

Specific binding of SsoII DNA methyltransferase to its promoter region provides the regulation of SsoII restriction-modification gene expression

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ABSTRACT

The regulation of the SsoII restriction-modification system from *Shigella sonnei* was studied *in vivo* and *in vitro*. In *lacZ* fusion experiments, SsoII methyltransferase (M.SsoII) was found to repress its own synthesis but stimulate expression of the cognate restriction endonuclease (ENase). The N-terminal 72 amino acids of M.SsoII, predicted to form a helix–turn–helix (HTH) motif, was found to be responsible for the specific DNA-binding and regulatory function of M.SsoII. Similar HTH motifs are predicted in the N-terminus of a number of 5-methylcytosine methyltransferases, particularly M.EcoRII, M.dcm and M.MspI, of which the ability to regulate autogenously has been proposed. *In vitro*, the binding of M.SsoII to its target DNA was investigated using a mobility shift assay. M.SsoII forms a specific and stable complex with a 140 bp DNA fragment containing the promoter region of SsoII R-M system. The dissociation constant (K_d) was determined to be 1.5×10^{-8} M. DNaseI footprinting experiments demonstrated that M.SsoII protects a 48–52 bp region immediately upstream of the M.SsoII coding sequence which includes the predicted –10 promoter sequence of M.SsoII and the –10 and –35 sequences of R.SsoII.

INTRODUCTION

The type II restriction-modification (R-M) system consists of two enzymatic components, restriction endonuclease (ENase) and methyltransferase (MTase). The ENase makes a double-strand DNA break within or near the specific recognition sequence. The cognate MTase modifies a specific base within the recognition sequence to prevent it from cleavage. Many R-M systems, e.g. EcoRII (1,2) and SsoII (3), are carried on self-transmissible plasmids. In the host cells, it is necessary that methylation precedes the endonuclease action. Therefore, the initial level of MTase expression should be higher compared to that of the ENase. Subsequently, the level of MTase is expected to be

reduced in order to provide effective protection of the host cells against bacteriophage infection.

To date, many R-M systems have been cloned, sequenced and expressed in heterologous systems, but, in general, there has been a paucity of information on their regulation. In PvuII and BamHI R-M systems, regulation is provided by a small protein, the encoding gene being found within the respective R-M intergenic space (4,5). In EcoRII, expression of M.EcoRII is autoregulated at the transcriptional level (6). A similar mechanism was proposed for M.MspI (7). Here, we examine the regulation of the SsoII R-M system *in vivo* and *in vitro*. In addition, we propose a common regulatory mechanism for a number of related 5-methylcytosine (m5C) MTases.

MATERIALS AND METHODS

Construction of the pACYC-SsoIIM and pACYC-SsoIIR plasmids

Plasmid pMS2 carrying the total nucleotide sequence of SsoII R-M system (8) was used as a template for PCR synthesis of a 140 bp DNA fragment containing the intergenic region between the *ssoIIM* and *ssoIIR* genes. The nucleotide sequences of the forward and reverse primers were:

5'-CTTAAGGATCCATAAAAAATAACCTTTTATATC-3'
BamHI

and

5'-CTGCGATATCAGATCTCATGCAITGTCTACCAGAA-3',
EcoRV BglII

respectively.

CAT in boldface is the complement of the translational start codons of R.SsoII (upper primer) and M.SsoII (lower primer), respectively. The BglII, EcoRV and BamHI sites were introduced in the primer sequences. These oligonucleotides were synthesized on an ASM 102U DNA synthesizer (Novosibirsk, Russia) using standard phosphoramidite chemistry. The conditions of PCR reactions were as follows. The first five initial cycles: 94°C for 1 min, 55°C for 1 min, 72°C for 40 s; and the following 25 cycles: 94°C for 1 min, 60°C for 1 min, 72°C for 40 s. The amplified

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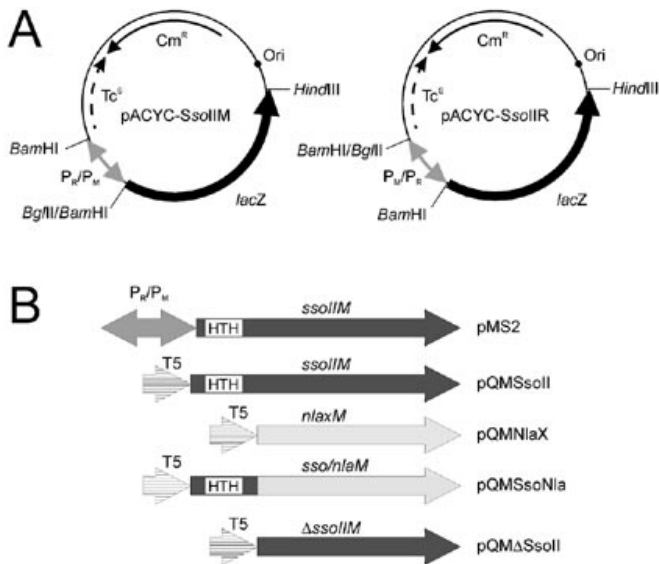


