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*Alw*26I, *Eco*31I and *Esp*3I – type IIs methyltransferases modifying cytosine and adenine in complementary strands of the target DNA

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ABSTRACT

The specificity of three DNA methyltransferases M.*Alw*26I, M.*Eco*31I and M.*Esp*3I, isolated from *Acinetobacter lwoffi* RFL26, *Escherichia coli* RFL31 and *Hafnia alvei* RFL3+, respectively, was determined. All the enzymes methylate both strands of asymmetric recognition sites yielding m^5C in the top-strand and m^6A in the bottom-strand, as below:

5′-GTm^5CTC	5′-GGTm^5CTC	5′-CGTm^5CTC
3′-Cm^6AGAG	3′-CCm^6AGAG	3′-GCm^6AGAG
(M.*Alw*26I)	(M.*Eco*31I)	(M.*Esp*3I)

They are the first members of type IIs methyltransferases that modify different types of nucleotides in the recognition sequence.

INTRODUCTION

Many sequence-specific methyltransferases, the constituent part of type II restriction-modification (R-M) systems, are able to modify bases of the same nature (either C or A) symmetrically positioned in the complementary strands of the palindromic recognition sequence (2). Much more complicated and poorly investigated are the enzymes which recognize asymmetric sequences. Because of the ability of restriction endonuclease to cut DNA outside the recognition site, these enzymes were classified as a sub-class of R-M enzymes, named type IIs (3).

In contrast to type II modification enzymes, for the double-strand methylation of the target DNA by some type IIs methyltransferases, two different sequences should be recognized. There have been only a few reports about the specificity of type IIs modification methylases (4–8). In the case of M.*Fok*I, it has been demonstrated that the same enzyme methylates adenine residues in both strands of the target DNA (7). However, different protein segments of M.*Fok*I are involved in the base modification of complementary strands (9). Another type IIs R-M system—*Hga*I—consist of two separate cytosine-methyltransferases which are responsible for methylation of different DNA strands (10). The third type of site-specific methylation was determined for M.*Mbo*II (4,11), which, like type III R-M enzymes (12,13), modifies only one strand of the recognition site. One-strand methylation was postulated also for M.*Ngo*BI (8).

Three new type IIs R-M systems—*Alw*26I, *Eco*31I and *Esp*3I—were discovered in *Acinetobacter lwoffi* RFL26, *Escherichia coli* RFL31 and *Hafnia alvei* RFL3, respectively (14,15,16). The restriction endonucleases and cognate methyltransferases were isolated and their substrate specificity was determined. The *Alw*26I enzymes recognize a 5-base pair sequence 5′-GTCTC/3′-CAGAG. The other two recognize hexanucleotides: *Eco*31I–5′-GGTCTC/3′-CCAGAG and *Esp*3I–5′-CGTCTC/3′-GCAGAG. The *Eco*31I and *Esp*3I recognition sequences include a common pentanucleotide 5′-GT-CTC which is also an *Alw*26I recognition site. All the restriction endonucleases cut DNA in the same manner, i.e. one nucleotide away from the recognition sequence in the 5′→3′ direction and 5 bases away in the 3′→5′ direction, in the opposite strand.

In this paper, we report the substrate specificity of methyltransferases *Alw*26I, *Eco*31I and *Esp*3I. All of them methylate both strands of their recognition sequences and show different strand-specificity, i.e. a cytosine residue is methylated in the top-strand and an adenine residue in the bottom-strand.

MATERIALS AND METHODS

Bacterial strains and substrates

The strains *Acinetobacter lwoffi* RFL26, *Escherichia coli* RFL31 and *Hafnia alvei* RFL3 were originally isolated in our laboratory from natural sources. Plasmid pBR322 DNA was a commercial product of Fermentas MBI.

