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Mutagenic potential of O^4-methylthymine *in vivo* determined by an enzymatic approach to site-specific mutagenesis

(alkylating agents/DNA polymerase/bacteriophage φX174/*Escherichia coli*/chemical carcinogenesis)

BRADLEY D. PRESTON*, B. SINGER†, AND LAWRENCE A. LOEB*‡

*Joseph Gottstein Memorial Cancer Research Laboratory, Department of Pathology, SM-30, University of Washington, Seattle, WA 98195; and †Donner Laboratory, Lawrence Berkeley Laboratory, University of California, Berkeley, CA 94720

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ABSTRACT **O^4-Alkylthymine-DNA adducts have been implicated as causative lesions in chemical mutagenesis and carcinogenesis. To directly assess the mutagenic potential of these adducts *in vivo*, we have designed an enzymatic technique for introducing nucleotide analogues at predetermined sites of biologically active DNA. *Escherichia coli* DNA polymerase I was used *in vitro* to incorporate a single O^4-methylthymine residue at the 3′ terminus of an oligonucleotide primer opposite the adenine residue of the amber codon in bacteriophage φX174 *am*3 DNA. After further extension of the primer with unmodified nucleotides, the partial-duplex product was transfected into *E. coli* spheroplasts. Replication of the site-specifically methylated DNA in *E. coli* deficient in O^4-methylthymine-DNA methyltransferase (*ada*$^-$) yielded 10-fold more mutant progeny phage than replication of nonmethylated DNA; no increase in mutation frequency was observed after replication in repair-proficient (*ada*$^+$) *E. coli*. The DNA from 20 independently isolated mutant plaques all contained A·T → G·C transitions at the original site of O^4-methylthymine incorporation. These data demonstrate that O^4-methylthymine induces base-substitution mutations in *E. coli* and suggest that this adduct may be involved in mutagenesis by *N*-nitroso methylating agents. This enzymatic technique for site-specific mutagenesis provides an alternative to the chemical synthesis of oligonucleotides containing altered bases.**

Alkylating agents are mutagenic, carcinogenic, or both in a variety of prokaryotic and eukaryotic organisms. Although the precise molecular mechanisms underlying mutagenesis by these agents are unknown, there is strong evidence suggesting that base-substitution mutations arise via the formation of alkyl-DNA adducts that direct the misincorporation of nucleotides during DNA replication (see ref. 1 for review). Recent studies have implicated O^4-alkyl-dT residues as important intermediates in mutagenesis (2–7) and carcinogenesis (8, 9) by *N*-nitroso alkylating agents. Theoretical considerations indicate that alkylation of the O^4 atom of thymine will disrupt the hydrogen-bonding properties of the base to favor the formation of O^4-alkyl-T·G base pairs, resulting in A·T → G·C transition mutations (10). Investigations by Singer *et al.* (2–4) and Saffhill (5) have shown that purified DNA polymerases frequently incorporate dG residues opposite O^4-alkyl-dT adducts in synthetic polynucleotide templates *in vitro*. The induction of A·T → G·C transitions in *Escherichia coli* treated with *N*-nitroso compounds (7, 11) provides indirect evidence that O^4-alkyl-dT adducts may also play an important role in mutagenesis *in vivo*. However, in these studies it is difficult to determine which adduct (or adducts) is responsible for mutagenesis, since a large number of potentially mutagenic adducts are formed by alkylating agents *in vivo* (1).

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Previous studies have shown that the error rate of a DNA polymerase *in vitro* is proportional to the ratio of incorrect to correct nucleotides in the reaction mixture (12). This dependence of DNA replication fidelity on dNTP "pool bias" has been used to generate site-specific mismatches in DNA by copying primed, single-stranded DNA in the presence of single, noncomplementary dNTPs (13). It has also been shown that certain alkyl-dNTPs (2, 14–16) and other dNTP analogues (ref. 17, pp. 119–120) will serve as effective substrates for DNA polymerases *in vitro*. Together, these data suggest that it should be possible to use DNA polymerases to incorporate modified nucleotides to give single-base substitutions at predetermined positions in DNA and thus to determine the mutagenic potential of specific base analogues.

