Identification of the Binding Site for the Extrahelical Target Base in N⁶-Adenine DNA Methyltransferases by Photo-cross-linking with Duplex Oligodeoxyribonucleotides Containing 5-Iodouracil at the Target Position*

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DNA methyltransferases flip their target bases out of the DNA double helix for catalysis. Base flipping of C⁴-cytosine DNA methyltransferases was directly observed in the protein-DNA cocrystal structures of M.HhaI and M.HaeIII. Indirect structural evidence for base flipping of N⁶-adenine and N⁴-cytosine DNA methyltransferases was obtained by modeling DNA into the three-dimensional structures of M.TaqI and M.PvuII in complex with the cofactor. In addition, biochemical evidence of base flipping was reported for different N⁶-adenine DNA methyltransferases. As no protein-DNA cocrystal structure for the related N⁶-adenine and N⁴-cytosine DNA methyltransferases is available, we used light-induced photochemical cross-linking to identify the binding site of the extrahelical target bases. The N⁶-adenine DNA methyltransferase M.TaqI and M.CviIII, which both methylate adenine within the double-stranded 5'-TCGA-3' DNA sequence, were photo-cross-linked to duplex oligodeoxyribonucleotides containing 5-iodouracil at the target position in 50–60% and almost quantitative yield, respectively. Proteolytic fragmentation of the M.CviIII-DNA complex followed by Edman degradation and electrospray ionization mass spectrometry indicates photo-cross-linking to tyrosine 122. In addition, the mutant methyltransferases M.TaqI/Y108A and M.TaqU/F196A were photo-cross-linked with 6-fold and 2-fold reduced efficiency, respectively, which suggests that tyrosine 108 is the primary site of modification in M.TaqI. Our results indicate a close proximity between the extrahelical target base and tyrosine 122 in M.CviIII or tyrosine 108 in M.TaqI. As both residues belong to the conserved motif IV ((N/D/S)(P/I)P(Y/F/W)) found in all N⁶-adenine and N⁴-cytosine DNA as well as in N⁶-adenine RNA methyltransferases, a similar spatial relationship between the target bases and the aromatic amino acid residue within motif IV is expected for all these methyltransferases.

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1 The abbreviations used are: Mtase, methyltransferase; AdoMet, S-adenosyl-L-methionine; DTT, dithiothreitol; ESI-MS, electrospray ionization mass spectrometry; M.CviIII, N⁶-adenine DNA methyltransferase from Chlorella virus NC-1A; M.EcoRI, N⁴-adenine DNA methyltransferase from E. coli; M.HhaI, C⁴-cytosine DNA methyltransferase from Haemophilus aphrophilus; M.HaeIII, C⁴-cytosine DNA methyltransferase from Haemophilus aphrophilus; M.PvuII, N⁴-cytosine DNA methyltransferase from Proteus vulgaris; M.TaqI, N⁶-adenine DNA methyltransferase from T. aquaticus; N-DNA Mtases, N⁶-adenine and N⁴-cytosine DNA methyltransferases; N-DNA/RNA Mtases, N⁶-adenine and N⁴-cytosine DNA as well as N⁶-adenine RNA methyltransferases; ODN, oligodeoxyribonucleotide; PAGE, polyacrylamide gel electrophoresis.
Photo-cross-linking of N6-Adenine DNA Mtases

N6-adenine DNA Mtases M.EcoRV (13) and M.EcoRI (14) to duplex ODNs carrying base analogues with reduced Watson-Crick hydrogen bonding potential at the target positions was observed and attributed to a reduced energetic cost to flip out the target base. Furthermore, a thymine residue placed at the target position within a duplex ODN showed an enhanced reactivity toward potassium permanganate oxidation in the presence of M.TaqI, which was interpreted by a higher accessibility of the thymine residue in the binary complex due to base flipping (15). A structural comparison of M.TaqI with M.HhaI showed that the cofactor binding domains of both enzymes have a very similar fold (16). In addition, the cofactors and the motifs IV ((N/D/S)PP(Y/F/W)-motif of N-DNA Mtases and PCQ-motif of C6-cytosine DNA Mtases) as well as the motifs VIII (FY/YW-motif in N-DNA Mtases and QXRQ-motif in C6-cytosine DNA Mtases) overlay very well. Thus, it was suggested that the extrahelical target adenine in M.TaqI is located in a similar position as the extrahelical cytosine in M.HhaI and that Tyr-108 (motif IV) and Phe-196 (motif VIII) in M.TaqI are responsible for proper orientation of the extrahelical adenine.

