Functional Roles of Conserved Amino Acid Residues in DNA Methylltransferases Investigated by Site-directed Mutagenesis of the EcoRV Adenine-\(N^6\)-methyltransferase*

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All DNA methyltransferases (MTases) have similar catalytic domains containing nine blocks of conserved amino acid residues. We have investigated by site-directed mutagenesis the function of 17 conserved residues in the EcoRV \(\alpha\)-adenine-\(N^6\)-DNA methyltransferase. The structure of this class of MTases has been predicted recently. The variants were characterized with respect to their catalytic activities and their abilities to bind to DNA and the \(S\)-adenosylmethionine (AdoMet) cofactor. Amino acids located in motifs X, I, and II are shown to be involved in AdoMet binding (Lys\(^{14}\), Glu\(^{37}\), Phe\(^{39}\), and Asp\(^{58}\)). Some of the mutants defective in AdoMet binding are also impaired in DNA binding, suggesting allosteric interactions between the AdoMet and DNA binding site. Asp\(^{78}\) (motif III), which was supposed to form a hydrogen bond to the AdoMet on its DNA binding and kinetic properties (33, 34, 36–39). Using its DNA binding and kinetic properties (33, 34, 36–39). Using

Methylation of DNA is essential in mammals. It is involved in regulation of gene expression, imprinting, development, chromatin structure, DNA replication, and origin of cancer (for reviews, see Refs. 1–5). DNA can be modified by two classes of DNA methyltransferases (MTases), viz., C-MTases modifying cytosines at the \(C^5\)-position and \(N\)-MTases transferring a \(N^6\)-methyl group to cytosine-\(N^4\)-, or adenine-\(N^6\)-positions. Prokaryotic DNA MTases comprise approximately 250–400 amino acid residues (for reviews, see Refs. 6–9). The C-terminal domain of eukaryotic enzymes (for a review, see Ref. 1) bears homology to the prokaryotic cytosine-\(C^5\)-MTases, which contain 10 conserved amino acid motifs (for reviews, see Refs. 8 and 10).

So far the structures of two prokaryotic C-MTases are known, M. \(HhaI\) (11–14) and \(HaeIII\) DNA methyltransferase (15). Both enzymes consist of two domains. One large, catalytic domain forms the binding site for the cofactor \(S\)-adenosylmethionine (AdoMet) and the catalytic center and harbors nine of the 10 conserved amino acid motifs. One smaller domain is responsible for DNA recognition. The catalytic mechanism of these enzymes involves flipping out the target base from the DNA helix (12). Then a conserved cysteine (motif IV) performs a nucleophilic attack on the \(C^5\)-position of the cysteine, forming a covalent enzyme-DNA intermediate. Thereby, the \(C^5\)-position is activated and accepts the methyl group from AdoMet. Subsequently, the covalent intermediate is deprotonated at \(C^5\) leading to the elimination of the cysteine (16, 12, 14).

\(N\)-MTases contain nine conserved amino acid motifs. An FXGXXG motif, also present in cytosine-\(C^5\)-MTases, and a DPPY motif (consensus sequence, \(S/N/D)PP(Y/F/W)\)) are moderately conserved (17–20), and the homologies of seven additional motifs are weak. Consequently, these additional motifs only recently could be identified in structure-guided alignments (21). Whereas some amino acid residues within the FXGXXG and DPPY motifs have already been investigated by site-directed mutagenesis (22–25), no mutational studies have been carried out so far to test the functional roles of conserved amino acid residues within the other motifs either in C- or N-MTases.

