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DNA synthesis with methylated poly(dA-dT) templates: possible role of O^4-methylthymine as a pro-mutagenic base

Peter J.Abbott and Roy Saffhill

Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester M20 9BX, UK

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ABSTRACT

The alternating copolymer poly(dA-dT) has been methylated with either dimethyl sulphate (DMS) or N-methyl-N-nitrosourea (MNU) and the levels of the various methylation products determined. In addition to the methylated adenines formed by both methylating agents, MNU resulted also in the formation of 3-methylthymine, O^4-methylthymine and phosphotriesters. The methylated polymers have been used as templates for E.coli DNA polymerase I in an in vitro assay and the incorporation of complementary and non-complementary nucleotides determined. With the DMS methylated template no wrong nucleotide incorporation was detectable, but with the MNU methylated polymer the incorporation of dGMP was observed. The amount of dGMP incorporated correlated with the level of O^4-methylthymine in the template over the range of methylation studied. The results indicate that O^4-methylthymine is capable of miscoding on a one-to-one basis while the products of DMS methylation (1-, 3- and 7-methyladenines), and also possibly the phosphotriesters, do not lead to any misincorporation.

INTRODUCTION

A role for alkylated bases in both mutagenesis and carcinogenesis has been considered for many years. More recently attention has been focussed on the O^6-alkylguanines as possible pro-mutagenic bases[1] and evidence is accumulating of some relationship between alkylation of the O^6-position of guanine in DNA and tissue susceptibility to tumour induction (refs 2-6). A second O-alkylated base, O^4-methylthymine, has now been detected in DNA following reaction, both in vitro[7] and in vivo[8], with carcinogenic methylating agents and it has been proposed that this base, like O^6-methylguanine, could also be pro-mutagenic[7]. Whilst bacterial studies[9,10] have revealed a predominance of GC->AT transitions after treatment with alkylating carcinogens presumably due to the point mutations arising from the O^6-alkylguanine, a smaller but significant number of AT->GC transitions are also observed. These could be due to miscoding by an alkylated adenine or thymine base.

Using the alternating copolymer poly(dA-dT) we have been

investigating the possibility of miscoding by methylated adenine and thymine bases during DNA synthesis. The polymer has been methylated *in vitro* with either dimethyl sulphate (DMS) or the potent carcinogen *N*-methyl-*N*-nitrosourea (MNU) and then used as template for highly purified *E.coli* DNA polymerase I, measuring the incorporation of complementary and noncomplementary nucleotides.

MATERIALS AND METHODS

Chemicals: Poly(dA-dT), deoxyribonucleoside 5'-triphosphates and *E.coli* DNA polymerase I (grade I) were purchased from the Boehringer Corporation (London) Ltd. Radioactive poly(dA-dT) was prepared according to the method of Schachman *et al*[11] using the appropriate labelled triphosphate. Radioactive monomers were obtained from the Radiochemical Centre, Amersham, England. MNU was prepared in these laboratories and was stored at -20°. DMS was purchased from BDH Chemicals Ltd and redistilled before use. Marker compounds were the gift of Dr P.J. O'Connor. O^4-methylthymidine as marker was prepared by the method of Lawley *et al*[7].

Methylation of Poly(dA-dT): Poly(dA-dT) was methylated at a concentration of 400nmole nucleotide phosphorus per ml in 0.25M sodium cacodylate buffer pH 7.0 and 0.5M NaCl. DMS (0 to 3µl/ml hourly for 3 hours) or MNU (0 to 40mg/ml) was added, the solution agitated and the reaction allowed to proceed overnight. The methylated polymer was then extensively dialysed against 0.01M Tris-HCl pH 7.4 and 0.02M NaCl at 4°. All samples (including non-methylated controls) were handled in the same manner: the only parameter that was altered for differing samples was the methylating agent and the amount.

Analysis of Methylation Products: Adenine methylation was determined using [^{14}C-adenine]-poly(dA-dT). The methylated polymer was lyophilised to dryness and hydrolysed with 0.3ml 72% perchloric acid for 1 hour at 100°. The acid hydrolysate was diluted to 10ml and applied to a Dowex-50 column (25 x 1.5cm) equilibrated with 0.3M triethylammonium formate pH 6.65. The adenine and methyladenine bases were eluted with a linear gradient (0.3 - 1.0M) triethylammonium formate pH 6.65 collecting 3ml fractions.