To identify the binding site of the extrahelical target base in N6-adenine DNA Mtases, we used ultraviolet light-induced photochemical cross-linking, which is a powerful technique to define specific contact points in nucleoprotein complexes, where direct structural information is not available (for a review, see Ref. 17). Non-substituted nucleic acids or nucleic acids containing 4-thiouracil, azido-substituted nucleobases, and halogenated pyrimidines like 5-bromouracil, 5-iouracil, or 5-iodocytosine are typically used in the photo-cross-linking reaction. In contrast to chemical cross-linking, which interposes spacers of varying length between the nucleic acid and the protein, photo-cross-linking produces zero-length cross-links. This allows the identification of contact points in nucleoprotein complexes at high resolution. Although thymine in non-substituted nucleic acids can be photo-cross-linked directly at 253 nm, the yields are generally low, which is most likely due to photodegradation of the proteins and nucleic acids (18). This problem can be minimized by incorporating photo-activatable analogues into nucleic acids, which can be excited above 300 nm. An additional advantage of using substituted nucleic acids is the fact that the site of modification within the nucleic acid sequence is already defined. The highest photo-cross-linking yields for different nucleoprotein complexes were obtained with nucleic acids containing 5-iodouracil, and yields of up to 95% were reported (19–22).

In this publication we show that the N6-adenine DNA Mtases M.TaqI from Thermus aquaticus (23, 24) and M.CviBIII from the Chlorella virus NC-1A (25), which both methylate adenine within the double-stranded 5'-TCGA-3' DNA sequence, give high photo-cross-linking yields with duplex ODNs containing 5-iodouracil at the target position (Scheme 1). Using a combination of Edman degradation and electrospray ionization mass spectrometry (ESI-MS) of a nucleopeptide obtained by proteolytic fragmentation of the covalent M.CviBIII-DNA complex we demonstrate that Tyr-122 (motif IV) is modified in the photo-cross-linking reaction. In addition, results from photo-cross-linking experiments with the mutant Mtases M.TaqI/Y108A (Tyr in motif IV replaced by Ala) and M.TaqUF196A (Phe in motif VIII replaced by Ala) are discussed.

**EXPERIMENTAL PROCEDURES**

Oligodeoxynucleotides—ODNs were synthesized on an Applied Biosystems 392 DNA/RNA synthesizer or purchased from MWG Biotech (Ebersfeld, Germany). The phosphoramidite for 5-iodo-2'-deoxyuridine was purchased from Glen Research. Due to the base lability of 5-iodo-2'-deoxyuridine (26), the syntheses were performed with Expedite phosphoramidites (Perseptive Biosystems) containing the more labile tert-butyldimethylsilyl protection group and allowing deprotection with concentrated ammonia at room temperature for 2 h. Purification, evaluation, and hybridization of ODNs were performed as described before (12). Sequences of synthesized ODNs are listed in Table I.

