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Regulation of *Eco*RII methyltransferase: effect of mutations on gene expression and *in vitro* binding to the promoter region

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ABSTRACT

***Eco*RII Methyltransferase (M.*Eco*RII) which methylates the second C in the sequence CCWGG (W = A/T) is autogenously regulated by binding to the 5′ regulatory region of its gene. DNase I footprinting experiments demonstrated that purified M.*Eco*RII protected a 47–49 bp region of DNA immediately upstream of the *ecoRIIM* coding region. We have studied this interaction with mutants of the enzyme, *in vitro* by DNA binding and *in vivo* by investigating the repression *in trans* of expression of β-galactosidase from an *ecoRIIM–lacZ* operon fusion. Two catalytically active mutants failed to repress expression of the fusion whereas catalytically inactive mutants had repressor activity. However, with one of the catalytically inactive mutants, C186S, in which the catalytic Cys was replaced with Ser, and which bound unmethylated CCWGG sequences, repression could only be demonstrated when those sequences in cellular DNA were methylated by supplying a cloned *dcm* gene *in trans*. *In vitro* binding of the DNA fragment containing the *ecoRIIM* regulatory region was detected only with the mutants that showed repressor activity, including C186S. Results indicate that down-regulation of the gene *in vivo* and binding to the promoter *in vitro* are not dependent on the catalytic properties of M.*Eco*RII. Mobility shift experiments with C186S also revealed that it could bind either the promoter or unmethylated CCWGG sites, but not both. We conclude that the concentration of unmethylated CCWGG sites controls expression from the *ecoRIIM* promoter.**

INTRODUCTION

Gene regulation of restriction-modification (RM) systems is important for the survival of the host organism. The cellular DNA has to be protected from restriction by its own endonuclease. Since RM systems are often transferable, their regulatory mechanism is expected to be associated with the system itself. However, the mechanism involved in the control of the RM genes differs from system to system. In some systems, such as *Pvu*II (1) or *Bam*HI (2), expression of the RM genes are regulated by a separate small open reading frame within the system, whereas in the *Eco*RII RM system, expression of the methylase is autogenously regulated at the transcriptional level (3,4). We have shown that *in vitro*, *Eco*RII methyltransferase (M.*Eco*RII) binds to the DNA fragment containing the promoter and the presumed regulatory sequences which lie upstream of the *ecoRIIM* structural gene and inhibits transcription (3). *In vivo*, cells containing an intact *ecoRIIM* promoter and the transcription start site, but with a deletion in the coding region, showed a marked increase in mRNA initiated from the *ecoRIIM* promoter (3). In addition fusion of the *ecoRIIM* promoter to the *lacZ* structural gene caused expression of high amounts of β-galactosidase (βGal) (4). In both cases supplying M.*Eco*RII *in trans* inhibits the transcription initiated from the *ecoRIIM* promoter. These results suggest a direct relationship between protein–promoter binding and regulation of the gene.

Our findings (3,4) imply two DNA binding activities of M.*Eco*RII: one with CCWGG sites which it methylates in presence of S-adenosyl methionine (AdoMet) and the other with a sequence in the promoter region where it acts as a repressor.

Two other enzymes that are autogenously regulated at the transcriptional level have been described in detail (reviewed in references 5,6). In one instance, proline dehydrogenase, PutA, which controls proline utilization, enzyme activity is not required for the regulation, whereas in the other, biotin-protein ligase, BirA, enzyme activity is necessary for autogenous regulation, since the product formed by the BirA protein, biotinyl-AMP, acts as a co-repressor. In both cases, however, regulation is mediated by the titration of functional sites by the enzyme; a functional electron transport chain in the case of PutA and a biotin acceptor protein in the case of BirA. When the functional sites are full the enzyme regulates its own expression.

In this paper we identify the *ecoRIIM* regulatory region that binds M.*Eco*RII. By studying the effect of mutants of the enzyme on expression from the *ecoRIIM* promoter *in vivo* and the promoter–protein interactions *in vitro*, we demonstrate that catalytically inactive mutants can repress expression and bind to

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the promoter region. We have also determined that unmethylated CCWGG sequences can serve as functional sites for regulation of *ecoRIIM*.

MATERIALS AND METHODS

Enzymes and chemicals

M.*Eco*RII was purified as described before (7). Restriction enzymes were from BRL and New England Biolabs. *Bam*HI linkers were from New England Biolabs. DNA polymerase I large fragment and DNA ligase were the products of Boehringer Mannheim. DNase I and nucleotides were purchased from Pharmacia. The DNA sequencing kits were from United States Biochemicals and the radionucleotides were from NEN.

