Substitutions of a cysteine conserved among DNA cytosine methylases result in a variety of phenotypes

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ABSTRACT

The proposed mechanism for DNA (cytosine-5)-methyltransferases envisions a key role for a cysteine residue. It is expected to form a covalent link with carbon 6 of the target cytosine, activating the normally inactive carbon 5 for methyl transfer. There is a single conserved cysteine among all DNA (cytosine-5)-methyltransferases making it the candidate nucleophile. We have changed this cysteine to other amino acids for the *EcoRII* methylase; which methylates the second cytosine in the sequence 5'-CCWGG-3'. Mutants were tested for their methyl transferring ability and for their ability to form covalent complexes with DNA. The latter property was tested indirectly with the use of a genetic assay involving sensitivity of cells to 5-azacytidine. Replacement of the conserved cysteine with glycine, valine, tryptophan or serine led to an apparent loss of methyl transferring ability. Interestingly, cells carrying the mutant with serine did show sensitivity to 5-azacytidine, suggesting the ability to link to DNA. Unexpectedly, substitution of the cysteine with glycine results in the inhibition of cell growth and the mutant allele can be maintained in the cells only when it is poorly expressed. These results suggest that the conserved cysteine in the *EcoRII* methylase is essential for methylase action and it may play more than one role in it.

INTRODUCTION

DNA (cytosine-5)-methyltransferases (C5 methylases) are found in a wide variety of organisms including bacteria, fungi, plants and mammals. Recent work on the genes of these enzymes has led to the realization that they share several amino acid sequence motifs (1, 2). The extent of the sequence conservation and the organization of these conserved motifs within the enzymes is such that the enzymes are likely to have a common structural organization. They may also interact with the common cofactor, S-adenosyl-methionine (SAM) in similar ways and may have a common mechanism of catalysis. What distinguishes different C5 methylases that recognize and methylate different DNA sequences, is a segment of approximately 90 to 275 contiguous amino acid residues that is different in each enzyme (1, 2). This

segment may contribute to the ability of different enzymes to recognize different DNA sequences (3).

Santi and his colleagues have proposed a mechanism for the transfer of methyl group to C5 of cytosine (4) that is analogous to the known mechanism of thymidylate synthase [TS, (5)]. It is outlined in Fig. 1A. A key step in this mechanism is the nucleophilic attack on C6 of cytosine by the enzyme. The nucleophile in TS is known to be a cysteine (5) and this cysteine is preceded by a proline in that enzyme. Wu and Santi have proposed (6) that the nucleophile for C5 methylases may also be a cysteine. There is a single conserved cysteine among C5 methylases; it lies within the conserved sequence block IV (2) and it is also preceded by a proline in all the methylases. Therefore this cysteine is the likely nucleophile in the methylase reaction.

Although no data have been presented that support such a role for the conserved cysteine, two pieces of experimental evidence suggest an important role for cysteine(s) in the action of C5 methylases. *HhaI* methylase is rapidly inactivated by N-ethyl maleimide (NEM) and is protected from inactivation by the substrate DNA (6). The target cysteine(s) in the NEM attack has not been identified. Recently, a cysteine in the *EcoRII* methylase was shown to be in the vicinity of the cofactor SAM (7). This is the same cysteine that is conserved among C5 methylases and it can be photolabeled with [methyl-³H]-SAM. Upon UV treatment, the cysteine, Cys-186, is converted to S-[methyl-³H]-cysteine (7). Although the methyl transfer to Cys-186 may be an artifact resulting from UV irradiation rather than a normal step in catalysis, this result suggests that the conserved cysteine is near the transferable methyl group.

To clarify the role of the conserved cysteine in the methylase action, we have undertaken mutational analysis of Cys-186 in the *EcoRII* methylase. By replacing the cysteine with other amino acids and studying the properties of mutant proteins, we hope to understand the normal biochemical function of this residue.

MATERIALS AND METHODS

Strains

E. coli strain GM31 (*dcm-6 thr-1 hisG4 leuB6 rpsL ara-14 supE44 lacY1 tonA31 tsx-78 galK2 galE2 xyl-5 thi-1 mtl-1*) was obtained from M.G. Marinus (U. of Massachusetts School of