**DNA Methyltransferases**—M.TaqI and the mutant Mtases M.TaqI/Y108A and M.TaqUF196A were prepared as described earlier (12, 27). M.CviBIII was expressed in DS1312 Esherichia coli cells harboring pNC1A-14lac (25), which carries the gene for M.CviBIII under the inducible lac promoter. The expression system was kindly provided by Dr. Waltraud Ankenbauer, Roche Molecular Biochemicals. Cells were grown at 37 °C to an optical density of 0.6 at 600 nm and induced with isopropyl-β-D-thiogalactoside (0.1 mM final concentration) at 30 °C for 4 h. Cells were harvested by centrifugation (15 min at 4,000 × g), resuspended in buffer A (20 mM Tris hydrochloride, pH 7.6, 10 mM β-mercaptoethanol, 20 mg/liter phenylmethylsulfonil fluoride, and 5% glycerol) supplemented with potassium chloride (500 mM) and lysed by sonication. After centrifugation (1 h at 30,000 × g), the supernatant was diluted with buffer A to give a potassium chloride concentration of 100 mM and loaded onto an anion-exchange column (DEAE-Sepharose FF, Amersham Pharmacia Biotech). M.CviBIII was eluted with buffer A containing potassium chloride (170 mM). The resulting protein solution was diluted with buffer A to yield a potassium chloride concentration of 100 mM and loaded onto a heparin column (heparin-Sepharose CL 6B, Amersham Pharmacia Biotech). A potassium chloride gradient (0.1 to 1.0 M) in buffer A was applied and M.CviBIII eluted at a potassium chloride concentration of 200–250 mM. Fractions containing M.CviBIII were combined and concentrated by ultrafiltration (Centriprep 30, Amicon). The concentrated protein solution was loaded onto a gel filtration column (Superdex 200, Amersham Pharmacia Biotech) and eluted with a Tris hydrochloride buffer (20 mM, pH 7.4) containing potassium chloride (600 mM, EDTA (0.2 mM), and DTT (2 mM)). Fractions containing M.CviBIII were pooled, 2-fold diluted with glycerol, and stored at −20 °C. M.CviBIII was at least 90% pure by sodium dodecyl sulfate gel electrophoresis in the presence of sodium dodecyl sulfate and staining with Coomassie Blue. Protein concentrations were estimated by the method of Bradford (28) (Coomassie Protein Assay Reagent, Pierce) using bovine serum albumin as standard.

**Ultraviolet Light-induced Photocross-linking**—Typically, photo-cross-linking reactions were performed with a light source (314 nm for analytical and 1 ml for preparative scale) of duplex ODNs (1.2–1.5 μM) and DNA Mtases (2.0–3.0 μM) in Tris acetate buffer (20 mM, pH 7.9) containing potassium acetate (50 mM), magnesium acetate (10 mM) and DTT (1 mM). Photo-cross-linking reactions were induced by a HeCd laser (Licoci, Santa Clara, CA) operating at 325 nm with an intensity of 25 miliwatts/mm². Samples were irradiated in Q5 quartz cuvettes (Helma, Mülheim, Germany) at 4 °C for 2 h or indicated times. Photo-cross-linking reactions were either analyzed by SDS-polyacrylamide gel electrophoresis or anion-exchange chromatography. The long ODNs (36 nucleotides) were 32P-labeled by treatment with T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP (110 TBq/mmol, 0.4 MBq/μl), Hartmann Analytik, Braunschweig, Germany) in Tris hydrochloride buffer (70 mM, pH 7.6) containing magnesium chloride (10 mM) and DTT (5 mM) prior to the hybridization and photo-cross-linking reactions. Photo-cross-linked and un-cross-linked duplex ODNs were separated on 16% SDS-polyacrylamide gels (29), and radioactive bands were quantified using a phosphorimager (Molecular Imager System GS 525, BioRad). Reaction mixtures with the short duplex ODN (14 base pairs) were loaded onto an anion-exchange column (Poros 200, Perseptive Biosystems) and compounds were eluted with a Tris hydrochloride buffer (70 mM, pH 7.4) containing potassium chloride (0 mM for 5 min followed by a linear gradient to 1 M within 15 min and 1 M for 10 min) at a flow rate of 2 ml/min. Absorption was detected at 260 nm.

**Determination of the Photo-cross-linked Amino Acid Residue**—Proteolytic fragmentation was investigated using a crude reaction mixture obtained with M.CviBIII and [32P]labeled TCGA/TCGA(S). The buffer of