Enzymes and chemicals

Methylases *Alw*26I, *Eco*31I and *Esp*3I were isolated using described procedures (17). Restriction endonucleases *Alw*26I, *Eco*31I, *Esp*3I, *Gsu*I, DNA polymerase I large fragment (Klenow), T4 polynucleotide kinase were commercial products of Fermentas MBI. Snake venom phosphodiesterase (VPDE) was purchased from Boehringer Mannheim, whereas nuclease P1 was

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+The bacterial strain was primarily identified as *Erwinia species* whereas R-M enzymes discovered were designated *Esp*3I. Closer examination of the taxonomy of microorganism caused its reidentification as *Hafnia alvei*. The name of R-M enzymes, however, following the practice used by Dr. R.Roberts in regularly prepared compilations of the restriction enzymes (1), was not changed.

obtained from Pharmacia. S-adenosyl-L-methionine (Ado-Met) was obtained from Serva, [^{3}H]Ado-Met (15Ci/mmol) was from Amersham. [γ-^{32}P]dATP were purchased from Izotop (Sankt Peterburg). m^{5}dC, m^{4}dC were synthesized in our laboratory (18) and m^{6}dA was purchased from Sigma. All oligodeoxynucleotides were synthesized using phosphoramidite chemistry on Biosearch synthesizer.

All other chemicals were analytical grade commercial products.

DNA methylation

Methylation of DNA or oligodeoxynucleotides was performed in a 0.1 ml of reaction buffer (25 mM Tris-HCl, pH 8.0, 20 mM NaCl, 1 mM DTT, 0.1 mg/ml BSA) containing 0.1 mM Ado-Met or 0.3 nmol [^{3}H]Ado-Met. To the reaction volume, 10–20 units of the corresponding methylase was added and incubated at 37°C for 4–16 hours. The methylase was then inactivated either by heating for 15 min at 70°C or by phenol-chloroform extraction. Digestion of the methylated DNA was carried out by adding 20 units of the corresponding restriction endonuclease and incubating for 1 hour at 37°C.

Determination of the nature of the methylated bases

pBR322 DNA was used as a substrate to determine the bases modified by M.*Eco*31I. 10 mg of DNA was methylated in the presence of [^{3}H]Ado-Met for 4 hours. The methylated bases yielded by M.*Alw*26I and M.*Esp*3I were determined using synthetic oligonucleotide duplexes containing the corresponding recognition sites (underlined):

5′-CCGCGAGACCCACGCT and 5′- CGCGAGACGCC
3′- GCGCTCTGGGT 3′-ATGCGCTCTGCGGT

The synthetic substrate (0.1 A_{260} units) was methylated with the corresponding methylase in the presence of [^{3}H]Ado-Met, for 16 hours. After methylation, DNA or synthetic oligonucleotide duplex was hydrolysed to deoxynucleosides (19). The hydrolysate was analysed by HPLC using m^{5}dC, m^{4}dC and m^{6}dA as standards, as previously described (20).

Determination of the position of the methylated bases

The position of the modified bases within the M.*Alw*26I, M.*Eco*31I and M.*Esp*3I recognition sequences was determined by using the strategy described previously (19, 20). The procedure involves 5′- [^{32}P]-end and [^{3}H]-methyl labeling of synthetic oligonucleotide duplex followed by its examination by two-dimensional mapping (21). The double-stranded substrates (see above) were designed in such a way that two-dimensional maps of the complementary oligonucleotides would not overlap with each other. The oligonucleotides were 5′- [^{32}P]-end labeled with T4 polynucleotide kinase, annealed and methylated with the corresponding methylase in the presence of [^{3}H]Ado-Met for 16 hours. The double-labeled substrates were then partially hydrolysed with snake venom phosphodiesterase (VPDE). The products obtained were separated by two-dimensional mapping by means of electrophoresis and homochromatography (21) and analysed for the presence of [^{3}H]-radioactivity (19).

RESULTS

Site-specificity of *Alw*26I, *Eco*31I and *Esp*3I methylases

Chromosomal DNA isolated from *A. lwoffi* RFL26, *E.coli* RFL31 and *H. alvei* RFL3 is resistant to cleavage by the restriction endonucleases *Alw*26I, *Eco*31I and *Esp*3I, respectively. Furthermore, DNA from *A. lwoffi* RFL26 is resistant to cleavage by any of the three restriction endonucleases (data not shown). This indicates *in vivo* modification of DNA sites recognized by the corresponding restriction endonuclease and shows the relationship between the methylation specificity of all the three methyltransferases.

