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SsoII-Like DNA-Methyltransferase Ecl18kI: Interaction between Regulatory and Methylating Functions

E. A. Fedotova[1], A. S. Protsenko[2,3], M. V. Zakharova[3], N. V. Lavrova[4], A. V. Alekseevsky[5], T. S. Oretskaya[1], A. S. Karyagina[4], A. S. Solonin[3], and E. A. Kubareva[5]*

[1]Chemical Faculty, Lomonosov Moscow State University, 119991 Moscow, Russia
[2]Pushchino State University, pr. Nauki 3, 142290 Pushchino, Moscow Region, Russia
[3]Skryabin Institute of Biochemistry and Physiology of Microorganisms, pr. Nauki 5, 142290 Pushchino, Moscow Region, Russia
[4]Gamaleya Research Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences, ul. Gamalei 18, 123098 Moscow, Russia
[5]Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University, 119991 Moscow, Russia; fax: (495) 939-3181; E-mail: kubareva@belozersky.msu.ru

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Abstract—The interaction of DNA-methyltransferase Ecl18kI (M.Ecl18kI) with a fragment of promoter region of restriction–modification system SsoII was studied. It is shown that dissociation constants of M.Ecl18kI and M.SsoII complexes with DNA ligand carrying a regulatory site previously characterized for M.SsoII have comparable values. A deletion derivative of M.Ecl18kI, Δ(72-379)Ecl18kI, representing the N-terminal protein region responsible for regulation, was obtained. It is shown that such polypeptide fragment has virtually no interaction with the regulatory site. Therefore, the existence of a region responsible for methylation is necessary for maintaining M.Ecl18kI regulatory function. The properties of methyltransferase NlaX, which is actually a natural deletion derivative of M.Ecl18kI and M.SsoII lacking the first 70 amino acid residues and not being able to regulate gene expression of the SsoII restriction–modification system, were studied. The ability of mutant forms of M.Ecl18kI incorporating single substitutions in regions responsible for regulation and methylation to interact with both sites of DNA recognition was characterized. The data show a correlation between DNA-binding activity of two M.Ecl18kI regions—regulatory and methylating.

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Restriction–modification (R–M) systems are now attracting greater attention of researchers in the context of characteristics of gene expression regulation, which plays an extremely important role during the functioning of these systems in bacteria cells. This is because restriction endonucleases (RE) are proteins potentially dangerous for the host cell expressing them. Violation of a specific methylation of host cell DNA can lead to DNA fragmentation by restriction endonucleases and subsequent cell death. Because many R–M systems are encoded by independently replicating elements (plasmids and viruses), we can assume that when they are transferred from one organism to another the regulation of methyltransferase (MTase) and RE gene expression should be accomplished by the system itself.

However, although regulation of R–M system gene expression obviously exists, not much is known today about mechanisms of the process [1]. This determines the relevance of detailed study of the process of gene expression regulation in R–M systems. The only common thing between characterized R–M systems is "tight linkage" of MTases and RE genes that can be located in two variants with respect to each other (endonuclease gene → methyltransferase gene, methyltransferase gene → endonuclease gene) and in different orientations: tandem, convergent, and divergent. The organization variety of R–M system

Abbreviations: a. a., amino acid residue; AdoHcy, S-adenosyl-L-homocysteine; AdoMet, S-adenosyl-L-methionine; HTH, "helix–turn–helix"; MTase, DNA-methyltransferase; N is A, G, T, or C; RE, restriction endonuclease; R–M, restriction–modification. Prefix "d" (deoxy) in oligodeoxyribonucleotide and DNA duplex names is skipped.

* To whom correspondence should be addressed.

genes points to the possibility of existence of different ways of regulation of expression in different systems.

For a number of R–M systems (PvuII, EcoRV, BamHI) it was shown that protein product of gene *C* (control), in many cases situated in the same direction with a restriction endonuclease gene, stimulates expression of the latter [2]. Namely, the expression of a restriction endonuclease gene is observed in cases when there is enough C-protein produced in the cell. In EcoRII and MspI restriction–modification systems, methyltransferase works not only as a modification enzyme, but also as a transcription repressor that is able to bind with the promotor region of its own R–M system and regulate the expression of its gene [3, 4].

