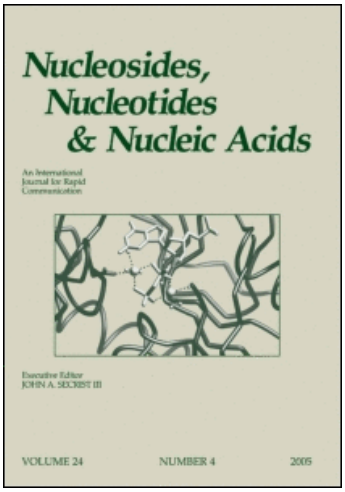

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AFFINITY MODIFICATION OF *Eco*RII DNA METHYLTRANSFERASE BY THE DIALDEHYDE-SUBSTITUTED DNA DUPLEXES: MAPPING THE ENZYME REGION THAT INTERACTS WITH DNA

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ABSTRACT

Affinity modification of *Eco*RII DNA methyltransferase (M·*Eco*RII) by DNA duplexes containing oxidized 2′-O-β-D-ribofuranosylcytidine (Crib*) or 1-(β-D-galactopyranosyl)thymine (Tgal*) residues was performed. Cross-linking yields do not change irrespective of whether active Crib* replaces an outer or an inner (target) deoxycytidine within the *Eco*RII recognition site. Chemical hydrolysis of M·*Eco*RII in the covalent cross-linked complex with the Tgal*-substituted DNA indicates the region Gly^{268}-Met^{391} of the methylase that is likely to interact with the

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DNA sugar-phosphate backbone. Both specific and non-specific DNA interact with the same M·*Eco*RII region. Our results support the theoretically predicted DNA binding region of M·*Eco*RII.

INTRODUCTION

C5-cytosine DNA methyltransferases (C5-Mtases) catalyze the transfer of a methyl group from S-adenosyl-L-methionine (AdoMet) to C5 carbon of cytosine within the recognition sequence of DNA. Methylation in bacteria serves to protect the DNA against restriction endonucleases. In eukaryotes DNA methylation plays an important role in the control of a number of cellular processes such as gene expression, genomic imprinting, etc. Three-dimensional structures are available for the C5-Mtases *Hha*I[1] and *Hae*III[2] in complex with DNA. Recently the crystal structure of the human C5-Mtase homologue DNMT2 has been determined.[3] These enzymes consist of two domains forming a positively charged cleft where DNA binds. Eukaryotic and prokaryotic enzymes share a set of ten conserved motifs.[4] A large variable region between motifs VIII and IX which lies in a small domain, according to resolved crystal structures of Mtases *Hha*I[1] and *Hae*III,[2] is supposed to serve to define the sequence specificity of C5-Mtases. An important feature of the C5-Mtase-DNA complexes is an extrahelical position of a target cytosine which is placed into the catalytic pocket in the large domain of Mtases.[1,2]

*Eco*RII DNA methyltransferase (M·*Eco*RII) recognizes the DNA sequence 5′...C<u>C</u>A/TGG...3′ and methylates carbon atom C5 of the inner cytosine residue (<u>C</u>). The primary structure of M·*Eco*RII was determined.[5] M·*Eco*RII has been found to contain a conserved Pro-Cys motif; the cysteine residue (Cys186) is required for methyl transfer.[6–8] It has been shown by photoaffinity modification of M·*Eco*RII that the cofactor is covalently attached to the same cysteine residue.[9] M·*Eco*RII discriminates between its canonical recognition site and the site containing a G/C base pair in the center of the recognition sequence by interactions in the DNA minor groove.[10] Both crystal structure of M·*Eco*RII and the protein region involved in DNA binding have not yet been determined.