![Scheme 1. Photo-cross-linking reaction of the N6-adenine DNA Mtase M.TaqI and its isoschizomer M.CviBIII with duplex ODNs containing 5-iodouracil at the target position.](image-url)
the reaction mixture was changed to Tris hydrochloride (10 mm, pH 8.0) and calcium chloride (20 mm) by repeated ultrafiltration (Centricon 10, Amicon), chymotrypsin (50% of total protein weight) was added, and the mixture incubated at 37 °C. Samples were taken after different incubation times and analyzed on a 16% SDS-polyacrylamide gel. For the isolation of the nucleopeptide, a preparative amount of the crude reaction mixture containing approximately 700 pmol of the covalent M.TaqI/TCGI/TCGA(S) complex was enzymatically fragmented for 16 h, as described above. The nucleopeptide was purified on an anion-exchange column (Pores H-Q10, 4.6 × 100 mm, Perseptive Biosystems) using a linear gradient of potassium chloride (0–1.0 M) in Tris hydrochloride buffer (10 mm, pH 7.4). Fractions containing the nucleopeptide (detection at 260 nm) were pooled and desalted by repeated ultrafiltration (Microsep 3K, Pall-Gelman Sciences). Peptide sequencing of the isolated nucleopeptide was performed with an Applied Biosystems 473A protein sequencer using standard conditions. The electrospray ionization (Microsep 3K, Pall-Gelman Sciences). Peptide sequencing of the isolated nucleopeptide was performed using an Applied Biosystems 473A protein sequencer using standard conditions. The electrospray ionization mass spectrometry of the isolated nucleopeptide was acquired using a double focusing sector field mass spectrometer MAT 90 (Finnigan MAT) equipped with an ESI II electrospray ion source.

RESULTS

Formation of a Photo-cross-link with M.TaqI—Initial photo-cross-linking experiments were performed with a hemimethylated duplex ODN (36 base pairs) in which the target adenine within the recognition sequence of M.TaqI was replaced by 5-iodouracil, in the presence of M.TaqI. A reaction mixture containing 32P-labeled duplex ODN TCGI/TCGA(Me)(L) (1.2 μM) and M.TaqI (3.0 μM) obtained after irradiation at 325 nm for the indicated times, B, photo-cross-linking yields after different reaction times were determined from A using a phosphorimagier, and the data were fitted to a single exponential function.

Isolation of a Covalent DNA-M.TaqI Complex—For the isolation of a covalent DNA-M.TaqI complex, the photo-cross-linking reaction was performed with TCGI/TCGA(S), a 14-base pair duplex ODN containing 5-iodouracil at the target position, and the reaction mixture was separated by anion-exchange chromatography (Fig. 2). M.TaqI did not bind to the anion-exchange column under the conditions used and eluted in the void volume, whereas TCGI/TCGA(S) eluted after 18.6 min. In addition, a new compound eluting after 12.0 min was observed. Such a lower retention time compared with the free ODN is expected for a DNA-M.TaqI complex, because binding of M.TaqI should lead to some steric shielding of the negatively charged phosphodiester backbone. From the peak areas of the compounds eluting after 12.0 and 18.6 min, a photo-cross-linking yield of 53% with regard to the duplex ODN TCGI/TCGA(Me)(L), in which the thymine within the recognition sequence is replaced by 5-iodouracil, in the presence of M.TaqI did not lead to a significant formation of a photo-cross-linked product. This result indicates that the photo-cross-linking reaction is DNA sequence-specific. Additionally, irradiation of the control duplex ODNs TCGA/TCGA(Me)(L) and TCGA/TCGA(Me)(L), which do not contain 5-iodouracil, in the presence of M.TaqI yielded no photo-cross-linked product. In addition, electrophoretic mobility shift experiments under native conditions were performed and revealed that binding of M.TaqI to canonical duplex ODNs is in the low nanomolar range and not altered significantly upon 5-iodouracil substitution.