According to the amino acid sequences and order of the conserved motifs, N-MTases can be subdivided into three distinct groups, viz., \(\alpha\)-N-MTases (e.g., M. \(EcoRV\), an adenine-\(N^6\)-MTase), \(\beta\)-N-MTases (e.g., \(PvuII\) DNA methyltransferase, a cytosine-\(N^4\)-MTase), and \(\gamma\)-N-MTases (e.g., M. \(TaqI\), an adenine-\(N^6\)-MTase) (20, 21). Eukaryotic enzymes involved in mRNA processing, double-stranded RNA adenosine deaminase (26), and \(mRNA\) (adenosine-\(N^6\)-)methyltransferase (27) are similar to DNA \(N\)-MTases. So far, structures are available only for \(\beta\)- and \(\gamma\)-type apoenzymes (M. \(TaqI\) (28) and \(PvuII\) DNA methyltransferase (29)). Like C-MTases, N-MTases are built up of two domains. Surprisingly, the catalytic domain of N-MTases has a similar structure as the catalytic domains of C-MTases, although the chemistry of catalysis is different (30). Interestingly, the conserved DPPY motif structurally corresponds to motif IV in C-MTases, which contains the catalytic cysteine residue. It is likely that N-MTases, like C-MTases, flip their target base out of the DNA double helix (31–34). The \(EcoRV\) DNA methyltransferase specifically methylates the first adenine within the \(EcoRV\) recognition sequence GATATC (35). The enzyme is well characterized with respect to its DNA binding and kinetic properties (33, 34, 36–39). Using

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‡ The abbreviations used are: MTase, DNA methyltransferase; M. \(HhaI\), \(HhaI\) DNA methyltransferase; M. \(EcoRV\), \(EcoRV\) DNA methyltransferase; M. \(TaqI\), \(TaqI\) DNA methyltransferase; AdoMet, \(S\)-adenosylmethionine; PCR, polymerase chain reaction; GST, glutathione S-transferase; R. \(EcoRV\), \(EcoRV\) restriction endonuclease.

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FIG. 1. Alignments of M. EcoRV with other \( \alpha \)-N-MTases. Amino acid exchanges introduced in M. EcoRV in this work are indicated by vertical arrows, which point to the identity of the new amino acid. Strongly and moderately conserved residues are boxed and shaded darkly and lightly, respectively. The position of the putative DNA binding domain is indicated by vertical arrows. A, alignment of the catalytic domains of all \( \alpha \)-N-MTases. On the basis of this structure-guided multiple alignment, 10 conserved amino acid regions have been defined (21). B, alignment of M. EcoRV with the seven most closely related \( \alpha \)-N-MTases (40). The approximate locations of conserved amino acid motifs as defined in Ref. 21 are indicated.
Fig. 1—continued
multiple sequence alignments (Fig. 1) as well as secondary structure predictions the structure of M. EcoRV and other α-type N-MTases was predicted to contain a similar catalytic domain as the other MTases (Fig. 2) (21, 40). On the basis of these predictions, putative functional roles for several conserved amino acid residues (Fig. 1) can be derived, e.g. involvement in DNA binding, AdoMet binding, or catalysis (Fig. 2). In particular, a putative binding site for the flipped target base can be derived by comparison with C-MTases (31, 21). Here we report the results of an extensive mutational study of 17 conserved amino acid positions of M. EcoRV that allow us to draw conclusions as to (i) the validity of the structural model for M. EcoRV and α-N-MTases, (ii) the functional roles of some amino acid residues within the conserved motifs of N-MTases, and (iii) the catalytic mechanism of N-MTases. To our knowledge, this work represents the first comprehensive analysis of a DNA MTase by site-directed mutagenesis that covers positions located in all conserved motifs as well as in the DNA recognition domain. Thus, it complements the detailed structural information available for this important family of enzymes.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—Site-directed PCR mutagenesis was carried out on pRVMetH6, encoding the N-terminal His$_6$-M. EcoRV fusion protein (34, 40). In this vector, the M. EcoRV gene is controlled by a synthetic promotor-operator sequence obtained by a combination of the EcoRV gene and the desired mutation. All mutant proteins purified in this study were obtained at similar concentrations as wild type M. EcoRV (viz. 5–25 μM). The specific activities of different GST-M. EcoRV preparations (50–100 units/μg) were slightly higher than those reported for untagged M. EcoRV (35). His$_6$-M. EcoRV could be purified by a 10–50-fold higher specific activity (34).