Figure 1. Plasmid constructs used to study the regulatory function of *M.SsoII* *in vivo*. (A) In pACYC-SsoIIM and pACYC-SsoIIR plasmids, expression of the *lacZ* gene is placed under the control of P_M and P_R promoters, respectively. (B) Derivatives of *ssolIM* and *nlaXM* genes used in cotransformation of *E.coli* strains harboring pACYC-SsoIIM and pACYC-SsoIIR plasmids. HTH is helix–turn–helix; T5 is bacteriophage T5 promoter. Construction of the pMS2, pQMSsoII, pQMNlaX, pQMSsoIa and pQMΔSsoII plasmids is detailed in our previous papers (8,11).

DNA was subcloned into the pACYC184 plasmid (9) in both orientations. In one case, both pACYC184 plasmid and PCR fragment were treated with *Bam*HI and *Eco*RV; in the other, the pACYC184 DNA was cleaved with *Bam*HI and the PCR fragment treated with *Bgl*III and *Bam*HI. The ligated DNA was transformed in *Escherichia coli* NW-22 [$\Delta(lac-proAB)$ [*F'* *tra*Δ36 *proA*⁺*B*⁺ *laq*^I *lacZ*ΔM15] *endA*1 *gyrA*96 (Nal^R) *hsdR*2(or17) *mcrA* *mcrB*-1 *mrr*? *relA*1 *supE*44 *thi*-1}. Colonies with Cm^R and Tc^S phenotype were selected. The resulting plasmids were then treated with *Bgl*III and *Hind*III or *Bam*HI and *Hind*III and ligated with *Bam*HI–*Hind*III fragment from p10 plasmid, a derivative of pCL47 (10) containing the full-length *lacZ* gene, to generate pACYC-SsoIIM and pACYC-SsoIIR plasmids, respectively (Fig. 2). Thus, the first ATG codon of *ssolIM* or *ssolIR* gene appeared to be the start codon of *lacZ* gene in resulting pACYC-SsoIIM and pACYC-SsoIIR constructs, respectively. Blue colonies were selected on Luria–Bertani (LB) plates containing X-gal and IPTG (isopropyl- β -thiogalactopyranoside) after transformation in *E.coli* NW-22.

Determination of β -galactosidase activity

The plasmid pMS2 (8) bearing the native *M.SsoII* gene (Fig. 2), and plasmids pQMSsoII, pQMNlaX, pQMSsoIaX and pQMΔSsoII carrying the genes of *M.SsoII*, *M.NlaX*, hybrid *SsoII/NlaX* MTase and a deletion variant of *M.SsoII*, respectively (11), were co-transformed in *E.coli* NW-22 cells harboring the pACYC-SsoIIM and pACYC-SsoIIR plasmids. In the listed pQM plasmids the methyltransferase genes are under the control of the artificial hybrid *lac/T5* promoter (11), while in the case of pMS2 the *ssolIM* gene is transcribed from its own *M.SsoII* promoter (8). β -Galactosidase activity in these strains was