Isolation of a Covalent DNA-M.TaqI Complex—For the isolation of a covalent DNA-M.TaqI complex, the photo-cross-linking reaction was performed with TCGI/TCGA(S), a 14-base pair duplex ODN containing 5-iodouracil at the target position, and the reaction mixture was separated by anion-exchange chromatography (Fig. 2). M.TaqI did not bind to the anion-exchange column under the conditions used and eluted in the void volume, whereas TCGI/TCGA(S) eluted after 18.6 min. In addition, a new compound eluting after 12.0 min was observed. Such a lower retention time compared with the free ODN is expected for a DNA-M.TaqI complex, because binding of M.TaqI should lead to some steric shielding of the negatively charged phosphodiester backbone. From the peak areas of the compounds eluting after 12.0 and 18.6 min, a photo-cross-linking yield of 53% with regard to the duplex ODN TCGI/TCGA(S) was calculated. The material eluting after 12.0 min was isolated, desalted, 32P-labeled by treatment with T4 polynucleotide kinase and [γ-32P]ATP, and analyzed by denaturing PAGE (Fig. 3). Again, a strongly reduced electrophoretic mobility of the product formed. Furthermore, irradiation of the duplex ODN ICGA/TCGA(Me)(L), in which the thymine within the recognition sequence is replaced by 5-iodouracil, in the presence of M.TaqI did not lead to a significant formation of a photo-cross-linked product. This result indicates that the photo-cross-linking reaction is DNA sequence-specific. Additionally, irradiation of the control duplex ODNs TCGA/TCGA(Me)(L) and TCGA/TCGA(Me)(L), which do not contain 5-iodouracil, in the presence of M.TaqI yielded no photo-cross-linked product. In addition, electrophoretic mobility shift experiments under native conditions were performed and revealed that binding of M.TaqI to canonical duplex ODNs is in the low nanomolar range and not altered significantly upon 5-iodouracil substitution. The recognition sequence of M.TaqI and M.CviIII is shown with larger spacing, and the following symbols are used: I = 5-iodo-2'- deoxyuridine, A(Me) = N6-methyl-2'-deoxyadenosine, L = long (36 base pairs), S = short (14 base pairs).  

| x  | y  | z  | Abbreviation                     |
|----|----|----|----------------------------------|
| T  | I  | A(Me)| TCGI/TCGA(Me)(L)               |
| T  | I  | A   | TCGI/TCGA(L)                   |
| I  | A  | A(Me)| TCGA/TCGA(Me)(L)              |
| T  | A  | A(Me)| TCGA/TCGA(Me)(L)              |
| T  | A  | A   | TCGA/TCGA(L)                   |

| x  | y  | z  | Abbreviation                     |
|----|----|----|----------------------------------|
| I  | TCGI/TCGA(S)                           |

FIG. 1. Photo-cross-linking of a duplex ODN containing 5-iodouracil at the target position and M.TaqI as a function of time. A, denaturing PAGE of the reaction mixture containing 32P-labeled duplex ODN TCGI/TCGA(Me)(L) (1.2 μM) and M.TaqI (3.0 μM) obtained after irradiation at 325 nm for the indicated times. B, photo-cross-linking yields after different reaction times were determined from A using a phosphorimagier, and the data were fitted to a single exponential function.

TABLE I

Sequences of duplex ODNs and their abbreviations used in this report

The recognition sequence of M.TaqI and M.CviIII is shown with larger spacing, and the following symbols are used: I = 5-iodo-2'-deoxyuridine, A(Me) = N6-methyl-2'-deoxyadenosine, L = long (36 base pairs), S = short (14 base pairs).

| x   | y   | z   | Abbreviation                     |
|-----|-----|-----|----------------------------------|
| 5'- | GCCCGGC | T  | G × CCAGG-3'                      |
| 3'  | GCGCGG| A  | G × T GCGG-5'                     |

| x  | Abbreviation |
|----|--------------|
| I  | TCGI/TCGA(S) |
Photo-cross-linking of N6-Adenine DNA Mtas

**Fig. 2.** Analysis of the photo-cross-linked DNA-M.TaqI complex by anion-exchange chromatography. The complex was produced by irradiation of the short duplex ODN TCGI/TCGA(S) (1.5 µM) and M.TaqI (2.0 µM) at 325 nm for 2 h.

**Fig. 3.** Denaturing PAGE of the isolated photo-cross-linked DNA-M.TaqI complex. Lane 1, 32P-labeled duplex ODN TCGI/TCGA(S) alone; lane 2, isolated (material eluting after 12.0 min in Fig. 2) and 32P-labeled photo-cross-link between M.TaqI and the duplex ODN TCGI/TCGA(S); lane 3, non-covalent complex between M.TaqI and 32P-labeled duplex DNA-M.CviBIII (S).

mobility (Fig. 3, lane 2) compared with the free ODN TCGI/TCGA(S) (Fig. 3, lane 1) was observed. In addition, a mixture of M.TaqI and TCGI/TCGA(S) did not show a band with reduced mobility under denaturing conditions (Fig. 3, lane 3), demonstrating that the material eluting after 12.0 min during anion-exchange chromatography is a covalent DNA-M.TaqI complex.

