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Detection of DNA Methylation by Dnmt3a Methyltransferase using Methyl-Dependent Restriction Endonucleases

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Abstract—DNA methylation at cytosine residues in CpG sites by DNA methyltransferases (MTases) is associated with various cell processes. Eukaryotic MTase Dnmt3a is the key enzyme that establishes the *de novo* methylation pattern. A new in vitro assay for DNA methylation by murine MTase Dnmt3a was developed using methyl-dependent restriction endonucleases (MD-REs), which specifically cleave methylated DNA. The Dnmt3a catalytic domain (Dnmt3a-CD) was used together with KroI and PcsI MD-REs. The assay consists in consecutive methylation and cleavage of fluorescently labeled DNA substrates, then the reaction products are visualized in polyacrylamide gel to determine the DNA methylation efficiency. Each MD-RE was tested with various substrates, including partly methylated ones. PcsI was identified as an optimal MD-RE. PcsI recognizes two methylated CpG sites located 7 bp apart, the distance roughly corresponding to the distance between the active centers of the Dnmt3a-CD tetramer. An optimal substrate was designed to contain two methylated cytosine residues and two target cytosines in the orientation suitable for methylation by Dnmt3a-CD. The assay is reliable, simple, and inexpensive and, unlike conventional methods, does not require radioactive compounds. The assay may be used to assess the effectiveness of Dnmt3a inhibitors as potential therapeutic agents and to investigate the features of the Dnmt3a-CD function.

Keywords: DNA methylation, methyl-dependent restriction endonucleases, eukaryotic DNA methyltransferases, methylation efficiency

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INTRODUCTION

Methylation of DNA plays a key role in regulating gene expression in eukaryotic cells and is thought to act as a major carrier of epigenetic information [1–3]. Cytosine residues of CpG sites are methylated at position C5 in mammalian cells. Methylated CpG sites located in certain positions form a methylation pattern, which is presumably established by de novo DNA methyltransferases (MTases) Dnmt3a and Dnmt3b during embryo development and is reproduced in somatic cells by maintenance MTase Dnmt1 [3, 4]. The cofactor S-adenosyl-L-methionine (AdoMet) is used as a methyl group donor by MTases. The methylation pattern is often changed in cancers, including general DNA hypomethylation and hypermethylation of the promoter regions of tumor suppressor genes [5]. Hence, it is of importance to search for new efficient inhibitors of MTases, including Dnmt3a, to add to a few inhibitors that have long been used in treating cancer [5].

Abbreviations: MTase, C5-cytosine DNA methyltransferase; RE, restriction endonuclease; MD-RE, methyl-dependent restriction endonuclease; AdoMet, S-adenosyl-L-methionine.

The mechanism of action of Dnmt3a is still incompletely understood. Active Dnmt3a is a tetramer of two Dnmt3a molecules and two molecules of the regulatory factor Dnmt3L [6]. In the absence of Dnmt3L, Dnmt3a alone produces an active form [6], which presumably consists of four monomers in the case of short oligonucleotide duplexes as substrates [7]. A model of the Dnmt3a/Dnmt3L tetramer in complex with DNA suggests that the active centers of the two central Dnmt3a subunits are 8–10 bp apart on DNA [6]. The C-terminal Dnmt3a catalytic domain (Dnmt3a-CD) remains active in the absence of the N-terminal domain [3]. Mutations have recently been found to arise in Dnmt3a in hematological disorders, but the mechanism of the process remains unknown [8].