Earlier we studied the SsoII R–M system that was found in *Shigella sonnei* strain 47. Complementary regulation of expression of RE and MTase genes that are transcribed divergently is a distinctive feature of this system. DNA-methyltransferase (M.SsoII) not only inhibits its own synthesis, but also activates synthesis of SsoII restriction endonuclease [5]. Regulation is due to the interaction of M.SsoII N-terminal region (amino acid residues 1-71) with the promoter gene region of the SsoII R–M system [5, 6]. We have described part of the promoter gene region of the SsoII R–M system that methyltransferase interacts with [6, 7]. This double stranded region (further we will call it regulatory) is a 15-mer inverse repeat 5′-AGGACA*A*ATTGTCCT-3′/3′-TCCTGTT-*T*AACAGGA-5′. Amino acid residues 72-379 of M.SsoII control another function of this protein — DNA methylation. As a modification enzyme, M.SsoII recognizes in double stranded DNA the pentanucleotide sequence 5′-C**C**NGG-3′, totally degenerate by the central pair, and methylates the inner C residue of this sequence with formation of 5-methyl-2′-deoxycytidine [8].

Genes of the SsoII R–M system are encoded by a natural P4 plasmid 4250 nucleotides long. This plasmid is present in *Shigella sonnei* strain 47 as a part of a complicated plasmid complex of nine or more plasmids, including transmissible ones that are able to co-transfer P4 plasmid during conjugation. This feature is probably the reason for the high occurrence of the SsoII R–M system in nature. This system is a champion in the number of independent studies connected with determining nucleotide sequence of the DNA fragment encoding it. Data published by independent research groups on the ExPASy server (http://cn.expasy.org/) reveal characteristics of genetic organization of eight SsoII-like systems having 1-2 differences in nucleotides within the coding regions of the RE and MTase genes and intergenic region. Ecl18kI is one of these R–M systems [9].

A restriction–modification system of Ecl18kI type II was observed in an *Enterobacter cloacae* strain. The intergenic regions of Ecl18kI and SsoII R–M systems are identical. The nucleotide sequences of genes encoding restriction and modification enzymes of Ecl18kI and SsoII systems are practically identical. As a consequence, methyltransferase and restriction endonuclease Ecl18kI (M.Ecl18kI, R.Ecl18kI) differ from M.SsoII and R.SsoII only by one amino acid residue (a. a.). At position 56, M.Ecl18kI has Met and M.SsoII has Ile. The Val232 residue in R.Ecl18kI corresponds to Ile in R.SsoII. The mentioned differences do not influence the ability of M.Ecl18kI or R.Ecl18kI to methylate or hydrolyze substrate, correspondingly [10]. In the case of M.Ecl18kI, the "mutant" amino acid residue is in the N-terminal fragment of the enzyme.

The ability of M.Ecl18kI to bind with a regulatory site characterized for M.SsoII is shown in this work. Using deletion derivative of M.Ecl18kI, Δ(72-379)Ecl18kI, representing the N-terminal fragment of MTase (1-71 a. a.), it was found that the Ecl18kI region responsible for DNA methylation (72-379 a. a.) is necessary for the enzyme to exert its regulatory function. Properties of methyltransferase NlaX, which is actually a natural deletion derivative of M.Ecl18kI and M.SsoII lacking the first 70 a. a. and not able to regulate gene expression of SsoII restriction–modification system, were studied [5]. Mutant forms of M.Ecl18kI having single amino acid substitutions in regions responsible for regulation and methylation were obtained. Their ability to interact with both DNA recognition sites was characterized.

MATERIALS AND METHODS

Reagents. S-Adenosyl-*L*-homocysteine (AdoHcy) and S-adenosyl-*L*-methionine (AdoMet) were from Sigma (USA); poly(dI·dC) was from GE Healthcare (USA); protein molecular weight markers 10-200 kD were from MBI-Fermentas (Lithuania); EDTA and Tris from Amresco (USA); magnesium chloride, β-mercaptoethanol, ammonium persulfate, lithium perchlorate, formamide, and dithiothreitol (DTT) from Fluka (Switzerland); N,N,N′,N′-tetramethylethylenediamine (TEMED), acrylamide, N,N′-methylene-bis-acrylamide, and glycerol from Serva (Germany); dye markers bromophenol blue (BPB) and xylene cyanole (XC) from Reanal (Hungary); [γ-^{32}P]ATP with specific radioactivity 1000 Ci/mol from the Institute of Molecular Biology (Moscow, Russia). Oligodeoxyribonucleotides were kindly provided by E. A. Romanova (Belozersky Institute of Physico-Chemical Biology, Lomonosov Moscow State University). The concentration of nucleotide material was measured with a spectrophotometer.

