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Specificity of a deoxyribonucleic acid transmethylase induced by bacteriophage T2. I. Nucleotide sequences isolated from Micrococcus luteus DNA methylated in vitro

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ABSTRACT

Deoxyribonucleic acid from Micrococcus luteus was methylated in vitro in the presence of S-adenosyl-(^{14}C methyl)methionine with a DNA methyltransferase purified from extracts of E. coli infected with bacteriophage T2. The labelled DNA was degraded by enzymatic and specific chemical methods and the resulting short oligonucleotides were separated and characterized. The analytical data permit the conclusion that the DNA transmethylase reacts specifically with N-G-A-T-C-N sequences in which it converts adenine to a 6-methyl-aminopurine residue.

INTRODUCTION

During the past decade many enzymes from a variety of organisms have been described which methylate certain bases, notably adenine and cytosine after their incorporation into newly synthesized DNA. One of the earliest examples of such an enzyme was described by Gold, Hausmann, Maitra and Hurwitz[1], when they discovered a highly active DNA transmethylase (S-adenosyl-L-methionine: DNA (6-aminopurine) methyltransferase (E.C.2.1.1.37) in extracts of Escherichia coli infected with bacteriophage T2. In vitro, this enzyme methylates adenine residues in the DNA of several microorganisms, such as Micrococcus luteus.

The product of the methylgroup transfer in this case is a N(6) methyl-aminopurine residue in the substrate DNA. The first two authors[2] found it difficult to measure with any precision the extent of methylation of the various acceptor DNA's, but it seems clear from their results and those of others[3] that the number of adenine residues to be methylated is small compared to the total number of this base present in the substrate. It would seem plausible that certain rules regarding the specificity of this enzyme might be formulated in terms of the bases in the immediate vicinity of the adenine residue receiving the methyl group.

Falaschi and Kornberg[4] undertook such an investigation with the help of a series of synthetic DNA polymers: d(AT); dA : dT; d(AG) : d(TC) and d(AC) : d(TG). Using active enzyme, they failed to get any incorporation of

methyl groups. While the polymers mentioned contained all of the seven possible dinucleotide sequences involving adenine, and seven unique trinucleotide sequences with A as well, the conclusion was drawn that the nucleotide sequence requirement of the enzyme was not met with in these experiments.

In the present study we have attempted to establish the nucleotide sequence on both sides of the adenine residues which are methylated by the purified enzyme. These studies gained interest after Hattman and his colleagues[3,5] had isolated mutants of bacteriophage T2 which apparently induce a variant of the T2 DNA transmethylase with different specificity.

MATERIALS AND METHODS

Chemicals were reagent grade. Alumina C γ gel was a Calbiochem product, calciumphosphate gel was from Sigma Chem. Co., St. Louis (Mo). S-adenosyl methionine (SAM), labelled in the methyl group with ^{3}H (Spec. act. 500 mCi/mMol) or ^{14}C (Spec. act. 50 mCi/mMol) was obtained from the Radiochemical Centre, Amersham and from International Nuclear Corp., Irvine (California). Pancreatic deoxyribonuclease, snake venom phosphodiesterase and spleen phosphodiesterase were purchased from Boehringer and Sons, Mannheim. Bacterial alkaline phosphatase was from Sigma. M. luteus DNA was a product from Miles Laboratories, Inc.

Purification of bacteriophage T2 induced DNA methyltransferase (E.C.2.1.1.37). Our method is a modification of that of Gold and Hurwitz[6] designed for the purification of a DNA transmethylase from uninfected E. coli. The enzyme was assayed according to Hausmann and Gold[2]. Early logarithmic cells of E. coli B at 3 x 10^{8}/ml were infected with wild type T2 phage at m.o.i. ≈ 4. After 15 min the cells were chilled with ice cubes, harvested at 2^{o} and frozen at -20^{o} until being used.