Phosphate and thymine methylation was measured using [^{3}H-thymine]-poly(dA-dT). The methylated polymer was degraded to the nucleosides (and phosphotriesters) by enzymic hydrolysis with 0.02 units snake venom phosphodiesterase and 3 units alkaline phosphatase in 0.1M Tris-HCl pH 8.9 and the products separated on Dowex-50, ammonium form, column

(25 x 1.5cm) eluting with 0.1M ammonium formate pH 8.9 collecting 3ml fractions.

Radioactivity in the fractions from both columns was measured by counting in a Triton/toluene phosphor. Authentic marker compounds were used to identify the peaks of radioactivity corresponding to the alkylated bases and nucleosides. The two peaks (after methylation with MNU) thought to correspond to phosphotriesters were identified by pooling the fractions of each and reducing them in volume followed by overnight alkaline hydrolysis, pH 12.5 at 37° [8]. These hydrolysis products were analysed on a Dowex-1 column (20 x 1cm) eluting with a linear gradient of 0.01M ammonia to 0.02M ammonium formate pH 7.4 followed by a linear gradient of 0.02 - 0.25M ammonium formate pH 4.2. Marker compounds of the possible hydrolysis products of the phosphotriesters (i.e. dinucleoside phosphates and nucleoside methyl phosphates) were run in a similar manner.

Polymerase Assays: The assay (total volume 300µl) contained 20nmole each of dATP and dTTP, 2nmole each of dCTP and dGTP, 30nmole nucleotide phosphorus as poly(dA-dT), 0.05 units E.coli DNA polymerase and was 70mM in Tris HCl pH 7.4, and 7mM in $MgCl_2$. Concurrent assays were used to measure and incorporation of complementary and non-complementary nucleotides. Complementary nucleotide incorporation was measured using either [^{3}H]-dATP or [^{3}H]-dTTP (each at 50mCi/mmole) and non-complementary nucleotide incorporation measured using [^{3}H]-dGTP (6Ci/mmole) or [^{3}H]-dCTP (9Ci/mmole). Assays, along with the appropriate controls were incubated at 37° and the reaction stopped by the addition of the appropriate unlabelled deoxyribonucleoside 5'-triphosphate, carrier DNA and cold 5% (w/v) trichloroacetic acid. Acid-insoluble material was collected on a glass fibre GF/C disc, washed with cold trichloroacetic acid containing 2% (w/v) $Na_4P_2O_7 . 10H_2O$ and dried. Radioactivity was measured by counting the disc in a toluene phosphor.

Caesium Chloride Density Gradient Centrifugation of Newly Synthesised Poly(dA-dT): To obtain sufficient material for gradient analysis the miscoding assay with [^{3}H]-dGTP precursor and MNU methylated template was increased 4-fold. Following incubation, the assay was made 0.2M in NaCl, heated to 80° for 5 minutes to inactivate the enzyme and cooled in ice. The mixture was then extensively dialysed against 0.01 M NaCl at 4° to remove excess low molecular weight material. Solutions of CsCl containing the newly synthesised poly(dA-dT) were prepared in 0.01 M potassium

phosphate buffer pH 7.4 to a starting density of 1.600g/cm^3 and a total volume of 5ml. Centrifugation was carried out at 45,000 rpm in a 3 place titanium head (No. 59587) in an MSE Superspeed 65 centrifuge for 16 hours at 25°. Fractions of 0.125ml were collected from the bottom of the tubes and the acid insoluble radioactivity determined. The position of poly-(dA-dT) was monitored by absorbance at 254 nm.

RESULTS

Methylation of Poly(dA-dT): The amounts of the various methylation products formed were measured using radioactively labelled poly(dA-dT) as described in the methods. The methylated bases and nucleosides obtained after acidic or enzymic hydrolysis were identified by their chromatographic properties against authentic compounds. Following enzymic hydrolysis of MNU methylated [^{3}H-thymine]-poly(dA-dT) two broad peaks of radioactivity were observed, eluting after the nucleosidic material. These were identified as phosphotriesters (i.e. dinucleoside methyl phosphates): on alkaline hydrolysis[8] both yield thymidine, a dinucleoside phosphate and a thymidine methyl phosphate as the only radioactive (i.e. [^{3}H]-thymine containing) products.

The relative amounts of the methylation products formed are shown in Table I. Analysis of polymer methylated with varying amounts of DMS or MNU showed no change in these relative levels over the range studied. In addition to the 1-, 3- and 7-methyladenines formed by both methylating agents, MNU treatment resulted also in the formation of 3-methylthymine,

TABLE I

PRODUCTS OF METHYLATION OF POLY(dA-dT) BY DIMETHYL SULPHATE OR N-METHYL-N-NITROSOUREA

Methylation Product	Per cent relative yield	
	DMS	MNU
1-methyladenine	5.2 ± 1.7	1.5 ± 0.2
3-methyladenine	21.8 ± 1.8	17.3 ± 0.7
7-methyladenine	73.0 ± 3.6	45.6 ± 3.3
3-methylthymine	ND	4.2 ± 0.2
O^4-methylthymine	ND	1.0 ± 0.1
Phosphotriesters	ND	30.4 ± 3.4

ND, not detected.

