On the Substrate Specificity of DNA Methyltransferases

ALENINE-N⁶ DNA METHYLTRANSFERASES ALSO MODIFY CYTOSINE RESIDUES AT POSITION N⁴⁺

(Received for publication, February 2, 1999, and in revised form, April 14, 1999)

Albert Jeltsch‡, Frauke Christ, Mehrnaz Fatemi, and Markus Roth

From the Institut für Biochemie, Fachbereich Biologie, Heinrich-Buff-Ring 58, 35392 Giessen, Germany

Methylation of DNA is important in many organisms and essential in mammalian DNA. Nucleobases can be methylated at the adenine-N⁶, cytosine-N⁴, or cytosine-C⁵ atoms by specific DNA methyltransferases. We show here that the M.EcoRV, M.EcoRI, and Escherichia coli dam methyltransferases as well as the N and C-terminal domains of the M.FokI enzyme, which were formerly all classified as adenine-N⁶ DNA methyltransferases, also methylate cytosine residues at position N⁴. Kinetic analyses demonstrate that the rate of methylation of cytosine residues by M.EcoRV and the M.FokI enzymes is reduced by only 1–2 orders of magnitude in relation to methylation of adenines. This result shows that although these enzymes methylate DNA in a sequence specific manner, they have a low substrate specificity with respect to the target base. This unexpected finding has implications on the mechanism of adenine-N⁶ DNA methyltransferases. Sequence comparisons suggest that adenine-N⁶ and cytosine-N⁴ methyltransferases have changed their reaction specificity at least twice during evolution, a model that becomes much more likely given the partial functional overlap of both enzyme types. In contrast, methylation of adenine residues by the cytosine-N⁴ methyltransferase M.BamHI was not detectable. On the basis of our results, we suggest that adenine-N⁶ and cytosine-N⁴ methyltransferases should be grouped into one enzyme family.

In higher eukaryotes post-replicative, enzymatic methylation of DNA is involved in genomic imprinting (1, 2), X-chromosome inactivation (3), gene regulation (4), and carcinogenesis (5–7). In eukaryotic cells methylation is the only known covalent modification of DNA. At least in mammalian DNA it is absolutely essential (8). In prokaryotes, methylation of DNA is involved in processes like post-replicative repair (9) and control of DNA replication (10). In addition, DNA methylation is required in restriction-modification systems found in almost all bacteria (11). Today, over 200 prokaryotic DNA methyltransferases (MTases) have been identified and characterized; most of which are components of restriction-modification systems (12–14). These enzymes recognize short DNA sequences and specifically methylate these sequences at a defined position yielding 6-methyladenine, 4-methylcytosine, or 5-methylcytosine. All MTases contain a weakly conserved F_G_G amino acid motif responsible for the interaction with the cofactor AdoMet. Amino acid sequence comparisons show that cytosine-C⁵ MTases share nine additional conserved amino acid motifs (12, 15, 16). In contrast, adenine-N⁶ MTases are much more heterogeneous. Besides the F_G_G motif, they only contain one moderately conserved (D/N)PP(Y/F) motif, which forms part of the active center of these enzymes. Some additional weakly conserved motifs could only be identified in structure-guided alignments (17). Interestingly, cytosine-N⁴ MTases also contain a set of motifs comparable with adenine-N⁶ MTases. However, most of them contain an SPP(Y/F) motif instead of the (D/N)PP(Y/F) motif present in adenine-N⁶ MTases (17). The conserved motifs of adenine-N⁶ and cytosine-N⁴ MTases may appear in variable distance and order. According to the arrangement of the motifs these enzymes can be subdivided into three groups, α, β, and γ (15, 17). Most cytosine-N⁴ MTases belong to the β group, however, some are members of the α group.

MTases of all classes show structural similarity in their catalytic domains comprising the overall fold and topology, the cofactor binding site and location of the catalytic center (18–22). The structure of the cytosine-C⁵ MTase M.HhaI-DNA complex has shown that these enzymes flip their target base out of the DNA helix during catalysis (23), which is also very likely to occur in adenine-N⁶ and cytosine-N⁴ MTases (20, 24–32). All DNA MTases studied so far prefer binding to substrates that contain a mismatch base pair or even an abasic site at the target position (27, 30, 32–34). This result can be rationalized because base flipping requires disruption of the Watson-Crick hydrogen bonds of the target base, which is facilitated if the target base forms weaker hydrogen bonds to its partner base. However, these results also imply that the target base may not be recognized very accurately by MTases, at least in the ground state of the enzyme-DNA complex. Given this finding and the structural similarity of adenine-N⁶ and cytosine-N⁴ MTases as well as the similarity of the active site sequence motifs of both enzyme families, the question arises whether adenine-N⁶ MTase might also be able to methylate cytosine residues and vice versa.

