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Determination of the recognition sites of cytosine DNA-methylases from Escherichia coli SK

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ABSTRACT

Two different cytosine DNA-methylases, M_I and G_{II}, are present in Escherichia coli SK. The G_{II} methylase recognizes the five-member symmetric sequence: 5'...NpCpCpApGpGpN...3'. This sequence is identical with the recognition site of the hsp II type determined by RII plasmide but, in contrast to RII methylase, the G_{II} enzyme methylates cytosine located on the 5' side of the site. By analogy with the isoschizomery of the restricting endonucleases, RII and G_{II} DNA methylaeses may be called isomethymers which recognize the same site but methylate different bases. Since the phage of the SK and hsp II phenotypes is effectively restricted in respective cells it may be assumed that the isomethymeric modification does not provide any protection against the corresponding restrictases. M_I methylase recognizes the five-member symmetric site which represents an inverted sequence of the G_{II} site: 5'...NpGpGpApCpCpN...3'. In this case cytosine at the 3'-end of the recognition site is methylated.

INTRODUCTION

It has been shown previously that fractionation of an extract of E.coli SK cells on carboxymethylcellulose (CM-70) reveals two peaks of the enzymatic activity which methylated cytosine: M_I and G_{II}[1]. Using the test of additional methylation we found that both enzymes were capable of methylating the acceptor DNA simultaneously and without interference, which indirectly indicated different specificity of the enzymes with regard to the methylated sequence[2]. This paper presents the results of the analysis of recognition sites for both cytosine methylases. A preliminary communication reporting some of the results has been published elsewhere[3].

MATERIALS AND METHODS

Bacterial strains - E.coli SK, E.coli (RII) and E.coli C, and bacteriophages S_d and PBV were obtained from the museum of the D.I. Ivanovsky Institute of Virology of the USSR Academy of Medical Sciences.

The enzymes: alkaline phosphomonoesterase, spleen phosphodiesterase and snake venom phosphodiesterase were acquired from Kochlight. Exonuclease A5 from an actinomyces was kindly supplied by Dr. R.I.Tatarskaya of the Institute of Molecular Biology of the USSR Academy of Sciences.

H^3-methyl-S-adenosyl-L-methionine with the specific activity of 8.4 Ci/mMol was acquired from Amersham.

Nucleotides and nucleosides were acquired from Calbiochem.

S_d bacteriophage was purified according to the standard method[4]. S_d phage DNA was prepared by the phenol method[5].

The total DNA-methylases preparation was a fraction precipitated with ammonium sulphate at 0.6 saturation and additionally purified on Sephadex G-100 column.

Individual M_I and G_{II} methylases were prepared by chromatography on carboxymethylcellulose-70[1].

Full DNA methylation was done under conditions described previously[2].

Enzymatic hydrolysis using phosphomonoesterase, spleen and snake venom phosphodiesterases was done under standard conditions[3].

Hydrolysis of trinucleotides with A5 actinomyces exonuclease was carried out in 0.05M tris-HCl buffer, pH 8.9, in the presence of 1 mM Mg for 3 hours at 37°C[6].

DNA depurinization was done by Burton's method[7].

Separation of oligopyrimidine blocks according to length and composition was done by thin-layer chromatography in solvents 1 and 2[8]. Solvent 1 contained 7M urea, 0.2M sodium acetate, and H_2O (7:1:2). Solvent 2 consisted of 0.01M sodium acetate buffer, pH 3.0, and 5M NaCl (33:1).

Paper chromatography was done in isopropanol - NH_4OH - H_2O (7:1:2)[9].

Separation of radioactive products by thin-layer and

paper chromatographies was done in the presence of a carrier. The carriers were prepared from thymus DNA by the appropriate enzymatic treatment.

Cross-titration of PBV phage with SK, hsp II and C phenotypes was done by the conventional method[10].

RESULTS

Table 1 presents the distribution of ^{3}H-5'-methylcytosine in oligopyrimidine blocks in DNA methylated with M_I and G_{II} enzymes. For the purposes of comparison, the Table includes the data on the rate of occurrence of individual isostichs for RII methylase from Hattman's report[13]. The bulk of radioactivity was in all cases localized in di- and trimucleotide blocks. In dimucleotide isostichs the bulk of the label was concentrated in C_2, and C_2T was the dominant labeled three-member isostich. Low radioactivity in other isostichs is due to experimental errors associated with DNA hydrolysis and oligonucleotide chromatography. In the analysis of the results noteworthy is the fact that the experimentally determined radioactivity of individual oligonucleotides for both E.coli SK enzymes corresponds to that for RII enzyme[13] within the limits of accuracy of the method.

