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# The cysteine conserved among DNA cytosine methylases is required for methyl transfer, but not for specific DNA binding

Michael W.Wyszynski, Sam Gabbara, E.A.Kubareva[1], E.A.Romanova[1], T.S.Oretskaya[1], E.S.Gromova[1], Z.A.Shabarova[1] and Ashok S.Bhagwat*
Department of Chemistry, Wayne State University, Detroit, MI 48202, USA and [1]Department of Chemistry, Moscow State University, Moscow 119899, Russia

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## ABSTRACT

**All DNA (cytosine-5)-methyltransferases contain a single conserved cysteine. It has been proposed that this cysteine initiates catalysis by attacking the C6 of cytosine and thereby activating the normally inert C5 position. We show here that substitutions of this cysteine in the *E.coli* methylase *M.Eco*RII with either serine or tryptophan results in a complete loss of ability to transfer methyl groups to DNA. Interestingly, mutants with either serine or glycine substitution bind tightly to substrate DNA. These mutants resemble the wild-type enzyme in that their binding to substrate is not eliminated by the presence of non-specific DNA in the reaction, it is sensitive to methylation status of the substrate and is stimulated by an analog of the methyl donor. Hence the conserved cysteine is not essential for the specific stable binding of the enzyme to its substrate. However, substitution of the cysteine with the bulkier tryptophan does reduce DNA binding. We also report here a novel procedure for the synthesis of DNA containing 5-fluorocytosine. Further, we show that a DNA substrate for M.*Eco*RII in which the target cytosine is replaced by 5-fluorocytosine is a mechanism-based inhibitor of the enzyme and that it forms an irreversible complex with the enzyme. As expected, this modified substrate does not form irreversible complexes with the mutants.**

## INTRODUCTION

Methylation of position 5 of cytosines is the most common modification of DNA found in nature. 5-Methylcytosine plays a variety of biological roles including distinguishing host DNA from foreign DNA, regulation of gene expression and tagging regions of sequence duplications for rapid evolution [Reviewed in (1, 2)]. Recent studies of DNA (cytosine-5) methyltransferases (C5 methylases) from a number of sources have clearly established that these enzymes evolved from a common ancestor. All C5 methylases carry several conserved sequence motifs and the organization of these motifs within these enzymes is similar (3–5).

D.Santi and his colleagues have pointed out that the known inhibition of C5 methylases from procaryotic sources (6) and eucaryotic sources (7) by 5-azacytidine, 5-fluorocytidine and other cytidine analogs could be explained by a common reaction mechanism for these enzymes (8). According to this mechanism, the reaction is initiated by a nucleophilic attack at the C6 position of cytosine by the enzyme. Production of this Michael adduct creates a negative charge at C5, thereby activating a normally inert carbon (8). The methyl group is transferred to C5 as a result of an electrophilic attack of a carbon from S-adenosyl-L-methionine (SAM). In the process, the cofactor is converted to S-adenosyl-L-homocysteine (SAH). When the target cytosine is replaced by 5-azacytosine or 5-fluorocytosine, the Michael adduct becomes stable causing an irreversible inactivation of the enzyme (8).

Wu and Santi have proposed (9) that a cysteine conserved among all C5 methylases is the nucleophile that initiates the methyl transfer reaction. This cysteine lies in the most conserved sequence motif for the methylases called motif IV (4). The proposal of Wu and Santi was based largely on similarities between the reaction carried out by thymidylate synthase (TS) and C5 methylases, and on the identification of the analogous nucleophile in TS as cysteine (10, 11). Two lines of evidence support this hypothesis. An irreversible covalent complex between M.*Hae*III and DNA substrate containing 5-fluorodeoxycytidine (5-FdC) was isolated by Chen et al. (12). When this complex was digested with either pronase or trypsin, the DNA was found to be linked to short peptides that contained the conserved cysteine. The cysteine itself appeared to be modified and could not be directly identified in the sequencing reactions. However, the lack of reactivity of this residue towards iodoacetamide was consistent with it being linked to DNA (12). Friedman and Ansari have presented parallel evidence (13) supporting a similar role for the conserved cysteine in the *E.coli* methylase M.*Eco*RII. These investigators isolated a covalent complex between M.*Eco*RII and 5-FdC-containing substrate and subjected it to protease V8 digestion. The peptide linked to the DNA was

* To whom correspondence should be addressed

sequenced and was found to contain the conserved cysteine (13). Once again, the cysteine itself appeared to be modified and could not be identified by the sequencing procedure.