Strains, plasmids and phages

A tetracycline resistant Dcm$^-$ strain, P90C (*dcm*), was prepared by transducing *E.coli* P90C (*ara* Δ(*lac-pro*) *thi*), obtained from R.W.Simons (8), with a P1 lysate grown on strain GM3888 (*dcm-6 zed501::Tn10*). GM3888 is a Dcm$^-$ strain containing Tn10 80% linked to *dcm*, obtained from M.G.Marinus. P90C (*dcm*) was used as the host in most of the operon fusion experiments. *E.coli* GM271 (*dcm-6 hsdR2*) was also obtained from M.G.Marinus. *E.coli* WP2, a *lon11 sulA* derivative of *E.coli* B was obtained from E.Witkin. Plasmids pT71-Ser and pT71-Trp (9) expressing M.*Eco*RII mutants, C186S and C186W, respectively, from a T7 promoter, were kindly provided by A.S.Bhagwat. In these mutants, the Cys-186 residue in the catalytic center of M.*Eco*RII was changed to Ser and Trp, respectively (9). The low copy number plasmid pAC59, bearing the *ecoRIIM* gene under the control of the *lac* promoter, and high copy number plasmids pSS12 and pSSQ, also carrying the *ecoRIIM* gene, were described previously (3). Restriction fragments containing the *ecoRIIM* promoter (Fig. 1A) were prepared from pSS12 DNA. These fragments do not contain CCWGG sites. The *dcm* locus (10) of *E.coli* K12 was isolated from cellular DNA as a 6.8 kb *Hin*dIII−*Bam*HI fragment and inserted into appropriately cut pBR322 to construct pBR-DCM. The construction of recombinant bacteriophage λRSPRII carrying an operon fusion of the *ecoRIIM* promoter to the promoter-less *lacZ* gene (*lacZ*$^+$) and integration of this fusion, *ecoRIIM'*−*lacZ*$^+$ into the chromosome of P90C was described previously (4). In a similar fashion the fusion was introduced into the chromosome of P90C (*dcm*) by lysogenization. Lysogens carrying a single copy of the prophage were isolated and designated as P90C (*dcm*) λRSPRII.

DNase I footprinting

A *Sal*I−*Sau*3AI fragment (see Fig. 1A) of pSS12 containing the *ecoRIIM* promoter was labeled separately at the recessed 3' ends of either strands using the Klenow fragment and an appropriate [α-^{32}P] deoxyribonucleotide in such a way that only the last incorporated nucleotide contained the label. Labeling the *Sal*I end produces the fragment which is labeled in the template strand. Labeling the *Sau*3AI end results in label in the non-template strand. DNase I footprinting of the DNA−M.*Eco*RII complex was done by adding DNase I directly to the binding mixture. The binding conditions were as describe before (3). Two tenth pmol of the labeled DNA (20 000 c.p.m.) was incubated with 40 pmols of purified M.*Eco*RII protein at 25°C for 30 min in 20 μl binding reaction mixture. The mixture was then supplemented with $MgCl_2$ to 6 mM and 1 unit of DNase I, and the incubation was continued for another 10 min. The reaction was stopped by adding 100 μl stop mix containing 0.3 M sodium acetate, pH 5.2, 20 mM EDTA and 10 μg tRNA, extracted with phenol−chloroform and the DNA fragments were precipitated with ethanol. The precipitated samples were dissolved in formamide loading buffer, heated to 90°C and resolved in 6% sequencing gel (11). The marker ladders were generated from the sample DNAs by G and G+A chemical sequencing reactions as described by Maxam and Gilbert (11).

Generation of mutants

Two types of mutants were used: (i) insertional and (ii) missense. The insertion mutagenesis was carried out by introducing a *Bam*HI linker, in-frame, at selective restriction sites within the wild type (WT) *ecoRIIM* gene of pAC59. For insertion at *Nru*I or *Hin*cII sites, the plasmid DNA was linearized by partial digestion with those enzymes and 12-mer linkers, 5'-CGCGG-ATCCGCG-3' were attached to the linearized DNA by T4 DNA ligase. After digestion with the *Bam*HI restriction enzyme and religation, the recombinants were used to transform competent *E.coli* cells. For insertion at *Ava*I sites the pAC59 DNA partially linearized with *Ava*I, was filled in with the Klenow fragment and dNTPs prior to the attachment of the 8-mer linkers, 5'-CGG-ATCCG-3'. Proper recombinants were identified by restriction mapping and sequencing the mutagenized region. Subcloning of the genes of missense mutants C186S and C186W in low copy

Table 1. Description of the mutants of M.*Eco*RII

No[a]	Name[b]	Type	Location[c]	Description[d]	Reference
1	Av2	insertion	31	K L L - - - - G Q V K L L G G S V G Q V	this paper
2	N6	insertion	56	E W S - - - - R E T E W S R A D P R E T	this paper
3	H4	insertion	186	F P C - - - - Q P F F P C S R I R Q P F	this paper
4	C186S	missense	186	F P C Q P F F P S Q P F	ref. no. 9
5	C186W	missense	186	F P C Q P F F P W Q P F	ref. no. 9

[a]In reference to Figure 1B.
[b]Designations used to describe the mutant proteins; low copy number plasmids carrying the mutant alleles are described in Table 2.
[c]The amino acid residue which is altered or after which the insertion is made.
[d]Top row, wild type sequence; bottom row, mutant sequence.

number plasmids was achieved by replacing an *AvaI* fragment internal to the *ecoRIIM* gene in pAC59 with corresponding fragments from pT71-Ser and pT71-Trp, respectively. The mutants are described in Table 1 and locations of the mutational changes within the primary structure of the protein is given in Figure 1B.