We then analysed the distribution of ^{3}H-CH_3 label in C_2 and C_2T oligopyrimidines. For this purpose, C_2 dimucleotides were recovered from phage S_d DNA methylated with purified M_I and G_{II} enzymes and C_2T trimucleotide was recovered from DNA methylated with a mixture of the enzymes.

In the former case the dimucleotide was dephosphorylated with phosphomonoesterase and aliquots of the resulting dimucleoside monophosphate were hydrolysed with snake venom and spleen phosphodiesterases. In snake venom enzyme treatment the nucleoside found at the 5'-end was cytosine, whereas spleen phosphodiesterase releases as nucleoside the 3'-end cytosine. The analysis of the data presented in Table 2 indicates that M_I methylase transfers methyl groups on cytosine located at the 3'-end of the site whereas G_{II} enzyme methylates the 5'-end cytosine. The absolute amount of the label in C_2 dimucle-

Table 1. Distribution of ^{3}H-5-methylcytosine in oligopyrimidine blocks after DNA methylation with E.coli SK M_I and G_{II} methylases

Isostich	% of total radioactivity*)		
	Experimental		Calculated**)
	M_I***)	G_{II}***)	
Mononucleotides	6.42	4.40	0.0
C	6.42	4.40	0.0
Dinucleotides	29.13	22.3	25.0
C_2	24.93	20.1	25.0
CT	4.2	2.2	0.0
T_2	0.0	0.0	-
Trinucleotides	36.83	46.2	37.5
C_3	4.82	12.6	6.2
C_2T	31.08	32.3	31.3
CT_2	0.74	0.92	0.0
T_3	0.5	0.35	0.0
Tetranucleotides	15.3	10.9	18.8
Pentanucleotides	11.4	6.7	18.7

*) Acceptor DNA was phage S_d DNA which after replication in E.coli SK cells contains no minor bases owing to the synthesis of the phage-specific enzyme destroying S-adenosylmethionine[11].

**)The rate of occurrence of individual oligopyrimidine blocks for hap II methylase (RTFII, N_3 plasmids) in accordance to Boyer[12] and Hattman[13].

***)Cytosine methylases of M_1 and G_{II} fractions obtained by chromatography on CMC-70.

otides after methylation with M_I and G_{II} methylases was practically the same (no data given). The fact that M_I and G_{II} enzymes methylate different cytosines in the same CC sequence makes it possible to use DNA methylated with the mixture of the enzymes for C_2T trinucleotide analysis.

Table 2. ^{3}H-label distribution in CC dinucleotides after DNA methylation with M_I and G_{II} methylases

Methylase	Phosphodiesterase*)	Radioactivity distribution (%%)	
		Nucleoside	Nucleotide
M_I	snake venom	9.2 (5')	90.8 (3')
	spleen	84.5 (3')	15.5 (5')
G_{II}	snake venom	88.5 (5')	11.5 (3')
	spleen	8.5 (3')	91.5 (5')

*) The incubation mixture for hydrolysis with snake venom phosphodiesterase (37°C, 3 hours) contained 0.1M tris-HCl buffer, pH 8.5, 0.1M $MgCl_2$, enzyme and substrate at a ratio of 1:1000. The same ratio was used with phosphodiesterase, the mixture was incubated under similar conditions in 0.1M ammonium-acetate buffer, pH 6.0, in the presence of 0.1M $MgCl_2$.