Recently, we reported the isolation of mutants of M.*Eco*RII that contain substitutions of the conserved cysteine (Cys-186) by six different amino acids—alanine, glutamine, glycine, serine, tryptophan and valine (14). Cell-free lysates containing these mutant proteins were unable to transfer methyl groups to DNA and a genetic test for the covalent linking of the enzyme to 5-azadeoxycytosine-containing DNA found evidence for complex formation with only the wild-type enzyme and the mutant with the serine substitution. These observations are consistent with an essential nucleophilic role for Cys-186 in the methyl-transfer reaction of M.*Eco*RII. Surprisingly, our studies with the mutant carrying glycine at position 186 found that the expression of this protein was highly toxic to *E.coli*. We describe here a biochemical study of some of these mutants that focuses on the non-covalent and covalent interactions between the enzyme and its substrate. It specifically addresses the question of whether the inability of the mutants to transfer methyl groups to DNA is the result of a lack of binding to the substrate or due to defect in a step subsequent to DNA-binding.

## MATERIALS AND METHODS

### Oligonucleotide synthesis

All the DNA oligomers except for the 5-FdC-containing 14-mer were synthesized at the Macromolecular Facility (Wayne State University). Synthesis of the 5-FdC-containing oligomer was done as follows: 5-Fluoro-2′-deoxyuridine was synthesized from 2′-deoxyuridine (Sigma Chemicals, St. Louis, MO) in the laboratory of D.Cech (Humboldt University, Berlin, Germany) together with C.-D Pein. 5-Fluoro-2′-deoxycytidine was obtained from 5-fluoro-2′-deoxyuridine using procedures that have been described before (15, 16). 5′-O-dimethoxytrityl derivative of the 5-fluoro-2′-deoxycytidine was obtained by standard methods (17). The products of the reaction were separated on a silica gel column and the desired product was eluted with a 0−10% gradient of ethanol in chloroform. Benzoylation at N4 position was achieved by adding benzoic anhydride to 5′-O-dimethoxytrityl-5-fluoro-2′-deoxycytidine dissolved in ethanol. The reaction was monitored by thin layer chromatography with chlroform-ethanol (9:1, v/v). The desired reaction product was separated from other products by silica gel chromatography. The yield of this reaction was 45%. 3′-N, N-diisopropylamidomethylphosphite of the 5′-O-dimethoxytrityl-N4-benzoyl-5-fluoro-2′-deoxycytidine was obtained by a previously described procedure (18). This modified nucleotide was incorporated in the nonamer 5′-**F**TGGATCCG (**F** = 5-FdC) using the DNA synthesizer 380B by Applied Biosystems (Boulder, CO). The procedure used to deprotect the oligomer is described in the 'Results'. This nonamer was phosphorylated at its 5′-end and was hybridized to the oligomer 5′-ACGGATCCAGGAGTGAC along with the oligomer 5′-CACTC. This created the nicked duplex

5′-CACTC **F**TGGATCCG
3′-CAGTGAG-GACCTAGGCA.

The nick was sealed with T4 DNA ligase and the resulting 14-mer with 5-FdC was purified by elution from a 20% polyacrylamide gel.

### Protein purification

The host for the overproducing plasmids carrying the genes for the wild type M.*Eco*RII and its mutants was the *E.coli* B strain BL21 (DE3) [$F^-$ *ompT hsdS*] with a phage λ lysogen (*imm21 int*) which contains T7 RNA polymerase gene under the *lac* UV5 promoter (19). The plasmid constructs pT71-Cys, pT71-Ser, and pT71-Trp carrying the genes for the wild type enzyme, C186S mutant, and C186W mutant, respectively, have been described before (14).