Enzyme activity is often necessary to assay when studying various issues related to the Dnmt3a function or investigating potential Dnmt3a inhibitors. Methylating activity of MTases in vitro is usually quantified with radiolabeled AdoMet wherein the methyl group is tritiated ([^{3}H]AdoMet) [9]. After carrying out the methylation reaction, tritiated DNA is to be separated from unused [^{3}H]AdoMet and quantitatively tested for radioactive isotope incorporation. Variants of the method include immobilization of DNA on DE filters

Table 1. DNA duplexes used in this work

No.	DNA duplex	Designation
I	5'-**FAM**-CTGAATACTACTT*G**C**G**C*TCTCTAACCTGAT-3' 3'-GACTTATGATGAA*C**G**C*GAGAGATTGGACTA-**FAM**-5'	fG**C**GC/C**G**C**G**Cf
II	5'-**FAM**-CTGAATACTACT*GC**C**G**GC*CTCTAACCTGAT-3' 3'-GACTTATGATGA*CG**GM**CG*GAGATTGGACTA-5'	fGC**C**GGC/CG**GM**CG
III	5'-**FAM**-CTGAATACTACT*GC**C**G**GC*CTCTAACCTGAT-3' 3'-GACTTATGATGA*CG**GC**CG*GAGATTGGACTA-5'	fGC**C**GGC/CG**GC**CG
IV	5'-**FAM**-TGCTGAATA*C**G**CTGTATA**MG***TCTAACCTG-3' 3'-ACGACTTAT*GM**G**ACATAT**GC***AGATTGGAC-5'	f**C**G-N_7-**MG/GM**–N_7-G**C**
V	5'-**FAM**-TGCTGAATA*C**G**CTGTATA**MG***TCTAACCTG-3' 3'-ACGACTTAT*GM**G**ACATAT**GM***AGATTGGAC-5'	f**C**G-N_7-**MG/GM**–N_7-**GM**

M, 5-methylcytosine; FAM (f), 6-carboxyfluorescein. CpG dinucleotides are in bold. Cytosines acting as methylation targets are underlined. RE (Hin6I, KroI, or PcsI) recognition sites are italicized.

[9], the interaction of biotinylated DNA with avidin [10], DNA attachment to cellulose [11], or thin-layer chromatography [12]. However, errors often arise in measurements because [^{3}H]AdoMet is absorbed or co-absorbed with proteins on the carrier, thus providing a radioactive background. Other drawbacks of the method are that [^{3}H]AdoMet is rather expensive and that [^{3}H] is a weak β-emitter and heterogeneous counting on a filter consequently underestimates the radioactivity and yields poorly reproducible results. Another group of methods makes use of the restriction endonucleases (REs) that have a CpG dinucleotide in the recognition sites, such as HhaI or Hin6I (G↓CGC, the DNA cleavage site is indicated with an arrow) [5, 13]. These REs specifically cleave DNA only when the CpG site is unmethylated. The reaction is usually performed with fluorescently labeled DNA duplexes, which are methylated by the MTase under study and then digested with RE that hydrolyzes only nomethylated substrates. The reaction products are then separated and detected by various techniques [5]. We have used this approach to study the inhibitors of MTases M.SssI and Dnmt3a with REs Hin6I [14] and HhaI [15], respectively.

Several methyl-dependent REs (MD-REs) have been characterized recently and differ from conventional REs in that they cleave only methylated DNA [16]. MD-REs offer an opportunity to develop a new MTase activity assay wherein these enzymes are used to cleave DNA substrates after their methylation. Examples of commercially available MD-REs that have CpG dinucleotides with C5-methylated cytosines in their recognition sites are provided by KroI (G↓C(5mC)GGC) [17] and PcsI ((5mC)GNNNN-N↓NN(5mC)G) [18], which have recently been isolated. The objective of this work was to develop a method to estimate the efficiency of DNA methylation by Dnmt3a with the use of MD-REs KroI and PcsI.

EXPERIMENTAL

Reagents. All oligonucleotides (Table 1) were synthesized by Syntol (Russia). The fluorescent dye 6-carboxyfluorescein (FAM, f) was covalently attached through the -NH-$(CH_2)_6$- amino linker to the 5'-terminal phosphate in some of the oligonucleotides. The oligonucleotide concentrations were measured spectrophotometrically according to a published protocol [19]. AdoMet was purchased from Sigma (Germany). The following buffers were used: A (20 mM HEPES-NaOH, pH 7.5, 100 mM KCl, 1 mM EDTA, 1 mM 1,4-DTT), B (33 mM Tris-CH_3COOH, pH 7.9, 10 mM $Mg(CH_3COO)_2$, 66 mM CH_3COOK, 0.1 mg/mL BSA), C (10 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM 1,4-DTT), D (10 mM Tris-HCl, pH 8.3, 20 mM NaCl, 3 mM $MgCl_2$, 1 mM 1,4-DTT), and E (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 1 mM 1,4-DTT).