In order to identify the photo-cross-linked amino acid in M.TaqI, the covalent DNA-M.TaqI complex was subjected to proteolytic degradation after purification by anion-exchange chromatography. However, enzymatic fragmentation of the covalent DNA-M.TaqI complex with different specific proteases (trypsin, chymotrypsin, elastase, and V8 endopeptidase) even under partially denaturing conditions (1 M guanidinium hydrochloride, 1% SDS, or 30% methanol) did not produce a small nucleopeptide complex. Thus, the resistance of the covalent DNA-M.TaqI complex toward proteases, presumably due to the thermophilic nature of M.TaqI, precluded further characterization of the photo-cross-linked amino acid in M.TaqI.

**Fig. 4.** Analysis of the photo-cross-linked DNA-M.CviBIII complex by anion-exchange chromatography. The complex was produced by irradiation of the short duplex ODN TCGI/TCGA(S) (1.5 µM) and M.CviBIII (2.0 µM) at 325 nm for 2 h.

photocross-linking reaction between M.CviBIII and the short duplex ODN TCGI/TCGA(S) by anion-exchange chromatography revealed that almost all the duplex ODN had reacted, and a new compound with a retention time of 7.9 min was formed (Fig. 4). M.CviBIII eluted in the void volume as observed for M.TaqI. As the photo-cross-linking reaction with M.CviBIII was almost quantitative with respect to the duplex ODN, a crude reaction mixture of 32P-labeled TCGI/TCGA(S) and M.CviBIII was subjected to proteolytic fragmentation with chymotrypsin, and the time course of the protease reaction was analyzed by denaturing PAGE (Fig. 5). After 16 h, almost all reaction intermediates had converted to a final product. The smaller electrophoretic mobility of this proteolytic end product compared with the free duplex ODN indicates that a peptide fragment remained bound to the DNA.

Identification of the Modified Amino Acid in the Covalent DNA-M.CviBIII Complex—A preparative amount of the photocross-linking reaction mixture was treated with chymotrypsin, and the resulting nucleopeptide was purified by anion-exchange chromatography. Fractions containing the nucleopeptide were concentrated and desalted by ultrafiltration, and the isolated nucleopeptide was subjected to automated peptide sequencing by Edman degradation. The peptide sequence of the first 13 residues was found to be DFIVGNPPXVR, and at position 9 (X) none of the 20 natural amino acids was observed in appreciable amounts. This peptide sequence corresponds to the amino acid residues 114–126 of M.CviBIII and contains a Tyr residue at position 122. The absence of Tyr at position 9 in the sequenced peptide suggests that Tyr-122 in M.CviBIII and contains a Tyr residue at position 122. The absence of Tyr at position 9 in the sequenced peptide suggests that Tyr-122 in M.CviBIII was modified in the photo-cross-linking reaction. The observed peptide sequence is preceded by a Phe residue in M.CviBIII, providing a cleavage site for chymotrypsin, which cleaves after aromatic amino acid residues. However, the non-aromatic residue Pro was found at the C terminus, which indicates that some C-terminal amino acid residues were missing. The C-terminal part of the nucleopeptide and its overall structure were verified by ESI-MS. The DNA strands of the nucleopeptide underwent dissociation in the transport region of the electrospray interface and the modified strand was detected as 5–9-fold negatively charged ions. A deconvoluted mass spectrum of the modified strand is shown in Fig. 6. The mass of the nucleopeptide was found to be 5900.5, and the higher observed masses in Fig. 6 represent sodium and potassium adducts. The observed mass corresponds well to the calculated mass of 5900.6 for a nucleopeptide containing the amino acid residues 114–129 of M.CviBIII, which has a chymotrypsin cleavage site at its C-terminal end (Fig. 7).