To unambiguously assess the mutagenic potential of O^4-alkyl-dT adducts *in vivo*, we have used *E. coli* DNA polymerase I (pol I) to incorporate a single O^4-methyl-2′-deoxythymidine (O^4-Me-dT) residue into a predetermined site in bacteriophage φX174 *am*3 DNA. Subsequent transfection and replication of the site-specifically methylated DNA in *E. coli* has permitted an evaluation of the mutagenicity of O^4-Me-dT *in vivo*. Our data demonstrate that O^4-Me-dT specifically induces A·T → G·C transitions in *E. coli* at the original site of adduct incorporation.

MATERIALS AND METHODS

Materials. O^4-Me-dTTP, O^4-ethyl-dTTP, O^4-isopropyl-dTTP, and O^2-Me-dTTP were prepared as previously described (2, 4). The O^4-Me-dTTP was further purified by HPLC on a Partisil 10 SAX column (4.6 mm × 25 cm; Whatman) eluted with a 60-min linear gradient of 0.025 M KH_2PO_4, pH 3.35, to 0.6 M KH_2PO_4, pH 4.1, at a flow rate of 1 ml/min. The fractions containing O^4-Me-dTTP (retention time of O^4-Me-dTTP = 62 min; retention time of dTTP = 56 min) were concentrated by lyophilization and desalted by HPLC on an Ultrasphere ODS column (4.6 mm × 25 cm; Beckman) isocratically eluted with distilled H_2O at a flow rate of 1 ml/min. The fractions containing O^4-Me-dTTP (retention time = 3.7 min) were concentrated by lyophilization and stored at −20°C in distilled H_2O. The O^4-Me-dTTP prepared in this way was >99.5% pure as determined by HPLC on the Partisil 10 SAX column monitored by UV absorbance at 254 nm.

pol I (18) and single-stranded φX174 *am*3 DNA (19) were purified as previously described. The HF4704 (nonsuppres-

Abbreviations: O^4-Me-dT, O^4-methyl-2′-deoxythymidine; pol I, *E. coli* DNA polymerase I; AMV pol, avian myeloblastosis virus RNA-dependent DNA polymerase; Sup$^+$, amber suppressor phenotype; Sup$^-$, nonsuppressor phenotype; 15-mer, 16-mer, and 17-mer, oligodeoxyribonucleotide pentadecamer, hexadecamer, and heptadecamer, respectively.

‡To whom reprint requests should be addressed.

sor phenotype; Sup$^-$), HF4714 (amber suppressor phenotype; Sup$^+$), and KT-1 (*ada*$^+$) *E. coli* strains are described elsewhere (19), and the GW5352 (*ada*$^-$) *E. coli* strain was kindly provided by Graham C. Walker (Department of Biology, Massachusetts Institute of Technology; see ref. 20). The large fragment of pol I, avian myeloblastosis virus RNA-dependent DNA polymerase (AMV pol), and T4 polynucleotide kinase were obtained from Bethesda Research Laboratories. dNTPs and custom-synthesized oligodeoxyribonucleotides were purchased from Pharmacia P-L Biochemicals. All other reagents were supplied by New England Nuclear, Bio-Rad, Sigma, or Bethesda Research Laboratories.

DNA Polymerization Assay. ϕX174 *am*3 (+) strand [^{3}H-dT]DNA (0.057 pmol/μl; 0.1 μCi/pmol; 1 Ci = 37 GBq) was primed (21) in 300 mM KCl/100 mM Hepes, pH 7.3, with a 5-fold molar excess of a (−) strand synthetic oligodeoxyribonucleotide pentadecamer [15-mer; 5′-d(AAAGCGAGGGTATCC)-3′] that hybridizes to nucleotide positions 588–602 of the ϕX174 genome (22); this yields a 15-mer/ϕX174 *am*3 partial-duplex molecule with the 3′-terminal dC residue of the oligonucleotide immediately adjacent to the dA residue at nucleotide position 587 of the amber codon. The primed template (0.0023 pmol/μl) was then incubated with the following components at 30°C for 15 min in a total volume of 25 μl: 20 mM Tris·HCl, pH 8.0; 4 mM Hepes, pH 7.3; 12 mM KCl; 10 mM $MgCl_2$; 5 mM dithiothreitol; either pol I (0.46 unit/μl; units as defined in ref. 18) or AMV pol (0.064 unit/μl; units as defined in ref. 23); 50 μM each of dATP, dGTP, and [α-^{32}P]dCTP (0.25 Ci/mmol); and 50 μM of either dTTP or O^4-Me-dTTP. The reactions were stopped by the addition of EDTA to 15 mM final concentration, and the average number of nucleotides incorporated per template was calculated from the perchloric acid-precipitable radioactive material (24).