EXPERIMENTAL PROCEDURES

Oligonucleotides—HPLC-purified oligodeoxyribonucleotides were purchased from Interactiva (Ulm, Germany). To anneal double-stranded substrates the complementary oligonucleotides were mixed, heated to 90 °C for 5 min, and slowly cooled to 20 °C. The following double-stranded oligonucleotide substrates were used: LR.L20, d(GATCGTAGATTACGATCGA)/d(TCGATCGATATCTGATCGA); ME.ME, d(GATCGTAGATTACGATCGA)/d(TCGATCGATATCGATCGA); MM.MM, d(GATCGTAGATTACGATCGA)/d(TCGATCGATATCGATCGA).

‡ To whom correspondence should be addressed. Tel.: 49-641-99-35404; Fax: 49-641-99-35409; E-mail: Albert.Jeltsch@chemie.bio.uni-giessen.de.

* This work was supported by the Deutsche Forschungsgemeinschaft (Pi 122/15-1) and a grant of the Justus-Liebig Universitat. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed. Tel.: 49-641-99-35404; Fax: 49-641-99-35409; E-mail: Albert.Jeltsch@chemie.bio.uni-giessen.de.

The abbreviations used are: MTases, DNA methyltransferases; AdoMet, S-adenosylmethionine; mA, N⁶-methyladenine; mC, N⁴-methylcytosine; HPLC, high pressure liquid chromatography.
I(1–367) and M. Fok (31). Cloning of M. I(335–647) is detailed by Fok (10 mM imidazole, 10% (v/v) glycerol, 0.01% (v/v) lubrol. All enzyme from Amersham Pharmacia Biotech. To avoid degradation, AdoMet was purified following the same protocol as M. Fok.

Methylation reactions with M. EcorV were carried out in 50 mM Tris/HCl, pH 7.5, 5 mM dithiorethrythirol, 2 mM EDTA, 1 mg/ml bovine serum albumin at an ambient temperature using a biphasic mixture of 100 mM Tris/HCl, pH 8.0, 5 mM MgCl2 for 30 min at an ambient temperature, and the released radioactivity was analyzed by liquid scintillation counting. As shown in Fig. 4 (only experiments with M. EcorV were carried out with the biotin/avidin assay), the assay is linear with respect to time and the signal/noise ratio is much better than in the curves determined with the thin layer chromatography assay. Further controls showed that the assay is very sensitive and accurate.

Methylation Reactions with the M. FokI Enzymes—Methylation reactions with the M. FokI enzymes were carried out in 50 mM Tris/HCl, pH 7.5, 5 mM dithiorethrythirol, 2 mM EDTA, 1 mg/ml bovine serum albumin at an ambient temperature as described (35) using a thin layer chromatography assay (31). Oligonucleotide concentrations were 0.2 μM; enzyme concentrations were between 0.5 and 9 μM. (methyl-14C)AdoMet was used at a concentration of 50 μM.

DNA Binding Analyses—DNA binding by M. EcorV was assayed in 50 mM HEPES, pH 7.5, 50 mM NaCl, 1 mM EDTA, 50 μg/ml bovine serum albumin in the presence of 1 mM s-methionin as described (31, 32).

RESULTS
Adenine-N6 and cytosine-N4 MTases have similar structures and catalytic centers. Moreover, biochemical evidence suggests that MTases do not very accurately recognize their target base (27, 30, 32–34). Hence, the question arose whether adenine-N6 MTases might be able to methylate cytosine residues at the N4 position, which is the subject of this study. Most of our experiments were carried out with the M. EcorV and M. FokI adenine-N6 MTases. M. EcorV recognizes GATAC and methylates the first adenine residue within this sequence (36). M. FokI is a tandem enzyme consisting of two fused MTases that are catalytically active independently of each other (35, 37, 38).

