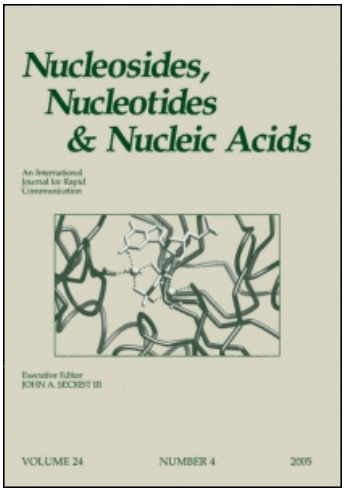


Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Probing the MvaI Methyltransferase Region that Interacts with DNA: Affinity Labeling with the Dialdehyde-Containing DNA Duplexes

O. M. Gritsenko[a]; S. N. Mikhailov[b]; E. V. Efimtseva[b]; A. Van Aerschot[c]; P. Herdewijn[c]; E. S. Gromova[a]
[a] Department of Chemistry, Moscow State University, Moscow, Russia [b] Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, Russia [c] Rega Institute, Katholieke Universiteit Leuven, Leuven, Belgium

To cite this Article Gritsenko, O. M. , Mikhailov, S. N. , Efimtseva, E. V. , Van Aerschot, A. , Herdewijn, P. and Gromova, E. S.(2000) 'Probing the MvaI Methyltransferase Region that Interacts with DNA: Affinity Labeling with the Dialdehyde-Containing DNA Duplexes', Nucleosides, Nucleotides and Nucleic Acids, 19: 10, 1805 — 1820
To link to this Article: DOI: 10.1080/15257770008045462
URL: http://dx.doi.org/10.1080/15257770008045462

NUCLEOSIDES, NUCLEOTIDES & NUCLEIC ACIDS, 19(10–12), 1805–1820 (2000)

PROBING THE *MVA*I METHYLTRANSFERASE REGION THAT INTERACTS WITH DNA: AFFINITY LABELING WITH THE DIALDEHYDE-CONTAINING DNA DUPLEXES

O.M.Gritsenko[a], S.N.Mikhailov[b], E.V.Efimtseva[b],
A.Van Aerschot[c], P.Herdewijn[c], E.S.Gromova[a]*

[a]*Department of Chemistry, Moscow State University, Moscow, 119899, Russia*
[b]*Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Vavilov str. 32, Moscow 117984, Russia*
[c]*Rega Institute, Katholieke Universiteit Leuven, Minderbroedersstraat 10, B-3000 Leuven, Belgium*

ABSTRACT: Affinity labeling of methyltransferase *Mva*I by DNA duplexes containing oxidized 2'-O-β-D-ribofuranosylcytidine or 1-(β-D-galactopyranosyl)thymine residues was performed. Partial chemical hydrolysis of the covalently bound methylase in the conjugates with the dialdehyde-containing DNA allowed us to determine the amino acid region in the C terminus of methylase *Mva*I that interacts with DNA.

INTRODUCTION

N^4-cytosine (N^4C) DNA methyltransferases (Mtases) belong to restriction-modification systems. *Mva*I DNA methyltransferase (M·*Mva*I) recognizes the DNA sequence 5'... $CC^A/_TGG$...3' and transfers methyl group from S-adenosyl-L-methionine (AdoMet) to nitrogen N4 of the "inner" cytosine residue[1]. The information concerning the M·*Mva*I crystal structure is not available and the enzyme region that interacts with DNA remains to be determined. As for other N^4C methylases, only the structure of *Pvu*II Mtase in the absence of DNA substrate has been reported[2].

This paper is dedicated to the memory of Professor A.Krayevsky.

Affinity modification of enzymes by modified substrates followed by identification of the cross-linking region is one of the methods used for determination of the amino acid residues involved in specific interactions with the target DNA. To identify the *Mva*I Mtase contacts with DNA, modified substrates containing sugar moieties with dialdehyde groups can be used. Dialdehyde-containing nucleic acids can specifically join to the proximal lysine residues in the protein. The affinity modification of *Eco*RII and *Mva*I DNA methyltransferases and *Eco*RII restriction endonuclease by the dialdehyde-containing DNA duplexes has been performed[3]. Besides, the DNA duplexes with dialdehyde groups have been employed for cross-linking to T7 RNA polymerase[4].

In this work, the amino acid region of M·*Mva*I interacting with the DNA sugar-phosphate backbone was determined for the first time for N^4C Mtases by affinity labeling with the dialdehyde-containing substrates.

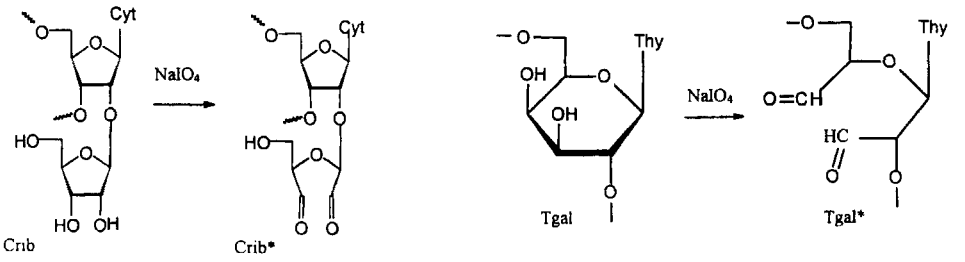
RESULTS AND DISCUSSION

(A) Design of substrate analogs for cross-linking to *Mva*I DNA methyltransferase

For probing the *Mva*I Mtase region interacting with DNA, it was necessary for us to introduce dialdehyde groups into the oligonucleotide substrate in regiospecific manner. One possibility is to replace cytidine or thymidine residues in oligonucleotides with 2′-O-β-D-ribofuranosylcytidine (Crib) or 1-(β-D-galactopyranosyl)thymine (Tgal)[3], respectively. These analogs contain *cis* diol groups. After periodate oxidation, one can obtain oligonucleotides with dialdehyde groups in any desired position of an oligonucleotide chain (SCHEME 1). All main functional groups in the dialdehyde-containing DNA duplexes are preserved.

