two because of incomplete cleavage of the site at the 2498 position. Thus, from the data on the methylated λ phage DNA and different plasmid DNAs we can conclude that the minimal MteI cleavage site is 5'-G(5mC)G(5mC)NG(5mC)GC-3'/ 3'-CG(5mC)GN(5mC)G(5mC)G-5'.

MteI cleavage position determination.

To confirm the recognition sequence and determine the DNA cleavage position in the recognition site, we have hydrolyzed some oligonucleotide duplexes with MteI.

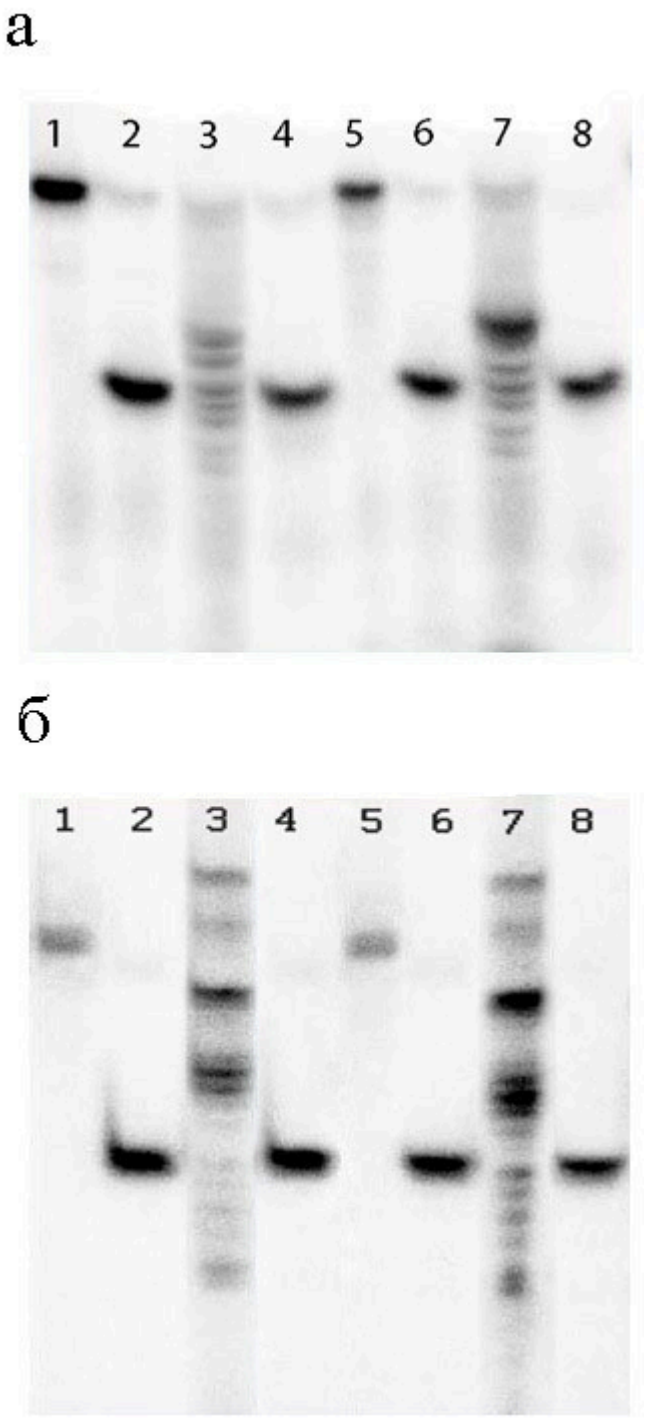

Fig. 6. MteI cleavage position determination on the DNA duplexes Mte3/Mte4 (a) and Glu7/Glu8 (b). Electrophoresis in 20% PAAG with 7 M Urea. The oligonucleotides were labeled with radioactive phosphorus [32P] at the 5'-end (marked with *). Lanes: (a) 1 - Mte3*/Mte4; 2 - Mte3*/Mte4 + MteI; 3 - Mte3*/Mte4 + ExoIII; 4 - Mte3*/Mte4 + GluI; 5 - Mte4*/Mte3; 6 - Mte4*/Mte3 + MteI; 7 - Mte4*/Mte3 + ExoIII; 8 - Mte4*/Mte3 + GluI. (b) 1 - Glu7*/Glu8; 2 - Glu7*/Glu8 + MteI; 3 - Glu7*/Glu8 + ExoIII; 4 - Glu7*/Glu8 + GluI; 5 - Glu8*/Glu7; 6 - Glu8*/Glu7 + MteI; 7 - Glu8*/Glu7 + ExoIII; 8 - Glu8*/Glu7 + GluI.

The cutting position determination was performed by comparing the lengths of the fragments produced after cleavage of oligonucleotide duplexes with MteI and GluI endonucleases

Mte3/Mte4 (includes A/T pair in the center of the site MteI) and Glu7/Glu8 (includes G/C pair in the center). As a marker of fragment lengths the products of partial cleavage of the same duplex with ExoIII were used. Fig. 6a represents the autoradiograph of electrophoregram of the Mte3*/Mte4 cleavage products. Fig. 6b shows the autoradiograph of electrophoregram of the Glu7/Glu8 cleavage products under the same conditions.

It is clear from the Fig. 6a that the MteI and GluI hydrolysis products of the Mte3*/Mte4 duplex (lanes 2 and 4), as well as the Mte4*/Mte3 duplex (lanes 6 and 8) have equal lengths. The same may be said about the bands in Fig. 6b for the Glu7*/Glu8 duplex (lanes 2 and 5) and for the Glu8*/Glu7 duplex (lanes 7 and 10). From the data presented in Figure 6 we can conclude that MteI cleaves DNA before the central nucleotide N: 5'-G(5mC)G(5mC)^NG(5mC)G(5mC)-3' and the positions of DNA hydrolysis by GluI and MteI enzymes are the same.

CONCLUSION

Presented in this paper a new methyl-directed site-specific DNA endonuclease MteI has a minimal recognition site of 5'-G(5mC)G(5mC)↑NG(5mC)GC-3'/3'-CG(5mC)GN↓(5mC)G(5mC)G-5' and cleaves DNA as indicated by the arrows. Replacement of the recognition site 5'-GC-3' dinucleotides by 5'-G(5mC)-3' dinucleotides and the appearance of additional 5'-G(5mC)-3' dinucleotides at the 5'-ends of nonanucleotide in both DNA strands leads to a significant increase in the enzyme activity. Previously, we have shown a significant increase in activity of a number of MD DNA endonucleases after replacing cytosine by 5-methylcytosine in the recognition site [3,6,11]. However, in case of MteI we observed a significant increase in the activity of the enzyme when the methylated recognition site was expanding and additional 5-methylcytosines replaced cytosines around the minimal site in both chains. This significant increase in enzyme activity with the expanding of the recognition site is a new phenomenon to MD DNA endonucleases and we will study it in detail in the subsequent papers.

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