The recognition sites of *Eco*31I (5′-GGTCTC) and *Esp*3I (5′-CGTCTC) completely overlap the sequence recognized by *Alw*26I (5′-GTCTC). There are three *Alw*26I recognition sites on plasmid pBR322 DNA. One of them is also the substrate for *Eco*31I while another one–for *Esp*3I. As expected, pBR322 DNA methylated with M.*Alw*26I became resistant to cleavage by all the three restriction endonucleases (fig.1A, lanes 4–6). The resistance of the modified DNA to restriction endonuclease cleavage should not be interpreted as an experimental artefact, since in a mixture of modified and unmodified DNAs, the latter was fragmented effectively by *Alw*26I (data not shown). Methylation of the same DNA with M.*Eco*31I or M.*Esp*3I makes it resistant to cleavage by the *Eco*31I (fig.1B, lane 9) or *Esp*3I (fig.1C, lane 14), respectively, and eliminates one (in each case–a different one) *Alw*26I cleavage site from the three existing (fig.1, lanes 10 and 15). M.*Eco*31I methylation does not protect DNA from *Esp*3I cleavage (fig.1B, lane 11). On the other hand, M.*Esp*3I methylated DNA is cleaved by *Eco*31I (fig.1C, lane 16).

From the obtained results, the following conclusions could be made: i) M.*Eco*31I and M.*Esp*3I recognize entire hexanucleotide sequences (5′-GGTCTC and 5′-CGTCTC, respectively), but not parts of them; ii) the possibility that a subset of the sequence 5′-GTCTC is a substrate for M.*Alw*26I can not be excluded.

Determination of the nature of the methylated bases

All the three methylases are able to catalyze the transfer of methyl groups from [^{3}H]Ado-Met onto substrate DNA. A standard procedure, before analyzing methylation products, includes functional purity control of the methylase. Generally, the inability of the restriction fragments generated by the complementary endonuclease to serve as a substrate for the methylase is controlled. Unfortunately, the above procedure is not applicable in the case of *Alw*26I, *Eco*31I and *Esp*3I, because the restriction endonucleases cleave DNA outside their recognition sites, and methylation sites remain intact and can serve as substrates for the methylases. This was confirmed by experiments with

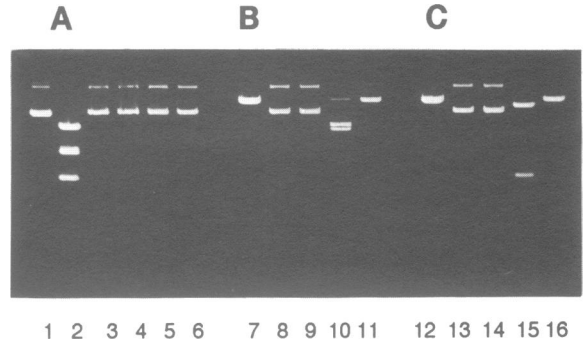

Figure 1. Resistance of pBR322 DNA methylated by M.*Alw*26I (A), M.*Eco*31I (B) and M.*Esp*3I (C) to restriction endonuclease *Alw*26I, *Eco*31I and *Esp*3I cleavage. pBR322 DNA (lane 1) was cleaved with *Alw*26I (lane 2), *Eco*31I (lane 7) and *Esp*3I (lane 12). (A)–DNA methylated with M.*Alw*26I (lane 3) and followed by digestion with *Alw*26I (lane 4), *Eco*31I (lane 5) and *Esp*3I (lane 6). (B)–M.*Eco*31I methylated DNA (lane 8) treated with *Eco*31I (lane 9), *Alw*26I (lane 10) and *Esp*3I (lane 11). (C)–M.*Esp*3I methylated DNA (lane 13) treated with *Esp*3I (lane 14), *Alw*26I (lane 15) and *Eco*31I (lane 16).