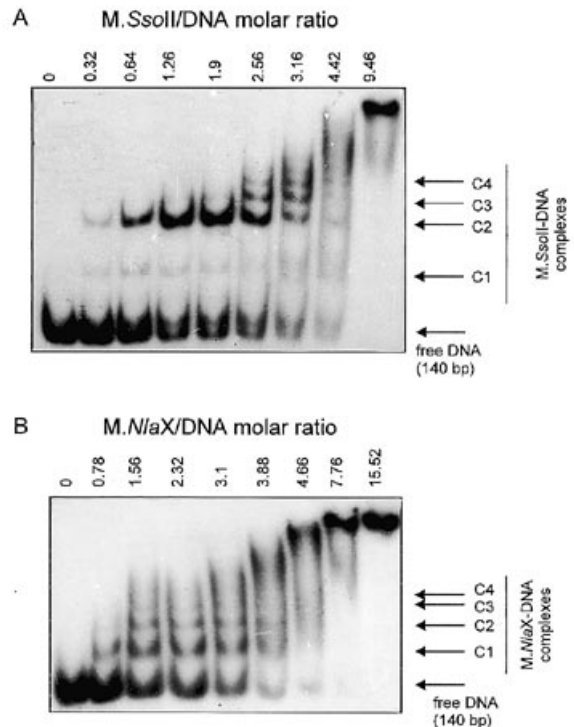


Figure 2. Gel retardation assays. A constant amount (413 nM) of the ³²P-labeled 140 bp DNA fragment corresponding to the *SsoII* regulatory region, was titrated with increasing amounts of homogeneous *M.SsoII* (A) and *M.NlaX* (B). (A) Lanes (left to right) represent increasing amounts of *M.SsoII* added to the reaction mixture. The molar ratio indicated represents concentrations of 0, 130, 260, 520, 780, 1040, 1300, 1820 and 3900 nM, respectively. (B) Lanes (left to right) represent increasing amounts of *M.NlaX* added to the reaction mixture. The molar ratio indicated represents concentrations of 0, 320, 640, 960, 1280, 1600, 1920, 3200 and 6400 nM, respectively. C1, C2, C3 and C4 represent various complexes.

calculated as described by Miller (12) or analysed on LB plates containing X-gal and IPTG.

Non-specific DNA for mobility shift assay

Plasmid pB6490 (13) was used as a template to synthesize a 140 bp DNA fragment which contains the promoter region of Bovine Leucosis Virus (BLV) as a non-specific DNA fragment for mobility shift assay. The primers for PCR synthesis are: 5'-CTCTATCT-CCGGTCTCTG-3' and 5'-GAAGGAGAGAGCGCGGGCC-3'. This DNA fragment as well as the 140 bp specific DNA described above were labeled with T4 polynucleotide kinase (Fermentas, Lithuania) and [γ -³²P]ATP. After separation in 8% polyacrylamide gel, these fragments were purified by electroelution.

Equilibrium binding of *M.SsoII* and DNA

M.SsoII and *M.NlaX* containing the 6×His tag were purified using a two-step procedure involving heparin–Sephacrose and Ni–NTA–agarose chromatography (11). Complexing of *M.SsoII* or *M.NlaX* with its target DNA was carried out in the buffer containing 50 mM Tris–HCl pH 7.5, 5 mM β -mercaptoethanol, 150 mM NaCl and 8% glycerol in a 10–20 μ l reaction volume, for 10–20 min at room temperature. For the determination of equilibrium binding constant, K_d , 80 nM of *M.SsoII* and the

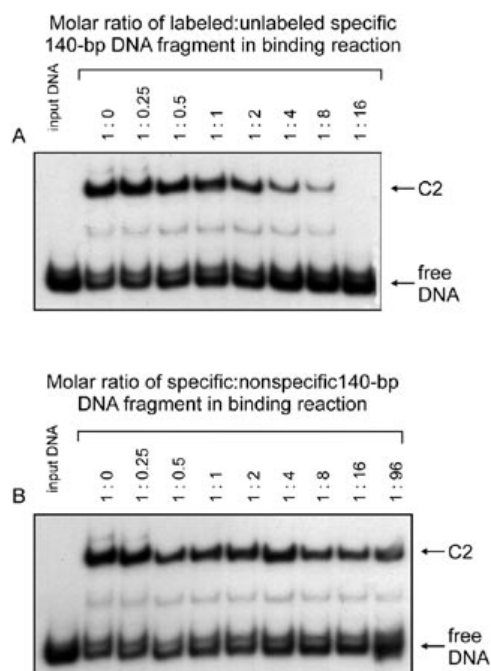


Figure 3. Competitive binding between specific and non-specific substrates to *M.SsoII*. *M.SsoII* (130 nM) and the specific ^{32}P -labeled 140 bp DNA fragment (25 nM) were incubated in standard binding conditions in the presence of increasing concentration of the unlabeled specific (A) and non-specific (B) 140 bp DNA fragments.

140 bp ^{32}P -labeled DNA fragment in the concentration range from 0 to 12.5 nM were incubated under the above conditions in a 10 μl volume. The free and bound DNA fractions were separated on a 6% PAGE (acrylamide:bisacrylamide = 19:1 w/v, 50 mM Tris-borate EDTA) and visualized by autoradiography.