*E.coli* BL21(DE3) harboring the appropriate overproducer was first grown overnight in 5 ml of Luria broth (LB) medium containing 50 μg/ml carbenicillin. Two and one half ml of the overnight culture was then transferred to a 2-liter flask containing one liter of fresh LB medium and 50 μg/ml carbenicillin. The flask was incubated at 37°C on a shaking platform. When $OD_{550}$ of the culture reached between 0.3 to 0.4, isopropyl-β-D-thiogalactoside was added to a final concentration of 120 μg/ml, and growth was continued for another 3 to 4 hrs. Cells were collected by centrifugation in a Sorvall GSA rotor at 4000g for 20 min. Typically, one liter of cells yielded about 2 grams of wet cells. To prepare cell-free extracts, 1.6 grams of cells were resuspended in 25 ml of 'low salt buffer'-buffer A (10 mM potassium phosphate, pH 7.0; 1 mM EDTA; and 10 mM 2-mercaptoethanol) with 0.1 M NaCl. The suspension was treated with 0.4 mg/ml lysozyme on ice for 10 min, quickly frozen in a dry-ice/ethanol bath, thawed at 37°C, and then returned to ice. Cells were then sonicated five times with 20 seconds pulses, and the cell debris was removed by centrifugation in a SS-34 rotor (Sorvall) at 12,000g for 20 min. The supernatant was diluted two-fold with low salt buffer and phenylmethylsulfonylfluoride was added to a final concentration of 25 μg/m.

A cellulose phosphate (SIGMA, St Louis, MO.) column (2.5×20 cm) was equilibrated with the low salt buffer and the cell-free extarct was loaded onto it. The column was washed with 70 ml of the low salt buffer at 0.5 ml/min. The enzyme was eluted from the column with a linear gradient of 0.1M to 0.8M NaCl in buffer A. The methylase and its mutants eluted as a single peak at about 0.5 M NaCl. Fractions (20 to 30 ml) within this peak were pooled and dialyzed for 5 hrs against 45% glycerol solution containing 10 mM potassium phosphate, pH 7.0, and 10 mM 2-mercaptoethanol. After dialysis, bovine serum albumin was added to a final concentration of 200 μg/ml, and the purified enzyme was stored at −20°C or −70°C.

### Assay for methyl transfer to DNA

The procedure for the methyl transfer assay has been described before (14).

### Radioactive labeling of DNA and the formation of duplexes

The labeling reaction typically contained 20−50 pmole of DNA, 60−150 mCi of ($\gamma$-$^{32}$P)-ATP (3000 Ci/mmol, NEN/Dupont, Boston, MA), and 10 units of T4 polynucleotide kinase (New England Biolabs, Beverly, MA) in 50 mM Tris−HCl (pH 7.8), 10 mM $MgCl_2$ and 5 mM DTT in final volume of 50 μl. The reaction was performed at 37°C for 30 min and was terminated by adding 2 μl of 0.5 M EDTA followed by heating to 70°C for 5 min. A two-fold molar excess of unlabeled DNA was added to this reaction mixture, and the volume of the hybridization mixture was brought up to 100 μl with TE (10 mM Tris−HCl, pH 7.8 and 1 mM EDTA). The hybridization mixture was then

heated at 90°C for 3 min and the two strands were annealed by allowing the mixture to cool slowly to 30°C. This solution was then extracted once with phenol/chloroform and once with chloroform. Finally, the unincorporated ($\gamma$-$^{32}$P)-ATP was removed from the reaction by spin-dialysis (20) through a Sephadex G-50 column (Pharmacia-LKB, Piscataway, NJ).

### DNA binding reactions and gel electrophoresis

The DNA binding reactions contained 100 mM Tris−HCl (pH 7.8), 20 mM EDTA, 3 mM 2-mercaptoethanol, 100 μg/ml BSA, 1−2 pmole of labeled duplex, 30 ng poly-dI:dC, appropriate cofactor at 10 mM, and 73 pmole of protein (~4 μg), except where noted. Reaction volumes were typically 50 μl. The reactions were incubated at 37°C for 30−90 min, and analyzed by electrophoresis performed on 10% polyacrylamide gels at 25°C for approximately four hours at 150 volts. Denaturing gels were performed with the inclusion of 0.1% SDS both in the gel and in the electrophoresis buffer. Following electrophoresis, the gels were fixed using a 30% methanol, 10% acetic acid solution and autoradiographs were prepared using X-ray film RX (Fuji Film, Japan).