The partially thawed cells (24 g) were then ground with 48 g Alumina A-301 powder (Alcoa) in a prechilled mortar kept on ice and subsequently suspended in 160 ml 0.05 M triethanolamine buffer pH 8.8 containing 10 mM $MgCl_2$, 1 mM EDTA and 5 mM mercaptoethanol (TMEM buffer). The suspension was centrifuged for 20 min at 12,000 x g to remove debris and then clarified by centrifuging for 2 hr at 80,000 x g in a Spinco refrigerated centrifuge (extract). The extract at 6 mg protein/ml was stirred for 30 min with 1/3 volume of a suspension of Alumina C γ containing 20 mg solids/ml. After centrifugation at 5,000 x g for 10 min the supernatant solution was poured off and saved (Al sup). The gel was washed with 12 ml TMEM buffer and centrifuged at 5,000 x g (Al wash). The gel was eluted twice with 10 ml 0.5 mM potassium phosphate buffer, pH 7.5 containing 10 mM mercaptoethanol

and recentrifuged. All fractions (extract, Al sup, Al wash, Al eluate) were assayed for enzyme activity. If necessary, the adsorption procedure was repeated. The combined active eluates were diluted tenfold with 10 mM mercaptoethanol and stirred with 1/5 volume of a freshly prepared suspension of calcium phosphate gel in 50 mM potassium phosphate, pH 7.5 (20 mg solids/ml) overnight. The gel was centrifuged off and the supernatant was assayed for remaining enzyme activity. When adsorption was found to be satisfactory, the gel was washed once with 50 mM phosphate buffer and then eluted with two 10 ml portions of 0.5 M potassium dimethyl glutarate buffer, pH 7.5 containing 10 mM mercaptoethanol. The active eluates were dialysed against 20 mM potassium phosphate, pH 7.5 - 10 mM mercaptoethanol, adjusted to pH 6.5 and applied to a 20 x 1 cm column of Biorex 70 resin (titrated to pH 6.5 and washed extensively with 50 mM KPO_4 buffer, pH 6.5). The enzyme was eluted by applying a 250 ml gradient with 0.6 M buffered NaCl in the reservoir, fractions of 6 ml being collected. Active enzyme was usually eluted half way the gradient; the most active fractions were dialyzed briefly against 50 mM KPO_4, pH 7.5 and stored on ice. The enzyme was used immediately for preparative purposes.

Large scale preparations of (^{14}C-methyl-6-aminopurine) DNA were made by scaling up the amounts of ingredients used in the assay of the enzyme. In a typical incubation 42 A_{260} units of M. luteus DNA were incubated with (^{14}C-methyl) labelled SAM and sufficient purified enzyme (Biorex fraction) to obtain incorporation of 5 x 10^5 c.p.m. of labelled methyl groups in 45 min at 37° (as determined by scintillation counting in a Tracerlab Corumatic apparatus). This incorporation is equivalent to the methylation of one out of fifty adenine residues present. The labelled DNA was then freed from excess SAM by exhaustive dialysis against 50 mM Tris Cl, pH 8.0 - 10 mM $Na_4P_2O_7$ - 10 mM EDTA and water; this was checked by precipitating a small aliquot with $HClO_4$ and counting.

Enzymic degradation of (^{14}C-methyl) DNA. The (^{14}C-methyl)-labelled DNA prepared by enzymic methylation was degraded with pancreatic DNase. To this end, 5 x 10^4 c.p.m. of DNA and 50 µg of DNase contained in 100 µl of 5 mM Tris-Cl (pH 7.4), 5 mM $MgCl_2$ were incubated overnight at 37°. Occasionally, more DNase (25 µg) was added after 16 hours, and the digestion continued for another two hours. The resulting oligonucleotides were fractionated by two-dimensional electrophoresis, as described by Sanger and Brownlee[7].

The oligonucleotides were eluted from the DE 81 paper, and had their 5'-terminal phosphate groups removed by treatment with bacterial alkaline

phosphatase. The dephosphorylated oligonucleotides were fractionated by one-dimensional electrophoresis on DE 81 paper in pH 3.5 or pH 1.9 buffer. This dephosphorylation and subsequent electrophoresis sometimes led to the separation of sequence isomers which in the two-dimensional pattern had run as a single spot. The sequences of the dephosphorylated oligonucleotides were determined by partial and complete hydrolysis with spleen and snake venom exonuclease, as described earlier[8-10].