14 mer DNA duplex 1 is a canonical substrate of *Mva*I Mtase (TABLE 1). DNA duplexes 2-9 (14 mers) were synthesized, in which dC or dT of DNA duplex 1 were replaced with oxidized 2′-O-β-D-ribofuranosylcytidine (Crib*) or oxidized 1-(β-D-galactopyranosyl)thymine (Tgal*) residues, respectively. Both types of modifications were used in order to have

chemically active groups of different "length" (Crib*, "extended" label, and Tgal*, "zero-length" label), which should favour trapping the DNA-binding region of M·*Mva*I. Crib* or Tgal* were introduced into definite positions of the M·*Mva*I recognition site (2-5 and



SCHEME 1.

8) or into the 3′ (6 and 9) or 5′ (7) end flanking nucleotide sequences. Our goal was to probe the M·*Mva*I contacts with different functional groups of DNA, first of all with DNA phosphates, by Lys-dialdehyde cross-linking (TABLE 1).

14 mer non-modified DNA duplex 10 5′-GCCAACCGGCTCTA

3′-CGGTTGGCCGAGAT

and 21 mer Crib*-containing DNA duplex 11 (TABLE 1), both lacking the *Mva*I recognition site were used in control experiments.

(B) Interaction of *Mva*I methylase with the substrate analogs containing Crib*

(a) Thermal stability of modified duplexes

Before using the DNA duplexes with dialdehyde groups for affinity modification of M·*Mva*I, it is necessary to evaluate their thermal stability, substrate properties, and the ability to specifically bind to the enzyme.

Melting temperatures (T_m) of the Crib*-containing DNA duplexes 2-7 were determined at pH 7.0 in the presence of Mg^{2+} (buffer A) or at pH 9.0 in the buffer for the cross-linking reaction lacking DTT and BSA (buffer B) (TABLE 1). In both cases, incorporation of Crib* into the canonical DNA duplex 1 (DNA duplexes 2-7) led to a slight destabilization (3-4°C) of double-stranded structure. We could not determine T_m of the Tgal*-containing DNA duplexes 8 and 9 due to their rapid decomposition under melting conditions[3].The Crib*-containing DNA duplexes were chemically stable under the cross-linking conditions in buffer C at pH 9.0.

TABLE 1. Properties of the Crib* or Tgal*-containing DNA duplexes as substrates of M·*Mva*I. Cross-linking of M·*Mva*I to DNA duplexes containing Crib* or Tgal* residues

DNA duplex	T_m[a]		Relative V_0[b]	Cross-linking yield, %[d]
	Buffer A	Buffer B		
1 5' GCCAA**CCTGG**CTCT 3' CGGTT**GGACC**GAGA	65	44	1[c]	-
2 5' GCCAA**X**CTGGCTCT 3' CGGTT**GGACC**GAGA	65	-	0.20±0.05	3
3 5' GCCAAC**X**TGGCTCT 3' CGGTT**GGACC**GAGA	62	-	0.04±0.02	4
4 5' GCCAA**CCTGG**CTCT 3' CGGTT**GGAC**XGAGA	63	-	0.06±0.02	3
5 5' GCCAA**CCTGG**CTCT 3' CGGTT**GGA**XCGAGA	63	41	0.03±0.01	4
6 5' GCCAA**CCTGG**XTCT 3' CGGTT**GGACC**GAGA	62	40	0.44±0.10	2
7 5' GC**X**AA**CCTGG**CTCT 3' CGGTT**GGACC**GAGA	61	-	0.42±0.09	6
8 5' GCCAA**CCYGG**CTCT 3' CGGTT**GGACC**GAGA	-	-	0.01[c]	2
9 5' GCCAA**CCTGGC**YCT 3' CGGTT**GGACC**GAGA	-	-	0.41[c]	3
11 5' CACAGAATTXTAGATATCACA 3' GTGTCTTAAGATCTATAGTGT	-	-	-	3

X = Crib*; Y = Tgal*.

[a]Accuracy of T_m determination ±1^{0}C.

[b]Defined as the ratio of V_o of DNA duplexes 2-7 to V_o of DNA duplex 1. For methylation conditions see Materials and Methods.

[c]Relative methylation was defined for 30 min at 20^{0}C as the ratio of ^{3}H radioactivity incorporation into DNA duplexes 8 and 9 to ^{3}H radioactivity incorporation into DNA duplex 1 [3].

[d]Cross-linking reactions were performed at the [enzyme] / [DNA duplex] ratio 1.6.

(b) Methylation

The substrate properties of the Crib*-containing DNA duplexes 2-7 strongly depended on the location of the oxidized disaccharide residue. DNA duplexes 6 and 7 containing the modified sugar residues in the sequences flanking the recognition site retained their ability to be methylated (TABLE 1). Substitution of dC in the recognition site by the oxidized disaccharide-containing analog (DNA duplexes 2-5) resulted in a significant loss of the *Mva*I Mtase activity. Similar effect of Tgal* incorporation into the *Mva*I recognition sequence or into the flanking nucleotide sequence (DNA duplexes 8 and 9, respectively) on M·*Mva*I activity has been observed[3].