To identify contacts of restriction-modification enzymes with DNA, affinity modification of the enzymes by modified substrates, containing sugar moieties with aldehyde groups, can be used.[11,12] Dialdehyde-substituted nucleic acids can specifically join to the proximal lysine residues of the protein. In contrast to the photoaffinity modification of proteins by DNA with a photoactive group which proceeds under irradiation, no degradation of protein or DNA has been observed in conditions of chemical cross-linking with dialdehyde-substituted substrate analogs.[11–13] It was shown that

*Eco*RII and *Mva*I Mtases and *Eco*RII endonuclease can be covalently joined to DNA containing oxidized galactose residues.[11] The region of N4-Mtase *Mva*I interacting with the DNA sugar-phosphate backbone has been determined using affinity modification of the Mtase by the dialdehyde-substituted substrates.[12] In addition, DNA duplexes with aldehyde groups have been employed for cross-linking to T7 RNA polymerase.[13]

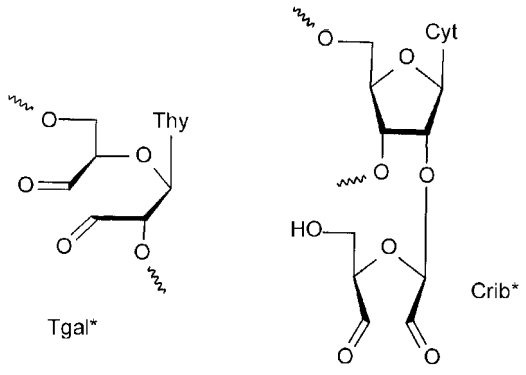
In this work, affinity modification of *Eco*RII Mtase with two types of the dialdehyde-substituted substrate analogs was studied. The region of M·*Eco*RII interacting with the DNA sugar-phosphate backbone was determined.

RESULTS AND DISCUSSION

Substrate Analogs Used for Cross-linking to M·*Eco*RII

In order to obtain an aldehyde group in any position of the oligonucleotide, an additional *cis* diol group has been introduced into sugar residues. Cytidine or thymidine residues in oligonucleotides have been replaced with 2′-O-β-D-ribofuranosylcytidine (Crib)[12] or 1-(β-D-galactopyranosyl)thymine (Tgal),[11] respectively. After periodate oxidation, oligonucleotides with oxidized Crib and Tgal residues (Crib* and Tgal*, respectively) were obtained.

In this way, chemically active aldehyde groups were introduced into the M·*Eco*RII recognition site (duplexes **2**–**5** and **8**) or into the 3′ (duplexes **6** and **9**) or 5′ (duplex **7**) end flanking nucleotide sequences (Table 1). Each pyrimidine nucleoside of the recognition site was replaced by an oxidized disaccharide (duplexes **2**–**5**) or oxidized galactose (duplex **8**) containing nucleoside. Crib* replaced both the outer and the inner (target) dC residues.



Scheme 1. Dialdehyde derivatives of nucleosides.

Table 1. Properties of the Crib*-Containing DNA Duplexes as Substrates of M·*Eco*RII. Cross-linking of M·*Eco*RII to DNA Duplexes Containing Crib* or Tgal* Residues

DNA Duplex[a]	Relative V_0[b]	Relative Binding Affinity[c]	Cross-linking Yield, %
1 5′- GCCAA**CCTGG**CTCT 3′- CGGTT**GGACC**GAGA	1	1	–
2 5′- GCCAA**X**CTGGCTCT 3′- CGGTT**GGACC**GAGA	0.62 ± 0.14	0.1	4
3 5′- GCCAAC**X**TGGCTCT 3′- CGGTT**GGACC**GAGA	0.90 ± 0.10	0.2	5
4 5′- GCCAA**CCTGG**CTCT 3′- CGGTT**GGAC**XGAGA	0.46 ± 0.10	0.1	5
5 5′- GCCAA**CCTGG**CTCT 3′- CGGTT**GGA**X**C**GAGA	0.66 ± 0.10	0.2	3
6 5′- GCCAA**CCTGG**XTCT 3′- CGGTT**GGACC**GAGA	0.45 ± 0.15	0.1	4
7 5′- GCXAA**CCTGG**CTCT 3′- CGGTT**GGACC**GAGA	0.95 ± 0.05	0.1	3
8 5′- GCCAA**CCY**GGCTCT 3′- CGGTT**GGACC**GAGA	–	–	10[d]
9 5′- GCCAA**CCTGG**CYCT 3′- CGGTT**GGACC**GAGA	–	–	15[d]
10 5′- TAATACGACTCACTAYAG 3′- ATTATGCTGAGTGATATC	–	–	6

[a]X = Crib*; Y = Tgal*.
[b]Defined as the ratio of V_o of DNA duplexes **2**–**7** to V_o of DNA duplex **1**. For methylation conditions see ''Experimental Section''.
[c]Defined as normalized values of the efficiencies of non-covalent complexes [M·*Eco*RII-AdoHcy-DNA duplex **2**–**7**] formation as described in ''Experimental Section''.
[d]The covalent joining of Tgal*-substituted DNA-duplexes **8** and **9** to M·*Eco*RII was reported in.[11] The cross-linking yields for **8** and **9** adduced in Table 1 were determined in slightly different conditions (see Experimental Section).