Both of the M. FokI enzymes recognize the asymmetric DNA sequence GAGTC/CATCC (39, 40), the C-terminal domain has a strong preference for the lower CATCC-strand (35, 37, 38). In this study we have used purified preparations of both individual domains, viz. M. FokI (1–367) comprising amino acid 1–367 and M. FokI (335–647) comprising amino acids 335–647 (35).

First we tested whether M. EcorV might flip a cytosine residue located at the target position out of the DNA helix, a prerequisite for enzymatic activity. To this end, the binding constants of M. EcorV to the canonical hemimethylated substrate RV_ME containing a GATACG/GATACATC site and to RV_CA were determined. RV_CA contains a GCTAGC/GAGATC site in which the unmethylated target adenine of RV_ME is replaced by cytosine and the “target” cytosine is bound with binding constants determined for an unmethylated 20-mer (31) and 30/33-mer (41). In contrast the RV_CA substrate is bound with Ka = 6 × 105 M−1. This preference of M. EcorV for binding to the mismatch substrate is very similar to results obtained with other mismatch substrates (30, 32). It suggests that M. EcorV flips cytosine residues if they are located in a GCTAGC/GAGATC sequence.

To find out if cytosine residues can be modified by ade-
nine-N6 MTases, the nature of the methylated base must be identified. To this end oligonucleotides containing adenine and cytosine residues at the MTase target position were methylated in vitro using labeled [methyl-3H]AdoMet. In most experiments we used hemimethylated oligonucleotide substrates in which one DNA strand is already methylated at the target position. The target adenine in the other DNA strand is replaced by cytosine such that the resulting C/A substrates bear a C:T mismatch at the target site, viz., GCTATCG/GATATC (RV_C/A) for M.EcoRV, GGGTCGG/GATATC (Fok_up_C/A) for M.FokI(1–367) and CCTCC/GGAGATG (Fok_low_C/A) for M.FokI(335–647). As a control hemimethylated substrates, viz., GATATCG/GATATC (RV_ME) for M.EcoRV, GGATGCG/GATCC (Fok_up_ME) for M.FokI(1–367) and CATCC/GGAGATG (Fok_low_ME) for M.FokI(335–647) were incubated with M.EcoRV and M.FokI enzymes. In each case a hemimethylated canonical substrate (—) and a C/A substrate in which the target adenine in one DNA strand is replaced by cytosine (—) were methylated with the corresponding MTase, degraded with nucleases, and the hydrolysates subjected to the HPLC analysis. A, methylation of RV ME and RV_C/A by M.EcoRV, B, methylation of Fok up_ME and Fok_up_C/A by M.FokI(1–367). C, methylation of Fok_low_ME and Fok_low_C/A by M.FokI(335–647). Note the different scales of the ordinates.

Fig. 1. Calibration of the HPLC separation of mononucleosides. Panel A, OD260 nm profiles of the separation of a mixture of all 4 nucleotides (trace labeled with dA, dT, dG, dC), of a hydrolysate of RV_L20 (trace labeled with GATATC/GATATC) after incubation with M.EcoRV and AdoMet as well as of a hydrolysate of RV_ME (trace labeled with GATATC/GATATC) after incubation with M.EcoRV and AdoMet. The peak at 5.5 min corresponds to the AdoMet. Methylation was substoichiometric in each case, however, the 6-methyladenine peak at 46.5 min is clearly visible in the OD260 nm profile of the hemimethylated substrate (RV_ME). Panel B, radioactivity profiles of the separation of hydrolysates of RV_L20 after incubation with M.EcoRV and Bam_L19 after incubation with M.BamHI. In addition, an analysis of AdoMet after incubation in M.EcoRV methylation buffer and treatment with nucleases is shown. Whereas AdoMet elutes after 5.5 min, 4-methylcytosine, the product of the M.BamHI MTase, elutes after 16.5 min, and 6-methyladenine, the product of M.EcoRV, elutes after 46.5 min, as already shown in panel A.

