# *In vitro* DNA replication implicates O²-ethyldeoxythymidine in transversion mutagenesis by ethylating agents

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## ABSTRACT

A 36-nucleotide oligomer containing a single O²-ethyldeoxythymidine (O²-Et-dT) adduct at a specific site was synthesized. The oligomer, which corresponds to a specific DNA sequence in gene G of bacteriophage φX174, was used as a template by T7 DNA polymerase to investigate the *in vitro* mutagenic specificity of O²-Et-dT. At 10 μM dNTP and 5 mM Mg⁺⁺, the progress of T7 DNA polymerase was interrupted by O²-Et-dT: 80% 3′ to O²-Et-dT and 14% after incorporating a nucleotide opposite O²-Et-dT (incorporation-dependent blocked product). DNA synthesis past the lesion was low (6%). Incorporation of a nucleotide opposite O²-Et-dT and subsequent postlesion synthesis were enhanced by increasing the dNTP concentration, with postlesion synthesis reaching 30% at 200 μM. Postlesion synthesis was further increased to 45% by addition of 10 mM dAMP to the polymerization reactions. DNA sequencing revealed that both dA and dT were incorporated opposite O²-Et-dT with dA incorporation impeding the progress of DNA synthesis. dT incorporation was efficiently extended implicating O²-Et-dT in transversion mutagenesis *in vivo*. These studies provide a basis for understanding the molecular mechanisms by which ethylating agents contribute to cytotoxicity, A·T transversion mutagenesis and activation of the oncogene *neu* by an A·T→T·A transversion event in rat neuroblastomas.

## INTRODUCTION

N-nitroso-alkylating agents (directly or after metabolic activation) modify DNA bases to form a diverse set of covalent adducts both *in vitro* and *in vivo* (1–3). Major alkylation sites include nucleophilic oxygens, such as the O⁶-position of dG, the O⁴- and O²-positions of dT, and phosphodiester groups as well as ring-nitrogens. Alkylation of DNA bases produces both miscoding and noncoding lesions. The miscoding mutagenic lesions alter the precision of base-pairing during DNA replication, stimulating the formation of mispairs such as O⁶-alkyl-dG·dT and O⁴-alkyl-dT·dG (reviewed in 4; 5). The noncoding lesions usually block

DNA replication and presumably distort the DNA structure sufficiently to interfere with geometric recognition mechanisms preventing effective base insertion opposite the lesion. At a highly distorted site, even a correctly inserted base may not be acceptable to the replication complex, leading to inhibition of DNA synthesis. Support for this hypothesis is provided by *in vitro* DNA replication studies of the lesions that block DNA synthesis such as N3-Et-dT, where incorporation of dA opposite the lesion terminates *in vitro* DNA replication (6,7). Presumably, alignment of the N3-Et-dT·dA base pair inhibits formation of a phosphodiester bond 5′ to the base pair. Mutagenesis by DNA replication-blocking lesions in bacteria is SOS dependent (8,9).

N-ethyl-N-nitrosourea (ENU) is one of an important class of potentially mutagenic and carcinogenic nitroso-alkylating agents. ENU is a potent carcinogen in animals (10,11) and humans (12). Tumors of the nervous system induced by transplacental treatment of rats with ENU, contained *neu* oncogene activated by an A·T→T·A transversion mutation (13,14). The biological significance of ENU-induced A·T transversions in other systems has also been demonstrated (15–17). The ENU-induced DNA lesions responsible for A·T transversions are not known.

In *Escherichia coli* (*E. coli*), ENU predominantly induces G·C→A·T and A·T→G·C transition mutations (18–20). Transversion mutations at A·T base pairs are induced at low efficiency (18,19) and are increased several fold under conditions of SOS induction (19). The G·C→A·T and A·T→G·C transition mutations presumably result from the unrepaired miscoding lesions O⁶-ethyldeoxyguanosine (O⁶-Et-dG) (21,22) and O⁴-ethyldeoxythymidine (O⁴-Et-dT) (23), respectively, as a consequence of their capacity to mispair during DNA replication. In the human cell, ENU induces a significant number of transversion mutations at A·T base pairs (24) in addition to the same G·C→A·T and A·T→G·C transition mutations observed in *E. coli*. Again, the ENU-induced lesions responsible for transversion mutations at A·T base pairs in bacteria and human cells are not known.

Based on the comparison of base-substitution mutations observed among alkylating agents, it has been suggested (24) that O²-Et-dT may be a significant premutagenic lesion, capable of inducing A·T→T·A transversion mutations. However, the involvement of other lesions produced by ENU, such as A or

T adducts and/or a breakdown product of these adducts, cannot be ruled out. Recently, we studied the mutagenic potential of N3-Et-dT (formed both *in vitro* and *in vivo* by ethylating agents) site-specifically placed in a DNA template corresponding to a sequence in gene G of bacteriophage $\phi$X174. Our studies suggested a dual role for N3-Et-dT (6,7). The incorporation of dA opposite this lesion blocks DNA replication *in vitro* and may terminate DNA synthesis *in vivo*, contributing to the cytotoxicity of the ethylating agents. Incorporation of dT opposite N3-Et-dT permitted postlesion synthesis and suggested a promutagenic role for N3-Et-dT *in vivo*, leading to the induction of A·T→T·A transversion mutations.