Site-Specific Extension of Oligonucleotides. ϕX174 *am*3 DNA (0.057 pmol/μl) was hybridized (21) with a 2-fold molar excess of 5′-^{32}P-labeled 15-mer (ref. 25, pp. 122–123). The 15-mer/ϕX174 partial-duplex DNA (0.011 pmol/μl) was incubated at 37°C for 30 min (total volume = 25 μl) in the presence of 20 mM Tris·HCl, pH 8.0; 20 mM Hepes, pH 7.3; 60 mM KCl; 5 mM $MgCl_2$; 2 mM dithiothreitol; either pol I (0.14–0.28 unit/μl), the large fragment of pol I (0.08 unit/μl), or AMV pol (0.10 unit/μl); and 50 μM of a single dNTP or *O*-alkyl-dTTP. All reactions were stopped by the addition of EDTA to 20 mM final concentration, and the products were analyzed by polyacrylamide gel electrophoresis.

Polyacrylamide Gel Electrophoresis (PAGE) of Extended Oligonucleotides. The reaction mixtures were eluted with distilled H_2O through 0.5-ml Sephadex G-100 columns (ref. 25, pp. 464–467) to remove nonhybridized oligonucleotides. The column eluates were dried under reduced pressure at 23°C and analyzed by electrophoresis through 16% polyacrylamide sequencing gels (ref. 25, p. 478). The yields of extended oligonucleotides were determined from the autoradiographs by densitometry on a Hoefer GS-300 scanning densitometer (Hoefer, San Francisco).

Mutagenesis Assay. ϕX174 *am*3 DNA (0.028 pmol/μl) was hybridized (21) with a 5-fold molar excess of the synthetic 15-mer, and the 15-mer/ϕX174 partial-duplex DNA (0.0023 pmol/μl) was incubated at 30°C for 30 min (total volume = 50 μl) in the presence of 20 mM Tris·HCl, pH 8.0; 8 mM Hepes, pH 7.3; 24 mM KCl; 5 mM $MgCl_2$; 2 mM dithiothreitol; pol I (0.14 unit/μl); and 50 μM either dTTP or O^4-Me-dTTP. The 3′-terminal adduct was "sealed" into the nascent (−) strand by continued polymerization (15 min at 30°C) with 100 μM each of dATP, dCTP, dGTP, and dTTP, and the product partial-duplex molecules were transfected into spheroplasts prepared from KT-1 (*ada*$^+$) or GW5352 (*ada*$^-$) *E. coli* (19). The spheroplasts were lysed after incubation at 37°C for 2 hr, and the progeny phage were titered on HF4714 (Sup$^+$) and HF4704 (Sup$^-$) *E. coli* (19).

Nucleotide Sequence Determination. The DNAs from plaque-purified revertant phage were isolated by the procedure of Kunkel *et al.* (19) with the following modifications: (*i*) $MgSO_4$ was omitted from the culture medium (total volume = 20 ml), and the phage-infected HF4704 (*sup*$^-$) cells were permitted to lyse without the addition of lysozyme; (*ii*) the CsCl- and sucrose-gradient centrifugation steps were omitted; and (*iii*) the isolated DNA was incubated with heat-treated RNase A (50 μg/ml; ref. 25, p. 451) at 37°C for 10 min prior to sequencing. The nucleotide sequences were determined by the method of Sanger *et al.* (22), using a 5′-^{32}P-labeled 15-mer primer that hybridizes to nucleotides 597–611 of the ϕX174 genome (22).