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Medicine). For experiments involving phage M13 derivatives, a F⁺ derivative of the strain was constructed. F' carrying kanamycin-resistance gene of Tn903 was conjugated into GM31 to make GM31(F' *kan*). RP4182 [Δ (*supD-dcm-flaA*) *trp gal rpsL*] was obtained from J.S. Parkinson (University of Utah). BH129 is GM31 *recA56 srlC::Tn10* and was created by the transduction of the *recA* allele into GM31 with P1 phage. BH128, an independent isolate P1 transduction and with genotype identical BH129, has been described before (8). Frozen competent cells of DH10B [*mcrA* Δ (*mrr-hsdRMS-mcrBC*) 80*dlacZ* Δ M15 Δ *lacX74 deoR recA1 endA1 araD139 Δ (*ara, leu*)7697 *galU galK rpsL nupG*] were purchased from GIBCO/BRL (Gaithersburg, MD). BL21(DE3) is an *E. coli* B strain (F⁻ *ompT hsdS*) with a phage λ (*imm21 int*) lysogen which contains T7 RNA polymerase gene under the *lacUV5* promoter (9) and was obtained from W. Studier (Brookhaven National Laboratory).*

Assays for DNA methylation

The ability to methylate *EcoRII* recognition sites was assessed in two ways. M13 replicative form (RFI) DNAs and plasmid DNAs containing the mutant alleles were prepared from GM31(F' *kan*) and GM31 cells respectively and digested with *EcoRII* endonuclease (GIBCO/BRL, Gaithersburg, MD). The products were analyzed by agarose gel electrophoresis. Methylation at the *EcoRII* sites results in the protection of the DNA from cleavage. Digests using *BstNI* endonuclease (New England Biolabs, Beverly, MA) were also performed to assess the quality of the DNA preparation used. This enzyme cuts at *EcoRII* sites regardless of methylation by *EcoRII* methylase.

Quantitation of the methylase activity was done with extracts prepared from GM31(F' *kan*), GM31 or BL21(DE3) cells carrying the appropriate phage or plasmid by measuring the transfer of methyl groups from S-[methyl-³H]-adenosyl-L-methionine (NEN/Dupont, Boston, MA) to chromosomal DNA from RP4182. The procedure used here differed in some respects from the previously described procedure (10). Purification of the methylase on a heparin-sepharose column was not carried out; instead the crude cell lysate was used in the assay. In the earlier procedure, the methylase reaction was followed by the precipitation of chromosomal DNA with trichloroacetic acid and the washing of the unincorporated radioactive SAM from precipitated DNA on 3MM filters. For this work, the unincorporated SAM was removed using spin columns (11). The concentration of proteins in the extracts was determined using the Bio-Rad (Richmond, CA) protein assay kit.

Site-directed mutagenesis

Phage 1909-Cys was constructed by ligating the *EcoRV-XhoI* fragment in the plasmid pR215 (12) to *SmaI-SalI* cut DNA of the phage M13 mp19 (13). It contains all of the *M.EcoRII* gene including its promoter and a part of *R.EcoRII* gene and has been used to sequence the latter gene (12).

The Cys to Gly and Gly to Ser changes were achieved using defined mutagenic oligonucleotides as described by Kunkel (14). M13 phage derivatives 1909-Cys and 1909-Gly were the templates for the mutagenesis and GM31(F' *kan*) was the *ung*⁺ host used for the transfection. The mutagenic oligonucleotides were complementary to the methylase sequence from 753 to 775, as defined by Som et al. (15).

Random mutagenesis at codon 186 was performed as described by Taylor et al. (16) using a commercial kit (Amersham, Arlington Heights, IL). A mixture of oligonucleotides complementary to the

methylase sequence from 753 to 775 was synthesized and contained an equal representation of the four bases at the three positions in codon 186 (positions 763–765 in the sequence). The oligonucleotide mixture was annealed to the single-stranded template 1909-Gly and the second strand synthesis was completed using the large fragment of DNA Polymerase I of *E. coli*. The reaction mixture contained dATP, dCTP- α -S, dGTP and dTTP as well as T4 DNA ligase. The template strand was nicked with *NciI*. This enzyme does not cleave the strand containing dCTP- α -S (16). The nicks were converted to gaps using exonuclease III and the gaps were filled-in with the large fragment of DNA Polymerase I in the presence of T4 DNA ligase. The ligation products were digested with *SmaI* to linearize molecules which had retained the Gly-186 codon (GGG) and the digestion products were transfected into GM31(F' *kan*).

The presence of mutations was confirmed by dideoxy chain termination sequencing using a primer that was complementary to the sequence from 781 to 800 or from 862 to 879. Prior to cloning into pR300 Δ *BstBI*, the *BstBI* fragments from the mutants was sequenced using these primers in conjunction with a primer that was complementary to the methylase sequence from 688 to 714.

5-azacytidine sensitivity assay

Sensitivity of strains with the methylase mutants was performed largely as described previously (8). The only alteration in that procedure was the elimination of a centrifugation step following 5-azacytidine treatment. For this study, cells were diluted and plated on LB plates with ampicillin.