Proteins. A plasmid carrying the gene of Δ(72-379)Ecl18kI protein was obtained by deletion of EcoRV-HindIII fragment of pQE30(M.Ecl18kI-6His) plasmid containing a nucleotide sequence encoding amino acid residues 72-379 of MTase Ecl18kI. An M.SsoII mutant form in which Cys142 was changed for alanine was kindly provided by T. V. Tikhonova (All-

Russia Research Institute of Agricultural Biotechnology, Russian Academy of Agricultural Sciences). Plasmids containing genes M.Ecl18kI(R15A), M.Ecl18kI(R35A), M.Ecl18kI(R38A), M.Ecl18kI(R39A), and M.Ecl18kI(R42A) were constructed with using site-directed mutagenesis [11]. Plasmid pQE30(M.Ecl18kI-6His) and the following primer pairs were used: R15f (5′-AAAGAGAAG-CACTTCATATGACTC-3′), R15r (5′-GAGTCATAT-GAAGTGCTTCTCTTT-3′); R35f (5′-ATATGGA-GACGCAACCATAAGAAG-3′), R35r (5′-CTTCT-TATGGTTGCGTCTCCATAT-3′); R38f (5′-GACA-GAACCATAGCAAGATGGGAG-3′), R38r (5′-CTCC-CATCTTGCTATGGTTCTGTC-3′); R39f (5′-GAAC-CATAAGAGCATGGGAGCGCG-3′), R39r (5′-CGCGCTCCCATGCTCTTATGGTTC-3′); R42f (5′-GAAGATGGGAGGCCGGGGAAAC-3′), R42r (5′-GTTTCCCCGGCCTCCCATCTTC-3′).

Recombinant proteins M.Ecl18kI and M.NlaX and their mutant forms containing six histidine residues at the N-terminus of the protein molecules were isolated from *E. coli* cell culture by affinity chromatography on Ni-NTA-agarose. Monomeric protein concentrations in preparations were 5 μM (M.Ecl18kI), 55 μM (Δ(72-379)Ecl18kI), 3 μM (M.Ecl18kI(R15A)), 28 μM (M.Ecl18kI(R35A)), 69 μM (M.Ecl18kI(R38A)), 63 μM (M.Ecl18kI(R39A)), 8 μM (M.Ecl18kI(R42A)), 126.5 μM (M.SsoII(C142A)), and 5 μM (M.NlaX).

Oligonucleotides were ^{32}P-labeled using T4 polynucleotide kinase (10 activity units (a. u.); MBI-Fermentas) and [γ-^{32}P]ATP in 10 μl 50 mM Tris-HCl buffer (pH 7.6) containing 10 mM $MgCl_2$, 5 mM DTT, 0.1 mM spermidine, and 0.1 mM EDTA for 30 min at 37°C. The reaction was stopped by adding 2 μl 250 mM aqueous EDTA solution and heating at 65°C for 10 min. [γ-^{32}P]ATP excess was removed on a G-25 micro-column (GE Healthcare). Radioactivity of ^{32}P-labeled preparations was determined by the Cherenkov method as counts per minute on a Tracor Analytic Delta 300 counter (ThermoQuest/CE Instruments, USA).

Solutions of DNA duplexes I and II were prepared by mixing equimolar amounts of corresponding oligonucleotides. 5′-^{32}P-labeled duplexes with known specific radioactivity were obtained by adding corresponding ^{32}P-labeled oligonucleotide to a certain amount of DNA duplex. The mixture was heated to 90°C and slowly cooled to room temperature for annealing.

M.Ecl18kI, M.NlaX, Δ(72-379)Ecl18kI, M.Ecl18kI(R15A), M.Ecl18kI(R35A), M.Ecl18kI(R38A), M.Ecl18kI(R39A), M.Ecl18kI(R42A), M.SsoII, and M. SsoII(C142A) complex formation with DNA duplexes containing ^{32}P-label in one of the strands was performed in 20 μl 50 mM Tris-HCl buffer (pH 7.6) containing 150 mM NaCl, 5 mM DTT, and 50 ng/μl poly(dI·dC) for 20 min at 37°C. Protein–nucleic acid complex and free DNA duplex was separated by gel electrophoresis in flat 200 × 180 × 1.5 mm polyacrylamide gel containing 6.8% acrylamide and 0.2% N,N′-bis-acrylamide in TBE-buffer (50 mM Tris-HCl, pH 8.3, 50 mM H_3BO_3, 1 mM EDTA) at field intensity 8.5 V/cm. Preparations were loaded on gels in 20 μl of reaction mixture containing 10-20% glycerol. Visualization of radioactive bands in gels and calculation was done using a Molecular Dynamics PhosphorImager SI (Molecular Dynamics, USA) and computer program Image Quantum, version 5.0.

The extent of MTase binding to DNA duplex was counted as a ratio of radioactivity of a band corresponding to DNA–protein complex to the sum of radioactivity of bands corresponding to DNA–protein complex and to non-bound DNA. Constants of dissociation (K_d) of complexes M.SsoII, M.NlaX, Δ(72-379)Ecl18kI, and M.Ecl18kI and its mutant forms with single amino acid substitutions with DNA duplexes I and II was determined by Scatchard analysis [12]. The concentrations of DNA duplexes I and II were varied in the interval from 20 to 100 nM. Protein concentrations are presented in Table 1. Protein active concentration was determined using experimental data of its binding with duplex II by Scatchard analysis [12].