RESULTS

Strategy for Site-Specific Mutagenesis. As a target for mutagenesis, we selected nucleotide 587 of the amber codon of bacteriophage ϕX174 *am*3 DNA. The experimental approach involves two steps of DNA polymerization (Fig. 1). In the first step, a synthetic 15-mer [5′-d(AAAGCGAGGGTATCC)-3′] is hybridized to nucleotide positions 588–602 of single-stranded ϕX174 *am*3 DNA such that the 3′-terminal dC residue of the oligonucleotide is immediately adjacent to the dA residue at nucleotide 587 of the amber codon (TAG); a dT analogue is then incorporated onto the 3′ terminus of the 15-mer by incubating the primed DNA in the presence of polymerase and the corresponding purified dTTP analogue. In the second step, the incorporated analogue is "sealed" into the nascent oligonucleotide by additional polymerization in the presence of unmodified dATP, dCTP, dGTP, and dTTP. The partial-duplex DNA containing a single dT analogue at position 587 of the (−) strand is then transfected into *E. coli* spheroplasts, where the DNA is replicated by the cellular DNA polymerase III replication complex (ref. 17, pp. 172–178). The progeny phage can then be titered on Sup$^+$ and Sup$^-$ indicator bacteria to identify mutants (19). Under these conditions, nonmutants resulting from the incorporation of dA residues opposite the dT analogue in the DNA will retain the *am*3 codon and grow only on Sup$^+$ *E. coli*, whereas mutants resulting from the misincorporation of dC, dG, or dT residues opposite the analogue will revert the *am*3 codon to wild type and grow on both Sup$^+$ and Sup$^-$ *E. coli*. Studies on ϕX174 *am*3 DNA have shown that substitution of a dC, dG, or dT residue at position 587 of the (+) strand yields viable wild-type phage (12, 13). Therefore, this assay will

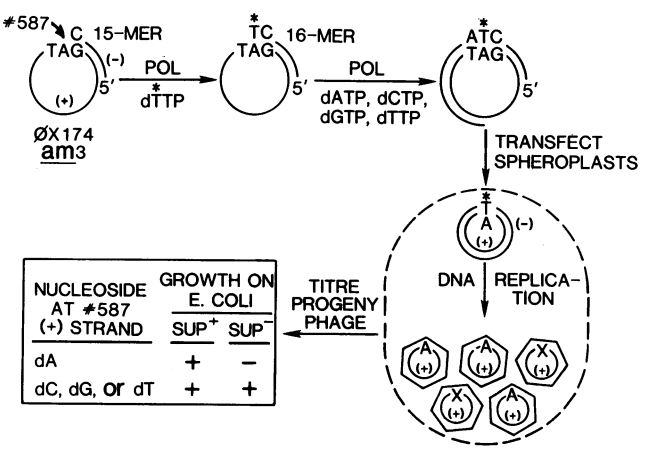

FIG. 1. Assay for the site-specific incorporation and mutagenicity of thymidine analogues. POL, DNA polymerase; T, thymidine analogue; X, C, G, or T.

Table 1. DNA polymerization in the presence of dTTP or O^4-Me-dTTP

dTTP or analogue	Nucleotide incorporation	
	pol I	AMV pol
Control (no dTTP)	2 ± 0.2	2 ± 0.8
dTTP	120 ± 21	94 ± 17
O^4-Me-dTTP	104 ± 8	67 ± 3

Single-stranded φX174 *am3* [^{3}H-dT]DNA was primed with a synthetic 15-mer and incubated at 30°C for 15 min with pol I or AMV pol in the presence of dATP, [α-^{32}P]dCTP, dGTP, and either dTTP or O^4-Me-dTTP. The extent of DNA polymerization was calculated from acid-precipitable radioactive material and is expressed as total nucleotides incorporated per template per unit of DNA polymerase (mean ± SD of four determinations from two separate experiments).

detect all possible base substitutions opposite the dT analogue.

The feasibility of this scheme, applied to the O^4-Me-dT analogue, relies on the ability of DNA polymerases to accept O^4-Me-dTTP as a substrate for the polymerization of natural, heteropolymeric DNA and then to extend primers containing the 3′-terminal analogue.

O^4-Me-dTTP as a Substrate for DNA Polymerases. To measure the ability of O^4-Me-dTTP to serve as a substrate for DNA synthesis, we examined the polymerization of φX174 DNA in the presence of dATP, dCTP, dGTP, and either O^4-Me-dTTP or unmodified dTTP (Table 1). Two polymerases were studied: pol I, a highly accurate DNA polymerase with an associated 3′→5′ exodeoxyribonuclease proofreading activity, and AMV pol, an error-prone DNA polymerase with no associated 3′→5′ exodeoxyribonuclease (ref. 12; ref. 17, pp. 221–225). Both polymerases yielded significant nucleotide incorporation in the presence of either dTTP or O^4-Me-dTTP. Replacement of dTTP with O^4-Me-dTTP decreased the extent of polymerization with pol I and AMV pol by only 13% and 29%, respectively.