Recombinant DNA work

Most of the recombinant DNA work involved standard procedures. Construction of pR300 and its derivatives is described in the 'Results'. The 'scrambling and ligation procedure' used in this study was somewhat elaborate and is described below. Approximately 2.5 micrograms of pR300(–)Gly was digested with *BstBI* and the digestion products were ligated to each other with T4 DNA ligase. The ligase was heat-inactivated and the DNA was digested with *XmaI*, an isoschizomer of *SmaI*. The digestion products were separated on a SeaPlaque GTG agarose (FMC BioProducts, Rockland, ME.) gel and the 6.8 kb linear fragment was purified from the agarose with GELase (Epicentre Technologies, Madison, WI) digestion. Following phenol extraction and ethanol precipitation, the linear fragment was circularized with T4 DNA ligase and the DNA transformed into DH10B cells. Plasmids from ampicillin-resistant colonies were screened for the expected recombinants.

The cloning of mutant alleles under the T7 promoter Φ 10 was accomplished as follows. An *EcoRI-HindIII* fragment containing the wild-type methylase gene lacking its endogenous promoter was obtained from pR234, which had been constructed by cloning a *PstI-HindIII* fragment from pSS114 (15) into pKK223-3 [(17); S. Gabbara and A. Bhagwat, unpublished]. This *EcoRI-HindIII* fragment was cloned into pT7-5 (18) to create pT71-Cys. A derivative of this plasmid, pT71 Δ *BstBI*, deleted for the *BstBI* fragment internal to the *M.EcoRII* gene was constructed similarly. PT71-Gly was constructed by ligating the 282 base pair *BstBI* fragment from 1909-Gly to *BstBI*-cut pT71 Δ *BstBI*. PT71-Ser and pT71-Trp were constructed by replacing a *PfMI-StuI* fragment internal to the *M.EcoRII* gene in pT71-Gly by the corresponding fragment from pR300(+)Ser and pR300(+)Trp, respectively.

RESULTS

Mutagenesis of the *EcoRII* methylase gene

Site-directed mutagenesis was used to replace the conserved cysteine (Cys-186) in *EcoRII* methylase to other amino acids. The overall strategy for mutagenesis is outlined in Fig. 2. The wild-type methylase gene was cloned in M13 mp19 to create 1909-Cys. Phage 1909-Cys was mutagenized with an oligonucleotide containing a pre-defined sequence to create a Cys (UGU) to Gly (GGG) codon change. The resulting mutant, 1909-Gly, was enriched by the method of Kunkel (14). The changes in codon 186 create an *SmaI* site (CCCGGG) in the gene and hence the mutants were identified by digesting the RFI DNA with *SmaI*. The mutation was further confirmed by DNA sequencing.

The Gly codon was changed to other codons in a second mutagenesis step. Change to a Ser codon was achieved with a mutagenic oligonucleotide and the mutant was again enriched by the method of Kunkel (14). In parallel experiments, 1909-Gly was mutagenized with a mixture of oligonucleotides containing an equal representation of all four bases at each of the three positions in the Gly codon. The resulting mutants were enriched *in vitro* in two steps. The first step in the enrichment consisted of preferentially copying the strand containing the mutagenic oligonucleotide by the method of Taylor et al. (16). In the second step, the molecular mixture containing double-stranded phage DNA was split with *SmaI* to select against the parental Gly codon, and the products were transfected into *E. coli*. DNAs from the resulting independent phage plaques were analyzed for the loss of the *SmaI* site, and a 300 bp segment including codon 186 was sequenced to determine the mutagenic change. Among the first fifteen plaques screened in this manner, altered codons were obtained for Ala (GCG; one isolate), Gln (CAG; four), Gly (GG-A and GGC; two and one, respectively), Trp (UGG; one) and Val (GUU; one). One isolate contained a two base deletion and the remaining four contained the original glycine codon.

Preliminary characterization of the mutants

Preliminary characterization of the mutants was done in two ways. RFI DNA of the mutants was subjected to *EcoRII* digestion and the products were analyzed by gel electrophoresis. While DNA from 1909-Cys was resistant to *EcoRII*, DNAs from all

the mutants were sensitive to the enzyme (not shown). Extracts were also prepared from phage-infected cells and DNA methylase activity in the extracts was determined by quantitating the transfer of ^3H -methyl groups from S-adenosyl-methionine (SAM) to DNA. The mutants showed 230 to 1000-fold reduction in enzyme activity and the levels of incorporation of ^3H -methyl groups in DNA due to many of the mutants were comparable to the incorporation with the vector alone (Table I). Since the assay uses crude cell extracts, it is unclear if the small amounts of ^3H incorporation seen with some of the mutants are significant. Further, it is not known if any of the mutant proteins are unstable inside the cell. When SDS-polyacrylamide gel electrophoresis of extracts from phage infected cells was carried out and the gel stained with coomassie brilliant blue, no bands corresponding to either the wild-type or the mutant proteins could be identified thereby preventing such an analysis (not shown). Therefore, we conclude that changing of Cys-186 to other amino acid codons leads to a large reduction in the methylase activity, but cannot eliminate the possibility that the mutants may retain a small amount of activity.