Fig. 2. HPLC analyses of methylation of adenine and cytosine residues by M.EcoRV and the M.FokI enzymes. In each case a hemimethylated canonical substrate (—) and a C/A substrate in which the target adenine in one DNA strand is replaced by cytosine (—) were methylated with the corresponding MTase, degraded with nucleases, and the hydrolysates subjected to the HPLC analysis. A, methylation of RV ME and RV_C/A by M.EcoRV, B, methylation of Fok up_ME and Fok_up_C/A by M.FokI(1–367). C, methylation of Fok_low_ME and Fok_low_C/A by M.FokI(335–647). Note the different scales of the ordinates.

lines). If fully methylated reference substrates are used, again no methylated cytosine is detectable (data not shown). In contrast, if the C/A substrates are incubated with the corresponding MTase no 6-methyladenine is detectable,5 but in all reactions with C/A substrates, a significant amount of 4-methylcytosine is formed (Fig. 2, continuous lines). This result is highly reproducible and the amount of 4-methylcytosine is far beyond any background fluctuations. We conclude that all three adenine MTases studied are able to methylate cytosine residues.

To find out if this behavior is a general property of adenine-N6 MTases, we have carried out additional experiments with M.EcoRI (recognition sequence GAGTTC, modified base is underlined) and E. coli dam MTase (recognition sequence GATC). In these experiments, unmethylated substrates con-

5 The small peak eluting after 45 min in the reaction with M.EcoRV represents a degradation product of AdoMet that is observed even in runs without oligonucleotide and enzyme under these conditions.
Substrate Specificity of Adenine DNA Methyltransferases

19541

Fig. 3. HPLC analyses of methylation of adenine and cytosine residues by M.EcoRI, E. coli dam MTase, M.EcoRV, and M.BamHI. In each case a canonical substrate (---) and a modified substrate in which the target base in one strand is replaced (---) were methylated with the corresponding MTase, degraded with nuclease S, and the hydrolysates subjected to the HPLC analysis. A, methylation of RI_L20 (GAATTC) and RI_CA (GACTTC) by M.EcoRI B, methylation of dam_L19 (GATC) and dam_C/A (GCTC) by dam MTase. In RI_CA and dam_C/A the target adenine is replaced by a cytosine in one DNA strand yielding a C/T mismatch. C, methylation of RV_ME (GATATC) and RV_CG (GCTATC) by M.EcoRV. RV_CG contains a GCTATC/ GATAGC site that differs in one base pair from the canonical EcoRV site. D, methylation of Bam_L19 and Bam_A/C by M.BamHI. In Bam_A/C the target cytosine of the M.BamHI MTase is replaced by an adenine in one DNA strand yielding an A/G mismatch. Whereas in A, B, and C significant 4-methylcytosine peaks appear in the analyses of the modified substrates, no 6-methyladenine is detectable after incubation of the Bam_A/C substrate with M.BamHI.

It was tempting to investigate whether the cytosine-N⁴ MTase M.BamHI (recognition sequence: GGATCC) could modify adenine residues. To this end a substrate was used in which the target cytosine of M.BamHI is replaced by an adenine in one DNA strand, i.e. GGATAC/GGATCC (Bam_A/C). This substrate is methylated by M.BamHI at the target cytosine in the lower DNA strand (data not shown), but no formation of 6-methyladenine could be detected (Fig. 3D). However, given the low amounts of 4-methylcytosine observed in the reactions with commercial enzymes, the quantitative meaning of this result remains unclear so far. Moreover, it is uncertain whether this result represents a common property of all cytosine-N⁴ MTases.

Taken together, all data presented so far demonstrate that adenine-N⁶ MTases can also modify cytosine residues at position N⁴, a result that has not been described before. Hence, we have tried to verify this conclusion by independent experimental evidence. To this end, we have measured the rate of methylation of the C/A substrates by M.EcoRV and the two M.FokI enzymes. These experiments were carried out with both M.FokI enzymes using the thin layer chromatography assay used in our previous work (31, 35, 42). This, however, was not possible with M.EcoRV, because in the case of M.EcoRV a high background of radioactivity was observed possibly due to AdoMet bound to the enzyme which obscured DNA methylation at low rates. Therefore, we have developed a new methylation assay. In this assay, oligonucleotide substrates that are biotinylated in one DNA strand are methylated using labeled [methyl-³²]AdoMet. After the methylation reaction, the substrates are bound to an avidin-coated microtiter plate. After extensive washing, the DNA is degraded by nuclease treatment. Thereby, the radioactivity incorporated into the DNA is released into solution and can be analyzed quantitatively by liquid scintillation counting. This assay proved to be very sensitive, reproducible, and accurate.