(c) Binding

Binding of DNA duplexes 1-9 to M·*Mva*I as well as cross-linking experiments have been performed in the presence of the reaction product S-adenosyl-L-homocysteine (AdoHcy). This compound is known to facilitate the formation of specific complexes. Complex formation was monitored by the gel mobility shift assays.

M·*Mva*I can form two types of complexes with the canonical substrate 1 depending on the enzyme concentration (FIG. 1). With increase of the enzyme excess, the amount of a slower-running complex was increased (FIG. 1A, lanes 4 and 5). Only the faster-running or the slower-running M·*Mva*I-DNA duplex 1-AdoHcy complexes were formed with a 1.6-fold or a 9-fold molar excess of the enzyme, respectively (FIG. 1A, lane 5 and 1B, lane 1). Only the slower-running complex was formed at any enzyme concentrations between M·*Mva*I and DNA duplex 10 without the recognition site (FIG. 1A, lanes 2 and 3).

The slower-running M·*Mva*I-DNA-AdoHcy complexes are likely formed due to non-specific binding and the faster-running complexes are likely formed due to specific binding. The faster-running complex mobilities are similar to that of the ternary complex M·*Eco*RII-DNA duplex 1-AdoHcy

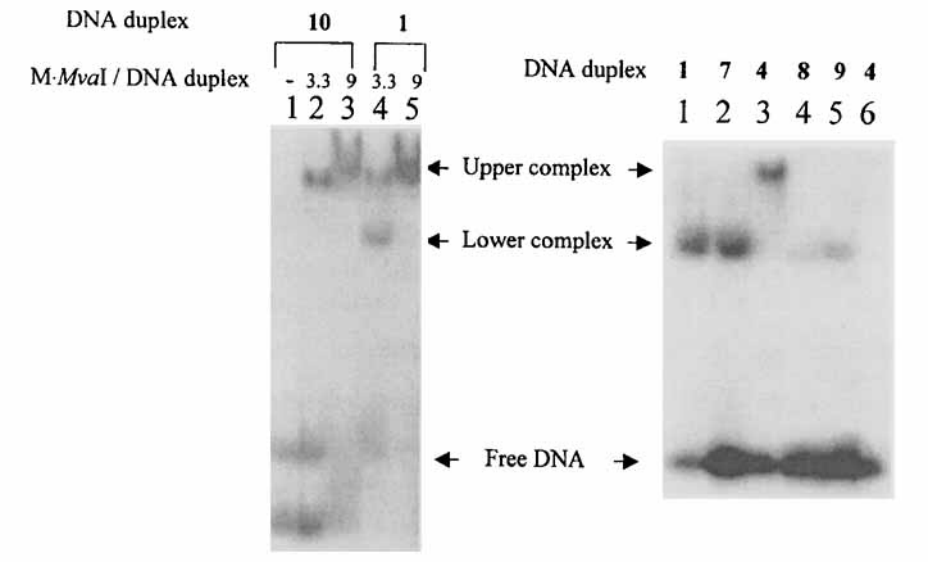


FIG. 1. Binding of DNA duplexes 1, 4 and 7-10 by M·*Mva*I in the presence of 0.1 mM AdoHcy. A: Binding of 0.35 μM of P^{32}-labeled DNA duplexes 10 and 1 with 1.16 and 3.2 μM of M·*Mva*I (lanes 2-3 and 4-5 respectively); lane 1: DNA duplex 10. B: Binding of P^{32}-labeled DNA duplex 1 and dialdehyde-containing DNA duplexes 4 and 7-9 (0.35 μM) by M·*Mva*I (0.58 μM) (lanes 1-5); lane 6: DNA duplex 4.

(data not shown). Molecular masses of *Mva*I and *Eco*RII Mtases are similar (about 54 kDa). One can suggest that the faster-running complex contains one molecule of M·*Mva*I bound to one molecule of DNA duplex. The slower-running complex probably contains two molecules of M·*Mva*I. It is known that some Mtases have the ability to form dimers and multimers at high concentrations[5-7]. Two types of complexes were observed by others in the case of Mtases *Msp*I and *Hha*I[5,8]. The authors confirmed that the faster-running complex was due to specific binding and the slower-running complex was due to non-specific binding.

Binding of modified DNA duplexes 2-9 to M·*Mva*I was studied under conditions when only the faster-running ternary complex was observed with

the canonical DNA duplex 1 (a 1.6-fold molar excess of M·*Mva*I). However, under these conditions modified substrates formed both types of complexes, and the ability to form each of them depended on the substrate properties of the duplexes. DNA duplexes 6 (data not shown) and 7 (FIG. 1B) which retained the ability to be methylated by M·*Mva*I formed only the faster-running complexes. DNA duplexes 3-5, which were poor substrates, formed the slower-running complexes (DNA duplex 4, FIG. 1B). DNA duplex 2 with an intermediate ability to be methylated by the enzyme gave two kinds of complexes (data not shown).

The Tgal*-containing DNA duplexes 8 and 9 formed only the faster-running complex with the 1.6-fold molar excess of the enzyme (FIG. 1B).

Only the slower-running complex was obtained at any enzyme concentrations with the Crib*-containing 21 mer duplex 11 lacking the *Mva*I recognition sequence (data not shown).

