Formation of $O^2$-methylthymine in poly(dA-dT) on methylation with N-methyl-N-nitrosourea and dimethyl sulphate. Evidence that $O^2$-methylthymine does not miscode during DNA synthesis

Roy Saffhill and Peter J. Abbott

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

Received 23 March 1978

ABSTRACT

The alternating co-polymer has been methylated with either N-methyl-N-nitrosourea (MNU) or dimethyl sulphate (DMS) and the levels of the various methylated thymidines ($O^2$-methylthymidine, 3-methylthymidine and $O^4$-methylthymidine) measured. MNU produced all three compounds whereas DMS only produced 3-methylthymidine and $O^2$-methylthymidine at detectable levels. These results have been combined with our earlier results concerning the misincorporation of dGMP with E. coli DNA polymerase using MNU-methylated poly(dA-dT). These results indicate that $O^2$-methylthymidine does not miscode during DNA synthesis.

INTRODUCTION

In a recent publication $^1$ we have reported that when the alternating co-polymer poly(dA-dT) that had been methylated in vitro with N-methyl-N-nitrosourea (MNU) was used as template for E. coli DNA polymerase I the incorporation of dGMP (but not of dCMP) into newly synthesised polynucleotide was observed. With template methylated with dimethyl sulphate (DMS) no misincorporation (of dGMP or dCMP) was detected. Since the amount of dGMP incorporation was similar to the level of $O^4$-methylthymine present in the template for all levels of methylation studied, the misincorporation of dGMP was attributed to the presence of the $O^4$-methylthymine (which is only produced at detectable levels in the template by MNU). Further, it is possible to construct a hydrogen-bonded base pair between $O^4$-methylthymine and guanine (but not adenine).

Since completing these experiments a new alkylated base, $O^2$-alkylthymine, has been reported in DNA after reaction with alkylnitrosourea $^2$ and on reaction of thymidine with a diazoalkane $^3$. It is possible that $O^2$-methylthymine may mispair with guanine and could be responsible for some, or all of the misincorporation of dGMP reported previously $^1$. This methylated base was not detected in our initial experiments as, firstly it was not looked for $^2$ and, secondly, it is unstable under the conditions...
of our analyses (pH 8.9) and it would have broken down. We have now investigated the presence of O\textsuperscript{2}-methylthymine in poly(dA-dT) methylated with either MNU or DMS and now report that it is produced on methylation with both agents. Our earlier results are reviewed in the light of these more recent analyses.

**MATERIALS AND METHODS**

**Chemicals:** DMS was purchased from BDH Chemicals Ltd. MNU was prepared in these laboratories and stored in the dark at -20\(^\circ\). Thymidine and all enzymes used were obtained from Sigma (London) Ltd. \([^{3}\text{H-Thymine}]\)-poly (dA-dT) was prepared according to the method of Schachman et al\(^4\) using \([^{3}\text{H-T}]\)-dTTP (obtained from the Radiochemical Centre, Amersham) as one of the precursors. Marker compounds, O\textsuperscript{2}-methylthymidine, 3-methylthymidine and O\textsuperscript{4}-methylthymidine were prepared by methylating thymidine with diazomethane\(^5\). Preparative paper chromatography on Whatman 3MM paper in solvent A\(^3\) (BuOH/EtOH/H\(_2\)O - 80:10:25) gave 2 UV-absorbing bands. The one of Rf 0.69 (Rf of thymidine is 0.60) was O\textsuperscript{2}-methylthymidine and the one of Rf 0.80 was a mixture of 3-methylthymidine and O\textsuperscript{4}-methylthymidine. The latter were separated by chromatography on a Dowex-50 column (25 x 1.6cm) eluting with 0.005 M potassium phosphate pH 7.0. The first compound to elute was 3-methylthymidine followed by the O\textsuperscript{4}-methylthymidine. The compounds had chromatographic and UV properties similar to those reported\(^3,5\).

**Methylation of Poly(dA-dT):** The \([^{3}\text{H-Thymine}]\)-poly(dA-dT) was methylated with either MNU or DMS as previously reported\(^1\). After methylation the polymer was extensively dialysed against 0.01 M Tris/HCl pH 7.4 and 0.02 M NaCl at 4\(^\circ\).