## RESULTS AND DISCUSSION

### Purification of the wild-type M.*Eco*RII and its mutants

A procedure for the purification of the wild-type M.EcoRII has been described by Friedman (21). The procedure involves several steps including five steps with chromatography. However, when we started the purification with cell extracts prepared from an overproducer of the enzyme (14), we found that the first two step of the procedure produced enzyme that appeared to be substantially pure (~90% homogeneous, Fig. 1 and data not shown). This improvement in the purification procedure was probably due to the fact that the strain used in the present study produces about 170-fold more methylase than the strain used by Friedman (14, 21). Mutants of M.EcoRII with substitutions of the conserved cysteine to serine (C186S) or to tryptophan (C186W) could also be purified by this procedure. A comparison of the protein compositions of crude lysates of the overproducing strains with the purified protein preparations is presented in Figure 1. Wild-type (WT) enzyme and the mutants prepared in this way were used in the assays described below.

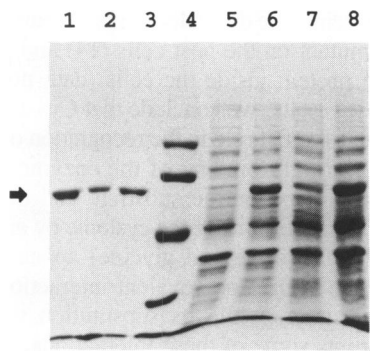


**Figure 1.** Protein compositions of cell lysates and purified preparations. To prepare lysates, cells from 200 μl of overnight cultures were suspended in 100 μl of buffer B (2% SDS, 50mM Tris−HCl, pH 6.8; 100 mM dithiothreitol; 10% glycerol, and 0.1% bromophenol blue) and boiled for 3 min. These samples and indicated amounts of purified proteins were electrophoresed on a 8% SDS-polyacrylamide gel and the proteins were visualized with the Coomassie Brilliant Blue stain. Lanes 1, 2, and 3 contained purified proteins. Lane 1—wild type (5μg), lane 2—C186S (10μg), and lane 3—C186W (5μg). Lane 5 contains extract from of cells containing the vector (pT7−5) used to produce the overproducing plasmids. Lanes 6, 7 and 8 contained starting cell lysates that were used in the purification. The overproducing plasmids in these cells were as follows: lane 6—pT71-Cys; lane 7—pT71-Ser and lane 8—pT71-Trp. Lane 4 contained molecular weight standards for 94,000; 67,000; 43,000 and 30,000.

When the ability of the purified proteins to transfer $^{3}$H-methyl groups from S-[methyl-$^{3}$H]-methionine to DNA was studied, no activity could be detected for the C186S and C186W mutants (Fig. 2). The amounts of $^{3}$H seen with these mutants were typically about 5,000 to 10,000-fold less than those seen with the WT enzyme and were indistinguishable from background. These results are consistent with our earlier report (14) in which no methyl transfer activity was detected in cell-free extracts of these mutants.

### Binding of the enzyme and its mutants to normal DNA

The lack of methyl-transferring ability in the mutants could be due to a variety reasons. For example, a loss of DNA binding ability as a result of the mutations could also prevent methyl-transfer to DNA. To test this possibility, we studied the specific binding of M.*Eco*RII and its mutants to substrate DNA by the gel mobility shift assay. This assay can detect the formation of specific stable protein−DNA complexes and has been used to study the interaction between a large number of DNA-binding proteins and their substrates. In particular, it has been used to study the interaction of the N6-adenine methylases Dam (22) and M.*Eco*RI (23), and several C5 methylases including M.*Msp*I (24) and the *Bacillus* phage-coded methylases M.*H*2I, M.*r*11sI (25) with their respective substrates.

The three DNA duplexes used in these experiments differ from each other only in their state of methylation (duplexes I, II and III; Table I). While the unmethylated DNA (duplex I) and the hemi-methylated DNA (duplex II) would be expected to form specific complexes with the WT enzyme under appropriate conditions, the fully-methylated substrate would be expected to

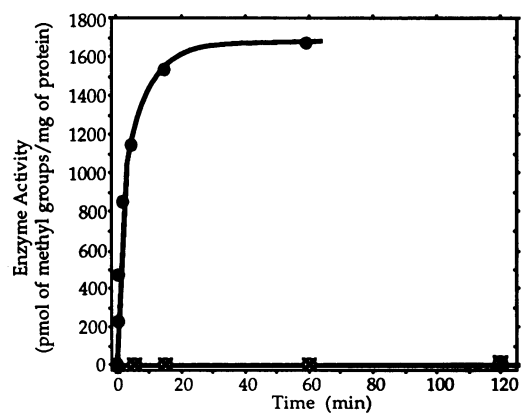


**Figure 2.** Methylase activity of the mutant proteins of M.*Eco*RII. The assay for enzyme activity has been described before (14). Symbols: filled circles—WT enzyme; open circles—C186W and 'X's—C186S.