Site-Specific Extension of 15-Mers with O^4-Me-dTTP as a Substrate. To determine the efficacy of using DNA polymerases for the site-specific incorporation of O^4-Me-dT residues, φX174 *am3* DNA was primed with 5′-^{32}P-labeled 15-mer, and the primed DNA was copied in the presence of single dNTPs or O^4-Me-dTTP and saturating amounts of polymerase and analyzed by PAGE (Fig. 2). As predicted from the nucleotide sequence of the template DNA, AMV pol efficiently extended the primer with the "correct" nucleotide, dTTP, to give about a 45% yield of 16-mer (Fig. 2 *Left*). Small amounts of 17-mer (11%) and 18-mer (3%) were also formed, presumably by the misincorporation of dT residues opposite the dT and dG residues on the template strand. The ability of AMV pol to insert mismatches during polymerization (12) was also demonstrated by the extension of primers in reactions containing the "incorrect" nucleotides dATP (5% yield) or dCTP (21% yield). Similar to dTTP, O^4-Me-dTTP supported primer extension to yield relatively large amounts of 16-mer (39%) and 17-mer (23%) as well as small amounts of 18-mer (1%). Other *O*-alkyl-dTTPs were also examined as substrates for primer extension by AMV pol (Fig. 2 *Left*), and in all cases the total yields of extended primers (52–63%) were similar to the yield with the "correct" nucleotide, dTTP (69%), and much greater than those with the "incorrect" nucleotides, dGTP, dATP, or dCTP (0–21%).

In contrast to AMV pol, extension of the primer with pol I and either dTTP or O^4-Me-dTTP was limited to the incorporation of single nucleotides to form exclusively 16-mers in almost quantitative yields (Fig. 2 *Right*). Similar yields of 16-mers were also formed from O^4-ethyl- and O^4-isopropyl-dTTPs (data not shown). In the absence of dNTPs or in the presence of the "incorrect" nucleotides dATP or dGTP, radioactive bands were not observed; this was presumably due to digestion of the oligonucleotides by the exonucleases associated with pol I (ref. 17, pp. 127–130, 134–142). The presence of dCTP prevented primer digestion by providing the correct nucleotide at position 15 of the 15-mer, but this nucleotide did not support significant primer extension to 16-mers. Similar results were observed with either the large fragment of pol I (Fig. 2 *Right*) or intact pol I (data not shown).

Evidence for the Presence of O^4-Me-dT in the Extended Primers. Examination of the electrophoresis patterns from the reaction mixtures containing O^4-alkyl-dTTPs and AMV pol (Fig. 2 *Left*) showed that the mobilities of the extended primers were altered compared to the primers extended by dTTP. As the size of the side group on the O^4-alkyl-dTTPs was increased from methyl to ethyl to isopropyl, there was a concomitant decrease in the electrophoretic mobilities of the extended primers. This was most dramatically seen in the primers derived from O^4-ethyl- or O^4-isopropyl-dTTP, particularly in the 17- and 18-mers, where the incorporated O^4-alkyl-dT residues would represent 12% and 17%, respectively, of the total nucleotides in the extended primers. In polymerizations with greater yields of extended primers (obtained by increasing the incubation time or the ratio of AMV pol to template), a distinct decrease in the mobility of the 18-mer derived from O^4-Me-dTTP was also observed (data not shown). No changes in mobility were observed in any of the 16-mer products, where the newly incorporated single O^4-alkyl-dT residues would comprise only 6% of the total nucleotides in the extended primers.