DNaseI footprinting

The 140 bp specific PCR fragment was labeled at either the top or bottom strand. The binding conditions were the same as described above, except that the binding buffer contained 10 $\mu\text{g}/\text{ml}$ poly (dI-dC)-poly (dI-dC). Fifty ng of DNA (100 000 c.p.m.) and 40 ng of *M.SsoII* were mixed together in binding buffer in a 20 μl volume and incubated at 30°C for 30 min. Then, 10 ng of DNaseI (freshly diluted from 2.5 mg/ml stock in 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 60 mM MgCl_2 , 15 mM CaCl_2 and 10% glycerol) in a 4 μl volume was added to the binding reaction. Digestion was terminated after 15 min incubation at room temperature by adding 170 μl of the stop solution (200 mM NaCl, 20 mM Na_2EDTA , 1% SDS and 100 $\mu\text{g}/\text{ml}$ tRNA). Control reactions were performed under the same conditions except for the addition of *M.SsoII*. The DNA was then extracted by phenol/chloroform and precipitated. The pellets were redissolved in the formamide loading buffer, heated to 90°C for 5 min and analyzed on 6% PAGE. The chemical sequencing reactions were performed as described previously (14).

RESULTS AND DISCUSSION

In vivo study of *SsoII* R-M gene regulation

The *SsoII* ENase and MTase encoding genes are divergently transcribed (8). The length of the intergenic space is 109 bp. To

investigate the effect of *M.SsoII* on *ssolIM* and *ssolIR* gene expression, the 109 bp promoter fragment was placed in either orientation in front of the *lacZ* gene in plasmids pACYC-*SsoIIM* and pACYC-*SsoIIR* (Fig. 1A). Thus, the *lacZ* gene is under the control of the *M.SsoII* promoter and the *R.SsoII* promoter, respectively. The activity of β -galactosidase produced in the *E.coli* cells harboring either pACYC-*SsoIIM* or pACYC-*SsoIIR* plasmid was measured in the presence or absence of *M.SsoII* or its derivatives provided *in trans* (Table 1). As a result, the level of β -galactosidase activity in bacterial cells harboring the pACYC-*SsoIIM* plasmid was found to be 540-fold higher than those harboring the pACYC-*SsoIIR* plasmid (Table 1). Such a situation probably occurs just after the R-M-bearing plasmid has been introduced into the host strain. Initially, an excessive level of MTase synthesis relative to a low level of ENase would enable the host DNA to be modified thus preventing digestion by the cognate restriction endonuclease. Upon introduction of pMS2 plasmid, which provides a constitutive *M.SsoII* synthesis, the level of β -galactosidase increased 8-fold in the cells harboring pACYC-*SsoIIR* plasmid and decreased 20-fold in the cells with pACYC-*SsoIIM* plasmid (Table 1). This likely reflects a further stage of the bacterial cell cycle, i.e., having the DNA methylated and yet requiring a high level of the ENase for providing effective protection of the host cell against bacteriophage infection. Here, *M.SsoII* is viewed to act as a regulatory protein, it binds to the promoter region suppressing the *M.SsoII* synthesis while enhancing the *R.SsoII* production.

Intriguing results were obtained when the plasmid pQMNlaX was introduced in the cells harboring either pACYC-*SsoIIM* or pACYC-*SsoIIR* plasmid. pQMNlaX produces *M.NlaX* which is closely related in amino acid sequence to that of *M.SsoII* (11) and recognizes the same DNA sequence (A.K., unpublished data). Contrary to *M.SsoII*, expression of *M.NlaX* does not appear to have an appreciable effect on β -galactosidase synthesis in either cell (Table 1). The *M.NlaX* lacks the N-terminal amino acid extension in comparison with *M.SsoII* although the remaining sequence has a high amino acid sequence identity (11). Thus, we deduce that the N-terminal portion of *M.SsoII* is responsible for promoter binding and forms the basis for *SsoII* R-M system regulation. Supporting evidence comes from the plasmids providing the synthesis of hybrid *SsoII/NlaX* MTase (consisting of the first 72 amino acids of the *M.SsoII* and the entire *M.NlaX*) and a deletion derivative of *M.SsoII* in which the 72 residue extraneous sequence has been deleted (Δ *SsoII* MTase) (Fig. 1B). Expression of *M.SsoII/NlaX* had a similar effect as the native *M.SsoII*, while neither *M.NlaX* nor *M. Δ SsoII* affected the activity of β -galactosidase.