Cloning of the mutant alleles into pBR322

To study these mutants further, we attempted to clone a *HindIII*-*EcoRI* fragment from the M13 phage derivatives into pBR322.

Figure 1. Proposed mechanism of methyl transfer to C5 of cytosine. **A.** Adapted from Santi et al. (4). **B.** Expected structure of the complex between the methylase and 5-azacytosine containing DNA.

Figure 2. Strategy for mutagenesis. **A.** Structure of 1909-Cys. This M13 mp19 phage derivative has been described previously (12). Filled-in rectangles represent *EcoRII* sequences. **B.** Outline of the procedure. Details of the procedure are given in Materials and Methods.

This is schematically outlined in Fig. 3A. Transformants of the strain BH129 resulting from the cloning experiments were screened for sensitivity to ampicillin and tetracycline, and plasmids from ampicillin-resistant (Amp^R) tetracycline-sensitive (Tet^S) clones were analyzed for the presence of the fragment containing the mutated gene. While we were successful in cloning some of the mutants of the methylase in this manner, repeated attempts to clone the mutant with Cys-186 to Gly-186 change (C186G; codon-GGG) failed. Few transformants were obtained in experiments involving the C186G mutant and when plasmid DNAs from a total of 29 Amp^R Tet^S clones from three experiments were analyzed, none were found to have the methylase gene. These plasmids were either head-to-head dimers of pBR322 or pBR322 with *Hind*III-*Eco*RI inserts of sizes substantially different than the expected size of 2.3 kbp.

The unexpected difficulties encountered in the cloning of the methylase gene containing the C186G change prompted us to attempt the cloning of the mutants in a different manner. It seemed possible that the apparent unclonability of this mutant allele was not due to the mutation in codon 186, but due to some other unidentified mutation(s) in the gene that arose during the process of site-directed mutagenesis. If there were secondary mutations in 1909-Gly, then these mutations would also be present in its derivatives, ie. 1909-Ser, 1909-Trp etc. To eliminate this possibility, all the plasmids obtained from the cloning of *Hind*III-*Eco*RI fragments into pBR322 were discarded and a new strategy for the cloning was adopted.

This strategy is outlined in Fig. 3B and 3C. First the wild-type methylase gene from 1909-Cys was cloned into pBR322 as a *Hind*III-*Eco*RI fragment. This construct, pR300(+)Cys, contains two *Bst*BI sites (Fig. 3B) and the smaller *Bst*BI fragment (282 bp) of this plasmid contains the codon 186. Next, a derivative of pR300(+)Cys missing the smaller *Bst*BI fragment was generated (pR300 Δ *Bst*BI, Fig. 3B). The *Bst*BI fragment from 1909-Gly was completely sequenced to confirm that it contained no mutations other than the changes in codon 186 and attempts were made to clone this fragment into pR300 Δ *Bst*BI (Fig. 3C). GM31 served as the host strain for these experiments. In parallel, attempts were also made to clone the small *Bst*BI fragment from each of the derivatives of 1909-Cys containing other mutations at codon 186 into pR300 Δ *Bst*BI. It was expected that in each case the cloning would generate two types of recombinants; one

with the *Bst*BI fragment in the correct ('+') orientation and one with the *Bst*BI fragment in the incorrect ('-') orientation and the number of isolates of the two types would be roughly equal. Consequently, the cloning of the *Bst*BI fragment in the (-) orientation would serve as an internal positive control for the ligation step in cloning.

While we were successful in obtaining several independent clones with the *Bst*BI fragment containing the C186S, C186W or C186V changes in the correct orientation [pR300(+)Ser, pR300(+)Trp and pR300(+)Val, respectively], no clones containing *Bst*BI fragment with the C186G change in the (+) orientation were obtained. When plasmids from 50 transformants resulting from the attempt to clone the C186G mutant were analyzed, 32 contained pR300 Δ *Bst*BI, nine contained the *Bst*BI fragment in the incorrect orientation [pR300(-)Gly] and the remaining nine appeared to have suffered some rearrangements. The pR300(-)Gly isolates contained a unique *Sma*I site within the *Bst*BI fragment confirming the presence of the mutation at codon 186 (not shown). We interpret these data to mean that the *Bst*BI fragment containing C186G change was competent for ligation, but that either the recombinants generated in the (+) orientation were unstable in *E. coli* or were toxic to the cells.

Figure 3. Cloning mutant alleles into pBR322. H — *Hind*III, E — *Eco*RI, B — *Bst*BI. Asterisk marks the site of the mutation at codon 186 in the methylase gene. The dotted areas are M13 sequences, the filled-in areas are *Eco*RI sites and the open areas are pBR322 sequences. **A.** Strategy 1. BH129 was the host used for the transformation. **B.** Construction of pR300 Δ *Bst*BI. The transformation step has been omitted from the figure. **C.** Strategy 2. GM31 was the host used for the transformation.