**Table 1.** Structures of the DNA Duplexes

| Number | Sequence |
|---|---|
| I | 5′-CACTCCTGGATCCG<br>3′-GTGAGGACCTAGGC |
| II | 5′-CACTCCTGGATCCG<br>3′-GTGAGGACCTAGGC<br>\|<br>me |
| III | 5′-CACTCCTGGATCCG<br>3′-GTGAGGACCTAGGC<br>\|<br>me<br><br>me<br>\| |
| IV | 5′-CACTCFTGGATCCG<br>3′-GTGAGGACCTAGGC<br>\|<br>me |

The recognition site for M.*Eco*RII (CCWGG) is underlined. The enzyme transfers a methyl group to the second cytosine in the sequence. 'me'—methyl group at position 5; **F**—5-fluorodeoxycytidine.

bind poorly to the enzyme. To assure that any observed complexes would not be due to non-specific binding of the enzyme to DNA, an excess of poly-dI.dC was included in these reactions.

In the presence of SAH, the WT enzyme formed specific stable complexes with duplexes I and II, but not with duplex III (Fig. 3, lanes 4, 5 and 6). This complex formation was substantially reduced if no cofactor was added or if SAM was present in the reaction (not shown). In the latter case, the enzyme probably bound duplexes I and II, methylated them during the binding reaction or during gel electrophoresis and then dissociated from the fully methylated DNA. To confirm this, we subjected an unmethylated DNA duplex to the binding reaction in the presence of SAM and then challenged it with *Eco*RII endonuclease. The DNA was resistant to *Eco*RII (not shown). This contrasts with the behavior of M.*Msp*I which forms complexes with substrate DNA in the presence of SAM or SAH as well as in the absence of added cofactor analogs (24). However, it should be pointed out that the gel mobility shift assay is only able to detect stable protein-DNA complexes and any unstable complexes formed by M.*Eco*RII with DNA would not be detected by this assay. Our preliminary studies using a nitrocellulose filter-binding assay do, in fact, suggest that M.*Eco*RII can bind tightly to substrate DNA in the absence of added cofactor analogs (not shown).

The C186S mutant discriminates between the substrate duplexes (duplexes I and II) and the product duplex (duplex III) in the same manner as the WT enzyme. It forms specific stable complexes with duplexes I and II and the complexes have gel mobilities comparable to the mobilities of the complexes formed by the WT enzyme (Fig. 3, lanes 7 and 8). Such complexes were not seen between C186S and duplex III (Fig. 3, lane 9). In contrast, no specific stable complexes could be detected between the C186W mutant and any of the duplexes under these conditions (Fig. 3, lanes 10, 11 and 12). In some experiments, trace amounts of complexes between C186W and duplex II were seen (see below); however, it is clear that the substitution of the cysteine by tryptophan reduces severely the ability of the protein to form specific stable complexes.

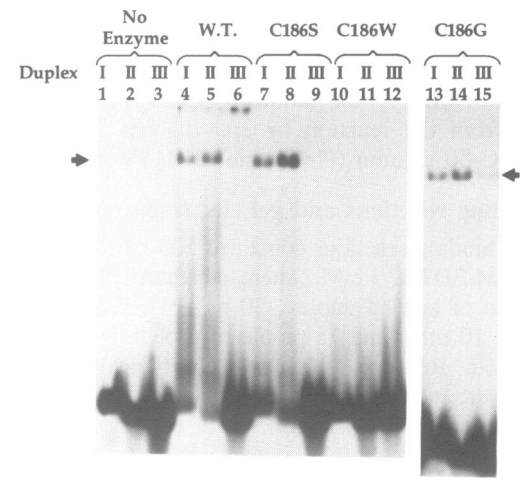


**Figure 3.** Binding of WT enzyme and mutants to duplexes I, II and III. The upper strands within the duplexes were labeled at their 5′-end with $^{32}P$ and the binding reactions were carried out as described in Materials and Methods. All binding reactions contained SAH at 10μM and 3 ng poly-dI-dC. Lanes 4 through 12 contain reactions in which ~4μg of purified protein was used. Lanes 13 through 15 are from a separate gel and contain reactions in which cell lysates containing ~10μg of total protein was used. The proteins in the various reactions are identified above the lanes. Autoradiographs of these gels are shown here. The location of the specific complexes is identified with an arrow.