Specific DNA-binding property of *M.SsoII* and *M.NlaX*

The DNA-binding property of *M.SsoII* or *M.NlaX* was analyzed using gel-electrophoresis mobility shift assay. The concentrations of purified *M.SsoII* and *M.NlaX* refer to the monomeric forms of M_r 45 000 and 38 000, respectively. The specific and non-specific 140 bp DNA fragments represent the intergenic region of the *SsoII* R-M system and the promoter region of BLV, respectively. There is no sequence similarity between the two DNAs and the *M.SsoII* recognition sequence is absent. In concentrations of *M.SsoII*, from 0.13 to 0.78 mM, the main shifted band is referred to as the C2 complex (Fig. 2A). When the concentration of DNA was higher than 1.3 mM, more than six discrete DNA bands were visualized on the autoradiograms.

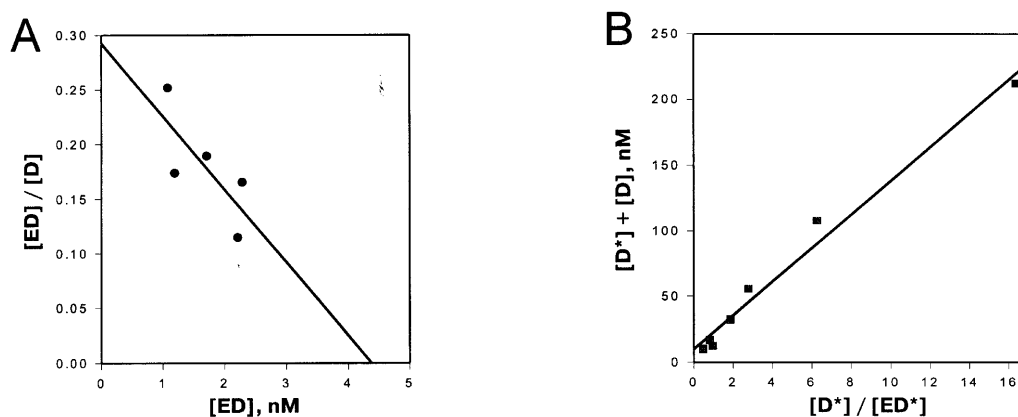


Figure 4. Determination of K_d of specific *M.SsoII*-140 bp DNA fragment complex and active concentration of the enzyme (E_0). (A) Scatchard plot of *M.SsoII*-DNA interactions. The magnitude of E_0 is determined as the length of segment cutting off on the abscissa axis by the straight line which represents the dependence of $[ED]/[D]$ from $[ED]$, while the K_d is determined from a value of tangent of the angle between the above line and the abscissa axis ($1/K_d$). (B) The concentration of the active form of enzyme (E_0) in competitive binding experiment can be determined as a tangent of the angle between the straight line representing the dependence of $[D^*] + [D]$ versus $[ED^*]/[ED^*]$ and the abscissa axis.

Table 1. Effect of *ssoIIM*, *nlaXM* genes and their derivatives on the expression of the *lacZ* gene in the model *in vivo* system

Host strain	Cotransformed plasmids	β -Gal activity ^a in Miller units (%)	Colour of colonies on the X-gal containing plates
<i>Escherichia coli</i> NW-22 [pACYC-SsoIIM]	–	269 (100)	dark blue
	pMS2	13.5 (5)	blue
	pQMSsoII	130.7 (49)	dark blue
	pQMNlaX	288.6 (107)	dark blue
	pQMSsoNlaX	232.7 (86)	dark blue
	pQM Δ SsoII	254.2 (95)	dark blue
<i>Escherichia coli</i> NW-22 [pACYC-SsoIIR]	–	0.5 (100)	white
	pMS2	4.1 (820)	blue
	pQMSsoII	10.8 (2160)	blue
	pQMNlaX	1.2 (240)	white
	pQMSsoNlaX	12.2 (2440)	blue
	pQM Δ SsoII	0.61 (122)	white

^aAll the plasmids used were introduced into the same host cells. Three independent sets of experiments were performed with different transformants and all assays were repeated twice.

Formation of the specific C2 complex is not affected by the presence of even a 96-fold excess of competitive DNA (Fig. 3). The patterns of *M.NlaX*/specific DNA binding (Fig. 2B) as well as *M.SsoII*/non-specific DNA binding (data not shown) appeared to be very similar and typical for non-specific DNA-protein interaction (15). In both cases, the quantity of shifted bands is a function of the added protein concentration. Moreover, the protein concentration required for multiple band formation is only slightly higher than that needed to produce the first shifted band.