The two types of modifications differ in size, representing ''extended'' (Crib*) or ''zero-length'' (Tgal*) labels. An 18-mer Tgal*-substituted DNA duplex lacking the recognition site (duplex **10**) was also used.

Incorporation of Crib* produced only a slight (3–4°) destabilization effect on double-stranded structure.[12] T_m data for the Tgal*-substituted DNA duplexes were not determined due to their decomposition at high temperatures.[11]

Our goal was to compare cross-linking yields as a function of location of aldehyde groups in the DNA substrate (in the case of Crib*-substituted DNA duplexes) and as a function of the type of modification (Crib* or Tgal*) following by mapping of the cross-linking region in the DNA-Mtase conjugates obtained with the highest yields.

DNA Binding and Methylation

The ability of DNA duplexes with aldehyde groups to specifically bind to M·*Eco*RII and their substrate properties have been evaluated. The formation of specific complexes of M·*Eco*RII with DNA duplexes **1**–**7** has been examined in the presence of the cofactor analogue S-adenosyl-L-homocysteine (AdoHcy). The formation of ternary complexes was monitored by the gel mobility shift assay. M·*Eco*RII binds to the modified DNA duplexes containing Crib* in the *Eco*RII recognition site (DNA duplexes **2**–**5**) or in the flanking nucleotide sequences (DNA duplexes **6**–**7**) with 10–20% affinity as compared to canonical DNA duplex **1** (Table 1). At the same time, incorporation of Crib* into the DNA substrates **2**–**7** does not strongly influence their ability to be methylated (Table 1). This suggests that conformational constraints of the sugar moiety of the oxidized disaccharide nucleoside residues become apparent rather at the DNA binding than at the catalytic step. The behavior of Crib*-substituted DNA duplexes differs from the previously studied Tgal* compounds. It was shown that M·*Eco*RII has high affinity for Tgal*-substituted DNA duplexes **8** and **9** which is comparable to the affinity for canonical DNA duplex **1**.[11] The DNA duplex with an oxidized galactose residue in the flanking nucleotide sequence (duplex **9**) retained its ability to be methylated while the DNA duplex with Tgal* in the center of the recognition site (duplex **8**) was not methylated.[11]

Cross-linking

Cross-linking of M·*Eco*RII to substrate analogs **2**–**7** containing oxidized disaccharide residues was performed in the presence of AdoHcy under conditions used for complex formation followed by the DNA-Mtase conjugate reduction with $NaBH_4$ according to the procedure developed earlier.[11] The DNA-duplexes with aldehyde groups are likely to react primarily with proximal lysine residues of restriction-modification enzymes.[14]

Covalent attachment of Crib*-substituted DNA duplexes **2**–**7** to M·*Eco*RII was monitored by 10% SDS PAGE (see Experimental Section). In all cases low cross-linking yields were observed (Table 1). Only small differences in cross-linking yields were found (Table 1). Therefore, the efficiencies of cross-linking do not change irrespective of whether active Crib* replaced outer or inner (target) deoxycytidine. This suggests that the ability of the Crib*-substituted DNA duplexes to form cross-linked complexes with M·*Eco*RII does not relate to a possible flipping out of the target cytosine from the DNA double helix but rather concerns the interaction of the M·*Eco*RII with the DNA sugar-phosphate backbone. Low cross-linking yields may be attributed to the reduced binding affinities of the Crib*-substituted DNA duplexes to M·*Eco*RII. Previously, covalent joining of M·*Eco*RII to the DNA

duplexes containing Tgal* residue in the central position of the recognition site (duplex **8**) or in the flanking sequence (duplex **9**) was performed.[11] Cross-linking yields in this case were higher than those for the Crib*-substituted DNA duplexes (Table 1). The Tgal*-substituted DNA duplex **10** lacking the recognition site was also cross-linked to M·*Eco*RII but with a lower yield as compared to specific Tgal*-substituted DNA duplexes (Table 1).