The rates of methylation of the C/A substrates (RV_C/A, Fok_up_C/A, and Fok_low_C/A) by M.EcoRV and both M.FokI enzymes were compared with the rates of methylation of hemimethylated substrates (RV_ME, Fok_up_ME, and Fok_low_ME) and, more importantly, with that of fully methylated substrates in which both target adenine residues are replaced by 6-methyladenine (RV_MM and Fok_MM). For each MTase, the C/A substrate and the fully methylated reference substrate differ from each other at only one nucleotide position, whereas in the C/A substrate, a cytosine is present at the target position of the MTase in one DNA strand, a 6-methyladenine is present at this position in the fully methylated substrate. If, therefore, the C/A substrate is modified faster than the fully methylated substrate, this methylation almost certainly takes place at the cytosine residue not present in the fully methylated DNA strand and is not dependent on the dimethylated AT base pair in the canonical recognition sequence. If this substrate is incubated with M.EcoRV 4-methylcytosine is formed although less than with the GCTATC/GATAGC site (Fig. 3C). This result shows that the methylation of cytosine residues can occur in regular DNA and is not dependent on the facilitated flipping of this base when it is located in a base mismatch.

FIG. 3. HPLC analyses of methylation of adenine and cytosine residues by M.EcoRI, E. coli dam MTase, M.EcoRV, and M.BamHI.
occur spontaneously. One example of this type is the methylation of adenine residues at the 3\textsuperscript{rd} position. In solution the 3\textsuperscript{rd}-atom of adenosines is more reactive than 6\textsuperscript{th}, and methylation would take place at 3\textsuperscript{rd}. Adenine-6\textsuperscript{th} MTases, however, selectively enhance the reactivity of the 6\textsuperscript{th} group leading to a highly regiospecific direct methylation of adenine residues at the 6\textsuperscript{th} position (43). It remains unclear how methylation is selectively directed to the 6\textsuperscript{th} atom of adenosines. One model to explain this regioselectivity would be that the enzyme positioned the flipped adenine in a precise stereoechemical configuration with respect to the methyl group donor, such that only the 6\textsuperscript{th} position is accessible for the methyl group.

Surprisingly, despite their catalytic efficiency and their DNA sequence specificity, we show here that adenine-6\textsuperscript{th} DNA MTases do not have a pronounced specificity for their target base, because these enzymes also catalyze methylation of cytosine residues. This result suggests mechanistic similarities between the methylation of adenine and cytosine residues. The catalytic mechanism of adenine-6\textsuperscript{th} and cytosine-6\textsuperscript{th} MTases is not precisely known, but models have been proposed. It has been suggested that a deprotonation of the target amino group could be catalyzed by the conserved Asn/Asp or Ser in the (D/N/S)PP(Y/F) motif (20). Alternatively, it has been suggested that the transition state of the methylation reaction is stabilized by cation-π interactions between the flipped base and aromatic amino acid residues (44). In a third model, the deprotonation of the exocyclic amino group of the adenine is catalyzed by a transient protonation of the adenine ring accompanied by a tautomeric change (45). This protonation could occur at N\textsuperscript{3}, which is the most basic nitrogen in the adenine ring. In the next step of the reaction, N\textsuperscript{3} could be deprotonated, and by a tautomeric shift, the N\textsuperscript{6} position would become more nucleophilic and could directly attack the AdoMet. Forming and breaking of a hydrogen bond to N\textsuperscript{7} could have a similar effect. As a similar protonation (or hydrogen bond contact) could occur in cytosines at N\textsuperscript{7}, this model is in accordance with our results, whereas important catalytic roles of the N\textsuperscript{3} or N\textsuperscript{7} atoms of the adenine ring are unlikely in light of our results, because these positions have no counterparts in cytosines. Note that these mechanistic models are not excluding each other.

DNA MTases can be subdivided according to the position that is modified into cytosine-C\textsuperscript{6} MTases, which methylate aromatic ring carbon atoms, and N-MTases comprising adenine-C\textsuperscript{6} and cytosine-N\textsuperscript{4} MTases, which methylate exocyclic amino groups. These groups of enzymes differ in the reaction mechanism and the sequences of conserved amino acid motifs involved in catalysis. Assigning adenine-C\textsuperscript{6} and cytosine-N\textsuperscript{4} MTases into a common group is reasonable, because cytosine-N\textsuperscript{4} and adenine-N\textsuperscript{6} MTases display some sequence similarities at the F_G_G as well as SPP(Y/F) and (D/N/S)PP(Y/F) motifs. In fact, these enzymes cannot be readily distinguished on the basis of their conserved amino acid motifs, because the cytosine-N\textsuperscript{4} MTase M.BamHI contains a DPPY motif, which is

---

\(\text{FIG. 4.}\) Methylation of C/A substrates and fully methylated reference substrates by M. EcoRV (RV_C/A, RV_MM), M.FokI(1–367) (Fok_up_C/A, Fok_MM), and M.FokI(335–647) (Fok_low_C/A, Fok_MM) (for details see the legend to Table I and “Experimental Procedures”).