The pattern of altered electrophoretic mobilities of the 17- and 18-mers derived from the O^4-alkyl-dTTPs provides

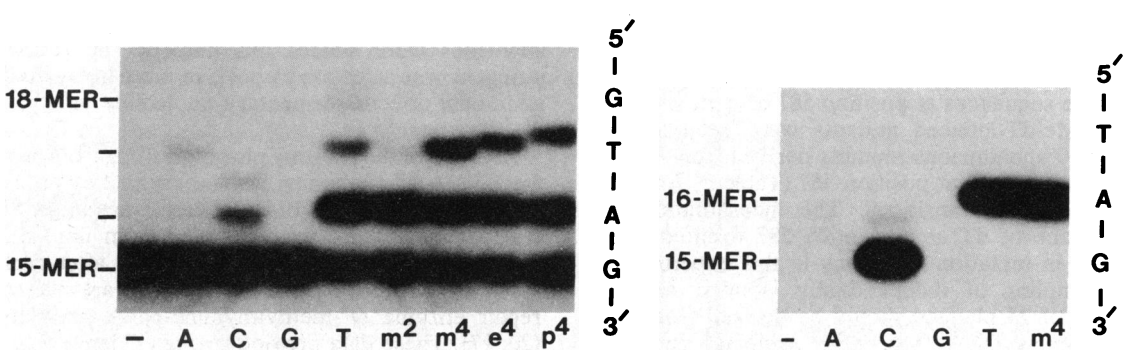

FIG. 2. PAGE of oligonucleotides site-specificially extended by AMV pol (*Left*) or the large fragment of pol I (*Right*). 5′-^{32}P-Labeled 15-mer was hybridized to single-stranded φX174 *am3* (+) strand DNA and incubated with polymerase and either no dNTPs (indicated as –) or a single dNTP (A, dATP; C, dCTP; G, dGTP; T, dTTP; m^2, O^2-Me-dTTP; m^4, O^4-Me-dTTP; e^4, O^4-ethyl-dTTP; p^4, O^4-isopropyl-dTTP). The nucleotide sequence in the right margin of each autoradiograph indicates the sequence of the template strand. The oligonucleotide lengths are indicated in the left margins.

Table 2. Mutagenicity of O^4-Me-dT

Nucleoside at position 587 in (−) strand	Reversion frequency × 10^6			
	Exp. 1	Exp. 2	Exp. 3	Mean
dT	1.2	2.5	2.0	1.9
O^4-Me-dT	30.2	6.6	21.7	19.5

dT or O^4-Me-dT residues were site-specifically incorporated by pol I at nucleotide position 587 of the (−) strand of φX174 *am*3 DNA (Fig. 2 *Right*), and the product duplex molecules were allowed to replicate *in vivo* after transfection into GW5352 (*ada*⁻) *E. coli* spheroplasts (Fig. 1). Progeny phage were titered on Sup^+ and Sup^- indicator bacteria, and the reversion frequencies were calculated as the ratio of Sup^- to Sup^+ titers (19). The average Sup^- titers from the DNAs containing dT or O^4-Me-dT at position 587 were 0.8 or 11.5 × 10^3 progeny phage per 100 ng of transfected DNA, respectively; both the dT- and O^4-Me-dT-containing DNAs yielded similar Sup^+ titers (4.5 and 4.7 × 10^8 progeny phage per 100 ng of transfected DNA, respectively). The data in the table represent the results of three separate experiments from three different preparations of dT- or O^4-Me-dT-containing 16-mers.

strong evidence that these oligonucleotides contain the O^4-alkyl-dT adducts. Characterization of the O^4-Me-dTTP-extended oligonucleotides by nearest-neighbor analysis (26), after further polymerization in the presence of [α-^{32}P]dATP, provided additional evidence that this adduct was incorporated by both AMV pol and pol I at position 587 of the nascent φX174 DNA strand (data not shown).

Mutagenicity of O^4-Me-dT. To determine the mutagenic potential of O^4-Me-dT *in vivo*, the 16-mer/φX174 *am*3 duplex DNA containing a single O^4-Me-dT residue at position 587 (prepared by pol I; Fig. 2 *Right*) was further extended by unmodified dNTPs and transfected into *E. coli* spheroplasts. Replication of this DNA in wild-type spheroplasts failed to yield an increase in mutation frequency above that observed from control DNA containing unmodified dT residues at position 587 (data not shown). However, about a 10-fold increase in mutagenesis was observed when the O^4-Me-dT-containing DNA was transfected into spheroplasts prepared from *E. coli* containing an inactivating mutation in the O^4-methylthymine-DNA methyltransferase gene, *ada* (Table 2). The cause of the variation of O^4-Me-dT-induced mutagenesis (3- to 25-fold above the dT control) is not clear. The three preparations of O^4-Me-dT-containing DNA used in these experiments contained virtually identical levels of adduct. Therefore, this phenomenon is not due to a simple dose effect. It is more likely that the variability in mutagenesis is somehow related to variations in the efficiencies of DNA transfection and expression in different preparations of spheroplasts. The detection of wild-type revertant phage from adduct-containing DNA required the use of highly competent spheroplasts (≥1.8 × 10^8 progeny phage per 100 ng of transfected DNA) and φX174 *am*3 DNA yielding low frequencies of spontaneous revertants (≤2.5 × 10^{-6}).