In Vitro Activity Assays—Kinetics of restriction protection were carried out using λ-DNA (21 EcoRV sites) and pAT153-DNA (one EcoRV site) in 50 mM NaCl, 50 mM HEPES (pH 7.5), 1 mM AdoMet at ambient temperature as described (42). 1 unit is defined as the amount of M. EcoRV required to protect 1 μg of DNA from R. EcoRV attack in 1 h. Specific activities (units/μg of protein) for GST fusion proteins were calculated using the molecular mass only of the M. EcoRV part (35,000 g/mol) in order to make the activities comparable with those measured with His$_6$ fusion proteins. Methyltransferase of a 20-mer oligonucleotide d(GATCGACATCGTCGATC) was carried out using $[^3]^{32}$P]AdoMet in 50 mM NaCl, 50 mM HEPES (pH 7.5) at ambient temperature as described (34). To determine $K_m$ values for DNA and AdoMet, DNA concentrations were varied between 0.2 and 4 μM and AdoMet concentrations were varied between 1 and 10 μM at 1 μM DNA, and the determined rates of methylation of DNA were fit to the Michaelis-Menten model.

DNA Binding—DNA binding was analyzed by nitrocellulose filter experiments carried out in a dot blot apparatus (Bio-Rad) as described (34) using a PCR product (352 base pairs) at 1 nM and a 20-mer oligonucleotide d(GATCGACATCGTCGATC) (5 ng) both containing one EcoRV site as substrates. The oligonucleotide substrate was labeled radioactively using [32P]ATP (Amersham Pharmacia Biotech) and polynucleotide kinase (MBI Fermentas). The DNA was incubated with differing amounts of M. EcoRV variants (10–50 units) in 50 μl of 50 mM Tris/HCl (pH 7.5), 2 mM MgCl$_2$, 50 mM NaCl, 1 mM EDTA, and 200 μM sinefungin for 30 min at ambient temperature. The nitrocellulose filter membrane (Merckey & Nagel, Düren, Germany) was prerinsed with 50 mM Tris/HCl (pH 7.5), 20 mM NaCl for 30 min. After membrane was transferred into the dot blot chamber, the slots were washed twice with 100 μl of 50 mM Tris/HCl, pH 7.5, 20 mM NaCl. The samples were transferred into the wells of the dot blot apparatus using a multiple encoding a DNA MTase are resistant to cleavage by the corresponding restriction endonuclease. This property can be used to assay the catalytic activity of MTases in vivo. To this end, pRVMetH6 (34, 40) or pGEXMRV (42) plasmids were grown in Escherichia coli LK1111(a) cells containing chromosomally encoded lacI. The transcription of the M. EcoRV gene was not induced. Plasmid preparations from overnight cultures and from growing cells (at 1 A$_{600}$) were carried out using DNA minipreparation kits (Qiagen) according to the instructions of the supplier, except that in the case of growing cells 20 ml of cell culture was used instead of 3–4 ml as used in the case of stationary overnight cultures. Plasmid DNA (1–2 μg) was incubated with 100–200 nM R. EcoRV (corresponding to approximately 100 units) in 10 μl of Tris/HCl (pH 7.5), 10 mM MgCl$_2$, 50 mM NaCl for 30–60 min. Subsequently, the samples were analyzed by agarose gel electrophoresis. Under these conditions, an unmethylated control plasmid was completely cleaved after 5 min, but no nonspecific DNA degradation of methylated plasmids was observed after incubation for 60 min.

Fig. 2. Location of mutated amino acid residues in the structural model of M. EcoRV (40). Strongly conserved amino acid residues are given in gray ovals, and less conserved residues are in white ovals. The positions of the putative AdoMet and adenine binding sites are indicated. The structure prediction is taken from Jeltsch et al. (40). It does not differ from that given by Malone et al. (21) in the region of amino acid residues 1–235. α-helices and β-strand are labeled A–F and 1–9. Localizations of the secondary structure elements in primary sequence are as follows: α-helix A, 18–26; β-strand 1, 42–46; α-helix B, 44–48; β-strand 2, 53–58; α-helix C, 61–63; β-strand 3, 64–70; α-helix D, 78–86; β-strand 4, 188–192; α-helix E, 211–222; β-strand 5, 224–229; α-helix F, 239–248; β-strand 6, 250–258; β-strand 7, 270–276; β-strand 8, 280–285; β-strand 9, 292–299.
RESULTS

We have used multiple sequence alignments to identify conserved amino acid residues in the EcoRV adenine-N6-MTase. Two alignments were analyzed for conserved residues, one including all α-type N-MTases (Fig. 1A) (21) and one in which only a subgroup of enzymes closely related to M. EcoRV are aligned (Fig. 1B) (40). On the basis of these alignments, we have chosen 17 amino acid residues that are strongly (Lys16, Tyr196, Ser229) or moderately conserved (Asp78, Asp211, Glu217, Glu37, Phe39, Asp58, Glu104, Arg128, Asn130, Glu178, Asp193, E37A, F39A, D58A, R128A, N130A, D193(A/N), Y196(A/F), Y196F) and moderately conserved (Asp180) residues (see Table II). All residues were substituted into the EcoRV adenine-N6-MTase. All mutants were tested for their ability to methylate DNA in vitro, and most of the variants were purified and also characterized in vivo with respect to their catalytic properties as well as DNA binding and AdoMet binding affinities.