Enzymes. REs Hin6I, KroI, and PcsI were from SibEnzyme (Russia). Murine MTase Dnmt3a-CD with a N-terminal hexahistidine tag was expressed in *Escherichia coli* BL21 (DE3) cells carrying pET28a with the MTase gene. The enzyme was isolated by metal ion affinity chromatography on Co^{2+}-containing TALON® resin as in [7]. The Dnmt3a-CD monomer concentration was measured by the Bradford assay. Protein preparations were stored at –60°C. Prokaryotic MTase M.SssI was isolated according to a published protocol [20].

Efficiency of DNA methylation by Dnmt3a-CD. FAM-labeled 30-mer DNA duplexes I–V (300 nM) were incubated with 2 μM Dnmt3a-CD in buffer A supplemented with 25 μM AdoMet at 37°C for 1 h.

Table 2. Methylation efficiency of DNA duplexes I–V with MTase Dnmt3a-CD as estimated with ERs Hin6I, KroI, and PcsI

DNA duplex	Restriction endonuclease	Duplex cleavage efficiency w, %*			Methylation efficiency M of DNA duplexes by Dnmt3a-CD, %**
		w_0	$w_{M.SssI}$	w_{Dnmt3a}	
I	Hin6I	88 ± 6	0	10 ± 7	89
II	KroI	27 ± 9	58 ± 3	21 ± 4	–***
III	KroI	0	58 ± 3	14 ± 1	24
IV	PcsI	4 ± 2	68 ± 14	68 ± 10	100
V	PcsI	71 ± 18	68 ± 14	74 ± 15	–***

* See Experimental for the definitions of w_0, $w_{M.SssI}$, and w_{Dnmt3a}. Each w value was obtained in at least two replicate experiments. Standard deviations are shown.
** The parameter was calculated from Eq. (1).
*** The parameter was not calculated for DNA duplexes II and V (see text).

DNA was precipitated with ethanol in the presence of 0.4 M sodium acetate. The precipitate was washed with 80% ethanol and dried in a SpeedVac concentrator. In parallel, duplexes were methylated with M.SssI (500 nM) in buffer E in the same conditions. The DNA duplexes were then digested with 2 units of Hin6I or 1 unit of KroI or PcsI at 37°C for 1 h; the reaction was carried out in 20 μL of buffer B (Hin6I), C (KroI), or D (PcsI). The reaction mixtures were evaporated, the pellets resuspended in 10 μL of 80% formamide supplemented with dyes, and samples analyzed by denaturing 20% PAGE with 7 M urea. Gels were scanned with a FUJIFILM FLA-3000 (Japan) or Typhoon FLA 9500 (Life Sciences) system, and fluorescence intensities were measured for the reaction products and original DNA. The DNA cleavage efficiency w was calculated as a ratio of the fluorescence intensity of the cleavage products to the sum of the fluorescence intensity of the uncleaved DNA and the cleavage products (in %). The methylation efficiency M for Dnmt3a-CD was calculated from the resulting w estimates, including that obtained for the DNA duplex after methylation with M.SssI (complete methylation):

$$M = \frac{w_{Dnmt3a} - w_0}{w_{M.SssI} - w_0} \times 100\%, \quad (1)$$

where w_{Dnmt3a} and $w_{M.SssI}$ are the efficiencies of DNA cleavage with MD-REs after treatment with Dnmt3a-CD or M.SssI, respectively, and w_0 is the DNA cleavage efficiency without preliminary methylation.