Methylation of DNA substrate II by M.Ecl18kI, M.NlaX, M.Ecl18kI(R15A), M.Ecl18kI(R35A), M.Ecl18kI(R38A), M.Ecl18kI(R39A), M.Ecl18kI(R42A), M.SsoII, and M.SsoII(C142A). The extent of DNA duplex methylation by MTases was evaluated by the extent of substrate "defense" from hydrolysis by restriction endonuclease Ecl18kI (R.Ecl18kI).

The same starting protein active concentrations of 14 nM were used in the experiments. The active concentrations of MTases were determined from graphs of dependence of DNA ligand part bound with protein on DNA–protein complex concentration (Scatchard coordinates) as the intersection on the abscissa axis [12].

^{32}P-labeled DNA duplex (concentration 350 nM) was incubated with methyltransferase in 50 mM Tris-HCl

Table 1. Protein concentrations used for calculating K_d of DNA–protein complexes

Protein	Protein concentration, nM	
	duplex I	duplex II
M.Ecl18kI	150	200
Δ(72-379)Ecl18kI	2000	–
M.NlaX	–	800
M.Ecl18kI(R15A)	800	300
M.Ecl18kI(R35A)	1600	400
M.Ecl18kI(R38A)	1600	1200
M.Ecl18kI(R39A)	900	600
M.Ecl18kI(R42A)	400	640
M.SsoII	200	400
M.SsoII(C142A)	300	400

buffer (pH 7.6) containing 150 mM NaCl, 5 mM DTT, and 1 mM AdoMet for 0.5-60 min at 37°C. Then the reaction mixture was held for 10 min at 65°C to inactivate the enzyme, and after cooling to 25°C, 10 mM $MgCl_2$ and 240 nM Ecl18kI restriction endonuclease (final concentrations in reaction mixture are given) were added and the mixture incubated for 1 h at 37°C. Hydrolysis products were analyzed in flat 200 × 200 × 1.0 mm polyacrylamide gels containing 19% acrylamide, 1% N,N′-methylene-bis-acrylamide, and 7 M urea. The electrode buffer was TBE and the field intensity was 50 V/cm. The preparations were loaded onto the gel in 10-15 μl of solution for oligonucleotide analysis: 0.025% bromophenol blue and xylene cyanole solution in formamide–water mixture (80 : 20). DNA bands in gel were determined with a Molecular Dynamics PhosphorImager SI instrument. The extent of duplex II hydrolysis after incubation with MTase and R.Ecl18kI was defined as a ratio of radioactivity band corresponding to hydrolysis product to the sum of radioactivity of bands corresponding to hydrolysis product and non-cleaved DNA. The extent of non-methylated duplex II hydrolysis by R.Ecl18kI was taken as 100%. The extent of duplex II methylation by the studied proteins was calculated based on this value, and kinetic curves were plotted. Initial methylation rate (v_0) of DNA duplex II by MTases was calculated from the slope of the initial rectilinear part of the kinetic curve.

RESULTS AND DISCUSSION

Interaction between M.Ecl18kI and DNA duplex, containing the M.SsoII regulatory recognition site. M.Ecl18kI binding with 31-mer synthetic ligand I containing the regulatory site identified for M.SsoII (bolded) was investigated [7]: 5′-ATCAAAAC**AGGACAA*A*TTGTCCT**AAAACCAA-3′/3′-TAGTTTTG**TCCTGTT*T*AACAGGA**TTTTGGTT-5′ (duplex I).

Complex formation of M.Ecl18kI and M.SsoII with duplex I was studied under conditions of specific binding in the presence of poly(dI·dC). DNA–protein complexes were traced by the polyacrylamide gel retardation method. For complexes of M.SsoII and M.Ecl18kI with ligand I, K_d were determined by the Scatchard method [12]. In this method, a fixed amount of protein is titrated with increasing amount of DNA duplex. The dissociation constant of M.Ecl18kI with duplex I complex was found to be 224 ± 24 nM, this being within the error range with K_d of complex M.SsoII with this duplex (248 ± 33 nM) (Table 2).