Catalytic Activity of the M. EcoRV Variants in Vivo—We have cloned all mutants as His6 fusion proteins in pRVMetH6. This plasmid contains one EcoRV recognition site. During propagation of the expression plasmids coding for the individual mutants in E. coli cells, residual expression of the MTase variant takes place even under repressing conditions. Thus, the MTase variant can modify the plasmid DNA in vivo, with the result that purified plasmids are resistant against R. EcoRV cleavage to a degree corresponding to the catalytic activity of the MTase variant. We have carried out this in vivo activity test in two ways by isolating plasmids either from stationary E. coli cells or from growing E. coli cells (Fig. 4, Table I). Plasmids encoding wild type M. EcoRV are fully protected against R. EcoRV cleavage under both experimental conditions. Generally, the degree of methylation of EcoRV sites is lower under conditions of active growth, because then DNA replication takes place and, in comparison with stationary E. coli cells, higher M. EcoRV activities are required to achieve the same degree of methylation. Five of the variants fail to methylate the DNA even in stationary E. coli cells (D58A, R128A, D193A, D193N, and Y196A). In growing cells, many variants show a strongly reduced level of DNA methylation indicating a strongly reduced catalytic activity (– or – in Table I) (K16A, E37A, F39A, D58A, R128A, N130A, D193(A/N), Y196(A/F), S229A, Y258A).

Catalytic Activity of the M. EcoRV Variants in Vitro—To investigate the mutant proteins in vitro, we have overexpressed and purified wild type M. EcoRV and 18 of the variants (Table I). E104A and E178A that are fully active in vivo were not purified and further investigated. Since many mutants could not be overexpressed as His6 fusion proteins or remained in the pellet after cell disruption and centrifugation, we decided to express and purify all mutants as GST fusion proteins. To this end, all mutants were also cloned as GST fusion proteins in pGEXRV. The catalytic activities of these variants in stationary E. coli cells are very similar to those of the His6 fusion proteins (data not shown). Wild type GST-M. EcoRV and the mutants were overexpressed in 500-ml scale and purified by
two chromatography steps (glutathione-Sepharose and phosphocellulose) to >90% purity as judged by Coomassie-stained SDS-polyacrylamide gels. The concentrations of all preparations were between 5 and 25 μM. We have determined the catalytic activity of wild type GST-M. EcoRV and all mutants by 1- and plasmid-DNA protection assays (Fig. 5; Table II). High catalytic activity could be detected only with wild type M. EcoRV and the D78A mutant. The Y196F, E217A, E238A, and D244A mutants displayed a reduced activity in vitro, and all other variants were catalytically inactive in vitro. In general, there is a very good agreement between the in vivo and in vitro data; most mutants with strongly reduced catalytic activities in vivo, (− or − in growing cells, Table I), are catalytically inactive in vitro, whereas most mutants with good in vivo activity (+ or ++ in growing cells, Table I) also display catalytic activity in vitro. There are only two exceptions to these rules; Y196F has a low catalytic activity in vivo (− in Table I) but is not inactive in vitro, and D211A is inactive in vitro but shows good activity in vivo (+ in Table I). These differences may be caused by different expression rates and/or different in vivo and in vitro stabilities of these two mutants.


**Kinetic Characterization of Catalytically Active M. EcoRV Variants**—The activities of the Y196F, E217A, E238A, and D244A variants were determined by the restriction protection assay at concentrations of AdoMet between 1 mM and 0.3 μM (data not shown). The catalytic activities of the variants were analyzed according to the Michaelis-Menten model to estimate a K_{m} value for AdoMet. According to this analysis, the Y196F, E217A, and E238A mutants had K_{m} values between 5 and 20 μM, similar to wild type M. EcoRV and the D78A variant (see below). In contrast, the D244A variant shows a significantly higher K_{m} value for AdoMet (>250 μM), indicating a reduced affinity for the cofactor.