To determine the M·*Eco*RII regions cross-linked to the dialdehyde-substituted DNA, we used the Tgal*-substituted DNA duplexes **8**–**9** because of the higher conjugate yields. Usually, determination of peptide fragments cross-linked to DNA includes enzymatic or chemical hydrolysis of the covalently bound enzyme in the oligonucleotide-enzyme conjugate followed by analysis of the obtained oligonucleotide-peptides. Covalent conjugates of M·*Eco*RII with DNA duplex **8** or **9** were obtained and the appropriate oligonucleotide-Mtase conjugates were subjected to chemical fragmentation by cyanogen bromide (BrCN) that cleaves peptide bonds of proteins at methionine residues. Chemical hydrolysis was performed under conditions of partial (Fig. 1 A, B) or complete (Fig. 1 C) cleavage of covalently bound M·*Eco*RII depending on time, temperature, and BrCN concentration. In the case of DNA duplex **8** the partial hydrolysis of the oligonucleotide-M·*Eco*RII conjugate under moderate conditions resulted in several oligonucleotide-peptides, the largest fraction of starting covalent complex being uncleaved (Fig. 1A). The hydrolysis of the conjugate under more severe conditions resulted in the appearance of additional oligonucleotide-peptides (Fig. 1B, line 1) and in disappearance of the starting covalent complex and accumulation of an oligonucleotide-peptide with molecular mass of 19 kDa (Fig. 1B, line 2). Finally, in the case of complete hydrolysis essentially only the 19 kDa product was observed (Fig. 1C).

Theoretically, cleavage of M·*Eco*RII-DNA conjugate can give seven sets of oligonucleotide-peptides depending on the location of the cross-link in the Mtase. Taking into account that Lys residues are the primary sites of the modification, only four theoretical cleavage patterns can be considered (an oligonucleotide may cross-link to one of the regions Ser^{2}-Met^{82}, Leu^{83}-Met^{251}, Gly^{268}-Met^{391}, or Gly^{421}-Ile^{477}). Figure 2 shows all possible oligonucleotide-peptides which can be derived from the covalent complex of M·*Eco*RII with DNA duplex 8, if cross-linking occurs at the region Gly^{268}-Met^{391}. Comparison of the products observed (Fig. 1) with the theoretical cleavage patterns permits mapping the cross-link between Met^{267} and Met^{391} (Fig. 3). It should be noted that the 19 kDa product of complete hydrolysis can be obtained only if the dialdehyde-substituted oligonucleotide had been cross-linked to this region. If one assumes other cross-linking sites, products of complete hydrolysis with masses 11, 14 or 23 kDa would be obtained.

The data on chemical cleavage of the conjugate M·*Eco*RII-DNA duplex **9** completely coincides with those for M·*Eco*RII-DNA duplex **8**. We also carried out partial and complete hydrolysis with BrCN of the covalent