Characteristics of M.Ecl18kI deletion derivative—Δ(72-379)Ecl18kI. The M.Ecl18kI regulatory function as well as that of M.SsoII is probably defined by its N-terminal part (1-71 a. a.). The presence of "helix–turn–helix" (HTH) structural motif, homological to HTH-motif of the HTH_3 protein family, is predicted in it [13]. We comparatively analyzed amino acid sequences of SsoII-like proteins (including M.Ecl18kI) and C-proteins available in REBASE (http://rebase.neb.com/rebase/rebase. html). Figure 1 shows that the SsoII-like MTases and C-proteins have a high level of amino acid sequence similarity in the HTH-motif region. There are about 80 a. a. in C-proteins. Structural and functional similarity of MTase N-terminal regions and C-proteins indicates that SsoII-like prokaryotic methyltransferases with N-terminal regulatory region could have emerged during confluence of a C-protein homolog with methyltransferase, consisting of single domain responsible for methylation. The presence of N-terminal regulatory region brings SsoII-like MTases closer with eukaryotic MTases, for which multi-domain organization is typical, including methylating and regulatory domains [14].

Table 2. Interaction of M.SsoII, M.NlaX, M.Ecl18kI, and their mutant forms with duplexes I and II containing regulatory and methylation sites, respectively

Protein	K_d of DNA–protein complexes, nM		Relative initial methylating rate of duplex II
	duplex I with regulatory site	duplex II with methylation site	
M.Ecl18kI	224 ± 24	87 ± 12	1
M.SsoII	248 ± 33	144 ± 14	1
M.NlaX	–	162 ± 18	0.3
M.SsoII(C142A)	35 ± 3	172 ± 10	no
M.Ecl18kI(R15A)	56 ± 13	103 ± 24	0.4
M.Ecl18kI(R35A)	>4000	140 ± 12	1
M.Ecl18kI(R38A)	>4000	96 ± 13	7
M.Ecl18kI(R39A)	93 ± 14	266 ± 4	14
M.Ecl18kI(R42A)	32 ± 2	256 ± 4	0.3

We obtained deletion mutant of M.Ecl18kI, Δ(72-379)Ecl18kI, in which the part of the protein responsible for DNA methylation was removed. No binding of Δ(72-379)Ecl18kI protein with 31-mer DNA duplex I containing the regulatory site was found (Fig. 2). Still, it is known that C-proteins and transcription factors from the HTH_3 family effectively interact with a recognition site in DNA. For example, K_d of C.AhdI complex with a 35-mer DNA fragment is 5-6 nM [15]. So, the M.Ecl18kI region responsible for methylation is needed for effective binding of the N-terminal fragment with the regulatory site.

Interaction of M.NlaX, a natural analog of Δ(1-70)Ecl18kI deletion mutant, with a methylation region in DNA. A construction containing the Ecl18kI methyltransferase gene with deletion in the region of 1-71 a. a., encoding the protein regulatory domain, was created. However, we did not obtain sufficient amount of Δ(1-71)Ecl18kI homogenous preparation for molecular bio-