From the fact that the only detected labeled dipyrimidine for both enzymes is CC, methylated C_2T trinucleotide may have nucleotide sequence 3'...TCC...5' or 3'...CCT...5' and cannot have the 3'...CTC...5' structure. For the analysis, dephosphorylated trinucleotide was treated with actinomyces A5 exonuclease and phosphodiesterases. The specificity of the exonuclease is such that it liberates 5'-mononucleotide from the 3'-OH end of trinucleoside diphosphate the rest being dinucleoside monophosphate[6]; this mononucleotide after phosphomonoesterase hydrolysis was detected as a nucleoside. The mixture was paper-chromatographed in alkaline isopropanol and localization of radioactivity was determined in the presence of witness compounds. It will be seen in Fig.1 that radioactivity is approximately evenly distributed between the spot of nucleoside and dinucleoside monophosphate. An increase in the dose of the enzyme and in time of incubation did not affect the radioactivity distribution (no data presented). Thus, half of the total C_2T contains labeled methylcytosine in the 3'-position. This means that the M_I enzyme which methylates cytosine in the 3'-end position (see above) recognizes tri-

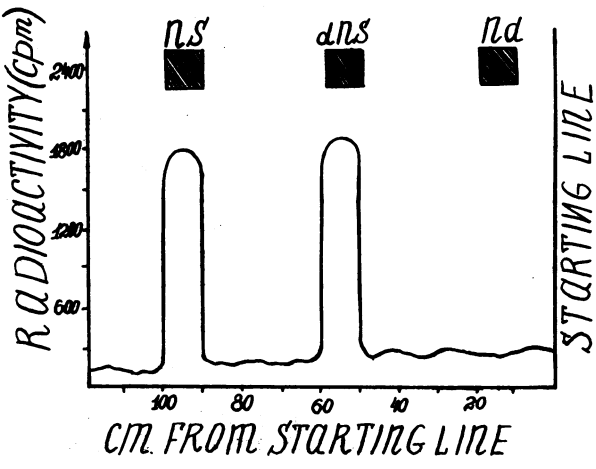

Fig.1. Radioactivity distribution in products of C_2T fraction exonuclease hydrolysis on paper chromatogram. Ordinate on the left - radioactivity, on the right - the start line; Abscissa - distance (cm) from the start. Trinucleoside diphosphates were hydrolysed with actinomyces A5 exonuclease and phosphomonoesterase in 0.05M tris-HCl buffer, pH 8.9 and 0.1M $MgCl_2$ at 37°C for 3 hours (volume 0.5 ml). The mixture was deproteinized, applied on the paper and chromatogram was developed in isopropanol - NH_4OH - H_2O (7:1:2).

nucleotide sequence 3'...CCT...5'. An alternative means thymine in the 3'-position which could not incorporate the ^3H-label under our conditions.

For further analysis, the labeled dinucleoside monophosphate was eluted and hydrolysed with snake venom phosphodiesterase resulting in the formation of 5'-mononucleotide from the 3'-end and nucleoside from 5'-end. The radioactivity distribution on subsequent paper chromatography (Fig.2) clearly demonstrated that all the label was localized in the nucleoside spot. These data suggest that the G_{II} enzyme methylates 5'-end cytosine in the 3'...TCC...5' sequence.

Thus the radioactive products of degradation of DNA methylated in the presence of ^3H-SAM with N_I and G_{II} enzymes have the following structures:

N_I - 3'...C-C...5' and 3'...C-C-T...5'

G_{II} -3'...C-C...5' and 3'...T-C-C...5'

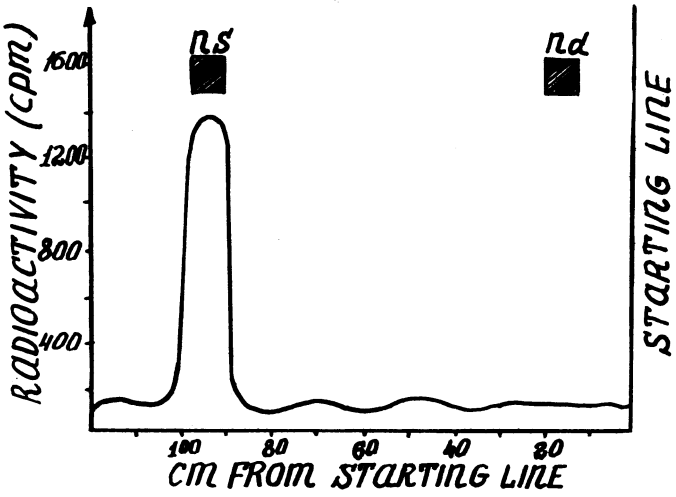

Fig.2. Radioactivity distribution after hydrolysis of dinucleoside monophosphates with snake venom phosphodiesterase. Designation, as in Fig.1. Dinucleoside monophosphates were incubated with snake venom phosphodiesterase at pH 8.5 in 0.1M tris-HCl buffer in the presence of Mg^{2+}, then deproteinized and chromatographed as described in Fig.1.