The nucleotide sequences at position 587 of both spontaneous and O^4-Me-dT-induced mutants were determined. Twenty out of 20 spontaneous mutants derived from DNA containing unmodified dT at position 587 (Table 2, Exp. 1) exhibited A·T → G·C transitions. The incorporation of O^4-Me-dT in place of dT at nucleotide 587 resulted in a 25-fold increase in mutation frequency in this experiment. Thus, in a sampling of independently isolated mutant plaques, 24 out of 25 plaques should be derived from the O^4-Me-dT-containing DNA. Nucleotide sequence determinations of the mutants induced by O^4-Me-dT showed that, in all cases, there was an A·T → G·C transition at position 587 (20 out of 20 revertants sequenced; Fig. 3). When O^4-Me-dTTP was replaced with dCTP during the first step of polymerization by pol I (Fig. 1), there was no increase in

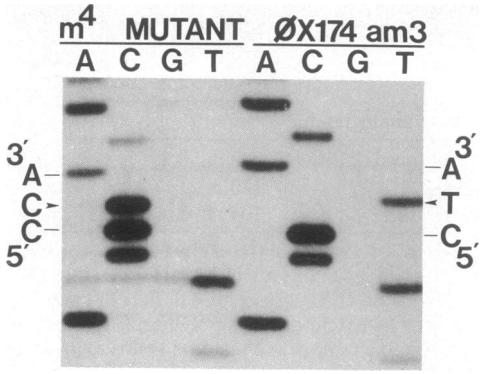

FIG. 3. Nucleotide sequence determination of DNA from φX174 *am*3 phage and mutant phage induced by O^4-Me-dT (m^4). The nucleotide sequences in the left and right margins correspond to the sequences of the (−) strands read directly from the autoradiograph. The nucleotides at position 587 are indicated with arrows.

mutation frequency. Therefore, the mutant phage from O^4-Me-dTTP-polymerized DNA were not the result of the misincorporation of contaminating dCTP *in vitro*. In control experiments in which dA residues were site-specifically incorporated in low yields by AMV pol (Fig. 2 *Left*), there was an 8-fold increase in mutation frequency; correspondingly, of the 40 revertants sequenced, 36 contained A·T → T·A transversions (i.e., induced mutations) and only 4 contained A·T → G·C transitions (i.e., spontaneous mutations).

DISCUSSION

We have adapted techniques for DNA polymerase-catalyzed, site-specific mutagenesis (13) to synthesize an oligonucleotide with a single O^4-Me-dT residue at a predetermined site. This procedure relies on the ability of polymerases to accept O^4-Me-dTTP as a substrate for incorporation opposite a dA residue in φX174 *am*3 DNA. Singer *et al.* (2, 4, 14) recently showed that O^4-alkyl-dTTPs can substitute for dTTP during pol I-catalyzed polymerization of activated salmon sperm DNA or poly(dA-dT) *in vitro*, and these investigators have postulated an O^4-alkyl-T·A base-pairing scheme to account for this phenomenon. Our data corroborate and extend these findings by showing that high concentrations of AMV pol, as well as pol I, can copy long stretches of natural, heteropolymeric DNA under conditions in which O^4-Me-dTTP is the only source of thymidine nucleotide substrate (Table 1). The PAGE data on the extension of the oligonucleotides (Fig. 2) provide direct evidence that polymerases can catalyze the incorporation of O^4-alkyl-dTTPs opposite dA residues in natural DNA. With AMV pol, extension occurred beyond a single nucleotide to yield oligonucleotides with multiple 3′-terminal analogues. However, with pol I, only single dT analogues were added; this may be the result of more stringent base selection by pol I, or more likely, hydrolysis of additional noncomplementary nucleotide analogues by the 3′→5′ exonuclease.