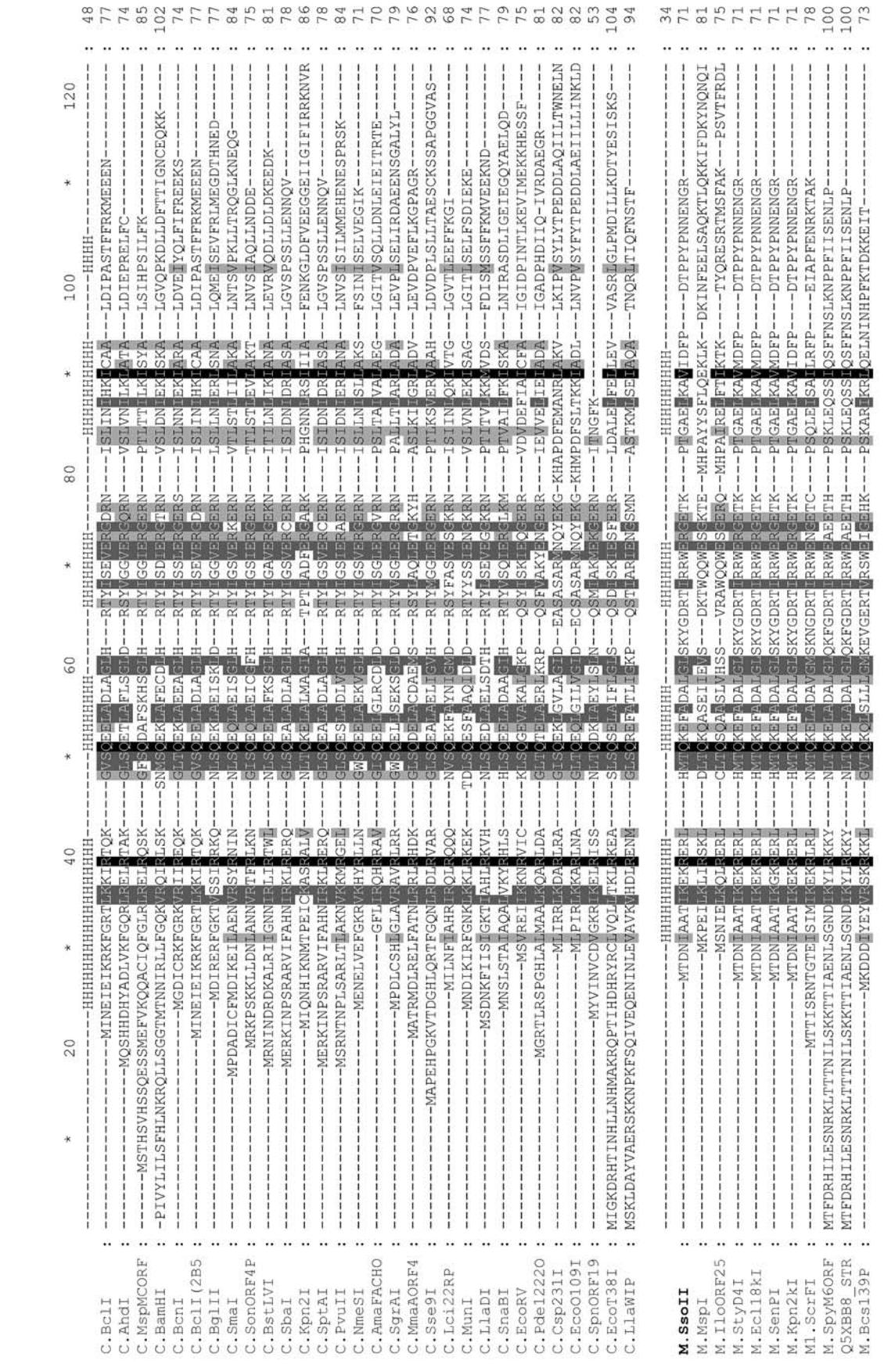


Fig. 1. Multiple alignment of amino acid sequences of C-proteins and N-terminal regions of SsoII-like methyltransferases. Letter "H" above alignments indicates amino acids possibly located in α-helixes. Amino acid residues whose conservation is above 50% are marked with background shading.

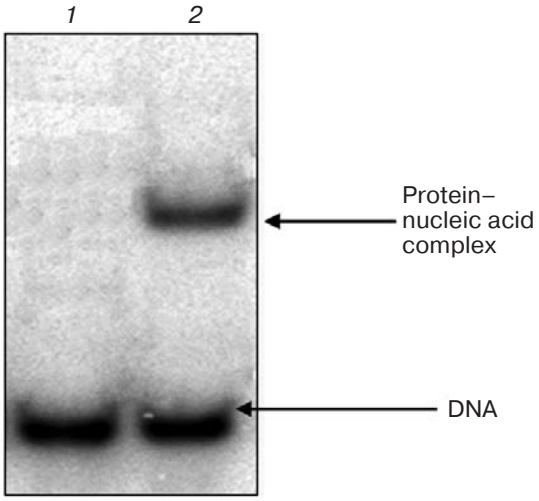


Fig. 2. Analysis of equilibrium binding of 5′-^{32}P-labeled DNA duplex I (concentration 20 nM) with Δ(72-379)Ecl18kI (2000 nM; lane *1*) and M.Ecl18kI (150 nM; lane *2*) by the gel retardation method.

logical studies. Previously the same result was achieved when trying to obtain the M.SsoII deletion derivative lacking the N-terminal regulatory domain [16]. Thus, for further work we used DNA methyltransferase NlaX from a *Neisseria lactamica* strain as SsoII-like DNA methyltransferase lacking the N-terminal region. It methylates 5′-CCNGG-3′ sequence at C5 location of an internal cytidine residue, the same as M.Ecl18kI, and has 65.5% identical and 7.7% similar amino acid residues in the region responsible for methylation with SsoII-like proteins [16, 17]. A 30-mer synthetic substrate II was used to characterize the affinity of M.Ecl18kI and M.NlaX to the methylation site (methylation region is bolded): 5′-GATGCTGCCAA**CCTGG**CTCTAGCTTCATAC-3′/3′-CTACGACGGTT**GGACC**GAGATCGAAGTATG-5′ (duplex II).