In addition to performing the restriction protection assays using macromolecular substrates, we have analyzed the in vitro activity of all mutants by determining incorporation of radioactively labeled methyl groups into a 20-mer oligonucleotide. However, apart from wild type M. EcoRV, only the D78A variant had sufficiently high activity to allow an unequivocal detection of catalytic activity with this assay. We have analyzed the D78A variant in more detail using the 20-mer substrate and determined its K_{m} values for DNA and AdoMet. Both kinetic constants (K_{m}, DNA = 0.4 ± 0.2 μM in the presence of 5 μM AdoMet; K_{m}, AdoMet = 13 ± 5 μM in the presence of 1 μM 20-mer) were very similar to those measured with wild type M. EcoRV (K_{m}, DNA = 0.3 ± 0.1 μM; K_{m}, AdoMet = 12 ± 5 μM) and wild type His_{6}-M. EcoRV (K_{m}, DNA = 0.3 μM; K_{m}, AdoMet = 12 μM (data taken from Ref. 34)) under these conditions.

**DNA Binding**—We have determined the binding constants of the GST-M. EcoRV variants to a 20-mer oligonucleotide substrate. Wild type GST-M. EcoRV binds to DNA with similar affinity (K_{d} = 5 × 10^{7} M^{-1}) as His_{6}-M. EcoRV (K_{d} = 6 × 10^{7} M^{-1}; Ref. 37) and untagged M. EcoRV (K_{d} to a 30/33-mer oligonucleotide = 2 × 10^{7} M^{-1}; Ref. 37). The D58A, D78A, D193(A/N), Y196(A/F), D211A, E217A, S229A, E238A, D244A, and Y258A variants bind to their DNA substrate with similar binding constants as wild type M. EcoRV (Table II, Fig. 6). Some of the mutants even bind slightly better to DNA than wild type M. EcoRV, which in some of the cases (e.g. D58A, D193N, or D211A) might be caused by the removal of negative charge due to the amino acid exchange. Binding to the 20-mer could not be detected with the K16A, E37A, F39A, R128A, and N130A. In addition, we have analyzed DNA binding using a 382-mer substrate, because we have shown previously that this substrate is bound better by many M. EcoRV mutants (42). Binding to the 382-mer was detectable with all mutants that were able to bind the 20-mer but in addition with the K16A, E37A, and F39A variants. Only the R128A and N130A en-
zymes did not bind to both substrates (Table II, data not shown). Thus, only amino acid substitutions introduced into the putative DNA binding domain of M. EcoRV prevent DNA binding of the enzyme, whereas all variants carrying mutations in the putative catalytic domain can bind to DNA.

AdoMet Binding—To determine AdoMet binding abilities of the GST-M. EcoRV variants, a filter binding assay was employed. In these experiments, 50 pmol of wild type GST-M. EcoRV retain 0.5 pmol of AdoMet on the nylon membrane. At present, we cannot explain this low retention efficiency. We do not think that most of the protein is inactive, because with His6-M. EcoRV the AdoMet retention efficiency was only 3-fold higher (42), although 80% of the protein in this preparation is active in DNA binding (34). It should be noticed that here a heterophasic assay is employed to analyze a binding equilibrium characterized by a relatively low equilibrium binding constant ($K_m$ for AdoMet is 12 $\mu$M; Ref. 34). Under such circumstances, often not all bound ligand molecules are retained on the filter, and stoichiometric data cannot be obtained. Consequently, the AdoMet binding efficiencies observed here (Fig. 7, Table II) were only interpreted qualitatively. Four of the GST-tagged variants displayed AdoMet binding comparable with wild type GST-M. EcoRV (D78A, R128A, D193N, and S229A). AdoMet binding was detectable with all other variants except the K16A, E37A, F39A, and D58A variants. It should be noted that all of these four variants carry an amino acid exchange in the putative AdoMet binding site of M. EcoRV. As shown in Fig. 7, the amount of AdoMet binding observed in the filter binding assay is significantly lower with the D244A variant than with D78A, Y196F, E217A, and E238A. This result is in good agreement with the results of the kinetic analyses of the AdoMet dependence of the D78A, Y196F, E217A, E238A, and D244A variants, because D244A has a significantly higher $K_m$ for AdoMet than all other variants that are active in vitro.