Thus, specific non-covalent complexes were formed between M·*Mva*I at the 1.6-fold molar excess and DNA duplexes 6 and 7, containing Crib* in the 3′-end flanking nucleotide sequence, adjacent to the recognition site (6) or separated from the 5′-end of the recognition site by two nucleotide residues (7) and DNA duplex 9 which contains Tgal* in the 3′-end flanking nucleotide sequence separated from the recognition site by a single nucleotide residue. All these DNA duplexes retained the ability to be methylated. DNA duplex 8 containing Tgal* in the center of the recognition site was almost not methylated by M·*Mva*I but also formed the specific non-covalent complex. These DNA duplexes were referred to group 1. This group was of prime importance for cross-linking experiments. DNA duplexes 3-5 with Crib* substituting for the "inner" or "outer" dC residues of the recognition site form non-specific non-covalent complexes with the 1.6-fold molar excess of M·*Mva*I. They lost the ability to be methylated by the enzyme. These DNA duplexes as well as DNA duplex 11 lacking the recognition sequence were referred to group 2.

(d) Cross-linking

To probe the M·*Mva*I region interacting with DNA, cross-linking of DNA duplexes 6-9 (group 1) to *Mva*I Mtase has been performed under the conditions favorable for the specific non-covalent complex formation. To compare the enzyme DNA binding sites in the faster-running (specific) and the slower-running (referred to as non-specific) complexes, affinity modification of M·*Mva*I by DNA duplexes 3-5 and 11 (group 2) was carried out under the same conditions.

DNA duplexes of groups 1 and 2 containing Crib* or Tgal* residues were cross-linked to the enzyme (TABLE 1, FIG. 2). Only small differences in reactivity were found between reagents 2-9 and 11. Introduction of dialdehyde group into DNA duplex 1 resulted in 2-6% cross-linking of total DNA (TABLE 1). However, the yields of cross-linking of dialdehyde-containing DNA duplexes to M·*Mva*I relative to bound DNA vary from 10 to 50%. In each case, one covalent DNA-enzyme conjugate was formed under any conditions. Molecular mass of the DNA-enzyme conjugates corresponded to total molecular mass of one M·*Mva*I subunit and the appropriate oligonucleotide strand. The cross-linking yields could be increased up to 12% relative to total DNA with the increase of the enzyme excess. However, in these cases the cross-linking reaction for DNA duplexes of group 1 would proceed under conditions favorable for the slower-running non-specific complex formation.

(C) Mapping the M·*Mva*I regions cross-linked to substrate analogs

One of the methods making possible determination of peptide fragments cross-linked to DNA is partial hydrolysis of the covalently bound enzyme in the oligonucleotide-enzyme conjugates with chemical reagents under conditions of the "single-hit cleavage" followed by analysis of the oligonucleotide-peptide conjugates obtained[9].

This method was used for the analysis of the M·*Mva*I conjugates with modified DNA duplexes. Several chemical reagents, such as, 2-nitro-5-thiocyanobenzoyc acid (NTCBA) and N-chlorosuccinimide (NClS) cleaving peptide bonds at cysteine or tryptophan residues, respectively, were used (FIG. 3A). *Mva*I Mtase has one cysteine and four tryptophans.

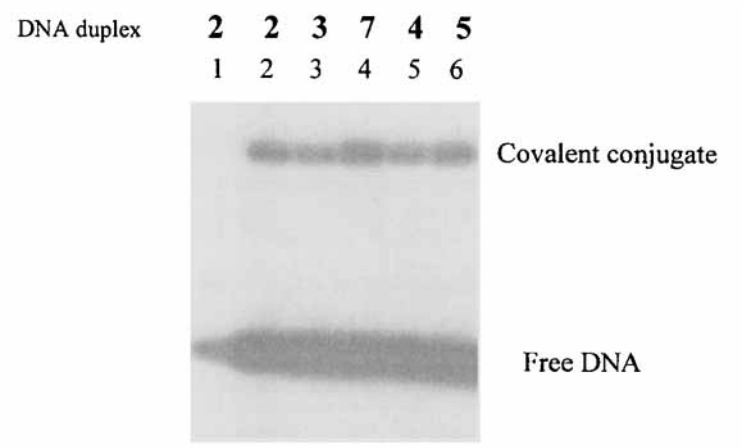


FIG. 2. Cross-linking of M·*MvaI* to DNA duplexes 1-5 and 7 in the presence of 0.1 mM AdoHcy. 10% SDS PAGE of the reduced reaction mixtures contaning P^{32}-labeled DNA duplexes 2-5 and 7 (0.35 μM) and M·*MvaI* (0.58 μM) (lanes 2-6); control: DNA duplex 2 (lane 1).