O^4-methylthymine and phosphotriesters, none of which were detectable after reaction with DMS.

E.Coli DNA Polymerase I Assays with Methylated Poly(dA-dT) Templates:

Polymers with differing degrees of methylation (up to 5% of all bases) were used as templates for E.coli DNA polymerase I in the in vitro assay. In all cases the incorporation of complementary nucleotides (from dATP and dTTP precursors) were found to be equal and linear with time for up to 6 hours incubation. After methylation with either DMS or MNU the incorporation of the two complementary nucleotides decreased from the level obtained with un-methylated template: the template activity of the polymer being more sensitive to DMS than to MNU methylation (Tables II and III). With DMS methylated template, at all levels of alkylation, no noncomplementary nucleotide incorporation was observed (Table II) indicating that

TABLE II

MISCODING BY DIMETHYL SULPHATE METHYLATED POLY(dA-dT)

DMS µl/ml/hr for 3 hrs	Per Cent Methylation[a]	Nucleotide Incorporation		
		(dTMP + dATP) pmole	dGMP pmole	dCMP pmole
0	0	3334	<0.03	<0.02
1.0	1.30	914	<0.03	<0.02
2.1	2.75	514	<0.03	<0.02
3.1	4.10	356	<0.03	<0.02
4.2	5.50	320	<0.03	<0.02

a Calculated as the amount of all methylation products expressed as a percentage of all bases in the poly(dA-dT).

TABLE III

MISCODING BY N-METHYL-N-NITROSOUREA METHYLATED POLY(dA-dT)

MNU mg/ml	Per Cent Methylation[a]	Nucleotide Incorporation		
		(dTMP + dATP) pmole	dGMP pmole	dCMP pmole
0	0	2260	<0.03	<0.02
7.3	0.78	1390	0.13	<0.02
13.0	1.40	994	0.15	<0.02
21.0	2.25	800	0.17	<0.02
32.8	3.50	680	0.21	<0.02

a Calculated as in Table II.

the presence of 1-, 3- and 7-methyladenine does not lead to any misincorporation. With the MNU methylated template the incorporation of deoxyguanosine 5'-monophosphate (dGMP) but not of deoxycytidine 5'-monophosphate (dCMP) was observed. Even though the total complementary nucleotide incorporation decreased on methylation, that of dGMP increased (Table III). Similar results have been obtained in a double labelling experiment using [^{3}H]-dTTP and [^{32}P]-dGTP[12]. When the amount of dGMP incorporated was calculated as a percentage of all nucleotides it was found to increase with methylation and it corresponded very closely to the amount of O^{4}-methylthymine present in the template poly(dA-dT) i.e. the wrong nucleotide appeared to be incorporated on a one-to-one basis with O^{4}-methylthymine (Figure 1).

CsCl Analysis of Newly Synthesised Poly(dA-dT): In neutral caesium chloride gradients newly synthesised poly(dA-dT) was found to band at a density of 1.65g/cm^{3}. The acid insoluble radioactivity from [^{3}H]-dGTP precursor in the assay with MNU methylated polymer as template was found to band at the same position indicating that the radioactivity was indeed associated with the newly synthesised polymer (Figure 2).

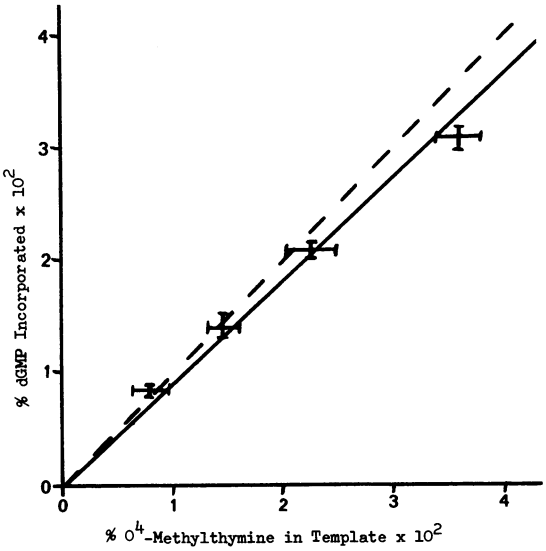

Figure 1
The incorporation of dGMP into newly synthesised polymer with increasing amounts of O^{4}-methylthymine in the poly(dA-dT) template.
The points are derived from several independent experiments.
----- Indicates slope = 1, the dGMP incorporation that would be expected if O^{4}-methylthymine miscoded with guanine on a one-to-one basis.