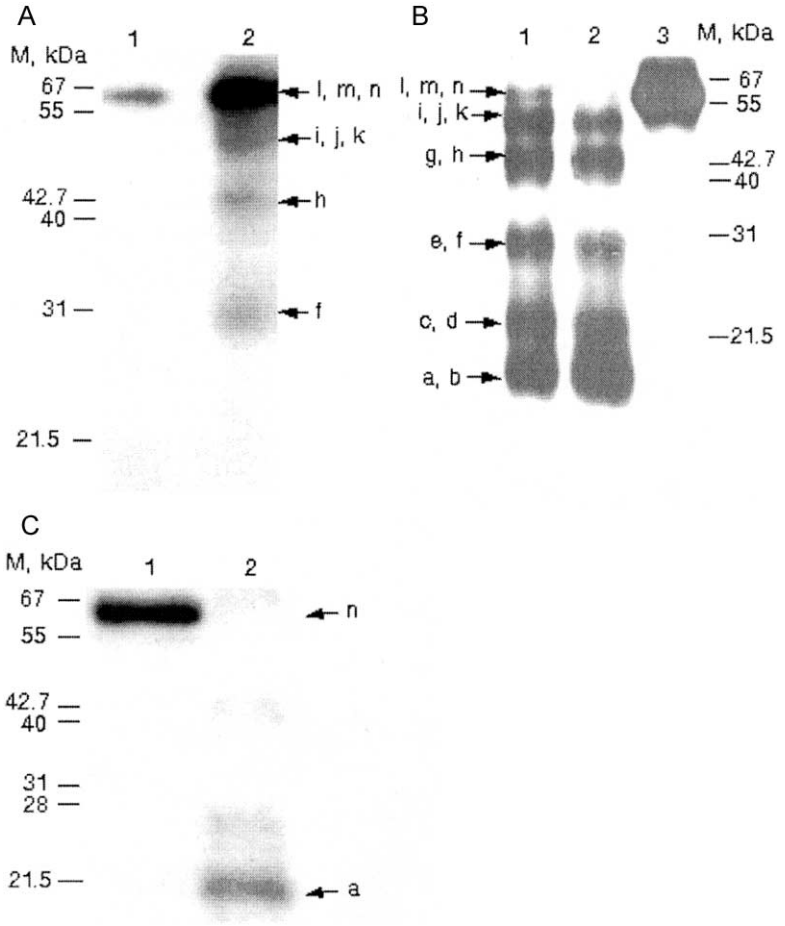


Figure 1. Hydrolysis with BrCN of the cross-linked complex of M·*Eco*RII with GCCAACCYGGCTCT (the modified strand of DNA duplex **8**). An autoradiogram of 10% SDS-PAGE showing products of hydrolysis by (A) 0.05 M BrCN at 25°C for 0 and 10 min (lanes 1 and 2, respectively); (B) 1 M BrCN at 0°C for 0, 2, and 10 min (lanes 3, 1, and 2, respectively) and (C) 0.3 M BrCN at 25°C for 0 and 840 min (lanes 1 and 2, respectively). Lettered products (a–m) correspond to fragments shown on Fig. 2; (n) represents conjugate GCCAACCYGGCTCT-M·*Eco*RII. Calibration protein standards are shown.

complex of M·*Eco*RII with the Tgal*-containing 18-mer DNA duplex **10** lacking the recognition site. The same cleavage patterns as those for DNA duplexes **8** and **9** were obtained under both conditions, only molecular masses of the cross-linked complex TAATACGACTCACTAYAG-M·*Eco*RII and the derived oligonucleotide-peptides were higher than those in the case of the 14-mer DNA duplexes **8** and **9.**

The data obtained suggest that the same M·*Eco*RII region Gly^{268}-Met^{391} interacts with the DNA sugar-phosphate backbone in the cases of specific and non-specific complexes. This region contains ten lysine residues