RESULTS

A method to assay the efficiency of DNA methylation by Dnmt3a was developed using MD-REs KroI and PcsI and fluorescently labeled substrate DNA duplexes containing the respective MD-RE recognition sites, which harbored CpG dinucleotides (Table 1). In our assay, DNA is first methylated with Dnmt3a and then digested with MD-RE. The reaction mixtures are resolved by denaturing PAGE, and the methylation efficiency is calculated. RE Hin6I, which cleaves unmethylated DNA, is used for comparison.

DNA Hydrolysis by Endonucleases Hin6I, KroI, and PcsI before and after Methylation with MTases

Hin6I. In contrast to MD-REs, Hin6I acts as a conventional RE and cleaves the unmethylated recognition sites G↓CGC in DNA (Fig. 1a). The 30-mer DNA duplex fG**C**G**C**/C**G**C**C**Gf (I) with FAM present in both of the strands was used as a methylation substrate for the first time (Table 1). DNA duplex I was treated with MTase Dnmt3a-CD in buffer A, precipitated from the reaction mixture with ethanol, resuspended in buffer B, and digested with ER Hin6I. A similar digestion reaction was carried with duplex I without methylation. The reaction mixtures were resolved by 20% PAGE in the presence of 7 M urea (Figs. 1b, lanes *2* and *4*). In a parallel positive control sample, duplex I was methylated with prokaryotic MTase M.SssI (Fig. 1b, *3*), which recognizes a CpG-containing site like Dnmt3a and completely methylates DNA. Hin6I cleaved nonmethylated duplex I to produce FAM-labeled products (Fig. 1a), while treatment with Dnmt3a-CD or M.SssI almost fully protected duplex I from cleavage. The methylation efficiency M of DNA duplex I with Dnmt3a-CD was 89% as calculated from Eq. (1) (Table 2).

KroI cleaves the methylated recognition site G↓C(5mC)GGC in DNA (Fig. 1a) [17]. We used a 30-mer hemimethylated (fGC**C**G**G**C/CG**GMC**G, II) and an unmethylated (fGC**C**G**G**C/CG**G**C**C**G, III) DNA substrates, which each contained FAM in one of the strands (Table 1). The experiment was carried out as with Hin6I, but substrate digestion with MD-RE KroI was performed in buffer C. As is seen from Fig. 1b (lane *6*) and Table 2, KroI cleaved the upper strand of the substrate fGC**C**G**G**C/CG**GMC**G (II) (w_0 = 27%) to produce a 13-mer FAM-labeled oligonucleotide. The result agrees with the data that KroI is capable of