**Analysis of Methylation Products:** The methylated polymer was enzymically digested with snake venom phosphodiesterase and phosphatase at pH 7.0 according to the method of Singer\(^2\). Each hydrolysate was divided into two halves and applied separately to Whatman 3MM paper along with marker compounds. The chromatograms were run overnight in solvent A. After drying, one chromatogram of each sample (i.e. half of each hydrolysate) was cut into 0.5cm strips and the radioactivity in each counted in toluene phosphor. The UV-absorbing spots from the remaining chromatogram were cut out and the material eluted with methanol. Samples from the thymidine and O\textsuperscript{2}-methylthymidine spots were rerun overnight in the same system and similarly cut into 0.5cm strips and the radioactivity
Figure 1. Methylation of $[^3]$H-Thymine]poly(dA-dT) with N-methyl-nitrosourea (MNU).

a. Paper chromatography of enzymic digest in solvent A.

b. Rechromatography of $O^2$-methylthymidine spot in solvent A.

c. Rechromatography of $3$-methylthymidine/$O^4$-methylthymidine spot on Dowex-50.

The material from the $3$-methylthymidine/$O^4$-methylthymidine spot was applied to a Dowex-50 column ($25 \times 1.6$cm) equilibrated with $0.005M$ potassium phosphate buffer pH 7.0 and eluted with the same buffer collecting 4ml fractions. Radioactivity was counted in a Triton/toluene phosphor.
Figure 2. Methylation of $[^3\text{H-}\text{Thymine}]-\text{poly(dA-dT)}$ with dimethyl sulphate (DMS).

a. Paper chromatography of enzymic digest in solvent A.
b. Rechromatography of $O^2$-methylthymidine spot in solvent A.
c. Rechromatography of $3$-methylthymidine/$O^4$-methylthymidine spot on Dowex-50.

RESULTS

Chromatography of Enzymic Digests: On initial paper chromatography of the enzymic digests of the $[^3\text{H-}\text{Thymine}]-\text{poly(dA-dT)}$ a very large peak of radioactivity was observed in the position of the thymidine UV-absorbing spot. See figures 1a and 2a. In the case of the MNU-methylated polymer this was followed by a shoulder coinciding with $O^2$-methylthymidine. No
TABLE I
PRODUCTS OF METHYLATION OF POLY(dA-dT) BY N-METHYL-N-NITROSOUREA (MNU) AND DIMETHYL SULPHATE (DMS)

<table>
<thead>
<tr>
<th>Methylation Product</th>
<th>Per Cent Relative Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DMS</td>
</tr>
<tr>
<td>1-methyladenine</td>
<td>5.1</td>
</tr>
<tr>
<td>3-methyladenine</td>
<td>21.6</td>
</tr>
<tr>
<td>7-methyladenine</td>
<td>72.2</td>
</tr>
<tr>
<td>(\text{O}^2)-methylthymine</td>
<td>0.4</td>
</tr>
<tr>
<td>(\text{O}^4)-methylthymine</td>
<td>0.7</td>
</tr>
<tr>
<td>phosphotriesters</td>
<td>ND</td>
</tr>
</tbody>
</table>

\(\text{a}\) Data taken from previously reported results.¹

ND Not detected (limit 0.1% relative yield)

similar shoulder was observed from the DMS-methylated polymer. Coinciding with the 3-methylthymidine/\(\text{O}^4\)-methylthymidine UV-absorbing spot (which run together in this system) both methylating agents yielded a peak of radioactivity. From the MNU-methylated polymer a very broad ill-defined peak of radioactivity was observed chromatographing between the origin and the thymidine. This is very likely phosphotriester (i.e. dinucleoside methyl phosphate) which is produced on reaction with MNU.¹ However, on account of the very diffuse nature of this material it was not characterised as such.