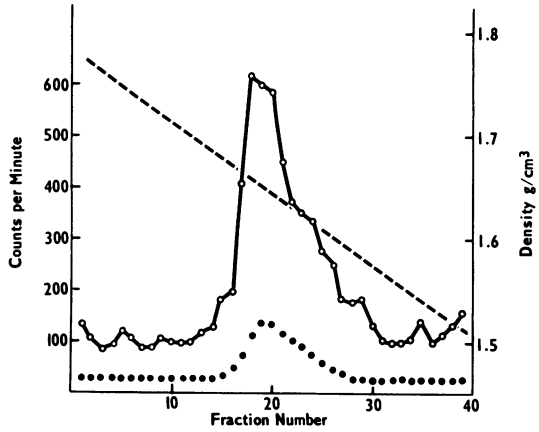

Figure 2
Caesium chloride density gradient centrifugation of newly synthesised polymer made on MNU methylated poly(dA-dT) template.
O, ^{3}H (from [^{3}H]-dGTMP precursor) counts per minute.
●, Relative A^{254}.

DISCUSSION

DNA polymerase I is normally very accurate in copying poly(dA-dT). In fact, an error frequency or less than one wrong base in 500,000 has been claimed[13] and the high fidelity of the enzyme has been utilised in these experiments.

The methylated bases produced by DMS failed to produce any wrong nucleotide incorporation. Methylation of adenine at the 1-position might be expected to interfere with normal hydrogen bonding to thymine, but this, presumably, is not sufficient to lead to any wrong incorporation. The reason for the decrease in template activity following both DMS and MNU treatment is uncertain. It may be due to the presence of methylated bases, e.g. 1-methyladenine, 3-methylthymine, or even apurinic sites, that are unable to form hydrogen bonded base pairs.

The incorporation of the dGMP into newly synthesised polymer following MNU treatment of the template seems very likely to be due to miscoding by O^{4}-methylthymine. A stable hydrogen bonded base pair between O^{4}-methylthymine and guanine but not adenine or cytosine can be constructed (Figure 3). The possibility of some noncomplementary nucleotide incorporation arising from the presence of 3-methylthymine cannot entirely be dismissed. This seems unlikely, however, since the tautomeric form of the base does not change on methylation at the 3-position and in any case the methyl group would interfere with normal

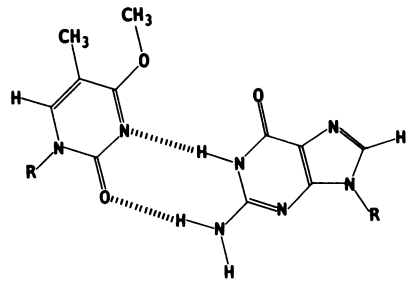

Figure 3.
The abnormal base pair O^4-methylthymine-guanine.

hydrogen bonding to adenine (c.f. 1-methyladenine). No hydrogen bonded base pair can be constructed with 3-methylthymine. Miscoding by 3-methylcytosine has been reported when present in templates for RNA polymerase[14,15]. In similar experiments, using methylated poly(dC-dG) as template for the DNA polymerase we have shown that whilst O^6-methylguanine miscodes during DNA synthesis, 3-methylcytosine does not[12]. The possibility of forming a stable hydrogen bonded structure, as in the case of the O^4-methylthymine-guanine and O^6-methylguanine-thymine base pairs, would appear to be essential if any miscoding is to arise from the action of the DNA polymerase. The evidence from polymers containing 1-methyladenine and 3-methylcytosine suggest that a mere weakening of the hydrogen bonded base pair is not sufficient to allow any misincorporation. The DNA polymerase, presumably having recognised in the template the major tautomeric form of a base, is able to provide with a high degree of accuracy a base partner to form a stable hydrogen bonded base pair[16]. The possibility of phosphotriesters leading to some miscoding cannot be excluded but, in view of the above results, it seems unlikely, especially as the misincorporation is specific (i.e. only one wrong base is concerned) and it corresponds to the amount of O^4-methylthymine present.

The evidence supports the idea that O^4-methylthymine can miscode with guanine during DNA synthesis and that this could lead to point mutations. In this regard it would play a role analogous to O^6-methylguanine in the mutagenic and carcinogenic processes and, as such, it is worthy of further study.

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