Chemical degradation of (^{14}C-methyl) DNA. (^{14}C-methyl)-labelled DNA was also degraded chemically by a hydrazinolysis procedure described earlier[11]. In some of these degradation studies conditions were chosen such that cytosine but not thymine was completely removed from the sugar-phosphate backbone. The resulting ^{14}C-methylaminopurine-containing fragments were dephosphorylated by phosphatase treatment, and separated into di-, tri- and tetranucleotide tracts by gradient chromatography on DE 81 paper. These tracts were fingerprinted and identified by hydrolysis with spleen and snake venom exonuclease.

RESULTS

The results of the purification procedure are given in Table I. The phage-induced enzyme eluted later from the Biorex column than the host DNA transmethylase from which it was well separated.

Table I. Purification of T2 induced DNA transmethylase

Step	Specific activity (u/mg prot.)	Yield (% of extract)
Clarified extract	3.6	100
Alumina C γ gel eluate	6.4	40
Ca-phosphate gel eluate	50	22
Biorex resin eluate	2250	--*

*Yield satisfactory, not accurately determined.

Fig. 1A is the autoradiograph of a fingerprint of in vitro methylated DNA digested with pancreatic DNase; fig. 1B is a diagram of the same fingerprint in which the numbered spots represent the methylated oligonucleotides that have been characterized. The results of this sequencing work have been summarized in Table II.

On inspection of fig. 1A, it was immediately evident that of the four possible 6-methylaminopurine (m^6A)-containing dinucleotides two are lacking in the fingerprint: p(m^6A,A) and p(m^6A,C) which according to a previous study[9] should be located in the right-hand bottom corner of the pattern are either

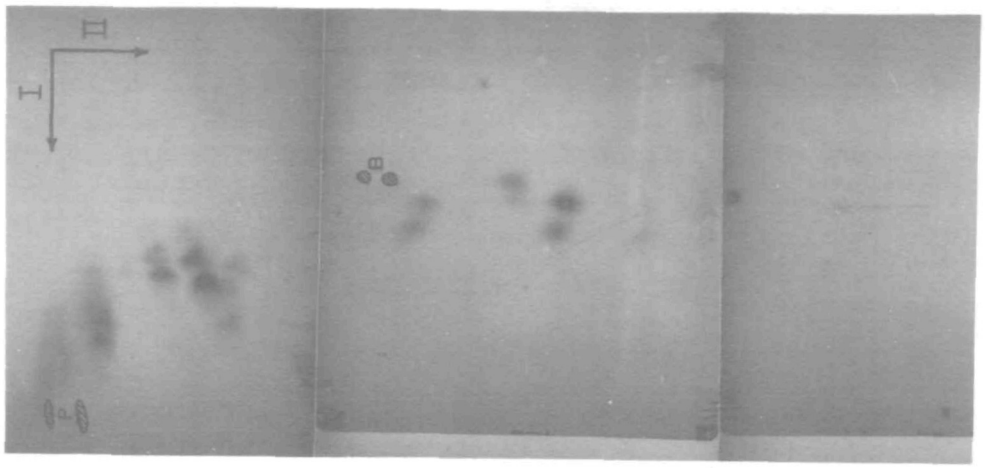

Fig. 1A. Two-dimensional electrophoresis of a pancreatic DNase digest of (^{14}C-methyl)-labelled micrococcal DNA. First dimension: electrophoresis on cellulose acetate at pH 3.5; second dimension: Whatman DE 81 paper at pH 1.9. P = pink dye (acid fuchsin); B = blue dye (xylene cyanol FF).