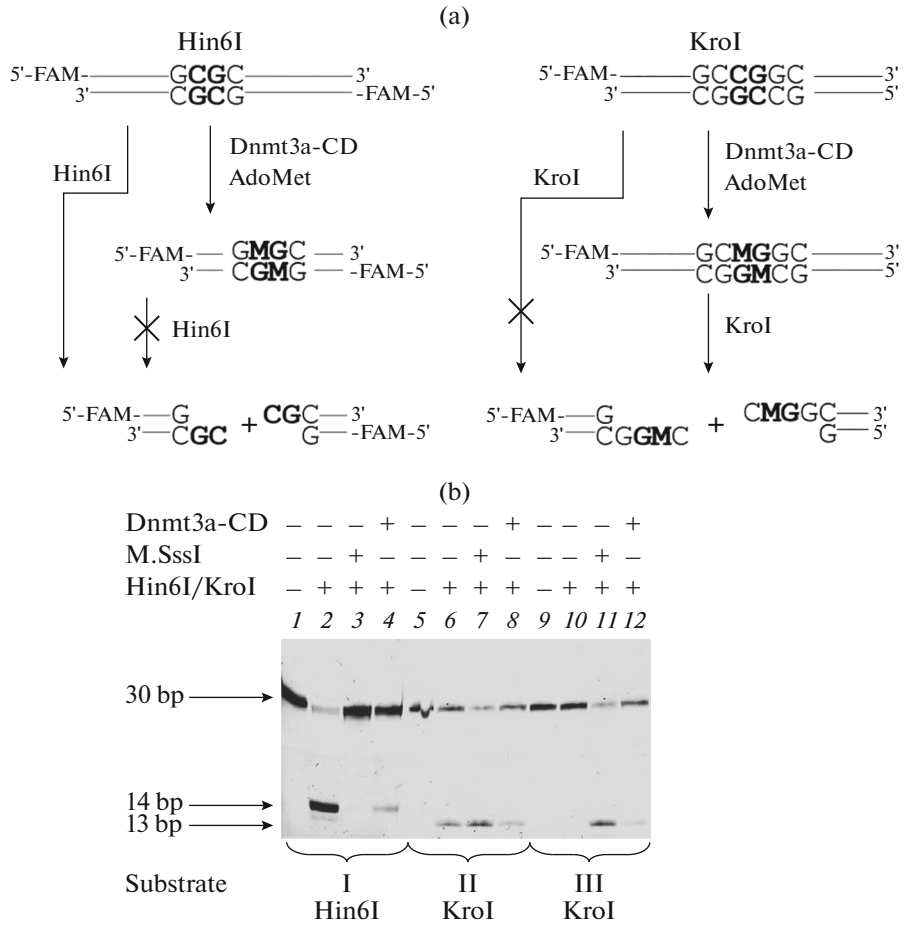

Fig. 1. Cleavage of DNA duplexes I–III with REs Hin6I and KroI before and after methylation with MTases Dnmt3a-CD and M.SssI. (a) Cleavage schemes for duplexes I and III. (b) Digestion products of duplexes I–III as resolved by 20% PAGE with 7 M urea.

hydrolyzing DNA substrates with hemimethylated recognition sites [17]. The addition of Dnmt3a-CD (Fig. 1, *8*) did not increase the efficiency of substrate cleavage by KroI, in contrast to the addition of M.SssI (Fig. 1, *7*). Thus, hemimethylated substrate II was unsuitable for assaying Dnmt3a-CD activity. KroI did not cleave the nonmethylated duplex fGC**C**G**G**C/CG**G**C**C**G (III) (Fig. 1b, *10*). Duplex III was cleaved after treatment with M.SssI or Dnmt3a-CD, but the cleavage efficiency was only 58% in the case of M.SssI, which fully methylated the substrate, and did not exceed 14% in the case of Dnmt3a-CD (Fig. 1b, lanes *11* and *12*, respectively; Table 2). As a result, the methylation efficiency *M* of duplex III by Dnmt3a-CD was estimated at 24% (Table 2). Incomplete cleavage has already been reported for synthetic oligonucleotide duplexes wherein both of the central cytosines are methylated in the KroI recognition site [17]. In addition, the methylation efficiency of duplex III with Dnmt3a-CD was low possibly because nonoptimal nucleotide sequences flanked the CpG site [21]. These sequences are characteristic of the KroI site. Thus, the unmethylated duplex fGC**C**G**G**C/CG**G**C**C**G (III) is principally possible to employ in estimating Dnmt3a-CD activity, but only at a qualitative level in view of the above limitations.

PcsI cleaves the substrates that harbor two fully methylated CpG sites located 7 bp apart (Fig. 2a) [18], the distance approximately corresponding to the distance between the active centers in the Dnmt3a-CD tetramer [6]. This feature makes PcsI appealing to use in a Dnmt3a-CD activity assay. The Dnmt3a-CD is known to bind DNA so that its two active centers are positioned to methylate the two target cytosines in adjacent CpG sites located approximately 8–10 bp apart; methyl groups are added to different strands of substrate DNA toward their 5' ends [3, 6]. With this tilted position, Dnmt3a-CD can add only two methyl groups to a DNA duplex with two target sites in one binding event, while four methyl groups are necessary for PcsI-dependent cleavage. To obtain a methylation product that provides a good substrate for PcsI, we constructed a DNA duplex that already has two methylated cytosine residues (f**C**G-N_7-**MG**/**GM**-N_7-G**C**,