Using cell-free extracts containing the C186G mutant, we were able show that this protein binds tightly and specifically to duplexes I and II (Fig. 3, lanes 13 and 14). In contrast, little complex formation could be detected between duplex III and C186G (Fig. 3, lane 15). Therefore, the C186G has substrate specificity similar to the WT enzyme and C186S mutant. The ability of this mutant to discriminate between the substrate and the reaction product was tested using cell-free extracts—rather than purified protein—because it has been exceedingly difficult to purify this protein. The difficulty stems, in part, from the lethal effects of this mutant on the host cells (14) and partly from the paucity of free protein inside the cells (data not shown).

Based on these results, we conclude that Cys-186 of M.*Eco*RII does not have an essential role in the recognition of base sequence by the enzyme, in tight binding of the enzyme to DNA, or in the discrimination between the substrate and the final product by the enzyme. Substitutions of the cysteine by amino acids with smaller side-chains (serine and glycine) appear to cause little qualitative change in the non-covalent interactions between the enzyme and substrate, while its substitution with the bulkier tryptophan disrupts some of these interactions. We predict that mutants such as C186A should bind tightly to the substrate, while mutants such as C186T and C186L should bind poorly to it.

### Synthesis of 5-fluorodeoxycytidine containing DNA

If the conserved cysteine in M.*Eco*RII is responsible for the attack of C6 of cytosine, some of the mutants of the enzyme with substitutions of this residue may not carry out this reaction. To test this, we decided to construct a substrate for M.*Eco*RII which can trap the covalent intermediate in the methyl transfer reaction. This was achieved by replacing the target cytosine with 5-fluorocytosine. The model of catalysis by Santi et al. (8) suggests that methyl transfer to C5 is essential for making a covalent complex between the enzyme and a substrate containing 5-FdC, irreversible. As all the mutants of M.*Eco*RII isolated so

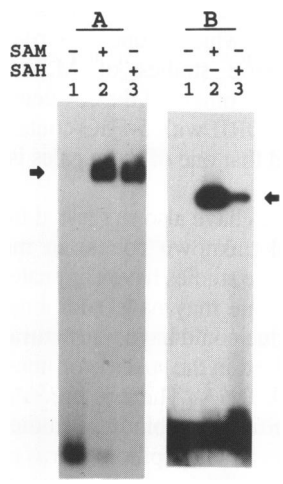


**Figure 4.** Binding of M.*Eco*RII to duplex IV. The reactions conditions and electrophoresis procedure for the gels is described in Materials and Methods. The cofactor used in the binding reactions are indicated above each lane. Following the binding reactions, the samples were divided in two halves. One half of each reaction was loaded on the native gel (part A) and the other half was denatured prior to its loading on a denaturing gel (part B). Autoradiographs of these gels are shown here. The location of the specific complexes is identified with an arrow.

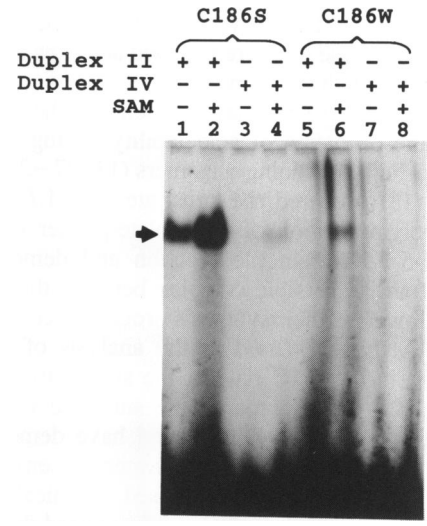


**Figure 5.** Binding of mutants of M.*Eco*RII to duplex IV. The upper strands within the duplexes were labeled at their 5′-end with $^{32}P$ and the binding reactions were carried out as described in Materials and Methods. Following the binding reaction, one half of each reaction was electrophoresed in a non-denaturing polyacrylamide gel and the other half was electrophoresed in a denaturing polyacrylamide gel. Only the native gel is shown here. The duplex used in the reactions, the presence or the absence of SAM and the mutants used in the reactions are indicated above the lanes. The location of the specific complexes is identified with an arrow.

far with substitution of Cys-186 are defective in methyl transfer [(14) and Fig. 2], any complexes formed by the mutants with such a DNA substrate should not be irreversible.