**DISCUSSION**

Implications of the Results on the Structural Model for a-type Adenine MTases—We have investigated 17 highly or moderately conserved amino acid residues of the EcoRV DNA methy-
AdoMet Binding—Four of the mutants investigated are unable to bind AdoMet in vitro (K16A, E37A, F39A, D58A). All of these mutants are catalytically inactive in vitro and show a strongly reduced catalytic efficiency in vivo. These variants are mutated at the following amino acid residues that are implicated in AdoMet binding of M. EcoRV. Lys^{16} (motif X) is analogous to Thr^{23} in M. TaqI and Asn^{104} in M. HhaI, both of which contact one of the carboxyl oxygens of the AdoMet. Glu^{37} (motif I) corresponds to Glu^{45} in M. TaqI and Asp^{16} in M. HhaI, which contact the peptide backbone within motif I (FXGXG), thereby stereoelectronically constraining this region. In addition, M. TaqI-Glu^{45} contacts the amino group of the methionine moiety of the AdoMet. Phe^{39} (motif I) is equivalent to Phe^{18} in M. HhaI, which forms a hydrophobic contact to the adenine ring of the AdoMet. R128A and N130A both are catalytically inactive in vitro with respect to their catalytic efficiency, D193(A/G), R128A and N130A both are unable to bind AdoMet and DNA but show a strongly reduced catalytic activity in vitro. Most variants were catalytically inactive in vitro. These results suggest that all of these contacts to the cofactor observed in the x-ray structure analyses of several methyltransferases (M. HhaI (7), catechol-O-methyltransferase (43), M. TaqI (28), HaeIII DNA methyltransferase (15), VP39 vaccinia protein RNA methyltransferase (44), CheR (45), and PvuII DNA methyltransferase (29)) are very important for binding and positioning the cofactor. The data obtained with R128A and N130A both correlate with the biochemical properties of the variants will be discussed in more detail.

DNA Binding—The DNA binding domain of M. EcoRV was predicted to comprise amino acid residues 90–180 (40). In this study, we have investigated two residues within this region (Arg^{128} and Asn^{130}). R128A and N130A both are not able to

![Diagram](https://example.com/diagram.png)
bind DNA but can bind the cofactor AdoMet. These are the only residues in this study that are unable to bind to DNA. This finding is comparable with similar results of a random mutagenesis study that identified 13 M. EcoRV single mutants that are catalytically inactive in vivo and in vitro. Five of these variants do not bind to DNA, four of which carry a mutation in the putative DNA binding domain (42).

Catalytic Mechanism—There is evidence that N-MTases flip out their target adenine prior to methyl group transfer (31–34). Thus, the active site of the enzyme must form a binding site for the flipped out adenine residue, providing a hydrophobic pocket and hydrogen bond partners for the adenine as well as catalytic residues. It should be noted that variants that are not able to flip out the target base are not expected to have a lower affinity toward DNA, because nonspecific binding of M. EcoRV to DNA is almost as strong as specific binding (34). Two of the most conserved mutants investigated here (D193N, S229A) are catalytically inactive but bind to DNA and AdoMet with similar affinities as wild type M. EcoRV. Ser229 and Asp193 consequently are prime candidate residues to be involved in catalysis. This conclusion is not surprising for Asp193, because the aspartic acid of the DPPY motif (motif IV) almost certainly is part of the active site of N-MTases (30, 31). Our results with the D193N and D193A variants are in agreement with mutational data obtained with EcoKI, BcgI, and E. coli Dam (22, 23, 25); a DPPY → NPPY exchange in E. coli Dam (an α-N-M.Tase) abolishes catalytic activity but leaves DNA binding of the enzyme intact. Interestingly, AdoMet binding could not be demonstrated with this variant (22). In EcoKI, an NPPF → DPPF exchange results in a catalytically inactive enzyme that still binds the cofactor (23) and variants of BcgI in which the NPPY Asn was exchanged by Ala, Asp, or Gln are catalytically inactive (25).