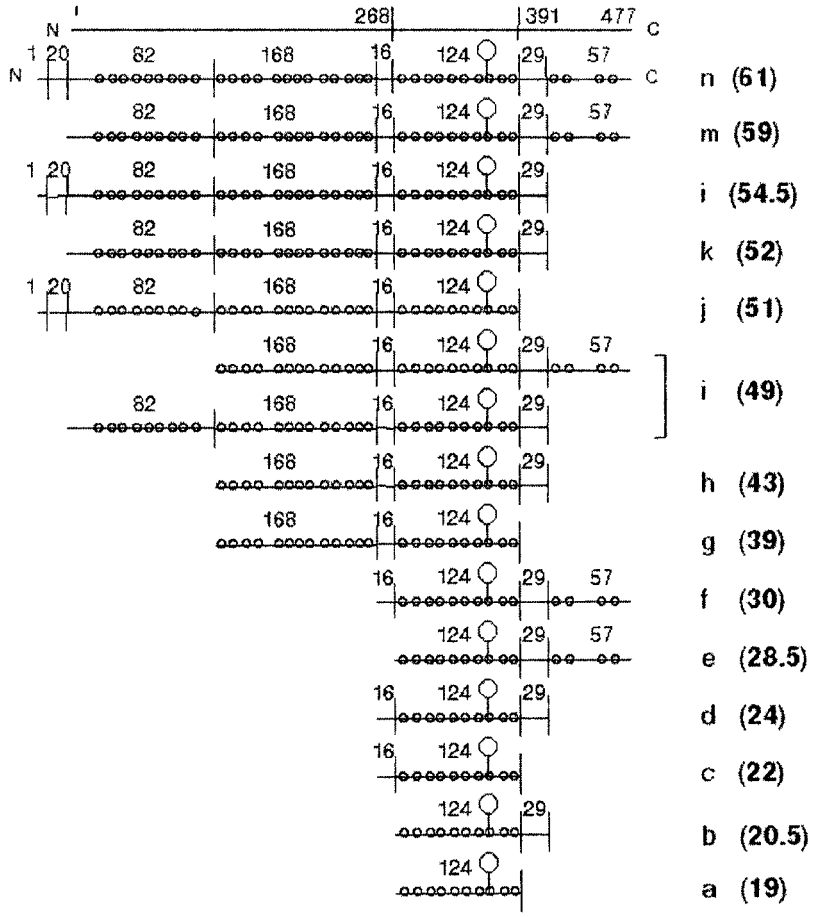


Figure 2. A theoretical pattern of oligonucleotide-peptides derived from the M·*Eco*RII-oligonucleotide conjugate after hydrolysis with BrCN, if an oligonucleotide is cross-linked to the M·*Eco*RII region Gly^{268}-Met^{391}. The horizontal line at the top symbolizes the wild type M·*Eco*RII. Lettered lines represent a conjugate of His_6-tagged M·*Eco*RII with oligonucleotide (n) and degradation products (a–m). Bold numbers in parentheses indicate molecular masses of a–n. Vertical bars represent sites of cleavage with BrCN. The numbers of amino acid residues in the cleavage products are indicated. ⚲-an oligonucleotide; o-a lysine residue.

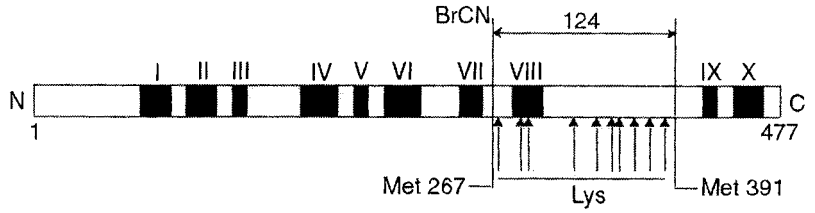


Figure 3. Location of the cross-linked region within the M·*Eco*RII molecule as determined by the results of hydrolysis with BrCN. The shaded regions represent conserved amino acid motifs I–X; numbers of amino acid residues of M·*Eco*RII are indicated.

(Fig. 3). Seven of them are located within a variable region between the conserved motifs VIII and IX (the target recognition domain of C5-Mtases, TRD). Two lysine residues belong to the conserved motif VIII and one lysine residue is located in the region preceding the conserved motif VIII.

Recently, a hypothetical model of three-dimensional structure of M·*Eco*RII and its complex with DNA has been proposed on the basis of homology between amino acid sequences of M·*Eco*RII and structurally studied Mtases *Hha*I and *Hae*III.[15] According to this model, several lysine residues from TRD in the region Gly^{268}-Met^{391} may interact with phosphates of the recognition site and with adjacent nucleotide sequences. It was proposed that lysine residues 351 and 378 interact with phosphates at the 3′- and 5′-ends of the methylated deoxycytidine residue and lysine residue 347 interacts with phosphate in the flanking nucleotide sequence. Our results support the location of the DNA binding region in the M·*Eco*RII molecule predicted by this theoretical model. Recently, conservation of Thr in the variable region of C5 Mtases and its role in catalysis for Mtase *Hha*I was reported.[16] Interestingly, that the M·*Eco*RII region Gly^{268}-Met^{391}contains a conserved Thr^{371}.

To sum up, it was shown that DNA duplexes containing an oxidized ribofuranosylcytidine residue are able to form covalent conjugates with M·*Eco*RII. The amino acid region Gly^{268}-Met^{391} of M·*Eco*RII interacting with the DNA sugar-phosphate backbone has been proposed on the basis of chemical cross-linking. Both specific and non-specific DNA interact with the same M·*Eco*RII region.