Osterman et al. (26) have noted that an intermediate in the chemical synthesis of 5-fluorocytosine containing oligonucleotides—5′-O-dimethoxytrityl-N4-benzoyl-5-fluoro-2′-deoxycytidine (DMT-Bz-5-FdC)—is unstable during the acidic treatment used to remove the dimethoxytrityl protecting groups. The removal of these protecting groups is essential for the elongation steps in the oligonucleotide synthesis. To avoid such instability, we changed the scheme of synthesis such that the 5-fluorodeoxycytidine (5-FdC) incorporated into the oligonucleotide was not subjected to further cycles of chemical linking and deprotection. DMT-Bz-5-FdC was introduced into the 5′ end of the oligonucleotides during their standard automated chemical synthesis. Then the modified oligonucleotide was subjected to acid treatment only after removal of the alkali-labile protecting groups and reverse-phase HPLC. The nonamer 5′-FTGGATCCG (F= 5-FdC) was phosphorylated at its 5′-end and ligated to 5′-CACTC (see Materials and Methods). The sequence of the resulting 14-mer was confirmed by the chemical sequencing method. The presence of 5-FdC in the oligomer was confirmed by separating the nucleosides generated by the enzymatic degradation of the oligomer by HPLC (not shown).

### Binding of M.*Eco*RII to a substrate containing 5-FdC

The substrate for the binding experiments was duplex IV (Table 1)—a hybrid between the 14-mer mentioned above and its (methylated) complement. It was identical to duplex II except for the substitution of the target cytosine by 5-fluorocytosine (Table 1). As the complementary strand was methylated, the enzyme was expected to transfer methyl group to only the fluorinated cytosine. If the target cytosine in the complementary strand were to be unmethylated, binding of the enzyme to such DNA may be the result of an attempt by the enzyme to transfer a methyl group to this cytosine. Further, any observed transfer of methyl groups to such a substrate could involve two separate methyl transfers to the two strands.

The WT enzyme forms specific stable complexes with duplex IV in the presence of SAM (Fig. 4, A, lane 2). In the absence of cofactor, little complex formation is detected (Fig. 4, A, lane 1). However, a small amount of complex could be detected in that lane upon longer exposures during autoradiography (not shown). Existence of tightly bound SAM to a small fraction of the enzyme preparation could explain such complexes. As expected, the complexes observed in the presence of SAM were stable to SDS and heat treatment (Fig. 4, B, lane 2). Therefore M.*Eco*RII forms an irreversible covalent complex with a substrate in which the target cytosine is replaced by 5-FdC.

The enzyme also forms specific stable complexes with duplex IV in the presence of SAH (Fig. 4, A, lane 3). These complexes were similar in mobility to the complexes formed in the presence of SAM (Fig. 4, A, lane 2) and were partially resistant to denaturation (Fig. 4, B, lane 3). The only difference between the complexes formed in the presence of SAM and SAH appeared to be their relative stabilities. The complexes formed by the enzyme in the presence of SAH were significantly reduced by the denaturation treatment. Further, a longer exposure of the gel to X-ray film revealed a smear of radioactivity between the complex and the free DNA (not shown). It has been noted above that the proposed reaction mechanism predicts that methyl transfer to C5 is required to make such complexes irreversible (8). Therefore, it was surprising to find that the complexes formed between duplex IV and the WT enzyme when SAH was present in the reaction were not completely dissociated by the denaturing treatment. It is unlikely that the resistant complexes were formed by the fraction of the enzyme carrying bound SAM. If it were so, then an equally strong signal should have appeared when no cofactor was added to the reaction. No such complex was seen (Fig. 4, B, lane 1). We suggest that the complexes formed in

the presence of SAH do involve covalent linking of the enzyme to DNA, and that these links are reversed at a slow rate following the denaturation of the enzyme.