Because E.coli SK cytosine methylases recognize only double-stranded DNAs[1] and the total composition of pyrimidine isostichs proved to be identical with that of E.coli RII methylase, it may be assumed that the site of recognition of E.coli SK enzymes is subject to the general regularities established for this group of enzymes and has symmetrical palyndrome structure. This be the case, from all the experimental results the only variant of the five-member symmetrical site of recognition for both enzymes may be postulated:

5'...NpCpCpApGpGpNp...3' in case of G_{II} methylase

3'...NpGpGpTpCpCpNp...5' and

5'...NpGpGpApCpCpNp...3' in case of N_I methylase

3'...NpCpCpTpGpGpNp...5'.

Thus G_{II} methylase of EcoSK recognizes the same sequence as methylase of EcoRII but methylates cytosine located at the 5'-end of the molecule. N_I methylase recognizes the sequence which is the inversion of the G_{II} site, and methylates cyto-

sine located at the 3'-end. Undoubtfully, when such sequences are degraded by Burton's method[7], oligonucleotide isosticks should be formed indistinguishable from analogous structures corresponding to the hsp II type of modification[11].

Analysis of modification-restriction. According to the above conclusion, G_{II} and RII methylases recognize the same nucleotide sequence but methylate it in different ways. From this point of view it seemed of great interest to elucidate the relationships of the restricting and modifying components of E.coli SK and E.coli RII host specificity systems. For this purpose we cross-titrated different phenotypes of phage PBV-I corresponding to E.coli SK, E.coli (RII) and E.coli C hosts. The latter host has no modification-restriction system and represents the h.s.(o) type of cells. The results of titrations (Table 3) showed that modification of the SK-type did not provide even partial protection against RII restriction. The efficiency of plating was not more than 0.01%. The phage of the RII phenotype was restricted in E.coli SK cells to a comparable degree.

DISCUSSION

Thus, among methylated oligopyrimidine blocks we identified the following nucleotide sequences:

5'...CpCp... and ...CpCpT...3 (G_{II} enzyme)

5'...CpCp... and ...TpCpCp...3 (N_I enzyme)

Table 3. Cross-titration of phage PBV-I with different phenotypes on various E.coli strains

Phage phenotype	Efficiency of plating on E.coli strains*)		
	SK	RII	C
PBV-I·SK	100	0.01	100
PBV-I·RII	0.005	100	100
PBV-I·C	0.001	0.01	100

*) In per cent to the titer of the phage with a given phenotype on the homologous host taken for 100%.

Theoretically, several six-, five-, and four-member symmetrical recognition sites containing the above nucleotide sequences may be constructed. Table 4 presents such sites and oligopyrimidine isosticha forming upon their degradation. Many of these variants may be rejected immediately since theoretically expected and actually determined oligopyrimidines (see Table 1) differ significantly. Thus, among six-member sites variants Nos. 2, 3, 5, and 8 do not produce methylated dipyrimidine C_2 at all. Variants Nos. 6 and 7 do not produce tripyrimidine isosticha and in variants Nos.1, 4, 6, and 7 tetrapyrimidine frac-

Table 4. The calculated composition of oligopyrimidine isosticha in possible recognition sites of G_{II} and N_I methylases

Sequence 5'N——N3'	Oligopyrimidine isosticha												
	Di-			Tri-			Tetra-						Total
	C_2	CT		C_3	C_2T	CT_2	C_4	C_3T	C_2T_2	CT_3			
Six-member													
1.CCATGG	8	-	8	4	4	-	8	-	-	-	-	-	16
2.CCTAGG	-	-	-	-	9	-	9	-	4	3	-	7	16
3.GGATCC	-	-	-	1	9	-	10	-	3	3	-	6	16
4.GGTACC	8	-	8	4	4	-	8	-	-	-	-	-	16
5.AGGCCT	-	-	-	-	8	-	8	-	3	5	-	8	16
6.TGGCCA	16	-	16	-	-	-	-	-	-	-	-	-	16
7.ACCGGT	16	-	16	-	-	-	-	-	-	-	-	-	16
8.TCCGGA	-	-	-	-	4	-	4	-	5	7	-	12	16
Five-member													
9.CCAGG	4	-	4	2	6	-	8	-	2	2	-	4,	16
10.CCTGG	8	-	8	3	3	-	6	-	1	1	-	2	16
11.CCGGG	4	-	4	6	2	-	8	2	2	-	-	4	16
12.CCCGG	4	-	4	6	2	-	8	2	2	-	-	4	16
Four-member													
13.CCGG	8	-	8	4	4	-	8						16

tion is lacking among the degradation products.