Table I. Methylase activity of the mutants in M13 mp19

Phage	Codon	Relative Methylase Activity
M13 mp19	—	0.11 ± 0.02
1909-Cys	UGU	(100) [@]
1909-Gly		
1	GGG	0.10
2	GGA	0.16
3	GGC	0.43
1909-Ala	GCG	0.11
1909-Gln	CAG	0.13
1909-Ser	UCU	0.10
1909-Trp	UGG	0.21
1909-Val	GUU	0.18

[@] The assay for enzyme activity is described in Materials and Methods. The number for M13 mp19 is the average (± S.D.) of results from three experiments, while the numbers for the wild-type gene and the mutants are averages of results from two experiments. The specific activity for the wild-type enzyme in the extract was 3.96 pmol of CH_3 -groups/min/mg of protein.

It should be noted that initial efforts to clone the C186A and C186Q alleles have also failed.

Characterization of mutants with C186S, C186W or C186V changes

Plasmids containing the methylase gene with C186S, C186W or C186V in the (+) orientation were characterized further. These plasmids could be stably maintained in strains of *E. coli* and these DNAs were sensitive to *EcoRII* endonuclease. Further, cell-free extracts prepared from cells containing these plasmids showed little or no methylase activity (Table II). These results are consistent with the apparent lack of methylase activity displayed by these mutants in their M13-based derivatives (Table I). The

design of the cloning experiment argues that the change in the codon 186 from a Cys codon to codons for other amino acids was the cause of the observed methylation defect. These data are consistent with an essential role for Cys-186 in catalysis by the *EcoRII* methylase.

C5 methylases are proposed to form transient covalent complexes with DNA as intermediates in the methyl transfer reaction (4). This complex would be formed as a result of an attack on C6 of the target cytosine by a cysteine (Fig. 1A, structure II). Such intermediates are expected to be stabilized when the target is 5-fluorocytosine or 5-azacytosine [Fig. 1B, structure VI, (4)]. The wild-type *EcoRII* methylase has been shown to form stable covalent complexes at *EcoRII* recognition sites in DNA containing 5-azacytosine (19, 20).

We used a genetic assay to study the ability of the mutant proteins to form covalent adducts with DNA. The assay has been described before (8) and it involves determining the sensitivity of *recA*⁻ cells carrying the different mutants to 5-azacytidine (5-azaC). In the cell, covalent complexes form between the methylase and DNA containing 5-azacytosine and the *recA* mutation prevents their efficient repair leading to cell death (8, 21, 22). If the role of the conserved cysteine within the C5 methylases is to carry out the nucleophilic attack on position 6 of cytosine, then its replacement by a non-nucleophilic amino acid should eliminate complex formation; i.e. *recA*⁻ cells carrying such mutant methylases should be resistant to 5-azaC.

The results of the 5-azaC sensitivity assay are summarized in Table II. As expected, the plasmid carrying the wild-type methylase gene confers sensitivity to 5-azaC upon its host. Cells carrying the wild-type gene are about 100 times as sensitive to 5-azaC as cells carrying pBR322. Both C186W and C186V changes reduce the sensitivity of the cells to 5-azaC to about the level of pBR322, but plasmids carrying C186S change confer about 10% of the sensitivity conferred by the wild-type gene

Table II. Properties of the mutants in pBR322

Plasmid	Codon	Relative Methylase Activity	Relative Sensitivity to 5-AzaC [‡]
pBR322	—	0.06 ± 0.08	(1.0)
pR300(+)-Cys	UGU	(100) [@]	104 ± 28
pR300(+)-Ser	UCU	0.14	12 ± 4
pR300(+)-Trp	UGG	0.16	0.58 ± 0.29
pR300(+)-Val	GUU	0.17	1.7 ± 0.5

[@] The assay for enzyme activity is described in Materials and Methods. The number for pBR322 is the average (± S.D.) of results from three experiments, while the numbers for the wild-type gene and the mutants are averages of results from two experiments. The specific activity for the wild-type enzyme in the extract was 0.41 pmol of CH₃-groups/min/mg of protein.

[‡] The assay for 5-azaC sensitivity has been described before [Bhagwat, 1987 #3]. Relative sensitivity of BH129 cells with different plasmids to 20 µg/ml 5-azaC is the ratio—(percent survival of cells with pBR322)/(percent survival of cells with the test plasmid). The numbers are averages (± S.D.) of results from three or more experiments. The percent survival for pBR322 carrying cells was 5.4 ± 1.7.