Overexpression of proteins

For *in vitro* binding studies, crude extracts were prepared from overproducing strains of *E.coli* WP2 carrying the compatible plasmids pGP1-2 (12) containing the gene for T7 polymerase under the control of a thermolabile λ*cIts*857 repressor and a pT7 (12) derivative carrying the gene for M.*Eco*RII (WT or mutants) under the control of a T7 polymerase promoter. A construct producing the WT protein from a pT7 derived plasmid, pRIIM, has been described (7). Plasmid pRIIM-N6, over-producing the mutant N6 (Table 1) was constructed by replacing an appropriate restriction fragment of the *ecoRIIM* gene in pRIIM with a similar fragment from pAC59-N6, the low copy number plasmid expressing the mutant protein (Table 2). WP2 (pGP1-2) was transformed with pRIIM-N6. Plasmids pT71-Ser and pT71-Trp, over-expressing the mutants C186S, and C186W, respectively, were also introduced into WP2 (pGP1-2). The cloned proteins were expressed when the P_L promoter was induced by elevating the culture temperature from 30 to 42°C as described (7). Cells from 10 ml of induced or uninduced cultures grown to an A_{600} of 3.5–3.75, were suspended in 1 ml buffer, sonicated and centrifuged to isolate clear lysates as described previously (13). The amount of total protein in the extracts was estimated by Lowry's method (14) and the extracts from induced cultures were diluted to the desired total protein concentration with 10 mM potassium phosphate, 1 mM EDTA, 7 mM β-mercaptoethanol, pH 7.0, just before use in the binding reaction described below.

Enzyme assays

Assays for methyltransferase activity in the crude extract measured the transfer of ^{3}H-methyl groups from AdoMet to *E.coli* B DNA as described previously (15). One unit is one pmol methyl group transferred per min per mg protein of crude extract.

βGal activities were measured with permeabilized cells as described by Miller (16) using aliquots of 20–100 μl cultures grown to a density of approximately 1.0 A_{600}.

Promoter–protein complex formation using crude extracts

For the promoter–protein binding studies, 1 μl of diluted crude extracts (1 μg total protein for the promoter fragment; 2.5 μg for control DNA) were incubated with 0.1 pmol DNA fragments in 10 μl binding mixture. A 289 bp *SalI–NruI* fragment (Fig. 1) from pSS12 was used as the M.*Eco*RII promoter containing DNA. A 194 bp *SalI–NruI* fragment from pSSQ served as the control DNA lacking promoter sequences. The conditions of the