IV) and carries FAM in the upper strand (Table 1). The orientation of the two target cytosines in duplex IV is optimal for their methylation by Dnmt3a-CD (Fig. 3). Duplex IV was not cleaved by PcsI, but a 15-mer product of its hydrolysis was detected in gel after its methylation with Dnmt3a-CD (Fig. 2b, lanes *2* and *4*, respectively). The same product was observed after methylation with M.SssI in a control experiment (Fig. 2b, *3*). The methylation efficiency *M* of duplex IV was 100% (Table 2). Therefore, Dnmt3a-CD is capable of efficiently methylating the duplex f**CG**-N_7-**MG**/**GM**-N_7-**GC** (IV). The DNA duplexes

5'-**FAM**-TGCTGAATA***CG***CTGTATA***CG***TCTAACCTG-3'
3'-ACGACTTAT***GM***GACATAT***GM***AGATTGGAC-5' and
5'-**FAM**-TGCTGAATA***MG***CTGTATA***CG***TCTAACCTG-3'
3'-ACGACTTAT***GC***GACATAT***GM***AGATTGGAC-5',

which contained two methylated cytosines in orientations differing from those in duplex IV, were efficiently cleaved by PcsI (data not shown). The duplexes are thereby unsuitable for our Dnmt3a-CD activity assay. Given the possibility of their hydrolysis, it is of interest to study how the PcsI specificity depends on the substrate methylation pattern because such a dependence has been reported for other MD-REs [16].

We additionally tested the DNA duplex f**CG**-N_7-**MG**/**GM**-N_7-**GM** (V), which contained three methyl groups in the CpG sites and had one target cytosine (Table 1, Fig. 3). We have previously observed that such substrate (with three methyl groups) is efficiently methylated by Dnmt3a-CD [22]. However, PcsI efficiently cleaved the trimethylated duplex (Fig. 2b, *6*) even in the absence of its further methylation by Dnmt3a-CD ($w_0 = 71\%$, Table 2), and the electrophoretic pattern did not change after duplex methylation with either MTase (Fig. 2b, *7* and *8*). Substrate V was therefore unsuitable for assaying the DNA methylation efficiency by Dnmt3a-CD. Thus, the DNA duplex f**CG**-N_7-**MG**/**GM**-N_7-**GC** (IV) provided an optimal substrate to be used in a Dnmt3a-CD activity assay with MD-REs.

DISCUSSION

Because Dnmt3a inhibitors are in increasing demand and the mechanism of the Dnmt3a function are of interest to study, an efficient assay is necessary for estimating the efficiency of DNA methylation by the enzyme [3, 5]. Our attention was attracted by MD-REs, which cleave methylated DNA and are used to assess the epigenetic status (presence of 5-methylcytosine residues) of DNA [16, 23, 24]. We used the murine Dnmt3a catalytic domain (Dnmt3a-CD) and MD-REs KroI and PcsI, which have, respectively, one or two CpG dinucleotides in the recognition site. Each RE was tested with a variety of substrates, including partly methylated ones. We found that KroI is of limited utility for estimating the efficiency of DNA methylation by Dnmt3a-CD because hydrolysis of a methylated substrate with KroI is incomplete and because the context of the CpG dinucleotide in the KroI recognition site is nonoptimal for Dnmt3a-CD, which is consequently low active (see above). A comparison of the *M* values (Table 2) obtained for KroI and Hin6I, which have one CpG dinucleotide in the recognition site, indicates that the conventional RE Hin6I is more promising.