**DISCUSSION**

DNA Binding and Base Flipping by M.TaqI—The crystal structure of M.TaqI in complex with the cofactor shows that the
enzyme consists of two domains forming a positively charged cleft with a diameter of 21 Å, which closely matches the diameter of B-DNA (9). However, modeling B-DNA into the structure showed that the distance between the amino group of the target adenine within the DNA helix and the methyl group of AdoMet is 15 Å, which is too far for a direct methyl group transfer (30). By rotating the adenine out of the DNA helix toward the cofactor, the distance between the methyl group donor and acceptor can be reduced significantly, and hence a base flipping mechanism, as observed in the crystal structures of the C5-cytosine DNA Mtases M.HhaI (7) and M.HacIII (8) in complex with DNA, was proposed for M.TaqI. Biochemical evidence for a base flipping mechanism of M.TaqI was obtained using a duplex ODN, in which the target adenine is replaced with the fluorescent base analogue 2-amino purine (12). The 2-aminopurine fluorescence is highly quenched in polynucleotides as a result of interactions with neighboring bases and is strongly enhanced upon binding of M.TaqI. In addition, a thymine residue placed at the target position within a duplex ODN showed an enhanced reactivity toward potassium permanganate oxidation in the presence of M.TaqI (15). This result can readily be explained by a higher accessibility of the thymine residue toward permanganate in the DNA-M.TaqI complex because of base flipping. As the Van der Waals radius of iodine (2.15 Å) is only slightly larger than that of a methyl group (2.0 Å), base flipping is also expected for the 5-iodouracil residue in a complex between M.TaqI and a duplex ODN containing 5-iodouracil at the target position.

Binding of M.TaqI to canonical and 5-iodouracil-modified duplex ODNs was analyzed in an electrophoretic mobility shift assay under native conditions, and revealed that substitution of the target adenine with 5-iodouracil exerted only little effect on the binding strength. This result is consistent with earlier binding studies, which showed that a duplex ODN carrying a mismatched thymine at the target position binds with a similar affinity as the canonical substrate (15). Recently, tighter binding of the C5-cytosine DNA Mtases M.HhaI (31, 32) and M.HpaII (31) as well as the N6-adenine DNA Mtases M.EcoRV (13) and M.EcoRI (14) to duplex ODNs carrying mismatched bases or base analogues with reduced Watson-Crick hydrogen bonding potential at the target positions was observed and attributed to less energy required to disrupt a mismatched base pair and flip out the target base. As the energetic contribution of base flipping to DNA binding should at least involve the energy needed to flip out the target base and the energy gained by binding the extrahelical base, replacement of the matched target base by a mismatched base or a base analogue can lead to tighter, unchanged, or even worse binding, depending on the sum of positive and negative energetic terms. Thus, the similar affinities of M.TaqI for duplex ODNs containing adenine, 5-iodouracil, or thymine at the target position are compatible with a base flipping mechanism.

Photo-cross-linking Experiments with the Mutant Mtases M.TaqI—M.TaqI was photo-cross-linked to the hemimethylated TCGI/TCGA(L) and the unmethylated TCGI/TCGA(AL) duplex ODNs carrying the photo-activable 5-iodouracil at the target position in 50–60% yield. The DNA sequence specificity of the cross-linking reaction is readily demonstrated by our observation, that the hemimethylated duplex ODN TCGA/TCGA(MeL), in which thymine within the recognition sequence of M.TaqI is replaced by 5-iodouracil, yielded no significant amounts of a photo-cross-linked product. In order to facilitate purification of the photo-cross-linked complex by anion-exchange chromatography, the short duplex ODN TCGI/TCGA(S) was photo-cross-linked with M.TaqI and the covalent complex isolated. However, all attempts to digest the covalent DNA-M.TaqI complex with various specific proteases did not result in short nucleopeptide fragments which precluded further analysis. This resistance toward proteases is presumably due to the thermophilic nature of M.TaqI. A relatively high resistance toward proteolytic cleavage was also reported for a photo-cross-link between the thermostable MutS protein from T. aquaticus and a duplex ODN (33).