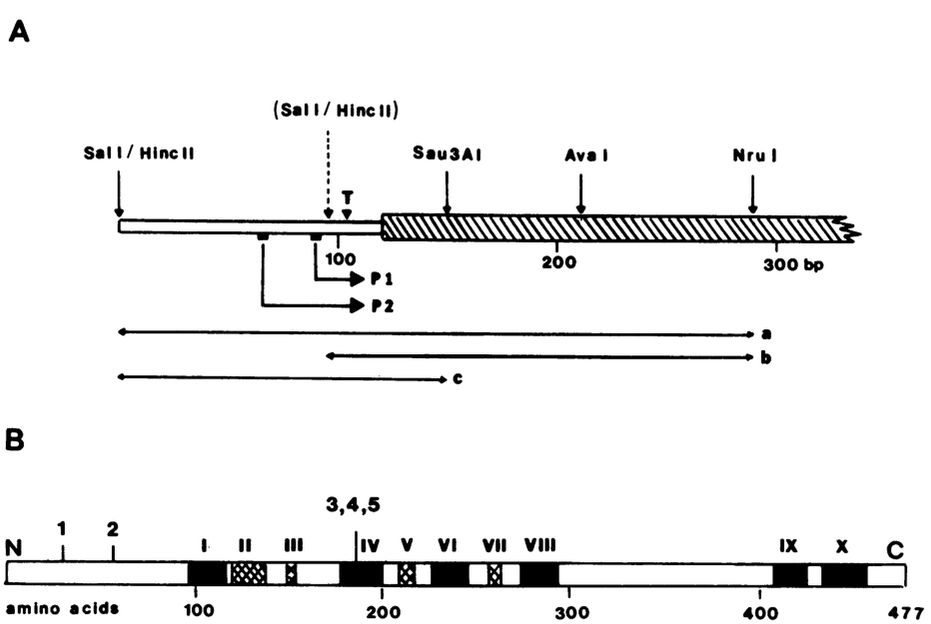

Figure 1. (A) Restriction map of the upstream regulatory region and partial coding region of the *ecoRIIM* gene from pSS12. Selected restriction sites are shown. The 5′ *SalI*/*Hin*cII site is the fusion joint between the pUC18 vector and the insert DNA sequences. This position in construct pSSQ is shown in parentheses. Hatched box, coding region; open box, upstream region; T, transcription start site(s); P1 and P2, −10 and −35 region promoters, respectively. The 289 bp *SalI–NruI* fragment (fragment a) of pSS12 was used in binding to M.*Eco*RII and its mutant proteins. The *SalI–NruI* fragment (fragment b) from pSSQ was used as the promoter-less control DNA in binding experiments. DNase foot-printing was done with the *SalI–Sau*3AI fragment (fragment c) from pSS12. The *Hin*cII–*Sau*3A fragment (fragment c) from pSS12 was used to prepare the *ecoRIIM′–lacZ*$^+$ fusion (see ref. 4). B, Primary structure of M.*Eco*RII and location of the mutations. The position of the mutations are numbered *1* to *5*. Conserved motifs are conventionally designated with Roman numbers (refs 19 and 20). Filled boxes, highly conserved; criss-crossed boxes, moderately conserved; open boxes, variant regions. An invariant Cys in motif IV is the active site of catalysis and binds to the recognition sequences in DNA. The variant region between motifs VIII and IX contains the domain responsible for recognition of the CCWGG sequences. The variant N-terminal region in which mutations *1* and *2* are located, is not essential for catalytic activity of the enzyme. Data from X-ray crystallography (ref. 20) have revealed that a pocket involved in binding of AdoMet is contributed by motifs I–V and motif X. Mutations are described in Table 1. The insertional mutations are *1*, at an *AvaI* site, *2*, at an *NruI* site, and *5*, at a *Hin*cII site. The missense mutations *3* and *4* (see ref. 9) also resulted in the loss of the *Hin*cII site.

binding reaction were the same as described before (3) except that AdoMet was omitted.

In separate binding experiments, crude extracts of cells containing approximately 0.5−1.0 pmol M.*Eco*RII mutants were pre-incubated for 30 min with DNAs containing 15 pmol methylated or unmethylated CCWGG sites, in 10 μl of the same binding buffer as above plus 50 μM AdoMet, prior to the addition of 0.1 pmol of the ^{32}P-labeled promoter DNA fragment. Further incubation was under the same conditions. Plasmid pUC19, isolated from the Dcm$^-$ strain GM271, served as unmethylated DNA and pSS59 (17) carrying the *ecoRIIM* gene, was used as a source of methylated DNA.

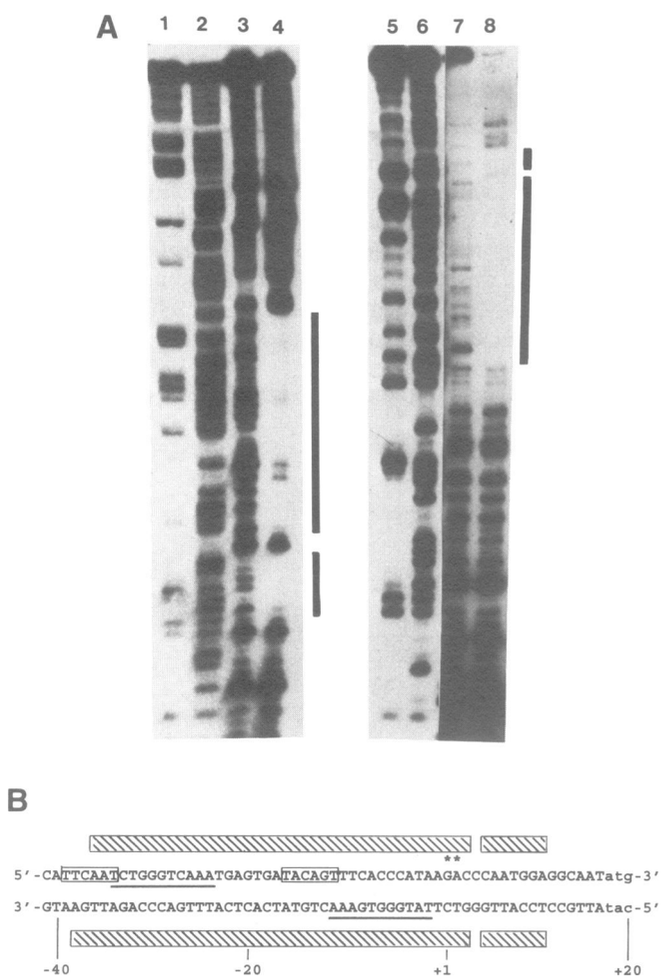

Figure 2. DNase I footprints of M.*Eco*RII bound to a DNA fragment containing the *ecoRIIM* promoter. (**A**) Autoradiogram of the gel. A *SalI*−*Sau*3AI fragment from pSS12 was end-labeled with ^{32}P on either strand, treated as indicated below and the products were resolved in an 8% sequencing gel. Lanes 1−4, label on the non-template strand; lanes 5−8, label on the template strand. Lanes 1 and 5, G ladder from Maxam and Gilbert's chemical sequencing reaction; lanes 2 and 6, G+A ladder; lanes 3 and 7, partial DNase I digestion of the uncomplexed fragment; lanes 4 and 8, partial DNase I treatment of the fragment complexed with M.*Eco*RII. The protected regions are shown by thick vertical lines. (**B**) Analysis of DNase I footprinting data. Partial DNA sequences are presented. The regions occupied by the M.*Eco*RII protein are shown by hatched boxes. The position +1 refers to the transcriptional start site. The translational start codon is shown in small case, and the promoter sequences are boxed. A gap within the protected region represents susceptibility to DNase I. Inverted repeat sequences are underlined.