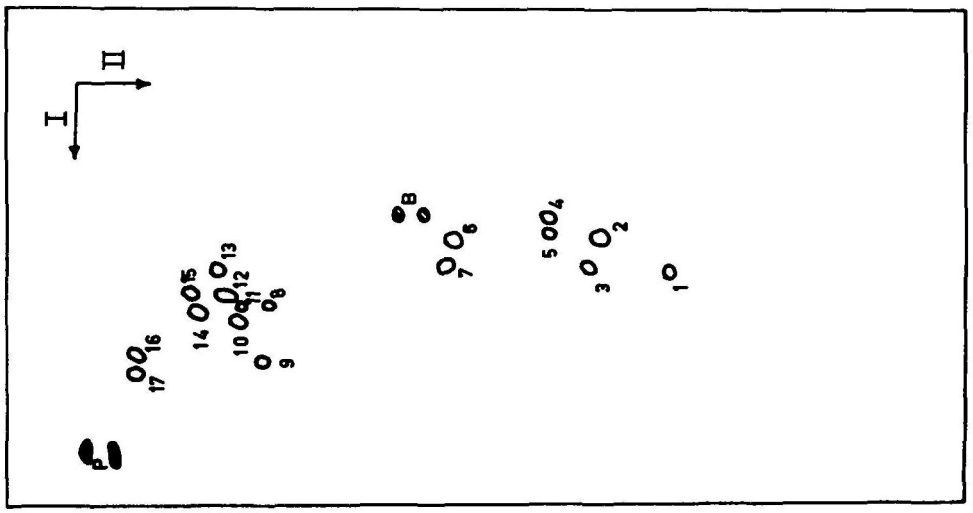

Fig. 1B. Line drawing of the same fingerprint; the numbered spots are those of which the sequence was determined (see Table II).

absent, or present in quantities so low as to escape detection by autoradiography. In that same study it became apparent that methylation of an adenine residue to 6-methylaminopurine did have a slight effect on the electro-

phoretic mobility of oligonucleotides affected by it, but not to such an extent as to invalidate the rules set forth by Sanger[7] for predicting the composition of an oligonucleotide from its position in the fingerprint.

So, spots 1 and 3 were shown to contain p(m^6A-T) and pG-m^6A, on the grounds of their place in the fingerprint, their electrophoretic behaviour in two different systems after phosphatase treatment, and the position of the methylated base in the dinucleotide; this last fact was established by determining whether the ^{14}C label after complete hydrolysis by spleen exonuclease and venom exonuclease was associated with a nucleotide or a nucleoside.

This complete hydrolysis was performed on most other oligonucleotides after their dephosphorylation, in order to establish whether the 6-methylaminopurine residue was in the 5' (m^6A-), 3' (-m^6A) or in a middle (-m^6A-) position. However, the main information that enabled us to establish the sequences was obtained from partial exonuclease hydrolysis of the dephosphorylated material, in combination with the positions in the original two-dimensional pattern. For instance, spot 2, according to its location with respect to spots 1 and 3, should have the composition p(m^6A,T,C) or p(m^6A,G,C). Its electrophoretic behaviour after dephosphorylation suggested that its composition was (m^6A,T,C); this was confirmed by partial hydrolysis with snake venom exonuclease which yielded (m^6A,T). Complete hydrolysis with the same enzyme giving the nucleoside 6-methyldeoxyadenosine showed the sequence to be m^6A-T-C.

By the same kind of reasoning longer sequences could be established; upon dephosphorylation and partial hydrolysis with snake venom exonuclease, spot 4 yielded a product with the same mobility as the phosphatase product of spot 2 (m^6A-T-C). On the basis of this fact, of its position in the fingerprint, and because of other data summarized in Table II, the sequence was deduced to be m^6A-T-C-C. Other arguments we used for the interpretation of the partial exonuclease digests were the characteristic shifts in mobility caused by the removal of a terminal nucleotide (M values)[7,12] and the experience gained in sequencing similar ^{32}P-labelled oligonucleotides from unmethylated DNA. A schematic representation of the partial exonuclease digests is given in fig. 2.

The chemically degraded DNA was separated chromatographically into a number of labelled isopliths. The labelled compound present in the dinucleotide fraction was identified as G-m^6A. The trinucleotide tract was shown to be a mixture of G-m^6A-T and T-G-m^6A. No other labelled trinucleotide was seen in these experiments. Analysis of the tetranucleotide tract added little