---

\(\text{FIG. 5.} A, \) groups of N-MTases. The locations of conserved amino acid motifs are given (17). TRD, DNA recognition domain. \(B,\) model of the molecular evolution of N-MTases. In this model the differentiation into the α, β, and γ groups by circular permutations and insertions of TRDs proceeds divergence into adenine-6\textsuperscript{th} and cytosine-6\textsuperscript{th} enzymes.

---

\(\text{A} \)

\begin{align*}
\text{α group} & \quad \text{N} \quad \text{I} \quad \text{H} \quad \text{TRD} \quad \text{N} \quad \text{α} \quad \text{α-C} \\
\text{β group} & \quad \text{N} \quad \text{I} \quad \text{H} \quad \text{TRD} \quad \text{N} \quad \text{β} \quad \text{β-C} \\
\text{γ group} & \quad \text{N} \quad \text{I} \quad \text{H} \quad \text{TRD} \quad \text{N} \quad \text{γ} \quad \text{γ-C} \\
\end{align*}

\(\text{B} \)

\begin{align*}
\text{α-N6} \quad \text{α-N4} \\
\text{β-N6} \quad \text{β-N4} \\
\text{γ-N6} \quad \text{γ-N4} \\
\end{align*}

 disproportion between groups of enzymes differ in the reaction mechanism and the sequences of conserved amino acid motifs involved in catalysis. Assigning adenine-C\textsuperscript{6} and cytosine-N\textsuperscript{4} MTases into a common group is reasonable, because cytosine-N\textsuperscript{4} and adenine-N\textsuperscript{6} MTases display some sequence similarities at the F_G_G as well as SPP(Y/F) and (D/N/S)PP(Y/F) motifs. In fact, these enzymes cannot be readily distinguished on the basis of their conserved amino acid motifs, because the cytosine-N\textsuperscript{4} MTase M.BamHI contains a DPPY motif, which is

---

\(\text{6 The rates observed in these experiments cannot be directly compared with the results of the HPLC analyses, because in the HPLC experiments DNA methylation was carried out at much higher DNA concentrations, and the incubation times were longer.}\)
characteristic for adenine-\textsuperscript{N6} MTases. Moreover the structures of all N-MTases are similar, and we have shown here that the catalytic activities of adenine-\textsuperscript{N6} and cytosine-\textsuperscript{N4} MTases are overlapping.

The overlap in the reaction specificities of adenine-\textsuperscript{N6} and cytosine-\textsuperscript{N4} MTases has implications on the molecular evolution of this class of N-MTases, because it opens an evolutionary pathway to change the reaction specificity of adenine-\textsuperscript{N6} MTases to cytosine-\textsuperscript{N4} and vice versa. So far, three groups of N-MTases are known (\(\alpha\), \(\beta\), and \(\gamma\)) (17). These groups differ in the positions of the integration of the DNA recognition domain into the catalytic domain and by a circular permutation of the amino acid sequence in the catalytic domain (46), which lead to different arrangements and distances of the conserved amino acid sequence motifs (Fig. 5A). So far, cytosine-\textsuperscript{N4} MTases have been found in the \(\alpha\) and \(\beta\) groups. Two scenarios could account for this the situation: N-MTases could have diverged into adenine-\textsuperscript{N6} and cytosine-\textsuperscript{N4} MTases first, and, then differentiated into the three groups. However, in light of the large number of possible ways for circular permutations and possible sites for integration of a DNA recognition domain into the catalytic domain, it is unlikely that adenine-\textsuperscript{N6} and cytosine-\textsuperscript{N4} independently diverged into \(\alpha\) and \(\beta\) groups having the same topology. Alternatively, N-MTases could have branched into the three groups first, and, afterward, members of the \(\alpha\) and \(\beta\) group could have changed their substrate specificity from adenine-\textsuperscript{N6} to cytosine-\textsuperscript{N4} (Fig. 5B). This evolutionary pathway implies that the substrate specificities of N-MTases must have changed at least twice. Our data support the latter model, because they demonstrate that the catalytic framework of N-MTases is, in principle, able to support methylation of adenine and cytosine residues and their derivatives. In the results obtained with adenine-\textsuperscript{N6} MTases, the cytosine-\textsuperscript{N4} MTase M.BamHI appears not to modify adenine residues at a detectable rate. Similar experiments with other cytosine-\textsuperscript{N4} MTases will reveal if this result represents a general property of cytosine-\textsuperscript{N4} MTases. The result obtained with M.BamHI can be interpreted in terms of molecular recognition, because the large active site of adenine-\textsuperscript{N6} MTases can also accommodate cytosine, whereas the converse is less likely. This situation may be analogous to that of aminoacyl-tRNA synthetases, because a proofreading mechanism is required to prevent misincorporation of tRNAs with amino acid residues, which are smaller than the canonical residue.