M.*Eco*31I. There is a unique *Eco*31I site on pBR322 DNA and it can be destroyed by cleavage with another type IIs restriction endonuclease, *Gsu*I. Thus, pBR322 DNA and the DNA precleaved with the restriction endonuclease *Eco*31I or *Gsu*I were probed as a substrates for M.*Eco*31I. No methylation was observed in pBR322 precleaved with *Gsu*I, whereas intact DNA incorporated [^{3}H]-methyl groups effectively (data not shown). Approximately 30% of the label was incorporated into the *Eco*31I fragments as compared to the intact DNA. The data above also proved the functional purity of M.*Eco*31I.

In the case of M.*Alw*26I and M.*Esp*3I, we used synthetic DNA substrates in order to avoid effects of possible contaminating methylation activities.

pBR322 DNA (in the case of M.*Eco*31I) or synthetic oligonucleotide duplexes containing the recognition site for either M.*Alw*26I or M.*Esp*3I were used for investigation of the nature of the methylated base. Each of the substrate was [^{3}H]-methylated with the corresponding methyltransferase in the presence of [^{3}H]Ado-Met. In control experiments no radioactivity was incorporated into the substrate if the methylase was not presented in the reaction mixture. So only methylase-dependent [^{3}H]-methylation was analysed. The [^{3}H]-methylated substrates were enzymatically hydrolysed to deoxynucleosides and HPLC analysis was performed using a mixture of unlabeled methyldeoxynucleosides as standards. The peaks corresponding to m^{4}dC, m^{5}dC and m^{6}dA were collected and counted for [^{3}H]-radioactivity (table I). The data obtained shows that the tritium radioactivity distributed between two peaks corresponding to m^{5}dC and m^{6}dA standards indicating that all three methyltransferases were able to modify both cytosine and adenine residues. In the case of M.*Alw*26I, approximately 20% of the label was detected as m^{5}dC and the rest as m^{6}dA. The other two—M.*Eco*31I and M.*Esp*3I—methylate both cytosine and adenine residues with equal efficiency (A/C ratio 1:1) (table I).

Table I. Identification of the M.*Alw*26I, M.*Eco*31I and M.*Esp*3I modified bases

Methylase	Substrate	[^{3}H]-radioactivity (cpm) m^{4}dC	m^{5}dC	m^{6}dA
M.*Eco*31I	pBR322 DNA	67	5096	5068
M.*Alw*26I	ds oligonucleotide	75	4270	20600
M.*Esp*3I	ds oligonucleotide	80	3675	3115

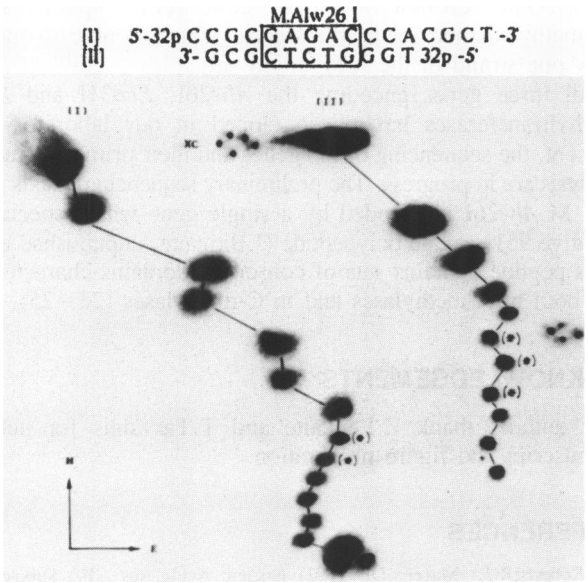

Figure 2. Two-dimensional map of the oligonucleotide duplex [^{3}H]-methylated with M.*Alw*26I. Spots containing [^{3}H]-radioactivity are marked by asterisk. E—Electrophoresis on cellulose acetate strip in pyridine acetate at pH 3.5; H—homochromatography on a DEAE-cellulose thin layer plate in homomixture VI (21); XC—xylene cyanol FF.