The tetranucleotide recognition site (variants No.13 or its inverted sequence) may be also excluded from consideration because of disagreement with the data in Table 1 concerning the actual frequency of occurrence of individual oligopyrimidine fractions. Indeed, a site of such composition would require, as minimum, 50% content of dipyrimidine fraction and an equal content of C_2 and C_2T isostichs.

Thus, five-member sequences are most real recognition sites for G_{II} and N_I methylases. The comparison of the really observed (Table 1) and theoretically expected frequencies of occurrence of oligopyrimidine isostichs (Table 4) permits to exclude three of the possible recognition sites under discussion. Thus, variant No.10 gives excessively high content of the C_2 fraction, variants Nos.11 and 12 contradict the experimental data on the predominance of the C_2T fraction among trinucleotide isostichs.

Accordingly, the only acceptable recognition site for G_{II} methylase is variant No.9 and the corresponding inverted pentanucleotide for methylase N_I respectively:

5'...NCCAGGN...3' 5'...NGGACCN...3'

3'...NGGTCCN...5' 3'...NCCTGGN...5'

As has already been mentioned in the "RESULTS", the first pentanucleotide is also recognized by EcoRII methylase, and the distribution of individual oligopyrimidine isostichs theoretically calculated for this enzyme[12,13] corresponds fairly well to the really observed values for G_{II} and N_I enzymes. Significant deviations are observed only in the content of the C_3 fraction in G_{II} enzyme site. This is most probably explained by a non-random distribution of nucleotides in the near vicinity of the recognition site under study. This possibility is also indicated by the results obtained by May and Hattman[13].

The phenomenon of isochizomery of restricting nucleases when several different enzymes recognize the same nucleotide sequence but hydrolyse in it different phosphodiesther bonds[14] is widely known at present. Since the modifying component of the host specificity system has been studied much less, thus

far it remained obscure whether or not such structural isomery in methylases is possible where different enzymes recognize the same nucleotide sequence but methylate different bases in it. The results presented in this paper indicate that such isomery may actually occur. Methylases G_{II} from E.coli SK cells and RII from E.coli cells carrying RII or N3 plasmid[13] recognize the same five-member site but methylate different cytosine residues in it. The former enzyme methylates proximal cytosine closer to the 5'-end of oligonucleotide, and the latter methylates distal cytosine in the same CC sequence closer to the 3'-end of oligonucleotide. By analogy with the isoschizomery phenomenon we suggest a term isomethymery for designation of isomery of modifying enzymes.

Although we carried out no in vitro experiments on restriction of DNA methylated with G_{II} enzyme from E.coli SK cells with EcoRII endonuclease, the in vivo experiments on cross titration of PBV-I·RII, PBV-I·SK, and PBV-I·C phages in the appropriate hosts gave quite definite results. There is no significant qualitative difference in titers, in E.coli SK cells, of phages with RII and C phenotypes, as well as there is no differences in the restriction of SK and C phenotype phages in cells with the hsp II type of specificity. Similar results were obtained in titrations of different phenotypes of phage DDVII[15].

A doubt may arise whether infection with phages PBVI and DDVII may affect the activity of cellular DNA methylases as is the case with phages S_d[11], and T3[16], which in one or another way could prevent methylation of DNA in infected cells. We showed previously, however, that neither phage PBVI nor phage DDVII[17] inhibited cellular methylation systems. After replication of both phages in E.coli SK, K12, and C cells both phage and cellular DNAs contain 6'-methylaminopurine and 5'-methylcytosine. Therefore it may be quite definitely concluded that G_{II} isomethymeric methylase does not protect the recognition site from RII restrictase. Unfortunately, thus far we cannot draw the same conclusion with respect to RII methylase, as no restrictase corresponding to G_{II} methylase has been found in E.coli SK cells. At the same time this

strain contains several new types of restricting endonucleases of which we have identified only two enzymes so far[18] protection against which is provided by adenine modification.

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