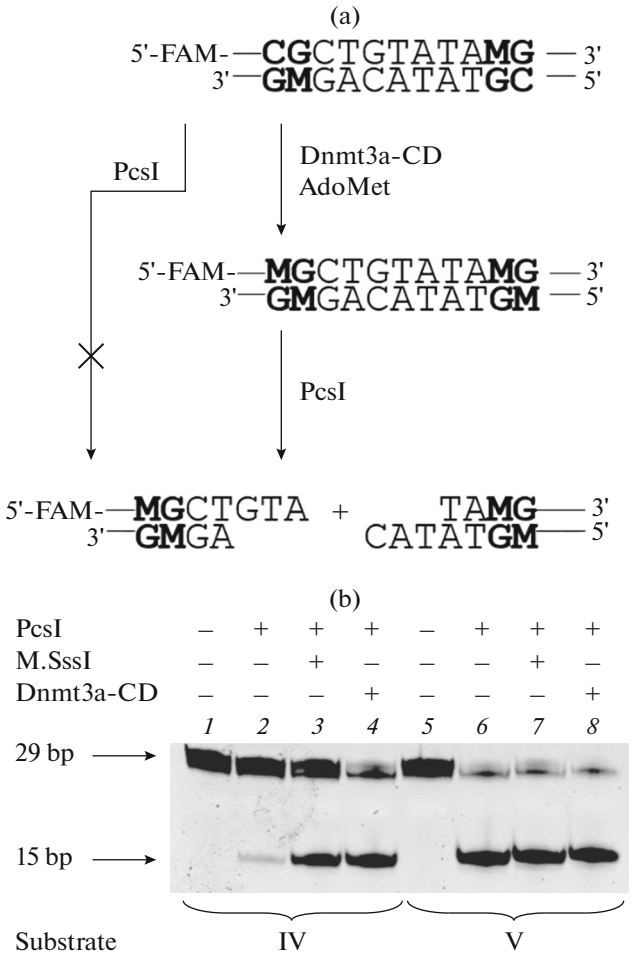

Fig. 2. Cleavage of DNA duplexes IV and V by RE PcsI before and after methylation with MTases Dnmt3a-CD and M.SssI. (a) Cleavage schemes for duplex IV. (b) Digestion products of duplexes IV and V as resolved by 20% PAGE with 7 M urea.

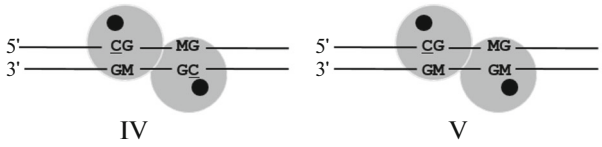

Fig. 3. Binding of Dnmt3a-CD with the DNA duplexes f**C**G-N_7-**MG**/**GM**-N_7-G**C** (IV) and f**C**G-N_7-**MG**/**GM**-N_7-**GM** (V). The schemes are based on a model describing a complex of the Dnmt3a-CD/Dnmt3L tetramer with DNA [6]. The Dnmt3a-CD dimers are shown gray; the enzyme active centers are black.

In the case of MD-RE PcsI, which has two CpG dinucleotides in the recognition site, substrates IV and V were designed with due regard to the mechanism of action known for Dnmt3a-CD [6]. The distance between the CpG dinucleotides in the substrates approximately corresponds to one turn of the DNA double helix, thus allowing a proper positioning of the two Dnmt3a-CD active centers for efficient methylation (Fig. 3). In addition, substrates IV and V are partly methylated so that only one or two cytosine residues, respectively, are available for methylation. Substrate IV allows for an optimal orientation of the target cytosines [3]. As shown above, substrate IV ensured the maximal efficiency *M*, which was comparable with the *M* value obtained with Hin6I and duplex I (Table 2), while duplex V proved unsuitable, as was discussed above.

To summarize, we developed a simple method to assay MTase Dnmt3a activity in vitro. The method obviates the use of radiolabeled AdoMet in contrast to conventional techniques [9–12, 25] and utilizes commercially available MD-REs. Substrate IV is possible to recommend for assaying Dnmt3a activity with MD-RE PcsI. An advantage of MD-RE is that the enzyme is principally possible to use simultaneously with MTase, which may be achieved in further optimization of the assay. New information on how the substrate specificity of PcsI depends on the orientation of methyl groups in the recognition site may be useful for further understanding the mechanism of action of Dnmt3a.

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