The complex formation of M.Ecl18kI and M.NlaX with DNA ligand II was studied in the presence of poly(dI·dC) and AdoHcy. Only specific binding of MTases with DNA substrate is observed under these conditions [18]. Complex formation was traced by the polyacrylamide gel retardation method under non-denaturing conditions. The K_d values of M.Ecl18kI and M.NlaX complexes with duplex II determined by the Scatchard method were 87 ± 12 and 162 ± 18 nM, respectively (Table 2). It is obvious that M.NlaX affinity to DNA ligand containing the methylation site is about twice lower than that of M.Ecl18kI. Taking this into account, we conclude that the presence of the first 70 a. a. in M.SsoII-like proteins may contribute to more effective enzyme binding to the methylation site.

The effectiveness of substrate II methylation by SsoII and NlaX enzymes was defined by the method of "reverse restriction". First, DNA duplex II was incubated with one of the MTases in the presence of AdoMet. Then $MgCl_2$ and restriction endonuclease Ecl18kI were added to the reaction mixture. R.Ecl18kI recognizes in DNA the same two-stranded sequence 5′-↓CCNGG-3′ as studied MTases and splits DNA in presence of magnesium ions in the place shown by the arrow. R.Ecl18kI is not able to hydrolyze the recognition site if the internal C residue is methylated [10]. In a control experiment, duplex II was not modified by MTases but was incubated with R.Ecl18kI.

M.Ecl18kI methylates substrate II about three times more effectively than M.NlaX (Table 2). This is probably connected with higher affinity of M.Ecl18kI to the methylation site. That means that the absence of the N-terminal region lowers M.NlaX functional activity but does not lead to its absence, as it was in the case with the deletion mutant Δ(72-379)Ecl18kI.

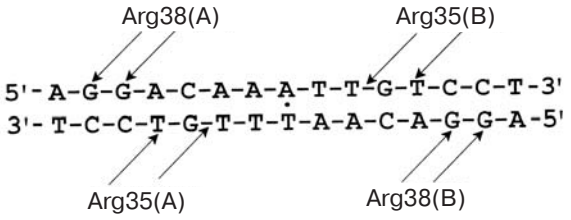
Therefore, the interaction between two DNA binding centers of M.SsoII-like proteins was demonstrated. The presence of the M.Ecl18kI region responsible for methylation is absolutely required for protein binding with regulatory site. The absence of the first 70 a. a. preceding the first conservative motif of (cytosine-5)-DNA methyltransferases leads to lowering protein affinity to the methylation site, and, as a consequence, to the reduction of effectiveness of catalytic transfer of a methyl group from cofactor to DNA.

Interaction of M.Ecl18kI and its mutant forms with recognition sites in DNA. According to computer modeling data [13], arginine residues 35, 38, 39, and 42 in M.SsoII are close to the regulatory sequence in DNA. We obtained Ecl18kI mutant forms where we have changed one of these residues to Ala. M.Ecl18kI(R15A) was used for control. The Cys142 residue is the only cysteine residue in the M.SsoII and M.Ecl18kI molecules. It is a part of conservative for all (cytosine-5)-DNA methyltransferases tripeptide that plays a key role in catalysis of transferring a methyl group from reaction cofactor AdoMet to DNA substrate.

Mutant forms of M.Ecl18kI containing a single amino acid residue substitution in the N-terminal region or in the catalytic center are presented in Table 2. The K_d values were defined for complexes of these proteins with 31-mer DNA duplex I and 30-mer substrate II.

Replacement of Cys142 by Ala in the M.SsoII molecule causes the protein to lose its enzymatic activity (Table 2). However, the mutant protein M.SsoII(C142A) retains its ability to bind with the methylation site, though about twofold less effectively compared to the initial M.Ecl18kI. "Turning off" of M.SsoII catalytic activity leads to substantial increase in its affinity towards the regulatory sequence (Table 2).

M.Ecl18kI(R35A) and M.Ecl18kI(R38A) practically do not interact with DNA ligand I, indicating that R35 and R38 residues play key roles in the binding regulatory



Scheme of presumed contacts of Arg35 and Arg38 of M.SsoII A and B subunits with DNA regulatory site (the symmetry center is marked with a dot)

site. According to computer modeling data done for M.SsoII [13], these amino acid residues are located in a second "recognizing" helix of the "helix–turn–helix" structural module. They can be involved in creating contacts with two G residues of trinucleotide 5′-GGA-3′ in one strand of a duplex (Arg38) and thymidine with the sugar-phosphate backbone of 5′-TGT-3′ trinucleotide in the opposite strand (Arg35) (see Scheme). M.Ecl18kI mutant forms where Arg15, Arg39, or Arg42 is replaced by alanine create steady complexes with DNA duplex I. Their affinity to ligand with the regulatory site increases 2-4-fold compared to the initial enzyme. Obviously, Arg39 and Arg42 do not make crucial contacts with the regulatory site, as was apparent from the results of molecular modeling [13]. According to the model of the M.SsoII N-terminal region with the regulatory site, Arg15 does not interact with DNA, which is confirmed by our experimental data.