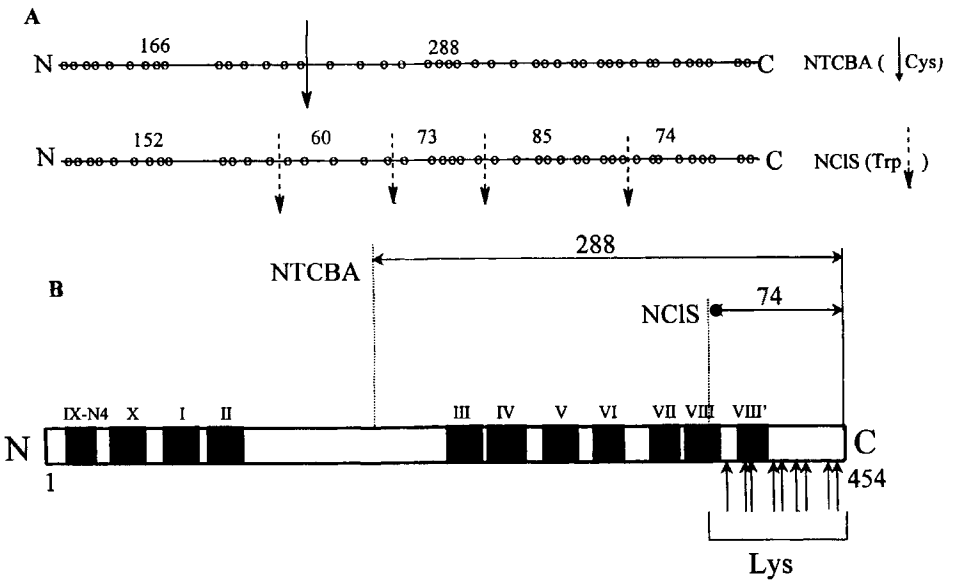


FIG. 3. Mapping the M·*MvaI* regions cross-linked to the dialdehyde-containing substrate analogs. A: Schematic presentation of M·*MvaI* cleavage with NTCBA and NClS; o – Lys residues. B: The cross-linking region identification by the results of hydrolysis with NTCBA and NClS; conserved motifs (I-X) and numbers of amino acid residues of M·*MvaI* are indicated.

First, the cross-linked products formed by M·*Mva*I and the Crib*-containing DNA duplexes of group 1 were analyzed. After cleavage of the M·*Mva*I-DNA duplex 6 conjugate with NTCBA, a single oligonucleotide-peptide conjugate was obtained corresponding to the C terminal enzyme region between Cys^{167} and Ser^{454} (FIG. 3B). The hydrolysis of the M·*Mva*I-DNA duplex 6 conjugate with NClS under conditions of "single-hit cleavage" resulted in four essential oligonucleotide-peptide conjugates (FIG. 4B). The theoretical cleavage patterns of the oligonucleotide-peptide conjugates strongly depend on the location of the cross-link. Comparison of the products obtained with the theoretical cleavage patterns (FIG. 4A) allows us to map the cross-link between Val^{381} and Ser^{454} (FIG. 3B). The same cleavage patterns of the cross-linked product of M·*Mva*I and DNA duplex 7 with NTCBA or NClS were obtained.

In the case of the M·*Mva*I covalent conjugates with the Tgal*-containing DNA duplexes 8 and 9, the single-hit cleavage at Cys or Trp residues was performed. Partial hydrolysis with NClS or NTCBA of the cross-linked products of M·*Mva*I and the Tgal*-containing DNA duplexes 8 and 9 resulted in the same cleavage patterns as those for DNA duplexes 6 and 7. Thus, in the case of DNA duplexes of group 1 the cross-linking site is located within the same amino acid segment in the C terminus of the protein.

Then, the covalent conjugates of *Mva*I Mtase with DNA duplexes of group 2 were investigated. The observed patterns of partial cleavage with NTCBA or NClS coincided with those for the cross-linked products with DNA duplexes of group 1. These data suggest that DNA interacts with the same C terminal region of M·*Mva*I both in the cases of specific and non-specific complexes. One can suppose that this *Mva*I region is involved in the interaction with the DNA sugar-phosphate backbone.

Some predictions concerning the structure of the DNA binding site of M·*Mva*I may be done based on N^4C Mtases amino acid sequence alignment[10] and X-ray structure of N^4C Mtase *Pvu*II[2]. According to the recent amino acid sequence alignment of 37 N^4C Mtases which takes into account the data on X-ray structures of N^6-adenine Mtase *Dpn*M[11] and N^4C Mtase *Pvu*II[2] nine sequence conserved motifs I-VIII and X were identified[10]. In addition, weakly conserved motif IX (referred to as IX-N4) was determined (FIG. 3B). N-methylases are subdivided into three groups (α, β and γ) depending on the order of the conserved amino acid motifs. The most of N^4C Mtases

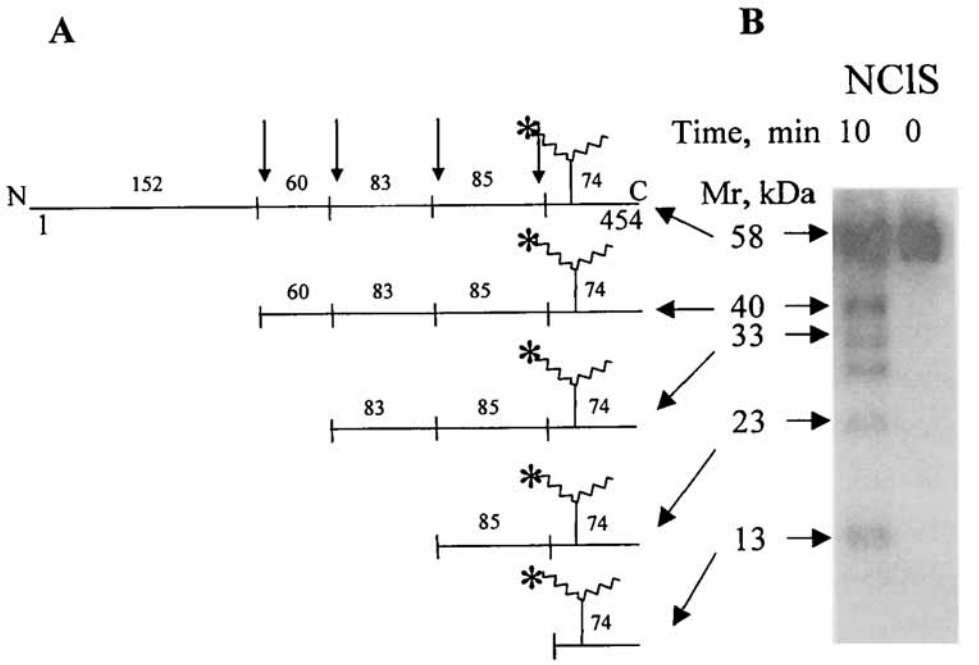


FIG. 4. Partial hydrolysis of the cross-linked M·*Mva*I-DNA duplex 6 complex with NClS. A: The theoretical set of oligonucleotide-peptides which should be obtained after hydrolysis with NClS if the oligonucleotide is cross-linked to the C terminus of M·*Mva*I. Vertical bars represent the sites of cleavage with NClS. The numbers of amino acid residues are indicated. B: The autoradiogram of 10% SDS-PAGE showing products of the M·*Mva*I-DNA duplex 6 conjugate partial hydrolysis with NClS for 0 and 10 min.