Equilibrium binding constant (K_d) of the *M.SsoII*-140 bp DNA fragment complex

To determine the constant of dissociation (K_d) of the *M.SsoII*-140 bp DNA complex the dependence of the ratio of bound to free DNA ($[ED]/[D]$) from DNA concentration (D_0) at a constant enzyme concentration (E_0) was studied (Fig. 4). Assuming that

the active enzyme specifically interacts with DNA substrate at a 1:1 ratio, then

$$K_d = [E][D]/[ED],$$

where $[E]$, $[D]$ and $[ED]$ are the equilibrium concentrations of *M.SsoII*, DNA and DNA-protein complex, respectively. For a Scatchard plot analysis this equation has to be rearranged into

$$[ED]/[D] = E_0/K_d - [ED]/K_d$$

The magnitude of E_0 is determined as the length of segment cutting off on the abscissa axis by the straight line which represents the dependence of $[ED]/[D]$ from $[ED]$, while the K_d is determined from a tangent of the angle between the above line and the abscissa axis ($1/K_d$) as shown in Figure 4A. Hence, the value of K_d was calculated as 15 ± 7 nM and E_0 was determined as 4.4 ± 0.6 nM. Taking into account the dilution factor it is possible to calculate E_0 in the enzyme sample being investigated. The value of E_0 is equal to 285 ± 40 nM instead of 5300 nM which was obtained using the Bradford technique (16). To calculate the

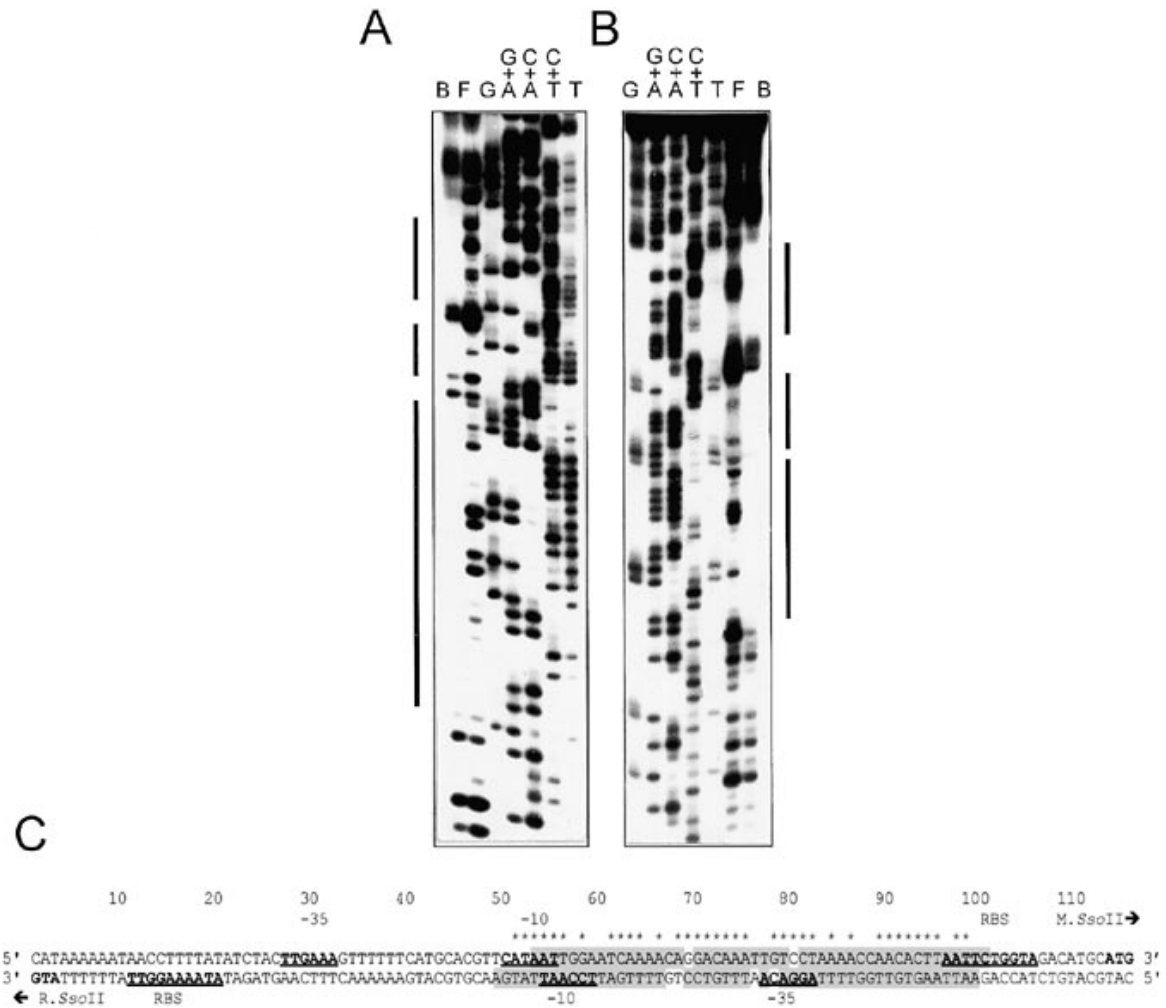
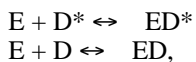