Photo-cross-linking Experiments with the Mutant Mtases M.TaqI/Y108A and M.TaqI/F196A—In order to test whether Tyr-108 or Phe-196 of M.TaqI are involved in the photo-cross-
linking reaction, two mutants of M.TaqI were investigated. In M.TaqI/Y108A Tyr at position 108 and in M.TaqI/F196A Phe at position 196 are replaced by Ala. The photo-cross-linking yield of TCGI/TCGA(L) and M.TaqI/Y108A was found to be 6-fold and that of M.TaqI/F196A 2-fold reduced compared with the wild-type enzyme. In an earlier study, we have already investigated DNA binding of these mutant Mtases using a fluorescence-based assay (27). Titrations of a duplex ODN, in which the target adenine was replaced by the fluorescent base analogue 2-aminopurine, yielded a slightly increased DNA binding affinity of M.TaqI/Y108A, whereas that of M.TaqI/F196A was unchanged compared with the wild-type enzyme. This demonstrates that the reduced photo-cross-linking yield with the mutant Mtases is not caused by a lower DNA binding affinity. However, the magnitude of the 2-aminopurine fluorescence increase observed in the titrations, which correlates with the ability of the Mtases to flip out the target base, was different. It was found that M.TaqI/F196A is impaired in base flipping, which offers a convenient explanation for its somewhat reduced ability to form a photo-cross-linked complex. In contrast, the mutant Mtase M.TaqI/Y108A is still able to flip the target base. Thus, the strongly reduced photo-cross-linking yield observed with M.TaqI/Y108A can directly be associated with the missing aromatic side chain and suggests that Tyr at position 108 is the primary site of modification in M.TaqI. A similar result was obtained in photo-cross-linking experiments with RNA containing 5-iodouracil and a variant of the MS2 coat protein deficient in forming phagelike capsid particles (21). The photo-cross-link was formed to a Tyr residue at position 85 in high yield, and a mutant containing Ala at this position failed to form a photo-cross-linked complex.

Photo-cross-linking Experiments with M.CviBIII—For fur-
Photo-cross-linking of N⁶-Adenine DNA Mtases

other photo-cross-linking experiments, we used the N⁶-adenine DNA Mtase M.CviBIII, a mesophilic isochizomer of M.TaqI. Photo-cross-linking of the short duplex ODN TCGI/TCGA(S) with M.CviBIII proceedded with an almost quantitative yield (Fig. 4). This represents the highest photo-cross-linking yield reported for double-stranded DNA and a protein. Furthermore, it was possible to fragment the DNA-protein complex enzymatically (Fig. 5), isolate the formed nucleoepitope, and determine its amino acid sequence by Edman degradation and its mass by ESI-MS (Fig. 6). We found that Tyr at position 122 in M.CviBIII is the site of modification in the photo-cross-linking reaction. Very high photo-cross-linking yields were also reported for the RNA-binding domain of the U1A spliceosomal protein (20) and the bacteriophage MS2 coat protein (21), where three-dimensional structures of the protein-RNA complexes are known (34, 35). In these complexes a Tyr residue is found in a π-stacking arrangement with a cytosine residue, and replacement of these cytosine residues by 5-iodouracil resulted in 67–90% photo-cross-linking yields. In fact, high photo-cross-link efficiencies with 5-iodouracil were attributed to such a π-stacking arrangement, in which an initial photoelectron transfer could take place before the resulting radical ion pair collapses to form a covalent bond (17, 36). Thus, the high yield of the photo-cross-linking reaction with M.CviBIII strongly indicates that Tyr-122 is in close proximity and most likely π-stacked with 5-iodouracil in the protein-DNA complex.

A Structural Model of M.TaqI in Complex with DNA—In an amino acid sequence alignment, Tyr-122 of M.CviBIII and Tyr-108 of M.TaqI were found in corresponding positions within a 20-amino acid fragment with 70% sequence identity (37). This supports our conclusion from the photo-cross-linking experiments with the mutant Mtases M.TaqI/Y108A and M.TaqI/F196A that Tyr at position 108 is the primary site of modification in M.TaqI. A model of M.TaqI in complex with TCGI/TCGA(S) is presented in Fig. 8. Docking B-DNA into the proposed DNA binding cleft of the M.CviBIII is presented in Fig. 8. Docking B-DNA into the F196A that Tyr at position 108 is the primary site of modification with the mutant Mtases M.CviBIII supports our conclusion from the photo-cross-linking experiment, 20-amino acid fragment with 70% sequence identity (37). This indicates that Tyr-122 is in close proximity and most likely π-stacked with 5-iodouracil in the protein-DNA complex.

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