Figure 4. Strategy for obtaining pR300(+)-Gly. Details of this 'scrambling and ligation' procedure are given in Materials and Methods. X—*XmaI* and B—*BstBI*. *XmaI* is an isoschizomer of *SmaI* and this site marks the position of the mutant Gly-186 codon. Other products include linear and circular molecules containing multiple copies of the two *BstBI* fragments. DH10B was the host for the transformation.

(Table II). Cells containing the methylase with C186S change are about 10-fold more sensitive to 5-azaC than cells carrying pBR322. This is true despite the apparent lack of methyl-transfer activity in the cell-free extracts prepared from these cells. These results suggest that the mutant methylase with C186S change may be able to form covalent adducts with 5-azaC containing DNA, but is unable to efficiently transfer methyl groups to DNA.

Further characterization of the mutant with C186G change

In our initial attempts to clone the *Bst*BI fragment containing C186G change into pR300Δ*Bst*BI, a majority of transformants (64%) were found to contain the original plasmid, i.e. pR300Δ*Bst*BI. To avoid this, we designed an alternate strategy for the cloning in which nearly all the resulting clones would be expected to contain either pR300(+)*Gly* or pR300(−)*Gly*. This procedure, referred to as 'scrambling and ligation procedure', had two features that were different from the earlier procedure. The procedure is outlined in Fig. 4. The ligation step in the new procedure involved only those two fragments that are expected to make up pR300(+)*Gly*. Specifically, M13 phage DNA sequences were not included in the reaction. Further, the ligation products were separated by electrophoresis and only the products of the expected size of pR300(+)*Gly* and pR300(−)*Gly* were used for the final transformation into the strain DH10B. Plasmids were extracted from a total of 43 independent clones and analyzed. While ten of these plasmid isolates were pR300(−)*Gly*, none of the remaining plasmids had structures expected of pR300(+)*Gly*. Only two of the plasmids were pR300Δ*Bst*BI and the rest of the plasmids appeared to have suffered some rearrangements. These results further support our earlier conclusion that it is not possible to clone into pBR322 the complete *Eco*RII methylase gene containing C186G change.

If the failure to clone the mutant allele with C186G is due to the change in codon 186, then reverting this codon to a cysteine codon should make it possible to clone this DNA into pBR322. To confirm this, 1909-*Gly* was mutagenized with an oligonucleotide to restore Cys-186 codon and phage clones

carrying the revertant were identified by the loss of *Sma*I site and by DNA sequencing. *Bst*BI fragment from the RFI DNA of one such revertant was ligated to pR300Δ*Bst*BI cut with *Bst*BI and the ligated DNA was transformed into *E. coli*. Four transformants were screened; two contained the *Bst*BI fragment in the (+) orientation and the other two contained plasmids with apparent rearrangements. Plasmids with the fragment in (+) orientation were resistant to *Eco*RII and extracts prepared from cells carrying these plasmids showed wild-type levels of methylase activity (not shown). Based on these results we conclude that the difficulties encountered in the cloning of the methylase gene containing C186G change were due to the alteration in codon 186 alone.

Cloning of mutant alleles under a regulated promoter

The *M.Eco*RII gene in pR300 contains the endogenous promoter of the gene. As this promoter is not regulated, the toxic effects of a mutant allele cloned in this plasmid are likely to be expressed constitutively, resulting in cell death. This may be the reason for our inability to replace Cys-186 in pR300-Cys by *Gly*, *Ala* or *Gln*. To avoid this, we attempted to clone a promoterless derivative of the gene with C186G substitution under a phage T7 promoter in the absence of any T7 RNA polymerase in the cell. This was successfully accomplished in the vector pT7-5 (18) and is described in Materials and Methods. The wild-type gene and the alleles with C186S and C186W changes were also cloned in this vector. These plasmids (pT71-Cys, pT71-*Gly*, pT71-Ser and pT71-Trp) were maintained in BL21(DE3), an *E. coli* B strain containing a chromosomal copy of the T7 RNA polymerase gene under the control of *lac* promoter (9).

For the wild-type gene, little methylase activity was seen in the absence of T7 RNA polymerase. Following the induction of synthesis of the polymerase with IPTG a large increase in methylase activity was seen (data not shown). When cell free extracts prepared under these conditions were electrophoresed, the presence of a band consistent with the known size of the methylase could be seen for the wild-type enzyme and the three mutants (Figure 5). When, the same cell-free lysates were tested for methylase activity, no activity was detected for any of the mutants above the background (Table III). Therefore, substitutions of the conserved Cys in *M.Eco*RII with *Gly*, *Ser* or *Trp* reduces enzyme activity by a factor of 3,000 or more. This is consistent with our results with the M13 phage-based and pR300-based clones (for C186S and C186W) of these mutants (Tables I and II). The level of production of the wild-type enzyme was much higher in the T7-based vector than in the M13 phage-based or pR300-based clones. This is reflected in the relatively