including *Pvu*II Mtase belongs to group β. M·*Mva*I as well as M·*Dpn*M belongs to group α of N-methylases. However, amino acid sequences of the *Mva*I individual conserved motifs more closely resemble amino acid sequences of the corresponding motifs of N^4C methylases of group β. According to the crystal structure of the N^4C Mtase *Pvu*II complex with AdoMet, the M·*Pvu*II polypeptide chain is folded into a structure with a V-shaped cleft where probably DNA binds[2]. The docking model of M·*Pvu*II-DNA complex shows that this and other N^4C methylases do not maintain DNA-recognizing elements in one distinct domain but gain target specificity by extension of flexible loops accommodating DNA in a V-shaped cleft[2,10]. Several loops containing a large number of positively charged amino acid residues, Lys and Arg, capable of interacting with the

DNA phosphate backbone are on the surface of this cleft[2]. One of these loops is localized between antiparallel β-strands (an antiparallel β-hairpin is formed). For N^4C Mtases, an antiparallel β-hairpin composes the conserved motif VIII[10]. This motif is subdivided into two submotifs VIII and VIII′ corresponding to either of the β-strands. The intervening loop corresponds to a variable region between submotifs VIII and VIII'. It was suggested for structurally characterized N-methylases (M·*Dpn*M and M·*Pvu*II) that this antiparallel β-hairpin besides the DNA backbone binding forms a part of a target nucleotide binding pocket[2,11]. Amino acid sequence of the M·*Mva*I cross-linking region found in this study corresponds to the conserved motif VIII and a variable region in the C terminus of M·*Mva*I (FIG. 3B). Based on the *Pvu*II structure we can suggest that the DNA binding region of M·*Mva*I contains a β-hairpin of the motif VIII. This M·*Mva*I region is rich with Lys residues.

In summary, the seventy four amino acid region Val^{381} -Ser^{454} in the C terminus of Mtase *Mva*I interacting with the DNA sugar-phosphate backbone has been proposed by chemical cross-linking (FIG. 3B). Both specific and non-specific DNA bind in the same DNA binding cleft. This is the first experimental probing the DNA binding region among N^4-cytosine methylases.

EXPERIMENTAL

Enzymes. T4 polynucleotide kinase was purchased from MBI Fermentas (Lithuania). DNA-methyltransferase *Mva*I (1.8 mg/ml) was purified by Dr. S.Klimašauskas.

N^4-Benzoyl-1-[5-O-dimethoxytrityl-2-O-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)-β-D-ribofuranosyl]cytosine. Following coevaporation with anhydrous pyridine an amount of 710 mg (1.03 mmol) of *N*^{4}-Benzoyl-1-[2-*O*-(2,3,5-tri-*O*-benzoyl-β-D-ribofuranosyl)-β-D-ribofuranosyl]cytosine[12] was dissolved in 30 mL of pyridine and dimethoxytrityl chloride (420 mg, 1.24 mmol) was added. The mixture was stirred for 21 h at ambient temperature when TLC (CH_2Cl_2-MeOH 95:5, Rf = 0.74) indicated the reaction to be completed. Following neutralization with aqueous sodium bicarbonate, the mixture was concentrated and partitioned twice between dichloromethane and aqueous sodium bicarbonate. The organic layer was purified on 35 g of silica gel with a methanol gradient (0 to 1%) in dichloromethane containing 0.2% of pyridine, affording 910 mg (0.83 mmol,

81%) of the title compound as a foam. For $C_{63}H_{55}N_3O_{15}$ calc. 1093.3633. LSIMS neg. (NBA) m/z 1092 ($M-H^+$, 5), 303 (DMTr, 100). ^1H NMR ($CDCl_3$): δ (ppm) 3.15 (brs, OH), 3.45 (m, 2H, H5'a, H5'b), 3.84 (s, 6H, 2xCH_3O), 4.02 (m, 1H, H4'), 4.45-4.90 (m, 5H, H2', H3', H4", H5"a, H5"b), 5.84-5.97 (m, 3H, H1", H2", H3"), 6.25 (s, 1H, H1'), 6.90 (d, J = 9 Hz, 4H, arom-H), 7.15-8.10 (m, arom-H, H5), 8.53 (d, J = 8 Hz, 1H, H-6), 8.65 (brs, 1H, NH). ^{13}C NMR ($CDCl_3$): δ (ppm) 165.9, 165.4 (4xCO), 162.3 (C4), 154.6 (C2), 149.7 (C6), 107.0 (C1"), 96.5 (C5), 89.3 (C1'), 87.0 (Ph_3C), 82.8, 82.3 (C4', C4"), 79.7 (C2'), 76.2 (C2"), 72.3 (C3"), 67.9 (C3'), 64.2 (C5"), 60.8 (C5'), 55.2 (2xCH_3O) + aromatic signals.