Amino acid substitutions in the M.Ecl18kI N-terminal region have practically no influence on complex formation of the mutant protein with substrate II containing the methylation sequence. The only exception is M.Ecl18kI(R39A); its affinity to the methylation region is three times less compared to the unmodified protein. However, M.Ecl18kI(R39A) very effectively methylates DNA substrate II. High level of duplex II methylation compared to M.Ecl18kI is also observed in the case of M.Ecl18kI(R38A) with "turned off" regulatory function. For M.Ecl18kI(R35A), the effectiveness of methylation does not change. Replacement of Arg15 and Arg42 by alanine is accompanied by 2.5-3-fold lower ability of the enzyme to methylate substrate.

Therefore, the interaction between functioning of two DNA-binding centers of SsoII-like proteins is evident. Amino acid substitutions in the regulatory part of the protein influence its ability to methylate substrate. There is also reverse dependence—the absence of enzyme activity in full-length protein leads to its higher affinity to the DNA regulatory site. However, deletion mutant Δ(72-379)Ecl18kI, lacking the whole protein region responsible for methylation, does not bind with the DNA regulatory site.

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REFERENCES

1. Kobayashi, I. (2001) *Nucleic Acids Res.*, **29**, 3742-3756.
2. Tao, T., Bourne, J. C., and Blumenthal, R. M. (1991) *J. Bacteriol.*, **173**, 1367-1375.
3. Som, S., and Freidman, S. (1994) *Nucleic Acids Res.*, **24**, 5347-5353.
4. Som, S., and Friedman, S. (1997) *J. Bacteriol.*, **179**, 964-967.
5. Karyagina, A., Shilov, I., Tashlitskii, V., Khodoun, M., Vasil'ev, S., Lau, P. C., and Nikolskaya, I. (1997) *Nucleic Acids Res.*, **25**, 2114-2120.
6. Shilov, I., Tashlitskii, V., Khodoun, M., Vasil'ev, S., Alekseev, Ya., Kuzubov, A., Kubareva, E., and Karyagina, A. (1998) *Nucleic Acids Res.*, **26**, 2659-2664.
7. Vorob'eva, O. V., Vasil'ev, S. A., Karyagina, A. S., Oretskaya, T. S., and Kubareva, E. A. (2000) *Mol. Biol. (Moscow)*, **34**, 1074-1080.
8. Nikol'skaya, I. I., Kartashova, I. M., and Lopatina, N. G. (1983) *Mol. Genet. (Moscow)*, **12**, 5-10.
9. Denjmuhametov, M. M., Zakharova, M. V., Kravecz, A. N., Pertsev, A. V., Sineva, E. V., Repyk, A. V., Beletskaya, I. V., Gromova, E. S., and Solonin, A. S. (1997) *Mol. Biol. (Moscow)*, **31**, 831-838.
10. Denjmuhametov, M. M., Brevnov, M. G., Zakharova, M. V., Repyk, A. V., Solonin, A. S., Petrauskene, O. V., and Gromova, E. S. (1998) *FEBS Lett.*, **433**, 233-236.
11. Kunkel, T. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 488-492.
12. Varfalomeev, S. D., and Gurevich, K. G. (1998) *Biokinetics* [in Russian], Fair Press, Moscow.
13. Karyagina, A. S., Alexeevski, A. V., Golovin, A. V., Spirin, S. A., Vorob'eva, O. V., and Kubareva, E. A. (2003) *Biophysics (Moscow)*, **48**, Suppl. 1, 45-55.
14. Fatemi, M., Hermann, A., Pradhan, S., and Jeltsch, A. (2001) *J. Mol. Biol.*, **309**, 1189-1199.
15. Streeter, S. D., Papapanagiotou, I., McGeehan, J. E., and Kneale, G. G. (2004) *Nucleic Acids Res.*, **32**, 6445-6453.
16. Karyagina, A. S., Lunin, V. G., Levtechenko, I. Ya., Labbe, D., Brousseau, R., Lau, P. C., and Nikolskaya, I. I. (1995) *Gene*, **157**, 93-96.
17. Kubareva, E. A., Walter, J., Karyagina, A. S., Vorob'eva, O. V., Lau, P. C. K., and Trautner, T. (2002) *BioTechniques*, **33**, 526-531.
18. Vorob'eva, O. V. (2004) *(Cytosine-5)-DNA Methyltransferase SsoII as Bifunctional Protein: Studying Interaction with Methylation Site and with Promoter Gene Region of SsoII Restriction-Modification System*: Candidate's dissertation [in Russian], Moscow State University, Moscow.