RESULTS

DNase I footprinting of M.*Eco*RII−promoter complex

Previously we have demonstrated by mobility shift assays that M.*Eco*RII binds to the DNA fragment containing sequences upstream of the *ecoRIIM* gene (3). The sequences bound by M.*Eco*RII were determined by DNase I footprinting after a complex was formed between the enzyme and a DNA fragment containing its promoter (Fig. 2A). M.*Eco*RII was used in excess. Mobility shift assays with an aliquot of the reaction mixture were performed to verify that all the DNA was bound (data not shown). Mapping of the DNAse I treated samples revealed protection of a specific region in both template and non-template strands. This region spans residues −38 to +11 in the template strand and residues −36 to +11 in the non-template strand. In both the strands, within the protected region, however, one base was found to be susceptible to DNase digestion. The protected region includes the putative −35 (partly) and −10 region promoter sequences as well as the transcriptional start site(s) (Fig. 2B).

Effect of M.*Eco*RII mutants *in trans* on expression from the *ecoRIIM′−lacZ*$^+$ fusion

There are ten conserved motifs that have been identified in DNA(cytosine-5) methyltransferases (m5C MTases) (18−20). Six of them are highly conserved and four are moderately conserved (20) (Fig. 1B). The catalytic center which contains an invariant Pro−Cys dipeptide, is in highly conserved motif IV (20). The cysteine residue binds to the target cytosine in the DNA and is actively involved in catalytic transfer of the methyl group from AdoMet (21,22). In three of the mutants, C186S, C186W and H4, the mutational change is at this catalytic center. In C186S and C186W (9), the cysteine residue (Cys-186) was replaced with a serine and a tryptophan residue, respectively (Table 1). While the replacement with serine does not destroy the ability of the protein to bind to the CCWGG sequences, replacement with the bulkier tryptophan residue severely affects such binding (23). In the insertion mutant H4, which also lacks the CCWGG-binding ability (unpublished data) the Pro−Cys dipeptide is present but the motif is disrupted by insertion of a tetrapeptide after the Cys residue (Table 1). Methylase activities were not detectable in strains carrying these three mutants (Table 2). On the other hand, deletions of M.*Eco*RII from the N-terminus of as much as one fourth of the total residues retain catalytic activity (24). This region does not contain any domain that is conserved among m5C MTases. In two of our insertion mutants, N6 and Av2, the insertion is in this region (Fig. 1 and Table 1). Both these mutants are catalytically active.

For evaluating the effect of mutations on gene regulation, *E.coli* P90C (*dcm*) λRSPRII lysogens carrying the *ecoRIIM′−lacZ*$^+$ fusion were transformed with low copy plasmid pAC59, or its derivatives expressing the mutants described above. The phenotypes of the transformants are shown in Table 2. The WT protein was found to suppress the expression *in trans* by 96%. Two of the catalytically inactive mutants, C186W, and H4, were found to be almost as effective as the WT protein in suppressing the expression of βGal. On the other hand another catalytically inactive mutant, C186S, *in trans* only inhibited expression by 13%. None of the two N-terminal mutants, Av2 and N6, were found to suppress βGal expression although N6 retained more than one third of the WT enzyme activity. These experiments prove that mutants lacking catalytic activity can be effective in

gene regulation and mutants with catalytic activity can be deficient in regulating the expression of its gene. The results also suggest that these two properties are independent functions of M.*Eco*RII and that the regulatory function is affected by the changes in the N-terminal region of the protein.

In vitro interactions between the *ecoRIIM* promoter and mutant proteins

Expression of M.*Eco*RII is controlled by its interaction with the regulatory region of *ecoRIIM* gene (3). Previously we have shown that transcription from the *ecoRIIM* promoter *in vitro* is inhibited by protein−promoter binding (3). If the mutant proteins are to regulate gene expression they must interact with the regulatory sequences. We tested such interaction with three mutants. Two of them, C186S and N6 are biologically inactive in regulating expression from the *ecoRIIM'−lacZ*$^+$ fusion, while the third one, C186W is as efficient as the WT in regulation of expression. The binding of the promoter-containing DNA to M.*Eco*RII could be demonstrated quite efficiently in cell-free crude extracts. Overproducing strains expressing the WT or mutant proteins were grown in liquid cultures and extracts were prepared under identical conditions. As can be seen from Coomassie blue staining (Fig. 3A), the WT and the mutant proteins are expressed in equivalent amounts in induced cultures.