Figure 5. DNaseI footprints of *M.SsoII* to the promoter region of *SsoII* R-M. (A and B) Autoradiograms of 10% sequencing gel. A 140 bp PCR fragment was end-labeled with ^{32}P on the top (A) and bottom (B) strands, bound to *M.SsoII*, treated with DNaseI, and resolved in a gel (lanes B). Lanes F, DNaseI treatment of the 140 bp DNA fragment uncomplexed with protein. Lanes G, A+G, A+C, T+C and T, the corresponding ladders from Maxam and Gilbert chemical sequencing reaction. The regions of protection are indicated by thick vertical lines. (C) Nucleotide sequence of the *SsoII* R-M intergenic region. The nucleotides protected by the *M.SsoII* from DNaseI digestion are shown by the shaded background. Asterisks indicate the invariant nucleotides in aligned *SsoII* and *ScrFI* promoter sequences. The start codons of *M.SsoII* and *R.SsoII* are in bold face. The putative -10 and -35 promoter sequences are in bold face and underlined. RBS, ribosome binding sequence.

active concentration of *M.SsoII* (E_0) more accurately the competitive binding between ^{32}P -labeled and unlabeled specific 140 bp DNA fragment to *M.SsoII* was employed (Fig. 3). The dependence of the ratio of bound to free labeled DNA (ED^*/D^*) on adding unlabeled DNA (D_0) concentration was analyzed. This process is described by the following schemes:



where E is an active form of enzyme, D^* and D represent labeled and unlabeled forms of the same 140 bp DNA fragment, respectively, and ED^* and ED represent the complexes of the protein with labeled and unlabeled DNA fragments, respectively. In this experiment the concentration of labeled specific DNA fragment (D^*_0) was 25 nM. The following equations represent the above scheme:

$$D^*_0 = [ED^*] + [D^*]$$

$$D_0 = [ED] + [D]$$

$$E_0 = [E] + [ED] + [ED^*]$$

$$K_d = [E][D^*]/[ED^*]$$

$$K_d = [E][D]/[ED]$$

$$\text{Hence, } [D] + [D^*] = E_0[D^*]/[ED^*] - K_d.$$

The concentration of the active form of enzyme (E_0) can be determined as a tangent of the angle between the straight line representing the dependence of $[D] + [D^*]$ versus $[D^*]/[ED^*]$ and the abscissa axis (Fig. 4B). The value of E_0 appeared to be 13 ± 1 nM. Considering the dilution factor in this experiment the value of E_0 is equal to 256 ± 16 nM which approximates to the 285 ± 40 nM value obtained above. The calculated active concentration (E_0) of *M.SsoII* is 20-fold less than the concentration determined experimentally. This may be explained either by the loss of enzyme activity during storage or by the fact that the active form of *M.SsoII* is dimeric or oligomeric protein. It should be noted that in this concentration range the DNA-protein complex dissociation constant K_d cannot be determined using the above method