Rechromatography of Individual UV-Absorbing Spots: As the \(\text{O}^2\)-methylthymidine appeared as a shoulder on the side of the very large thymidine peak it was necessary to rechromatograph the material running with the \(\text{O}^2\)-methylthymidine marker in order to get quantitative data. Also, there is the possibility that a small amount of \(\text{O}^2\)-methylthymidine is produced by the DMS and it cannot be detected on the tail of the large thymidine peak. When the \(\text{O}^2\)-methylthymidine spots were rechromatographed in solvent A two peaks of radioactivity were detected from both methylated polymers. These corresponded to thymidine and \(\text{O}^2\)-methylthymidine, the latter being very much larger after MNU-methylation than after DMS methylation. See figures 1b and 2b.

The material from the 3-methylthymidine/\(\text{O}^4\)-methylthymidine spot was rechromatographed on a Dowex-50 column. The MNU-methylated polymer
afforded 2 peaks of radioactivity corresponding to 3'-methylthymidine and 5'-methylthymidine whereas only one peak was detected from the DMS-methylated polymer, namely 3-methylthymidine. See figures 1c and 2c.

Relative Levels of Methylcation Products in Poly(dA-dT): From the amounts of radioactivity associated with the various peaks in the chromatography reported above we have calculated the relative amounts of the various methylation products. These have been combined with the data for the adenine and phosphate methylation products reported previously. The results are shown in Table I.

DISCUSSION

Our results show that 5'-methylthymine is produced on methylation of poly(dA-dT) with both MNU and DMS. The level being ca 5 times higher following MNU-treatment than following DMS-treatment. The levels of 3-methylthymine and 5'-methylthymidine following the MNU-treatment as measured by these methods agree with those previously reported. In the work reported here 3-methylthymine was also detected following DMS-treatment whereas previously it was not detected. However, the detection of small quantities of this base as previously measured is difficult as the 3-methyl thymidine elutes just after the very large thymidine peak of radioactivity in the Dowex-50/ammonium formate pH 8.9 system used. (Even after rechromatography the last traces of thymidine could not be removed.) The paper chromatography technique to separate the compound from the thymidine followed by Dowex-50 column chromatography in phosphate buffer as reported above is a better method for the determination of 3-methylthymidine and 5'-methylthymidine in such circumstances. The formation of 5'-methylthymine by DMS may well be analogous to formation of low levels of 5'-methylguanine by DMS and methyl methanesulphonate (MMS), although the relative amount of 5'-methylthymine produced is much greater than in the case of the 5'-methylguanine. This may reflect some single-stranded nature of the poly(dA-dT).

The fact that 5'-methylthymine and 3-methylthymine are both produced on methylation with DMS, coupled with our previous observation that DMS-methylation of a poly(dA-dT) template does not lead to any misincorporation of dGMP or dCMP with E. coli DNA polymerase I indicates that these two methylated bases do not in fact miscode during DNA synthesis when they are present in a polynucleotide template. It is possible to draw a hydrogen-bonded base pair between 5'-methylthymine and guanine indicating that there is a possibility that the former may lead to mis-
Figure 3. $\Omega^2$-methylthymine-guanine base pair showing steric hindrance between the $\Omega^2$-methyl group and the H(2') of the deoxyribose.

incorporation. However, in order to fit such a base pair into the DNA helix it is necessary for the $\Omega^2$-atom to take up a trigonal configuration (to accept the hydrogen bond) planar with the base pair. Molecular models show that this cannot happen on account of the steric hindrance between the $\Omega^2$-methyl group and the deoxyribose moiety. See figure 3. We have shown that 3-methylcytosine does not miscode with the DNA polymerase $^8$ whereas it is known to miscode in an RNA polymerase assay $^9,10$. A similar situation may exist with $\Omega^2$-methylthymine as RNA polymerase and DNA polymerase may have different steric requirements.

From these results we conclude that whilst it is possible to draw a hydrogen-bonded base pair between $\Omega^2$-methylthymine and guanine, in practice this does not occur during DNA synthesis as steric factors prevent such a base pair from fitting into the DNA helix. Further, the misincorporation of dGMP reported previously $^1$ from template poly(dA-dT) meth-
ylated with MNU is indeed due to miscoding of $^4\text{methylthymine}$ with guanine on a one to one basis.

**ACKNOWLEDGEMENTS**

We thank Mrs Anne Hough for skilled technical assistance. This work was supported by grants from the Medical Research Council and the Cancer Research Campaign.

**REFERENCES**