A wide range of independent studies indicate that the mammalian cells lack the well-defined *E. coli* mechanisms for the repair of alkyl-pyrimidine lesions (25,26), which may be processed differently in these organisms (19,24). No enzyme has been isolated from a eukaroytic source that can measurably repair or remove $O^2$-Et-dT from DNA (25). $O^2$-Et-dT is among the most persistent DNA alkylation product in both cultured mammalian cells and animal tissues (27).

DNA polymerase from bacterial and mammalian sources may act differently when encountered by a specific lesion (28). In the *in vitro* DNA replication studies reported here, DNA synthesis was catalyzed by T7 DNA polymerase. The polymerase is a highly-processive enzyme with a rapid rate of DNA synthesis. The results demonstrate that the $O^2$-Et-dT lesion present at a single site in a DNA template incorporates both dA and dT when encountered by T7 DNA polymerase. Incorporation of dA opposite $O^2$-Et-dT reduces the efficiency of DNA synthesis *in vitro*. Formation of the $O^2$-Et-dT·dT base pair at the replication fork is not inhibitory to DNA synthesis. Efficient bypass of the $O^2$-Et-dT lesion occurs leading to an A·T→T·A transversion mutation. Our results suggest that formation of the persistent $O^2$-Et-dT lesion in DNA is biologically significant and may contribute to cytotoxic, mutagenic and carcinogenic processes long after the original exposure has occurred.

## MATERIALS AND METHODS

Ultra-pure grade dNTP, deoxythymidine (dT), deoxyadenosine-5'-monophosphate (dAMP) and T7 DNA polymerase were purchased from Pharmacia P-L Biochemicals. One unit of T7 DNA polymerase catalyzes the incorporation of 50 nmoles of total nucleotide into an acid-insoluble product in 30 min at 37°C utilizing M13 mp 19 (+) DNA as template. $\gamma$-$^{32}$P-labeled adenosine 5'-triphosphate (ATP) was obtained from Du Pont-New England Nuclear. T4 polynucleotide kinase was obtained from New England Biolabs. Silica gel (Merck, grade 60, 230−400 mesh, 60Å), 1-ethyl-3-nitro-1-nitrosoguanidine, 1,8-diazabicyclo[5,4,0]undec-7-ene (DBU), N,N-diisopropyl-ethylamine and the reagents used in DNA sequencing were purchased from Aldrich. The ultra-pure electrophoresis reagents were purchased from BioRad. EM Science-prepared thin-layer chromatography (TLC) plates, silica gel 60 $F_{254}$, were obtained from VWR Scientific. Chloro-N,N-diisopropylaminocyano-ethoxyphosphine was purchased from American Bio-Nuclear. The diisopropylamine, tetrahydrofuran, ethyl alcohol and other high-purity solvents were dried by distillation from calcium hydride and stored over molecular sieves 4Å (Fisher Scientific) under an atmosphere of nitrogen. All other chemicals were of high-grade quality and purchased from different sources. Synthesis and site-specific incorporation of $O^2$-Et-dT into an

oligomer are described below. Silica gel column chromatography was performed in the presence of 0.1% pyridine. TLC plates were developed in the following solvent systems: I, chloroform:methanol (90:10); II, benzene:acetone (40:60); and III, chloroform:ethylacetate: triethylamine (45:45:10).

## Synthesis of $O^2$-Ethyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxy-thymidine [DMTr($O^2$-Et)dT] (Fig. 1)

Diazoethylation of 5'-O-dimethoxytrityl-2'-deoxythymidine (DMTr-dT) (29) was accomplished as described (6). The N3-, $O^4$- and $O^2$-ethyl derivatives of DMTr-dT produced in the reaction mixture were separated by two successive rounds of silica gel column chromatography. In the first chromatography, the column (2×50 cm) was eluted with chloroform containing increasing amounts of methanol (1−5%). The compounds eluted out of the column in the following order: DMTr(N3-Et)dT, DMTr($O^4$-Et)dT, DMTr($O^2$-Et)dT and DMTr-dT. The fractions (5 ml/5 min) containing DMTr($O^2$-Et)dT (TLC; solvent system II) were pooled and concentrated under reduced pressure. The TLC analysis showed that the material was not homogeneous. The contaminants, DMTr($O^4$-Et)dT and the starting material DMTr-dT, were removed by a second silica gel column (2×50 cm) chromatography. Benzene containing increasing concentrations of acetone (30−70%) eluted the compounds from the column in the following order: DMTr-dT, DMTr($O^4$-Et-dT) and DMTr($O^2$-Et)dT. The fractions (5 ml/5 min) containing DMTr($O^2$-Et)dT (single spot on TLC; solvent system II) were pooled and the product recovered as described for DMTr(N3-Et)dT (6). The yield was 22%. DMTr($O^2$-Et)dT was homogeneous as judged by TLC (solvent system II; RF = 0.16) and free from contaminants, including DMTr($O^4$-Et)dT (RF = 0.30), DMTr-dT (RF = 0.42) and DMTr(N3-Et)dT (RF = 0.64). DMTr($O^2$-Et)dT was characterized (see Results) by chemical ionization (CI) mass spectrometry (MS) using a VG-70SE high resolution mass spectrometer (30).