N^4-Benzoyl-1-[5-O-dimethoxytrityl-3-O-(β-cyanoethyl-N,N-diisopropylaminophosphinyl)-2-O-(2,3,5-tri-O-benzoyl-β-D-ribofuranosyl)-β-D-ribofuranosyl]cytosine (the synton for oligonucleotide synthesis). The dimethoxytritylated derivative (860 mg, 0.78 mmol) was dissolved in 10 mL dichloromethane under argon and diisopropylethylamine (0.41 mL, 2.36 mmol) and 2-cyanoethyl N,N-diisopropylchlorophosphoramidite (0.26 mL, 1.18 mmol) were added and the solution was stirred for 30 minutes when TLC indicated complete reaction. Ethanol (4 mL) was added, the solution was stirred for 10 min. and partitioned between dichloromethane (50 mL) and aqueous $NaHCO_3$ (30 mL). The organic phase was washed with aqueous sodium chloride (3x30 mL) and evaporation of the organics left an oil which was flash purified on 40 g of silica gel (hexane: acetone: TEA, 58:40:2) to afford the product as a foam after coevaporation with dichloromethane. Dissolution in 2 mL of dichloromethane and precipitation in 60 mL cold (-70°C) hexane afforded 802 mg (0.62 mmol, 79%) of the title product as a white powder. Rf (hexane: acetone: TEA 49:49:2): 0.53. For $C_{72}H_{72}N_5O_{16}P$ calc. 1293.4712. LSIMS (NBA) m/z 1294 ($M+H^+$, 1), 303 (DMTr, 100). ^1H NMR ($CDCl_3$): δ (ppm) 1.01-1.15 (m, NC$\underline{CH_3}$), 2.35 (t) and 2.55-2.71 (m) (2H, $\underline{CH_2}$CN), 3.35 (dt) and 3.46-3.91 (m, H5'a, H5'b, CH_3O, NCH, $POCH_2$), 4.17 (m, 1H, H4'), 4.45-4.61 and 4.70-4.77 (m, 5H, H2', H3', H4", H5"a, H5"b), 5.76 (2d, 1H, J = 4.7Hz, H2"), 5.85 (m, 1H, H3"), 5.88, 5.92 (2s, 1H, H1"), 6.21, 6.27 (2s, 1H, H1'), 6.85 (2 d, 4H, arom-H), 7.18-7.57 (m, 22H, arom-H, H5), 7.78-8.00 (m, 9H, arom-H,

H6), 8.45, 8.51 (2 d, 1H, NH). ^{13}C NMR ($CDCl_3$): δ (ppm) 166.0, 165.2, 165.1, 165.0 (4xCO), 162.2 (C4), 154.6 (C2), 145.0 (C6), 117.7, 117.3 (CN), 106.1, 105.9 (C1"), 96.2 (C5), 90.1, 89.8 (C1'), 87.1 (Ph_3C), 81.8, 80.7, 80.4 (C4', C4"), 79.2, 79.0 (C2'), 75.9, 75.5 (C2"), 72.5, 72.4 (C3"), 69.4, 68.9 (2d, J=14.6Hz, C3'), 65.0, 64.8 (C5"), 60.6, 60.3 (C5'), 58.1, 58.0 (2d, J=19.3Hz, $POCH_2$), 55.2 (2xCH_3O), 43.4 (d, J=12.5Hz, NCH), 24.7-24.3 (NCMe), 20.2, 20.1 (2xd, J=7.5Hz, CH_2CN), + aromatic signals. ^{31}P NMR d (ppm, external ref. = H_3PO_4 capil.) 150.53, 151.73

Oligonucleotide synthesis. Synthesis of oligonucleotides containing 2'-O-β-D-ribofuranosylcytidine was performed on an ABI 392 synthesizer at 1 mmol scale using commercial 2-cyanoethylphosphoroamidites and standard methodology, but for a longer coupling time (80 sec) and a higher concentration (0.15 M) for disaccharide amidite to ensure high coupling yields. The oligonucleotides obtained were deprotected and removed from the solid support by concentrated ammonia (55°C, 16 h) and purified by ion-exchange on a MonoQ column using NaCl gradient (0.3-0.7 M) in 10 mM NaOH and reverse phase HPLC on Nucleosil 100 C18, 5 m column (4 x 250 mm) using MeCN concentration gradient (10%-20% of MeCN in 25 min) in 0.1 M triethylammonium acetate, pH 6.9 with subsequent gel filtration of the products on the Toyopearl HW-40 column in water. The oligonucleotide structure was proved by MALDI spectra.

Synthesis of oligonucleotide derivatives containing the regiospecifically incorporated 1-(β-D-galactopyranosyl)thymine has been performed as described[3].

Oxidation of the 2'-O-β-D-ribofuranosylcytidine or 1-(β-D-galactopyranosyl)thymine containing oligonucleotides was carried out for 1.5 h at 37°C in 20 mM or 50 mM $NaIO_4$ solution, respectively.

Determination of T_m values. The thermal melting curves of DNA duplexes 1-7 were obtained in 40 mM Tris-HCl, pH 7.0, 15 mM $MgCl_2$ (buffer A) and 50 mM Tris-HCl, pH 9.0, 20 mM NaCl (buffer B) using a Hitachi 150-20 spectrophotometer (Japan); concentration per duplex (C_d) 1 μM.

Gel mobility-shift assay. M·*Mva*I (0.29-3.2 μM) was incubated with the ^{32}P-labeled DNA duplexes 1-9 (0.35 μM) in 10 μl of 50 mM Tris-HCl, pH 9.0, 20 mM NaCl, 1 mM DTT, 100 μg/ml BSA (buffer C) containing 5% glycerol and 0.1 mM AdoHcy at room

temperature for 5 min and at 0°C for 15 min. Complex formation was monitored by the gel retardation analysis [3].