Table II. [^{3}H]- and [^{32}P]-label distribution in the partial degradation products of top (I) and bottom (II) oligodeoxynucleotides methylated *in vitro* with M.*Alw*26I

Strand	Product of partial hydrolysis	Radioactivity (cpm) [^{3}H]	[^{32}P]	Ratio [^{3}H]/[^{32}P]
Top (I)	pCCGC	49	97	0.51
	pCCGCG	56	110	0.51
	pCCGCGA	59	89	0.66
	pCCGCGAG	63	87	0.72
	pCCGCGAGA*	351	152	2.31
	pCCGCGAGAC*	150	72	2.08
Bottom (II)	pTGG	148	410	0.36
	pTGGG	95	300	0.32
	pTGGGT	82	230	0.36
	pTGGGTC*	1230	350	3.51
	pTGGGTCT*	1580	420	3.76

Table III. [^{3}H]- and [^{32}P]-label distribution in the partial degradation products of top (I) and bottom (II) oligodeoxynucleotides methylated *in vitro* with M.*Eco*31I

Strand	Product of partial hydrolysis	Radioactivity (cpm) [^{3}H]	[^{32}P]	Ratio [^{3}H]/[^{32}P]
Top (I)	pCCGC	86	75	1.15
	pCCGCG	75	96	0.78
	pCCGCGA	132	103	1.28
	pCCGCGAG	97	82	1.18
	pCCGCGAGA*	1296	192	6.75
	pCCGCGAGAC*	2779	385	7.22
Bottom (II)	pTGG	166	353	0.47
	pTGGG	119	309	0.39
	pTGGGT	129	230	0.56
	pTGGGTC*	739	384	1.92
	pTGGGTCT*	420	315	1.33
	pTGGGTCTC*	793	428	1.85

Table IV. [^{3}H]- and [^{32}P]-label distribution in the partial degradation products of top (I) and bottom (II) oligodeoxynucleotides methylated *in vitro* with M.*Esp*3I

Strand	Product of partial hydrolysis	Radioactivity (cpm) [^{3}H]	[^{32}P]	Ratio [^{3}H]/[^{32}P]
Top (I)	pCGC	608	616	0.99
	pCGCG	607	1604	0.38
	pCGCGA	502	1610	0.31
	pCGCGAG	449	1185	0.38
	pCGCGAGA*	1358	674	2.01
	pCGCGAGAC*	2856	1527	1.87
Bottom (II)	pTGG	155	430	0.36
	pTGGC	131	289	0.45
	pTGGCG	83	232	0.36
	pTGGCGT	87	172	0.51
	pTGGCGTC*	230	242	0.95
	pTGGCGTCT*	295	345	0.86
	pTGGCGTCTC*	932	1066	0.87

Determination of the position of methylated bases in the recognition sequence

Two oligonucleotide duplexes, one of them {1} containing the M.*Alw*26I and M.*Eco*31I recognition sites and the other {2} containing the M.*Alw*26I and M.*Esp*3I recognition sites were synthesized:

{1} 5'-CCGCGAGACCCACGCT top strand
3'- GCGCTCTGGGT bottom strand
{2} 5'- CGCGAGACGCC top strand
3'-ATGCGCTCTGCGGT bottom strand

They were designed in such a way that two-dimensional maps of the complementary oligonucleotides obtained by fingerprinting technique (21) would not overlap with each other (see fig.2). So it was possible to analyse oligonucleotide duplexes without the additional separation of the complementary strands.

The oligonucleotide duplex {1} was used to analyse the positions of modified nucleotides yielded by M.*Alw*26I and M.*Eco*31I and duplex {2} was used in the case of M.*Esp*3I. They were 5'-[^{32}P]-end labeled, [^{3}H]- methylated with appropriate methylase and then subjected to partial 3'-exonuclease digestion. The hydrolysates were analysed by two-dimensional mapping by electrophoresis and homochromatography. Fig.2 represents an example of such analysis in the case of M.*Alw*26I. [^{32}P]-radioactivity containing partial degradation products of top (I) and bottom (II) strands were scrapped off separately and counted for [^{3}H]- and [^{32}P]-radioactivity (table II). [^{3}H]/[^{32}P] ratio was calculated for every of the partial 3'-exonuclease digestion product. The removal of [^{3}H]-methylated nucleotide from the 3'-end of oligonucleotide results in noticeable decrease of the [^{3}H]/[^{32}P] ratio. Evaluation of [^{3}H]/[^{32}P] ratio revealed that the shortest oligonucleotide of the top-strand (I) containing double label was octanucleotide 5'-^{32}pCCGCGAGA (fig.2, table II). The shortest oligonucleotide of the bottom strand (II) which contained [^{3}H]-methyl label was determined to be hexanucleotide 5'-^{32}pTGGGTC. These results prove that M.*Alw*26I methylates the internal cytosine residue in the sequence 5'-GTCTC and the second adenine residue in the sequence 5'-GAGAC:

5'-G T m^{5}C T C-3'
3'-C m^{6}A G A G-5'

The analogues procedure to that of M.*Alw*26I was followed to find out which of nucleotides are modified by M.*Eco*31I and M.*Esp*3I. The results presented in table III (M.*Eco*31I) and table IV (M.*Esp*3I) demonstrate that [^{3}H]-methylated nucleotides are positioned in the same way as in the case of substrate modified by M.*Alw*26I. Thus, the methylation specificity of the M.*Eco*31I and M.*Esp*3I was certified as follows:

5'-GGTm^{5}CTC-3'	5'-CGTm^{5}CTC-3'
3'-CCm^{6}AGAG-5'	3'-GCm^{6}AGAG-5'
(M.*Eco*31I)	(M.*Esp*3I)

DISCUSSION

Type II methyltransferases modify either adenine or cytosine residues in both strands of the recognition sequence. The same is true for type IIs methyltransferases M.*Fok*I (7,9) and M.*Hga*I (10). On the other hand, some type IIs enzymes recognize sequences where one strand contains no adenines and the other strand contains no cytosines (e.g. *Mbo*II−5'-GAAGA (4) or *Ksp*632I−5'-CTCTTC (22). It was postulated then that methylation in such cases should take place only at one strand or at both strands asymmetrically, i.e. yielding methylated adenine and cytosine (2,4). One-strand methylation was determined for type III methylases (12,13). A few members of type IIs enzymes possibly show the same specificity. M.*Mbo*II (5'-GAAGA) is determined as an adenine methylase (4,11), but one strand of its recognition sequence containes no adenines. M.*Ngo*BI (5'-GGTGA) is a cytosine methyltransferase (8) and apparently methylates only one strand, because the other strand contains no cytosines. All attempts to find the other strand-specific methylase in both cases were unsuccessful (4,8,11). In the latter case, however, there is no clear evidence yet how restriction-modification functions *in vivo*. If the complementary strand of the recognition sequence is not modified by methyltransferase, some other mechanisms should be involved to protect chromosomal DNA during replication against the restriction endonuclease cleavage.

Here we report a new phenomenon of methylation of the target DNA by type IIs methyltransferases M.*Alw*26I, M.*Eco*31I and M.*Esp*3I which catalyze a transfer of methyl groups onto different nucleotides (cytosine and adenine) in the complementary strands of the recognition sites. It is not clear yet whether one or two proteins are responsible for the different strand-specific methylation. At present, the amino acid (aa) sequences of many type II DNA methylases are determined and the conserved sequence motifs characteristic for m^{4}C-, m^{5}C- and m^{6}A-methyltransferases are identified (23−25). The enzymatic properties of a methylase can be provided from the conserved domains revealed in the aa sequence. To date, only three type IIs methylases (M.*Fok*I, M.*Hga*I and M.*Mbo*II) have been sequenced and all of them possess different gene and protein organization (9−11). M.*Fok*I is encoded by a single gene, but the aa sequence analysis of the protein shows that it contains separate conserved domains responsible for each strand methylation (7,9). Two separate genes code two *Hga*I cytosine methylases, which methylate different strands of the target DNA (10). The cloned *mboIIM* gene (11) specifies an N^{6}-methyladenine methyltransferase which is able to modify only one strand of the recognition site.

All three genes encoding the *Alw*26I, *Eco*31I and *Esp*3I methyltransferases have been cloned in our laboratory. At present, the sequencing of the genes and their primary structure analysis are in progress. The preliminary sequencing results show that M.*Alw*26I is encoded by a single gene which specifies a putative 951-residue polypeptide (J.Bitinaite, unpublished data). This peptide contains sets of conserved domains characteristic for both m^{6}A-methylases and m^{5}C-methylases (23−25).

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