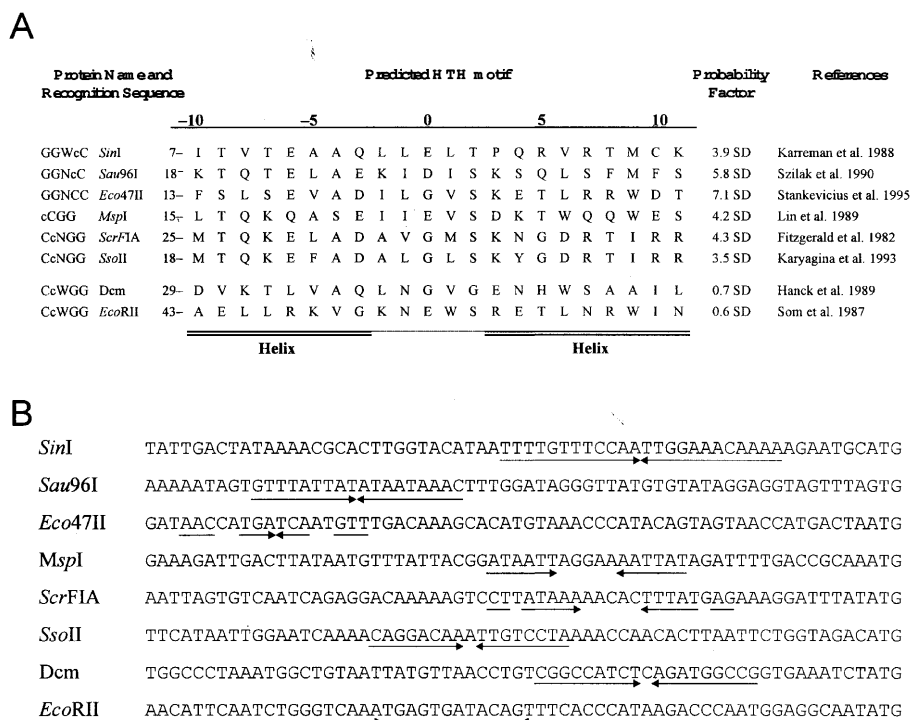


Figure 6. Potential sites of DNA–protein interaction in m5C-MTases. **(A)** Aligned HTH motifs identified in the N-terminal parts of m5C-MTases using the weight matrix method (18). The lowercase c in the recognition sequences of MTases represents the methylated residue. **(B)** The 5' non-coding sequence of various m5C-MTases showing regions of dyad symmetry (converging arrows) which may represent sites of regulatory protein binding.

because the K_d value thus obtained is comparable with the error of the experiment.

DNaseI footprinting of *M.SsoII*–promoter complex

A 140 bp DNA fragment containing the promoter region of *M.SsoII* was end-labeled on either the top or bottom strand, bound with a saturated amount of *M.SsoII*, and the resulting complex was treated with DNaseI. A control reaction was performed in the same manner except for the addition of MTase. The resulting DNA fragments were separated by electrophoresis and the positions protected by *M.SsoII* from DNaseI digestion were determined. Figure 5A shows that *M.SsoII* protects a specific region of 48 and 52 bp in top and bottom strands, respectively. The protected region includes a portion of the putative –10 region and RBS of *ssolIM* gene and both putative –10 and –35 regions of *ssolIR* gene (Fig. 5B).

A common regulatory mechanism

All m5C-MTases have a common architecture, 10 conserved amino acid blocks, referred to as motifs I–X (17) are typically found within the polypeptide. Motif I forms part of the binding site for AdoMet; block IV contains a conserved Cys which plays a key role in the methylation reaction; the variable region between motifs VIII and IX is responsible for the recognition of the target DNA sequence. Some m5C-MTases have an extended N-terminal portion preceding the conserved motif I. Som and Friedman (6) showed that the N-terminal portion of *M.EcoRII* binds to the promoter of the *ecoRIIM* gene in a region containing an inverted repeat.

In an earlier study, we analyzed the N-terminal sequence of two related m5C-MTases, *M.SsoII* and *M.ScrFI* which recognize CCNGG and found the presence of a typical prokaryotic DNA binding structure known as a helix–turn–helix (HTH) motif (11). Figure 6A shows that similar HTH motifs are predicted, the majority with high probability, in the N-terminal portions of eight out of the 10 m5C-MTases which possess an extended N-terminal sequence. It appears that these m5C-MTases can be divided into two groups, one recognizing the pentanucleotide DNA sequences, CC(N/W)GG (where W = A or T and N = A, C, G or T), except for the *M.MspI* which recognizes CCGG, the other recognizes the complement, GG(N/W)CC except for the ambiguous base in the middle of the recognition sequence. In all these systems, an inverted repeat sequence of variable length (Fig. 6B), presumably important for protein binding, can be found in front of the respective methyltransferase sequence. These suggest a common evolution of these R-M systems and a similar way of regulation. The possible mechanism of the regulation is based on the specific interaction of the MTase with its promoter, which provides the repression of the MTase synthesis during functioning of the R-M system in the cell.

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