EXPERIMENTAL SECTION

Enzymes. T4 polynucleotide kinase was purchased from MBI Fermentas (Lithuania). M·*Eco*RII (0.5 mg/mL) was overexpressed as an N-terminally His_6-tagged protein and purified by chromatography on a nickel chelate column as described.[17] The enzyme contained additional 20 amino acids at the N-end.

Oligonucleotides. Synthesis of an oligonucleotide containing the regiospecifically incorporated 2′-O-β-D-ribofuranosylcytidine and 1-(β-D-galactopyranosyl)thymine has been performed as described.[11,12] Oxidation of the 2′-O-β-D-ribofuranosylcytidine- or 1-(β-D-galactopyranosyl)thymine-containing oligonucleotides was carried out for 1.5 h or 3 h at 37°C in 20 mM or 50 mM $NaIO_4$ solution, respectively.

Gel Mobility-Shift Assay. M·*Eco*RII (1.8 μM) was incubated with the ^{32}P-labeled DNA duplexes **1**–**7** (0.35 μM) in 10 μL of 40 mM Tris-HCl (pH 7.9), 5 mM DTT, 1 mM EDTA (buffer A) 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 native 8% polyacrylamide gel electrophoresis (PAGE).[11] Bands with lower mobilities than those of DNA duplexes were observed for complexes. The efficiencies of non-covalent complex formation (R) were determined as the [c.p.m. bound DNA duplex / (c.p.m. free DNA duplex + c.p.m. bound DNA duplex)] ratio. To normalize data from different experiments, ratios of R for modified substrates **2**–**7** to R for canonical substrate **1** (relative affinities) in each experimental data set were defined.

Methylation Assay. The efficiency of methylation was monitored by the radioactivity ($C[^3H]_3$) incorporation into DNA duplexes **1**–**7** using DE 81 filters.[11] Methylation reactions were carried out at 10°C for 1, 2, 4, 8, 16, and 30 min in 10 μL reaction mixtures containing buffer A, 1 μCi of [methyl-^{3}H]-AdoMet (Amersham, 15 Ci/ mmole), 0.35 μM of DNA duplexes **1**–**7,** and 0.26 μM of M·*Eco*RII. The amount of methylation per minute was computed as:

V_o ($^{nM}/_{min}$) = $((cpm_t - cpm_0)\cdot[AdoMet])/(cpm_{100}\cdot t)$, 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·*Eco*RII (1.8 μM) to DNA duplexes **2**–**10** containing ^{32}P-label in the dialdehyde-substituted strand (0.35 μM) was performed in 10 μL of buffer A containing 0.1 mM 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-PAGE after heating samples in 0.1% SDS, 0.2% 2-mercaptoethanol at 95°C.[11] The product that moves more slowly than free oligonucleotide was observed. The cross-linking yield was determined as the ratio of the oligonucleotide-Mtase conjugate radioactivity to total radioactivity of the conjugate and unbound oligonucleotide. 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. For partial BrCN cleavage, lyophilized conjugates of M·*Eco*RII with dialdehyde-substituted strand of DNA duplexes **8**–**10** were dissolved in 20 μL of 1% SDS; 1 μL 1 M HCl and 1 μL 1 M BrCN or 2 μL 12 M BrCN was added. The reaction mixtures were incubated at 0–25°C for 0–10 min. The reactions were stopped by addition of 10 μL 0.06 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 5% 2-mercaptoethanol followed by incubation at 95°C for 2 min. For complete hydrolysis, lyophilized M·*Eco*RII-oligonucleotide conjugates were dissolved in 70 μL of formic acid; 30 μL of 1 M BrCN was added. The reaction mixtures were

incubated at 25°C for 12–16 h. 200 μL of water was added and the reaction mixtures were lyophilized. The cleavage products (oligonucleotide-peptides) were fractionated by 10% SDS-PAGE in Tris-glycine buffer system.[18] They were identified by comparing with masses of calibration protein standards and theoretical cleavage products which were calculated as a sum of the oligonucleotide mass and the corresponding peptide mass.

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