Extracts containing equal amounts of total protein were tested for binding to a DNA fragment containing the *ecoRIIM* promoter (Fig. 3B). Under the experimental conditions, with extract containing WT protein, almost complete binding of the DNA was observed (lane 3). As anticipated from the operon fusion experiments, no specific protein−DNA complex was formed with N6 (lane 6). Binding occurred with C186W (lane 5), although the amount of complex was less than that found with WT. However, as noted above, C186W is as efficient as WT in suppressing fusion-borne expression. One explanation for these observations could be that the amount of C186W expressed from the *lac* promoter on a plasmid is more than sufficient to suppress the single copy *ecoRIIM* promoter. Surprisingly, C186S was also found to form a complex with the promoter fragment (lane 4). In fact, the amount of complex formed with C186S was slightly more than that formed with C186W.

The binding is specific for the upstream regulatory region. The DNA fragment from pSSQ which contained sequences from two thirds of the pSS12 fragment but not the regulatory region (3), did not bind WT, C186S or C186W (lanes 9−11, respectively) although the amount of protein used was 2.5-fold greater than the amount used in the reactions with the pSS12 fragment.

The fact that C186S binds specifically to the *ecoRIIM* promoter suggests that some other factor might account for the lack of regulation associated with cells expressing C186S.

Table 2. Effect of plasmid borne M.*Eco*RII mutants *in trans* on *ecoRIIM'−lacZ*$^+$ fusion

Plasmid[a]	Methylation		βGal activity[b]
	in vivo[c]	*in vitro*[d]	
none (control)	−	0.0 (0)	1417 (100)
pAC59	+	22.8 (100)	58 (4)
pAC59-Av2	+	0.7 (3)	1275 (90)
pAC59-N6	+	8.2 (36)	1458 (103)
pAC59-H4	−	n.d	95 (7)
pAC59-C186S	−	n.d	1236 (87)
pAC59-C186W	−	n.d	62 (4)

E.coli P90C (*dcm*) λRSPRII lysogens carrying single copy fusion of *ecoRIIM'−lacZ*$^+$ were transformed with the plasmids listed above. Cells were grown in 20 ml LB medium containing 50 μg/ml Km to 1.0 A_{600}. Twenty to one hundred microliter culture was used to assay βGal activity. Cells from the remainder of the culture were harvested for assaying methyltransferase activity.
[a]Plasmids carrying the mutant alleles are all derivatives of pAC59.
[b]Mean values in Miller units (% of control) from three sets of assays are given.
[c]Based on whether the DNA, isolated from the strain, is sensitive or resistant to *Eco*RII restriction; −, sensitive; +, resistant.
[d]Mean values in assay units (% of pAC59 activity) from three determinations. For unit definition see text. n.d, not detectably different from background value.

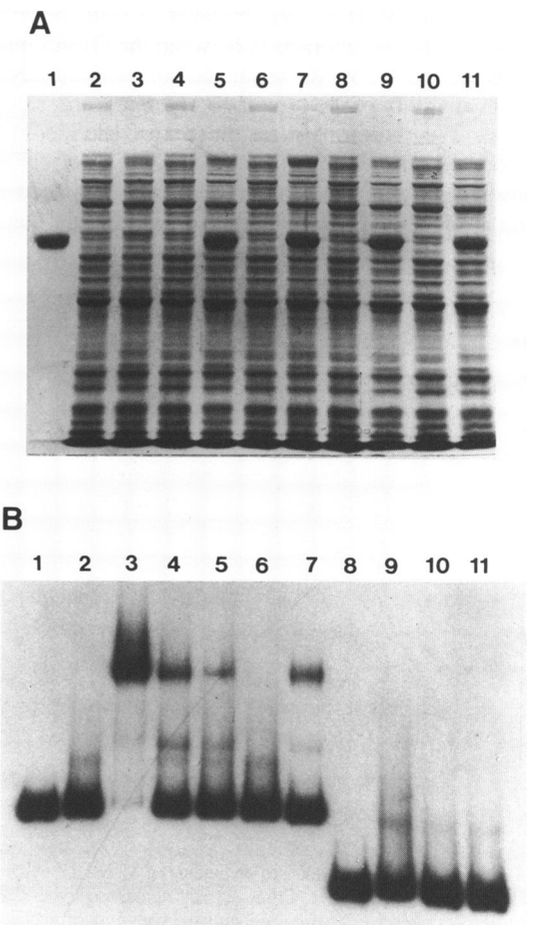

Figure 3. Binding of M.*Eco*RII to the *ecoRIIM* promoter. (**A**) Expression of the WT and mutant proteins. Six μl (25−30 μg of total protein) of crude extracts (lanes 2−11) from *E.coli* WP2 (pGP1-2) carrying compatible pT7-derivative plasmids were co-electrophoresed in a 7.5% SDS−polyacrylamide gel with 20 pmol purified M.*Eco*RII (lane 1) and stained with Coomassie Blue. The pT7-based plasmids are: lanes 2 and 3, pT7-5 (no protein expressed from T7 promoter); lanes 4 and 5, pRIIM (WT); lanes 6 and 7, pT71-Ser (C186S); lanes 8 and 9, pT71-Trp (C186W); lanes 10 and 11, pRIIM-N6 (N6). Lanes 2, 4, 6, 8 and 10, un-induced culture grown at 30°C; lanes 3, 5, 7, 9 and 11, induced at 42°C followed by incubation at 37°C. (**B**) Mobility shift assay for the promoter−protein complex. Aliquots of the crude extracts from induced cultures described above were diluted and incubated with DNA fragments as described in the text and the mixture was resolved in a 6% native polyacrylamide gel. Lanes 1−7, DNA fragment with promoter; lanes 8−11, control DNA fragment with promoter sequence deleted. Lanes 1 and 8, without protein; The crude extracts contained the following proteins: lanes 3 and 9, WT; lanes 4 and 10, C186S, lanes 5 and 11, C186W; and lane 6, N6; In lane 2, extract from pT7-5 carrying cells were used as negative control; lane 7, 0.5 pmol purified M.*Eco*RII as positive control.