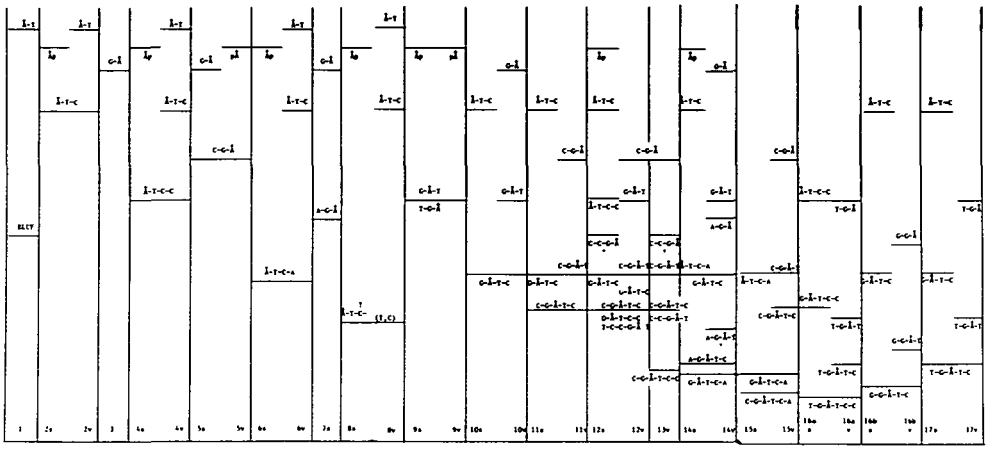

Fig. 2. Schematic representation of the electrophoretic separation on DE 81 paper in pH 3.5 of partial exonuclease digests. For space reasons m^6A is rendered as $\dot{A}$. The numbers at the bottom refer to the spots in fig. 1B; s = spleen exonuclease; v = venom exonuclease.

new information except that the last mentioned sequence can be extended at the 5' end with a thymidylate residue (T-T-G-m^6A).

DISCUSSION

As Table II shows, a number of m^6A-containing oligonucleotides have been sequenced. The only 5' neighbour to the methylated base was found to be a deoxyguanylate residue. At the 5' side of this guanylate residue all four nucleotides (see, for instance, spots 5, 7, 16 and 17) were found to occur. At the 3' side of m^6A only thymidylate was found, and next to that only deoxycytidylate. Beyond this last residue the sequences are seen to diverge: we found two different nucleotides at this position (spots 4 and 6). On the basis of these data we conclude that the phage T2-induced transmethylase recognizes the sequence N-G-A-T-C-N.

The structure of this recognition site has been deduced from the sequences of a number of oligonucleotides obtained by digestion with pancreatic DNase, an enzyme with little base specificity. Nevertheless, if its action is not entirely random there might be a bias against the appearance of certain oligonucleotides in the digest. The oligonucleotides resulting from chemical degradation of (^{14}C-methyl)-labelled DNA were shown to have sequences which are not in conflict with the DNase results. However, they themselves do not exclude the possibility of a deoxycytidylate residue next to the methylated nucleotide. Currently, we are investigating the in