Conclusions—We have shown here that five adenine-\textsuperscript{N6} DNA methyltransferases also methylate cytosine residues at position N4. The rates of cytosine methylation by M.EcoRV and both domains of M.FokI are reduced by only 1–2 orders of magnitude, demonstrating that these enzymes have a low specificity with respect to the target base. Our data confirm that adenine-\textsuperscript{N6} and cytosine-\textsuperscript{N4} belong to one enzyme family of N-MTases and suggest that these enzymes have repeatedly changed their specificity during evolution.

Acknowledgments—Expert technical assistance by H. Büngen as well as support, encouragement, and comments by A. Pingoud are gratefully acknowledged. Thanks are due to B. Connolly, W. Saenger, and E. Weinhold for helpful discussions.

REFERENCES

1. Razin, A. & Cedar, H. (1994) Cell 77, 473–476
2. Barlow, D. P. (1995) Science 269, 1610–1613
3. Migeon, B. R. (1994) Trends Genet. 10, 230–235
4. Tate, P. H. & Bird, A. P. (1993) in DNA Methylation: Molecular Biology and Significance (Jos, J. P. & Saluz, H. P., eds) Birkhauser Verlag, Basel
5. Cheng, X. (1995) Curr. Opin. Struct. Biol. 5, 4–10
6. Cheng, X. (1995) Annu. Rev. Biophys. Biomol. Struct. 24, 293–318
7. Wilson, G. G. (1992) Methods Enzymol. 216, 259–279
8. Kumar, S., Cheng, X., Klimasauskas, S., Sha, M., Posfai, J., Roberts, R. J. & Wei, G. (1994) Nucleic Acids Res. 22, 1–10
9. Malone, T., Blumenthal, R. M. & Cheng, X. (1995) Annu. Rev. Genet. 30, 441–464
10. Jones, P. A. & Gonzalgo, M. L. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2103–2105
11. Heintzen, J. M. (1993) in Genetic Engineering (N’Y, Setlow, J. K., ed) Vol. 15, pp. 57–108, Plenum Press, New York
12. Noyer-Weidner, M. & Trautner, T. A. (1993) in DNA Methylation: Molecular Biology and Significance (Jos, J. P. & Saluz, H. P., eds) Birkhauser Verlag, Basel
13. Cheng, X. (1995) Curr. Opin. Struct. Biol. 5, 4–10
14. Cheng, X. (1995) Annu. Rev. Biophys. Biomol. Struct. 24, 293–318
15. Wilson, G. G. (1992) Methods Enzymol. 216, 259–279
16. Kumar, S., Cheng, X., Klimasauskas, S., Sha, M., Posfai, J., Roberts, R. J. & Wei, G. (1994) Nucleic Acids Res. 22, 1–10
17. Malone, T., Blumenthal, R. M. & Cheng, X. (1995) J. Mol. Biol. 253, 618–632
18. Cheng, X., Kumar, S., Posfai, J., Pfahrgast, J. W. & Roberts, R. J. (1993) Cell 74, 299–307
19. Reinisch, K. M., Chen, L., Verdin, G. L. & Lipscomb, W. N. (1995) Cell 82, 143–153
20. Gong, W., O’Gara, M., Blumenthal, R. M. & Cheng, X. (1997) Nucleic Acids Res. 25, 2702–2715
21. Labahn, J., Grunzin, J., Schluckebier, G., Robinson, D. P., Jack, W. E., Schildkraut, I. & Saenger, W. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10957–10961
22. Tran, P. H., Kurz, Z. R., Cerritelli, S., Springhorn, S. & Saenger, S. A. (1998) Structure 6, 1563–1575
23. Klimasauskas, S., Kumar, S., Roberts, R. J. & Cheng, X. (1994) Cell 76, 357–369
24. Schluckebier, G., Labahn, J., Grunzin, J., Schildkraut, I. & Saenger, W. (1995) Gene 157, 131–134
25. Schluckebier, G., O’Gara, M., Saenger, W. & Cheng, X. (1995) J. Mol. Biol. 247, 16–20
26. Allan, B. W. & Reich, N. O. (1996) Biochemistry 35, 14757–14762
27. Allan, B. W., Beechem, J. M., Lindstrom, W. M. & Reich, N. O. (1998) J. Biol. Chem. 273, 2968–2973
28. Holz, B., Klimasauskas, S., Serva, S. & Weinhold, E. (1998) Nucleic Acids Res. 26, 1076–1083
29. Serva, S., Weinhold, E., Roberts, R. J. & Klimasauskas, S. (1998) Nucleic Acids Res. 26, 3473–3479
30. Cal, S. & Connolly, B. A. (1997) J. Biol. Chem. 272, 490–496
31. Jeltsch, A., Friedrich, T. & Roth, M. (1998) J. Mol. Biol. 275, 747–758
32. Jeltsch, A., Roth, M. & Friedrich, T. (1999) J. Mol. Biol. 285, 1121–1130
33. Klimasauskas, S. & Roberts, R. J. (1995) Nucleic Acids Res. 23, 1388–1395
34. Yang, S. A., Shen, J.-C., Zingg, J.-M., Mi, S. & Jones, P. A. (1995) Nucleic Acids Res. 23, 1380–1387
35. Leimann, O., Roth, M., Friedrich, T., Wende, W. & Jeltsch, A. (1998) Eur. J. Biochem. 251, 899–906
36. Nwosa, V. U., Connolly, B. A., Halford, S. E. & Garnett, J. (1988) Nucleic Acids Res. 16, 7351–7359
Substrate Specificity of Adenine DNA Methyltransferases