Effect of Dcm methylation on C186S mediated regulation of gene expression

We investigated the basis for the lack of autoregulatory activity of C186S despite its ability to bind to the promoter region *in vitro*. The C186S mutant differs from C186W in its affinity for the unmethylated CCWGG sequence (23). Binding between C186S and DNA containing CCWGG sequences can be detected by mobility shift assay if the DNA is either unmethylated or hemimethylated (23). *E.coli* K12 strains express M.*Eco*Dcm, which is an isoschizomer of M.*Eco*RII. In a Dcm$^-$ K12 strain, CCWGG sites of DNA remain unmethylated. Since, in the fusion experiments described above, we used a Dcm$^-$ host for studying the role of M.*Eco*RII mutants *in trans* on expression of the fusion gene, an interaction between the C186S mutant and the unmethylated CCWGG sequences of cellular DNA might limit the availability of the protein to regulate transcription from the fusion. We therefore determined if C186S could inhibit expression of the fusion in a host that carried either a chromosomal or a plasmid borne *dcm* gene. As seen in Table 3, in the absence of C186S, neither chromosomal nor plasmid mediated M.*Eco*Dcm activity have any detectable regulatory effect on the expression of the *ecoRIIM'−lacZ*$^+$ fusion. However, M.*Eco*Dcm, when expressed from a cloned gene in a multicopy plasmid as in pBR-DCM, can greatly modify the effect of C186S on expression of the *ecoRIIM'−lacZ*$^+$ fusion. In the presence of pBR-DCM, C186S was found to suppress the fusion based expression by 77% as against 17% in the presence of chromosomal M.*Eco*Dcm activity, or 11−13% in the absence of a functional *dcm* gene. In Dcm$^+$ cells, in which M.*Eco*Dcm is expressed from a single copy chromosomal gene, not all CCWGG sites are methylated (25−27). Methylation of CCWGG in Dcm$^+$ cells can be increased by supplying plasmids carrying either *ecoRIIM* (26) or the *dcm* (25) gene. This would explain the lack of regulation observed with C186S in the cells with only chromosomal M.*Eco*Dcm activity.

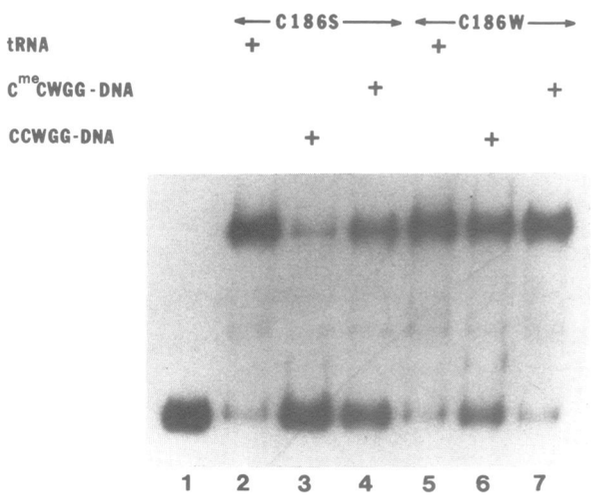

Figure 4. Effect of CCWGG sequences on binding of M.*Eco*RII mutants to DNA containing the *ecoRIIM* promoter. Crude extracts containing either C186S (lanes 2−4) or C186W (lanes 5−7) were pre-incubated with 5 μg of nucleic acids (the DNAs accounted for 15 pmol of either methylated or unmethylated CCWGG sites) as designated. A [α-^{32}P]-labeled 289 bp *SalI−NruI* fragment (do not have *Eco*RII sites) from pSS12 containing the *ecoRIIM* promoter was then added to the pre-incubation mix and incubation was continued as described in the text. The mixtures were electrophoresed on a 6% polyacrylamide gel Autoradiogram is shown. Lane 1 contains only labeled DNA fragment.