Figure 5. Overproduction of *M.Eco*RII and its mutants from a T7 promoter. Extracts from cells containing various T7-5-based constructs were electrophoresed on a 8% SDS-polyacrylamide gel and the gel stained with the Coomassie Brilliant Blue dye. Lane 1 contains molecular weight size markers. The sizes of the marker bands are indicated on the left of the gel. Lanes 2, 3, 5, 7 and 9 contain extracts prepared from cells grown in the absence of IPTG, while lanes 4, 6, 8 and 10 contain extracts from cells in which the T7 RNA polymerase gene was induced with 1 mM IPTG one hour prior to their harvesting. Lane 2—pT7-5; lanes 3 and 4—pT71-Cys, lanes 5 and 6—pT71-*Gly*; lanes 7 and 8—pT71-Ser; lanes 9 and 10—pT71-Trp. The expected position of the *M.Eco*RII band is indicated on the right side of the gel.

Table III. Methylase Activity of the Mutants Expressed from a T7 Promoter

Plasmid	Codon	Relative Methylase Activity
T7-5	—	0.021 ± 0.012
T71-Cys	UGU	(100) [@]
T71- <i>Gly</i>	GGG	0.033
T71-Ser	UCU	0.015
T71-Trp	UGG	0.030

[@] The assay for enzyme activity is described in Materials and Methods. The number for T7-5 is the average (± S.D.) of results from three experiments, while the numbers for the wild-type gene and the mutants are averages of results from two experiments. The specific activity for the wild-type enzyme in the extract was 83.6 pmol of CH₃-groups/min/mg of protein.

lower 'background' methylase activity and hence a higher level of confidence regarding the lack of methyl transferring ability by the mutants.

The effects of C186G allele on cell growth were studied by inducing the chromosomal T7 RNA polymerase gene with IPTG and monitoring the turbidity of the culture. The rate of growth of cells carrying pT71-Gly was reduced within 30 minutes after the induction of the polymerase gene and a complete halt in growth was seen within 90 minutes (Figure 6). The growth-arrested cells were elongated (data not shown), suggesting an inhibition of cell division. It should also be noted that attempts to transform cells carrying pGP1-2, a multi-copy plasmid containing the T7 RNA polymerase gene (18), with pT71-Gly were unsuccessful. This was despite the fact the T7 RNA polymerase gene in pGP1-2 was repressed by phage λ repressor cI present on the same plasmid. We conclude that the presence of even modest amounts of T7 RNA polymerase in cells carrying pT71-Gly results in cell death. In contrast, induction of the polymerase gene in BL21(DE3) cells carrying pT71-Cys resulted in no change in growth rate for 45 minutes. After this time, the cells continued to divide at a slightly reduced rate (Figure 6). The T7 promoter used for the methylase is very strong and is known to cause some growth inhibition (9, 18).

DISCUSSION

We have shown that the substitution of the conserved cysteine in *EcoRII* methylase with six different amino acids results in the loss of methylase activity in the cell and a lack of protection of these sites from endonuclease attack. Because our methylase assays were done with cell-free extracts, we cannot eliminate the possibility that some of the mutants may carry a small amount of methyl transferase activity. For three of the substitutions; Gly, Ser and Trp; the level of activity was found to be comparable to the background activity ($0.021\% \pm 0.012\%$ of the wild-type

enzyme) under conditions where all the proteins were overproduced in the cells. For the Ala, Gln and Val substitutions, the level of enzyme activity was also found reduced to the level of the background activity (about 0.1% of the wild-type enzyme), but due to the lower level of production of these proteins in the cells their presence in the cells was not directly demonstrated. These results suggest that Cys-186 plays a key role in the action of the methylase and is probably essential for it. The substitution of the conserved Cys has previously been reported for only one C5 methylase, the *Bacillus* phage methylase SPR (23), and the results have been similar to those reported here. A Cys to Ser substitution in this methylase was reported to lead to a reduction in methylase activity to less than 3% of the wild-type enzyme (23).

The sensitivity of cells with the C186S allele to 5-azaC suggests that this mutant is able to form a covalent link with DNA, although with a lower efficiency than the wild-type enzyme. It should be pointed out that our assay for covalent linking is indirect and it is possible that this mutant causes cell death in the presence of 5-azaC by a different mechanism. However, if this mutant is able to attack C6 of target cytosine, but is defective in methyl transfer to DNA, then Cys-186 must have a role(s) in the methylase action that is distinct from its role in covalent complex formation. Som and Friedman have shown (7) that Cys-186 in *EcoRII* methylase is close to S-adenosyl methionine (SAM). Perhaps this residue plays a role in SAM binding or in the transfer of methyl group to DNA.