37. Looney, M. C., Moran, L. S., Jack, W. E., Feehery, G. R., Benner, J. S., Slatko, B. E. & Wilson, G. G. (1989) *Gene* **80**, 193–208
38. Sugisaki, H., Kita, K. & Takanami, M. (1989) *J. Biol. Chem.* **264**, 5757–5761
39. Sugisaki, H. & Kanazawa, S. (1981) *Gene* **16**, 73–78
40. Landry, D., Looney, M. C., Feehery, G. R., Slatko, B. E., Jack, W. E., Schilknaut, I. & Wilson, G. G. (1989) *Gene* **77**, 1–10
41. Szczelkun, M. D. & Connolly, B. A. (1995) *Biochemistry* **34**, 10724–10733
42. Roth, M., Helml-Kruse, S., Friedrich, T. & Jeltsch, A. (1998) *J. Biol. Chem.* **273**, 17333–17342
43. Pogolotti, A. L., Jr., Ono, A., Subramanian, R. & Santi, D. V. (1988) *J. Biol. Chem.* **263**, 7461–7464
44. Schluckebier, G., Labahn, J., Granzin, J. & Saenger, W. (1998) *Biol. Chem.* **379**, 389–400
45. Mashhoon, N. & Reich, N. O. (1994) *Biochemistry* **33**, 7113–7119
46. Jeltsch, A. (1999) *J. Mol. Evol.*, in press
On the Substrate Specificity of DNA Methyltransferases: ADENINE-N 6 DNA METHYLTRANSFERASES ALSO MODIFY CYTOSINE RESIDUES AT POSITION N 4

Albert Jeltsch, Frauke Christ, Mehrnaz Fatemi and Markus Roth

J. Biol. Chem. 1999, 274:19538-19544.
doi: 10.1074/jbc.274.28.19538

Access the most updated version of this article at http://www.jbc.org/content/274/28/19538

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 44 references, 9 of which can be accessed free at http://www.jbc.org/content/274/28/19538.full.html#ref-list-1