Recently, several groups have presented other strategies to circumvent the problems of acid lability during the chemical synthesis of 5-FdC containing oligomers (13, 27–29). Friedman and Ansari (13) prepared the substrate for M.*Eco*RII by the enzymatic polymerization of a template-primer hybrid in the presence of 5-FdCTP in the reaction and demonstrated the formation of an irreversible complex between the enzyme and this DNA. However, their synthesis procedure could not ensure that the DNA that was used in the analysis of the covalent complex contained 5-FdC only at the site of methyl transfer. Smith et al. (29) have prepared the substrate for the human methyltransferase using 5-FdCTP and have demonstrated the formation of a covalent complex between the enzyme and the DNA. Finally, two groups have used chemical methods to synthesize substrates with 5-FdC and have used these DNAs to demonstrate the irreversible covalent linking of M.*Hae*III (12) and of the *E.coli* methylase Dcm (Hanck et al., this issue) with their respective substrates. Thus the key prediction of the proposed mechanism of C5 methylases (8) has been amply and independently confirmed.

**Binding of M.*Eco*RII mutants to a substrate containing 5-FdC**

Only trace amounts of complexes were seen when duplex IV was incubated with C186S in the presence of SAM (Fig. 5, lane 4) and no complexes were seen in the absence of SAM (Fig. 5, lane 3). The amounts of complexes seen between C186S and duplex IV were much lower than the amounts seen with duplex II (compare lanes 4 and 2 in Fig. 5). This suggests that the fluorine at C5 of cytosine causes some hindrance to the binding of the enzyme to DNA. In this sense the fluorine at position 5 may mimic a methyl group. Complexes were also observed when extracts containing C186G were incubated with duplex IV (not shown). Once again, C186W mutant served as a negative control. No complexes were detected between C186W and duplex IV (Fig. 5, lanes 7 and 8). The length of exposure of this autoradiograph was much longer than used for Figures 3 and 4 and as a result small amounts of SAM-dependent stable complex formation between C186W and duplex II (Fig. 5, lane 6). For the same reason, a small amount of complex formation was detected between C186S and duplex II in the absence of added cofactor.

As mentioned earlier, if the transfer of methyl group to C5 is required to make the complex between the enzyme and 5-FdC-containing substrate irreversible, then none of the mutants with substitutions of Cys-186 should form irreversible complexes with duplex IV. This was confirmed by splitting the binding reactions in two halves, boiling one half of each reaction in the presence of SDS and electrophoresing them under denaturing conditions. No complexes between C186S and duplex IV were detected by autoradiography of the denaturing gel (not shown). The undenatured halves of these reactions were used for the gel seen in Figure 5. In similar experiments, the binding of C186G to duplex IV was also found to be sensitive to denaturation (not shown). Hence the binding of C186S and C186G mutants with 5-FdC containing DNA must either be non-covalent interactions or involves covalent links that are easily reversible.

We have shown that substitutions of Cys-186 in M.*Eco*RII eliminate its ability to transfer methyl groups to substrate DNA. Therefore, Cys-186 must play an essential role(s) in the methyl transfer reaction. This is the only residue in DNA methyltransferases for which a functional role has been identified. Our previous genetic studies of M.*Eco*RII (14) and the biochemical studies of others of the covalent complexes between M.*Hae*III and M.*Eco*RII with 5-FdC-containing substrates (12, 13) have confirmed that one of these roles is in attacking the C6 of the target cytosine.

Our genetic studies have also suggested that this cysteine may have an additional unknown role(s) in methyl transfer (14). Further, none of these studies have eliminated the possibility that the conserved cysteine may have additional roles unrelated to catalysis. The residue could have a structural role in the protein, or it could be involved in the specific or non-specific interactions of the enzyme with DNA. There is precedent for a cysteine to have a role in specific DNA-binding. In the Fos-Jun families of transcription factors and in papilloma virus protein E2, the redox state of a conserved cysteine plays a key role in DNA-binding (30, 31). Further, in the case of fos-jun proteins a substitution of this cysteine to serine increases the affinity of these proteins to DNA (30). However, the data presented here argues that the conserved cysteine in M.*Eco*RII is not essential for the sequence-specific, cofactor-dependent interaction between the enzyme and its substrate.

We have shown that the mutants of M.*Eco*RII in which the conserved cysteine is replaced by other residues are useful for dissecting the methyl transfer reaction of C5 methylases. Further biochemical studies of these mutants should help one understand the reaction in a greater detail.

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