The most unexpected outcome of this study was our initial failure to clone into pBR322 methylase genes with C186G, C186A or C186Q changes and the subsequent demonstration of adverse effects of the C186G mutant on cell growth. We tested three different cloning strategies and several different strains of *E. coli* to try to clone the C186G allele; but without success. In each case, the majority of the recombinants obtained contained rearrangements of DNA. We have shown that the substitution at codon 186—and not a secondary mutation in the gene—was the cause of the failure to clone the mutant allele. The finding that the expression of C186G causes growth arrest in *E. coli*, explains our inability to clone the gene with its unregulated endogenous promoter into pBR322. It is possible that the C186A and C186Q alleles also cause growth arrest of the cells. Our success at obtaining these mutant alleles under the control of the endogenous promoter in the phage vector may have been the result of differing requirements for the two vector-host systems. In a phage infection, survival of the cell is not essential and hence some cytotoxic effects may be tolerated. For a plasmid vector, healthy growth of the host is essential for its own propagation.

The only lethal phenotype previously described for DNA methylases is referred to as modified cytosine restriction [Mcr, (24–26)]. Plasmids carrying genes for several cytosine methylases have been shown to be restricted by *E. coli*. *EcoRII* methylase is not subject to Mcr restriction, but if the mutations in this methylase were to alter its sequence-specificity, it may be subject to restriction. However, we do not think this to be the cause of our inability to clone the C186G, C186A or C186Q alleles. One of the hosts used in our unsuccessful cloning experiments, DH10B, is deleted for all the known Mcr functions. We have not investigated other possible reasons behind this unusual phenotype in a systematic fashion and it remains a mystery.

As the proposal for the mechanism of C5 methylases draws upon the known mechanism of thymidylate synthase (TS), it is

Figure 6. Growth inhibition due to C186G mutant. Cells carrying pT71-Cys or pT71-Gly were grown in LB medium and the turbidity of the cultures were monitored by measuring OD₅₅₀ with a Spectronic-21 spectrophotometer. When OD₅₅₀ reached 0.3 the cultures were divided in two halves and IPTG was added at a concentration of 1mM to one culture to induce synthesis of the methylase. The time of addition of IPTG is marked by a downward arrow. Squares represent cultures with pT71-Cys and circles represent cultures with T71-Gly. Open squares and open circles are cultures without IPTG.

instructive to compare the properties of TS mutants with the properties of the *EcoRII* mutants. Substitutions of the conserved cysteine in TS have been carried out for enzymes from *E. coli*, bacteriophage T4 and *Lactobacillus casei* (27–30). The replacement of the conserved cysteine by alanine, glycine or threonine resulted in the loss of catalysis for the enzymes from *E. coli* and *L. casei*. Similarly, substitutions of this residue in the *L. casei* enzyme with tryptophan or valine also lead to a loss of enzyme activity. Our results with these substitutions in *EcoRII* methylase are consistent with these results. Replacement of the conserved cysteine with serine results in somewhat different phenotypes for the different TS. The enzymes from *E. coli* and bacteriophage T4 with serine respectively retained 0.02% and 0.07% activity of the wild-type enzyme (27, 29). The activity of *E. coli* TS with serine was sufficient to partly complement *thyA* strains (27, 28). In contrast, a similar mutant of TS from *L. casei* failed to complement a *thyA* mutation in *E. coli* and no enzyme activity was detected in cell-free extracts (30). It will be interesting to find out if the C186S mutant of *EcoRII* methylase has any methyl transferase activity.

It is also interesting to note that the TS from *E. coli* with serine or alanine bound the substrate dUMP as well as the wild-type enzyme, but mutants with glycine or threonine bound the substrate poorly (27). It appears that the substitution of the catalytic cysteine in TS does not always lead to the loss of substrate binding. Finally, T4 TS with serine formed SDS-resistant complexes with 5-fluoro-dUMP (29). In contrast, despite its ability to carry out catalysis, no stable complex between the *E. coli* TS with serine and 5-fluoro-dUMP could be detected (27). Our results suggest that, *EcoRII* methylase with C186S should form a stable complex with DNA containing 5-azacytosine.

A number of aspects of the reaction mechanism for C5 methylases proposed by D. Santi and his colleagues (4) have been successfully tested over the past few years. These include the formation of tight complexes by these enzymes with DNA containing 5-azacytosine (19, 31, 32) or 5-fluorocytosine (33) and the exchangeability of the hydrogen at C5 (6). Indirect evidence has pointed to the importance of the conserved cysteine among the methylases for catalysis. Results presented here support a key role for this cysteine in the action of *EcoRII* methylase and are consistent with the notion that it plays roles in addition to its role as a nucleophile that creates a covalent intermediate. Further, these studies indicate that mutants with substitutions of this cysteine may help one to decipher these roles.

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