Table II

1.	2.	3.	4.	5.	6.	7.
	position of Å	mobilities on DE81 at pH 3.5	pH 1.9	products partial spd	products partial svd	deduced sequence
1.	Å-	Å-T	Å-T			Å-T
2.	Å-			Å-T-C Ap	Å-T-C Å-T	Å-T-C
3.	-Å	G-Å	G-Å			G-Å
4.				Å-T-C-C Ap	Å-T-C-C Å-T-C (2) Å-T	Å-T-C-C
5.	-Å			C-G-Å G-Å	C-G-Å pÅ	C-G-Å
6.				Å-T-C-A Ap	Å-T-C-A Å-T-C (2) Å-T	Å-T-C-A
7.	-Å			A-G-Å G-Å	?	A-G-Å
8.				Å-T-C-G Ap	Å-T-C-(T,C) Å-T-C (2) Å-T	Å-T-C-(T,C) ?
9.	-Å -Å-	(T,G,Å)	(T,G,Å)			T-G-Å G-Å-T
10.	-Å-			G-Å-T-C Å-T-C (2)	G-Å-T-C G-Å-T (9) G-Å	G-Å-T-C
11.	-Å-			C-G-Å-T-C G-Å-T-C(10) Å-T-C (2)	C-G-Å-T-C C-G-Å-T(10) C-G-Å (5)	C-G-Å-T-C
12.	-Å-			C-G-Å-T-C G-Å-T-C(10) Å-T-C (2)	C-G-Å-T-C C-G-Å-T(10) C-G-Å (5)	C-G-Å-T-C (see 11)
				Å-T-C-C (4)	G-Å-T-C(10) G-Å-T (9)	G-Å-T-C-C
				C-C-G-Å ?		T-C-C-G-Å ?
13.					C-G-Å-T-C-C C-G-Å-T-C (12) C-G-Å-T(10) C-G-Å (5)	C-G-Å-T-C-C
					C-C-G-Å-T (12) C-C-G-Å ?	C-C-G-Å-T-C ?
14.	-Å-			G-Å-T-C-A Å-T-C-A (6)	G-Å-T-C-A G-Å-T-C(10) G-Å-T (9) G-Å	G-Å-T-C-A
				A-G-Å-T-C G-Å-T-C(10) Å-T-C (2)	A-G-Å-T-C A-G-Å-T ? A-G-Å (8)	A-G-Å-T-C
15.	-Å-			C-G-Å-T-C-A G-Å-T-C-A (14) Å-T-C-A (6)	C-G-Å-T-C-A C-G-Å-T-C (12) C-G-Å-T(10) C-G-Å (5)	C-G-Å-T-C-A
16a.	-Å-			T-G-Å-T-C-C G-Å-T-C-C (15) Å-T-C-C (4)	T-G-Å-T-C-C T-G-Å-T-C (17) T-G-Å-T T-G-Å (9)	T-G-Å-T-C-C
16b.	-Å-			G-G-Å-T-C G-G-Å-T-C(10) Å-T-C (2)	G-G-Å-T-C G-G-Å-T G-G-Å	G-G-Å-T-C
17.	-Å-			T-G-Å-T-C G-Å-T-C(10) Å-T-C (2)	T-G-Å-T-C T-G-Å-T T-G-Å (9)	T-G-Å-T-C

Table II. Sequences of (^{14}C-methyl)-m^{6}A oligonucleotides obtained by pancreatic DNase digestion of labelled DNA; in column 1, the compounds are numbered as in fig. 1B.
In column 7, the sequence common to all oligonucleotides is underlined.
Column 2 gives the position of m^{6}A, as deduced from complete digestions with two exonucleases.
The numbers behind the partial products in columns 5 and 6 refer to identified oligonucleotides listed in column 1.

vitro methylation of unglucosylated, unmethylated phage T2 DNA with the same transmethylase; in this DNA hydroxymethylcytosine (hm^5C) substitutes for cytosine. Preliminary results indicate that pancreatic DNase digests of (^{14}C-methyl) T2 DNA contain pm^6A-hm^5C in addition to pm^6A-T. From these facts two conclusions can be drawn: firstly, the specific sequences for the transmethylase are different for the two types of DNA, and secondly, that pm^6A-C should not escape detection if present in DNA.

Whereas the sequences found by chemical degradation do not contradict the DNase data, several oligonucleotides are missing here which are expected to be present on the basis of the sequences given in Table II, notably G-G-m^6A and A-G-m^6A. It is conceivable that because of their higher purine content they run slower than T-G-m^6A during gradient chromatography on DE 81 paper and consequently, were overlooked.

The elucidation of the sequence recognized by the T2-induced transmethylase is of interest for the following reason: Hattman[3,5] isolated phage T2 mutants which are resistant to restriction by phage P1. These mutants were shown to induce an altered transmethylase. The authors suggested that this enzyme has a substrate specificity that differs from that of the wild-type transmethylase. Recently, the recognition sequence for the phage P1 modification transmethylase was established by Brockes, Brown and Murray[13]. to be A-G-m^6A-T-C-T. It is remarkable, that according to our data, the T2 wild-type enzyme should be able to methylate this sequence, at least in DNA containing cytosine. To resolve the question whether the mutationally altered enzyme methylates other nucleotide sequences than the wild-type enzyme does, we are collaborating with Dr. S. Hattman of the University of Rochester in studies on the specificites of both enzymes towards unglucosylated phage T2 hm^5C-DNA.

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