Analysis of the progeny phage resulting from replication of the O^4-Me-dT-containing DNA (prepared by pol I) in *E. coli* spheroplasts showed a 10-fold increase in mutation frequency when compared to phage derived from unmodified DNA (Table 2). Mutants were detected only after replication in spheroplasts from *ada*⁻ *E. coli*, which are defective in the repair enzyme O^4-methylthymine-DNA methyltransferase (20, 27). These data provide strong evidence that the mutations were induced specifically by the O^4-Me-dT residues. DNA sequencing showed that all of the mutants contained dGs in place of the dA residues that were originally present opposite the O^4-Me-dT adduct. The induction of A·T → G·C mutations at the original site of O^4-Me-dT incorporation is

consistent with a mechanism of "targeted" mutagenesis involving the formation of O^4-Me-T·G base pairs during DNA replication *in vivo*. Support for this mechanism comes from studies which have shown that O^4-Me-dT adducts frequently mispair with dG residues when copied by purified DNA polymerases *in vitro* (2–5). The replication of ϕX174 DNA in *E. coli* is catalyzed by the cellular DNA polymerase III replication complex, which is also responsible for the replication of *E. coli* DNA (ref. 17, pp. 172–178). Thus, it appears that the polymerase III complex *in vivo*, like purified pol I *in vitro* (2–5), frequently recognizes O^4-Me-dT residues as dC residues in template DNA. On the basis of the efficiencies of hybridization and subsequent expression of the (−) strand of oligonucleotide/ϕX174 duplex DNA in spheroplasts (28), we estimate that O^4-Me-dT nucleotides base pair with dG nucleotides approximately once per 2100 adducts copied. A higher frequency is likely, however, since this estimate assumes that the polymerase III complex encounters an adduct at position 587 in every viable (−) strand molecule transfected into the spheroplasts, and no correction has been made for the probable partial repair of these adducts by residual methyltransferase activity known to be present in the GW5352 (*ada*$^-$) *E. coli* (J. Essigmann, personal communication) or for the "spontaneous" conversion of O^4-Me-dT to dT that may occur during our procedures (29).

In summary, our data show that O^4-Me-dT adducts are mutagenic in *E. coli* and suggest that these adducts play a role in mutagenesis by methylating agents. Loechler *et al.* (30) have recently shown that O^6-Me-dG adducts are also mutagenic when site-specifically inserted into *E. coli* replication vectors. Thus, mutagenesis by methylating agents in *E. coli* may be induced by at least two different adducts. The relative contributions of each adduct to mutagenesis might be inferred from the frequencies of the mutations characteristic of each adduct; O^4-Me-dT nucleotides induce A·T → G·C transitions (data in this paper), whereas O^6-Me-dG nucleotides induce G·C → A·T transitions (30). Experiments characterizing mutations induced by *N*-nitroso methylating agents in *E. coli* suggest that both adducts contribute to mutagenesis, with the major contribution coming from O^6-Me-dG (7, 11). However, to directly assess the relative mutagenicities of these adducts, it will be necessary to study the frequencies of mutations induced by O^4-Me-dT and O^6-Me-dG after site-specific insertion in identical *E. coli* replication vectors.

Studies on site-specific mutagenesis by nucleotide adducts have relied on the chemical synthesis of adduct-containing oligonucleotides by methods often employing relatively harsh chemical treatments that may modify the adducts or adjacent nucleotides (31, 32). The protocol presented in this paper provides a rapid and simple alternative approach to site-specific mutagenesis that involves the enzymatic incorporation of nucleotide analogues under mild conditions (30–37°C, pH 8). Our data illustrate the applicability of this procedure for studying the O^4-Me-dT adduct and suggest that other analogues might be similarly studied. We have obtained good yields of site-specifically modified oligonucleotides containing O^2-Me-dT, O^4-Me-dT, O^4-ethyl-dT, O^4-isopropyl-dT, O^6-Me-dG, 1-Me-dA, 3-Me-dC, 5-Me-dC, 5-Br-dC, or 5-Br-dU residues (data in this paper and B.D.P. and L.A.L., unpublished data). In principle, this technique is limited only by the abilities of polymerases to accept dNTP analogues as substrates for DNA polymerization and to extend the product oligonucleotides containing 3′-terminal analogues.

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