Inhibition of promoter−protein interactions in the presence of CCWGG sequences

Data from Table 3 indicate a possible role of unmethylated CCWGG sequences in C186S mediated regulation of gene expression. We determined if pre-incubation of C186S or C186W with CCWGG sequences can affect the mutant proteins' interactions with the regulatory sequences. Data are presented in Figure 4. Binding of the C186S protein to the *ecoRIIM* regulatory sequences was markedly inhibited by pre-incubating the enzyme with unmethylated DNA (lane 3). Quantitation of the radioactivity associated with the bands shown in Figure 4 revealed that 80, 18 and 47% of the promoter DNA was complexed when C186S was pre-incubated with no DNA, unmethylated DNA and methylated DNA, respectively. Under similar conditions, these figures for C186W were 81, 57 and 79%, respectively. The C186S protein binds strongly to unmethylated DNA and this competitive binding can decrease binding to the promoter (Fig. 4, lane 3). Binding to the promoter is much stronger if the competing DNA is methylated (lane 4). These results imply that although the enzyme may have two DNA binding domains it can bind to CCWGG or the promoter, but not both. In these *in vitro* experiments, we also found that the binding of C186W to the promoter is slightly inhibited by competing unmethylated DNA. Weak binding of C186W to unmethylated DNA has been reported (23).

DISCUSSION

We defined the region upstream of the *ecoRIIM* gene that binds M.*Eco*RII and regulates its transcription. This 47−49 nucleotide region identified by DNase I footprinting, includes the −10 and −35 regulatory elements needed for transcription by *E.coli* RNA polymerase. M.*Eco*RII binds to this sequence which overlaps the promoters and inhibits initiation of transcription by RNA

Table 3. Effect of C186S on βGal activity expressed from an *ecoRIIM'−lacZ*$^+$ fusion in strains producing M.*Eco*Dcm

Lysogen	Plasmid 1	Plasmid 2	βGal activity[a]
P90C λRSPRII	none	none	1402 (100)
P90C λRSPRII	pAC59-C186S	none	1168 (83)
P90C (*dcm*) λRSPRII	none	none	1354 (100)
P90C (*dcm*) λRSPRII	pAC59-C186S	none	1205 (89)
P90C (*dcm*) λRSPRII	none	pBR-DCM	1335 (99)
P90C (*dcm*) λRSPRII	pAC59-C186S	pBR-DCM	311 (23)

[a]As described in Table 2.

polymerase (3). In the protected region is an inverted repeat of 11 base pairs with two mismatches, separated by 12 base pairs. The symmetry of this sequence suggests that the enzyme may bind as a dimer although the protein is a monomer in solution (13). The stoichiometry of the interaction and direct involvement of other factors, if any, that modify the protein–promoter binding remain unknown at this point.

Results presented here clearly demonstrate that the repression of expression of its gene is a function of the M.*Eco*RII protein and this is not associated with its catalytic function. This conclusion is based on the results obtained from *in vivo* experiment studying the effect *in trans* of the cloned mutants of M.*Eco*RII on expression of βGal from an *ecoRIIM'–lacZ*$^+$ fusion. While some mutants with catalytic activity such as N6 were unable to inhibit expression from the fusion, others such as C186W with no apparent catalytic activity due to substitution of the active Cys residue involved in binding to the CCWGG sequence, were highly efficient in repressing expression. This implies the presence of two DNA binding domains in M.*Eco*RII—one is the catalytic domain and the other is the promoter binding domain. With two mutants, N6 and C186W, findings from the genetic experiments were supported by the *in vitro* demonstration of the protein–promoter interaction, while with C186S, the *in vitro* interaction with the promoter conflicts with its inability to regulate the fusion-borne expression. We addressed this discrepancy by showing *in vivo* and *in vitro*, that this was due to an interaction of C186S with the cellular DNA at unmethylated CCWGG sites. High affinity of the C186S mutant for the recognition sequence is well documented in *in vitro* studies (23). The data presented in Figure 4 also imply that the enzyme exerts only one of its two DNA binding properties at a time since only one shifted band was observed.

When relatively stable CCWGG–M.*Eco*RII interactions occur, e.g., with C186S, or when cells containing M.*Eco*RII are exposed to 5-azacytidine, which, when incorporated into DNA forms stable complexes with M.*Eco*RII (13), the transcription from the *ecoRIIM* promoter is derepressed. For C186S to exert its repressor function, the cell DNA must be sufficiently methylated at the CCWGG sites. The studies with C186S (this paper) and with 5-azacytidine treated cells (3,4) indicate that in cells carrying the WT *ecoRIIM* gene, regulation of the latter might be mediated by binding of the enzyme to unmethylated CCWGG sequences. Unmethylated CCWGG sequences are generated as a result of DNA replication.

M.*Eco*Dcm and M.*Eco*RII, which methylate the same residue in the same sequence, have a strong resemblance in their primary protein structure (28). The homology between the two proteins is strongest (90% or more identical residues) in the conserved motifs. However, the cloned M.*Eco*Dcm enzyme has no cross-regulatory effect on the *ecoRIIM* promoter function. This indicates that the core regions of M.*Eco*RII are not primarily involved in the regulatory function. This presumption is also supported by the fact that both of the catalytically active mutants, Av2 and N6, have all the core motifs intact, yet neither of them demonstrates a regulatory role. These mutants have insertions in the N-terminal segment of the protein, which is not essential for enzyme activity (24). A catalytically active deletion mutant of M.*Eco*RII missing the first 33 amino acids (24) also failed to repress βGal expression from the fusion (data not given). Thus the N-terminal extension of M.*Eco*RII contributes to the regulatory functions of the protein and is a candidate for further mutagenic studies.

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