Methylation assay. The efficiency of methylation was monitored by the radioactivity ($C[^3H]_3$) incorporation into DNA duplexes 1-9 using DE 81 filters[3]. Methylation reactions were carried out at 10°C for 1, 2, 4, 8, 16 and 30 min in 10μl reaction mixtures containing buffer C, 1μCi of [methyl-^{3}H]-AdoMet (Amersham, 15 Ci/ mmole), 0.35 μM of DNA duplexes 1-9 and 0.58 μM of *Mva*I Mtase. The amount of methylation per minute was computed as: $V_o\ (^{nM}/_{min}) = ((cpm_t - cpm_0) \bullet [AdoMet]) / (cpm_{100} \bullet t)$,

where cpm_t – value of ^{3}H radioactivity incorporation into DNA after methylation reaction for t min (with washing procedure); cpm_0 and cpm_{100} – blank values of ^{3}H radioactivity incorporation (without enzyme) with or without washing procedure, respectively.

Cross-linking. Cross-linking of M·*Mva*I (0.58 or 3.2 μM) to duplexes 2-9 and 11 (0.35 μ M) was performed in 10 μl of buffer C containing 0.1mM AdoHcy at room temperature for 5 min and at 0°C for 15 min. 6 μl of 2 mM $NaBH_4$ was added and reaction mixtures were kept at 0°C for 40 min. Reactions were followed by 10% SDS-polyacrylamide gel electrophoresis (PAGE)[3]. The cross-linking yield was determined as the ratio of the covalent conjugate radioactivity to total radioactivity of the conjugate and unbound DNA. The cross-linked products were excised from the gel and eluted with 2 vol of 1% SDS at 37°C for 5-7 h.

Mapping the cross-links. Partial cleavage with NTCBA or NClS of M·*Mva*I conjugates with DNA duplexes 2-9 and 11 was performed as described[9,13]. The cleavage products were fractionated by 10% SDS-PAGE in Tris-glycine buffer system, identified by comparing with calibration protein standards, and compared with masses of the theoretical cleavage products, which were calculated as sum of the oligonucleotide and the corresponding peptide masses.

Acknowledgements

This research was supported by the Howard Hughes Medical Institute (HHMI 75195-545500), NATO (PST.CLG.976569), Russian Foundation for Basic Research (98-04-49108 and 99-04-49243), and INTAS (INTAS-97-11635). We thank Dr. S. Klimašauskas for generous gift of methylase *Mva*I. We are grateful to Dr. M.G. Brevnov for his

continuous support. A.V.A. is a research associate of the Fund for Scientific Research of Flanders.

REFERENCES

1. Butkus, V.; Klimašauskas, S.; Keršulytė, D.; Vaitkevièius, D.; Algimantas, L.; Janulaitis, A. *Nucleic Acids Res.* **1985**, *13*, 5727-5746.
2. Gong, W.; O'Gara, M.; Blumenthal, R.M.; Cheng, X. *Nucleic Acids Res.* **1997**, *25*, 2702-2715.
3. Brevnov, M.G.; Gritsenko, O.M.; Mikhailov, S.N.; Efimtseva, E.V.; Ermolinsky, B.S.; Van Aerschot, A.; Herdewijn, P.; Repyk, A.V.; Gromova, E.S. *Nucleic Acids Res.* **1997**, *25*, 3302-3309.
4. Tunitskaya, V.L.; Rusakova, E.E.; Memelova, L.V.; Kochetkov, S.N.; Van Aerschot, A.; Herdewijn, P.; Efimtseva, E.V.; Ermolinsky, B.S.; Mikhailov, S.N. *FEBS Lett.* **1999**, *442*, 20-24.
5. Dubey, A.K.; Roberts, R.J. *Nucleic Acids Res.* **1992**, *12*, 3167-3173.
6. Karyagina, A.; Shilov, I.; Tashlitskii, V.; Khodoun, M.; Vasil'ev, S.; Lau, P.C.K.; Nikolskaya, I. *Nucleic Acids Res.* **1997**, *25*, 2114-2120.
7. O'Gara, M.; Zhang, X.; Roberts, R.J.; Cheng, X. *J. Mol. Biol.* **1999**, *287*, 201-209.
8. Klimašauskas, S.; Roberts, R.J. *Nucleic Acids Res.* **1995**, *23*, 1388-1395.
9. Grachev, M.A.; Lukhtanov, E.A.; Mustaev, A.A.; Zaychikov, E.F.; Abdukayumov, M.N.; Rabinov, I.V.; Skoblov, Y.S.; Chistyakov, P.G. *Eur. J. Biochem.* **1989**, *180*, 577-585.
10. Bujnicki, J.M.; Radlinska, M. *Nucleic Acids Res.* **1999**, *27*, 4501-4509.
11. Tran, P.H.; Korszun, Z.R.; Cerritelli, S.; Springhorm, S.S.; Lacks, S.A. *Structure* **1998**, *6*, 1563-1575.
12. Mikhailov, S.N.; Efimtseva, E.V.; Gurskaya, G.V.; Zavodnik, V.E.; De Bruyn, A.; Rozenski, J.; Herdewijn, P. *J. Carbohydr. Chem.*, **1997**, *16*, 75-92.
13. Grachev, M.A.; Kolocheva, T.I.; Lukhtanov, E.A.; Mustaev, A.A. *Eur. J. Biochem.* **1987**, *163*, 113-121.