Ser229, which is highly conserved in α-N-MTases is located in motif VI and corresponds to Ghu119 in M. HhaI. This residue is involved in acid base catalysis in M. HhaI (13). It should be noted that the reaction catalyzed by M. EcoRV, methylation of an adenine N6, requires the abstraction of one proton from the target base. In contrast to α-N-MTases in β-type enzymes (mostly cytosine-N4-MTases), an acidic residue is located at a corresponding position in motif VI (Asp196 in PvuII DNA methyltransferase) (29). However, motif IV has a SPPF sequence in these enzymes. Thus, it appears as if the active site of β-type enzymes (DPPY . . . S) is analogous to the SPPF . . . D arrangement found in β-type enzymes. Hence, Ser229 possibly together with Asp193 could be involved in a proton relay system (ade... ...) . . . Asp193) similarly as suggested recently for the β-type N-MTase PvuII methyltransferase (29). Alternatively, Ser229 may form a hydrogen bond to the flipped adenine.

In addition, Asp211 is important for the catalytic activity of M. EcoRV, because the D211A variant is inactive in vitro, although this variant shows considerable activity in vivo. This result is in agreement with the structural model, according to which this residue is located in motif V, that participates in creating the adenine binding pocket. Asp211 is located at a position corresponding to Phe146 in M. TaqI that forms a hydrophobic contact to the AdoMet. However, the Phe146-AdoMet interaction is characteristic for γ-type N-MTases, and our data do not suggest involvement of the corresponding residue in M. EcoRV (Asp211) in AdoMet binding.

Adenine Binding Site—In addition to Asp193, Tyr196 is the second conserved amino acid residue in motif IV investigated in this study. In M. TaqI, a hydrogen bond is formed between the hydroxyl group of this tyrosine and the conserved asparaginase constraining the conformation of the NPPY motif (motif IV). Perhaps, in M. EcoRV Tyr196 serves to position Asp193 by having a buttressing role in catalysis. In agreement with this observation, we show here that the hydroxyl group of Tyr196 contributes to catalysis but is much less important than the carboxylate group of Asp193. However, our results obtained with the Y196A variant demonstrate that an aromatic residue is essential at this position, presumably because it is involved in binding the flipped adenine. These findings are in agreement with data obtained with EcoKI where the Phe residue of the NPPF motif was replaced by Tyr and Trp and the resulting NPPY and NPPW variants still had catalytic activity (1⁄4 of wild type in the case of NPPY, much less in the case of NPPW) (23). In BcgI, an NPPY → NPPA exchange abolishes catalytic activity but does not severely affect DNA binding and AdoMet binding. Similarly as found here, NPPY → NPPF or NPPW variants of BcgI retain catalytic activity (25). In contrast, a DPPY → DPPF exchange in EcoP15I inactivates this enzyme (24).

A second candidate residue to contribute to the adenine binding pocket is Trp231, which has been identified in a random mutagenesis approach (42). A W231R mutant binds to DNA and AdoMet similar to wild type M. EcoRV but is catalytically inactive in vivo and in vitro. According to the structure prediction for M. EcoRV, Trp231 is located at a position corresponding to Val121 in M. HhaI (40). This residue forms a hydrophobic contact to the flipped cytosine (12), suggesting that Trp231 in M. EcoRV may have a similar function in binding the flipped adenine.

Finally, Tyr458 (motif VIII) could participate in binding the flipped out adenine. This residue could provide an aromatic ring and/or hydroxyl group to interact with the adenine base. It corresponds to Phe139 in M. TaqI, which has been suggested to be involved in hydrophobic contacts to the flipped adenine ring (21, 31). Our data demonstrate that Tyr458 indeed is an important residue for catalysis, because the Y258A variant is catalytically almost inactive but binds to DNA and AdoMet, albeit with reduced affinity for the cofactor.

Conclusions—In this site-directed mutagenesis study covering 17 positions of M. EcoRV, we have provided evidence that a model for the structure of α-type N-MTases is largely correct. We could demonstrate the importance of amino acid residues in motifs X, I, and II in AdoMet binding. In addition, residues in motifs IV, V, VI, and VIII are important for catalysis and/or might contribute to the binding site for a flipped adenine. These data considerably extend our knowledge on DNA MTases, because so far results of site-directed mutagenesis studies were only published for some residues located in motifs I and IV. Together with more structural information, they will help us to understand better structure-function relationships and mechanism of DNA N-MTases.

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