The structure of DMTr($O^2$-Et)dT was confirmed by nuclear magnetic resonance (NMR) spectroscopic analysis using a General Electric QE 300 spectrometer (New York University Chemistry Department) operating at 300 MHz for protons. Chemical shift ($\delta$) assignments for the protons of DMTr($O^2$-Et)dT in dimethylsulfoxide are as follows: 1.28 (t, 3, J = 7.1 Hz, $O^2$-CH$_2$CH$_3$); 1.47 (s, 3, C5-CH$_3$); 2.17−2.37 (m, 2, 2'-CH$_2$); 3.15−3.23 (m, 2, 5'-CH$_2$); 3.72 [s, 6, (OCH$_3$)$_2$ of DMTr]; 3.89−3.95 (m, 1, 4'-H); 4.26−4.40 (q, 3, J = 6.9 Hz, $O^2$-CH$_2$-CH$_3$ and 3'-H); 5.38 (d, 1, J = 4.5 Hz, 3'-OH); 6.12 (t, 1, J = 6.5 Hz, 1'-H); 6.8−7.5 (m, 13, aromatic protons of DMTr); 7.61 (s, 1, C6-H). These assignments are based on one- and two-dimensional proton homonuclear correlation spectra.

## Removal of the Dimethoxytrityl (DMTr) Group from DMTr($O^2$-Et)dT to Yield $O^2$-Et-dT (Fig. 1)

DMTr($O^2$-Et)dT (0.5 mmol) was detritylated with zinc bromide (31). After desalting on a Sephadex G-25 column [1×40 cm; elution with ethanol:water (20:80)], the fractions containing $O^2$-Et-dT were evaporated to dryness and purified by two successive rounds of silica gel column chromatography as described for N3-Et-dT (6). The yield was 17%. Purified $O^2$-Et-dT was homogeneous as judged by its mobility as a sharp single spot on TLC (solvent system II; RF = 0.06). No contaminants such as $O^4$-Et-dT (RF = 0.21), N3-Et-dT (RF = 0.38) and dT (RF = 0.25) were detected. After crystallization from

isopropanol:acetone (12:88), $O^2$-Et-dT was compared by TLC, HPLC, UV and MS with the authentic sample prepared as described below. The TLC was developed in solvent system II. HPLC was performed using a Beckman Ultrasphere ODS 5 $\mu$m column (10 mm×25 cm) eluted with 5% acetonitrile in $H_2O$ at a flow rate of 2 ml/min. The UV spectra of aqueous solutions were obtained at pH 1 (1N HCl), pH 6 ($H_2O$) and pH 13 (0.1 N NaOH).

## Preparation of the Authentic Sample of $O^2$-Et-dT

The authentic sample of $O^2$-Et-dT was prepared as described for the authentic sample of N3-Et-dT (6). The isolated yield was 20%. The authentic sample was fully characterized by TLC, HPLC, UV and MS analyses.

## Preparation of $O^2$-Ethyl-5'-O-(4,4'-dimethoxytrityl)-2'-deoxythymidine-3'-[(2-cyanoethyl)-N,N-diisopropylphosphor-amidite] [DMTr($O^2$-Et)dT-3'-phosphoramidite] (Fig. 1)

The method described for phosphoramidites of normal nucleosides (32) and adapted for DMTr(N3-Et)dT (6) was used starting with 1 mmol of DMTr($O^2$-Et)dT. The phosphoramidite was isolated by silica gel column chromatography (6). The yield was 75%. The product was homogeneous (single sharp spot on TLC; solvent system III; $R_F$ = 0.55) and free from the phosphoramidites of DMTr($O^4$-Et)dT ($R_F$ = 0.65) and DMTr(N3-Et)dT ($R_F$ = 0.68).

## Synthesis of Site-modified Oligodeoxynucleotide

The 21-nucleotide-long oligomer, 5'AATAAAAGTCT*AAAA-CATGAT (T* = $O^2$-Et-dT) was synthesized on an Applied Biosystems Model 381A synthesizer using phosphite triester chemistry (33). $O^2$-Et-dT was introduced at the desired site during synthesis by the use of the DMTr($O^2$-Et)dT-3'-phosphoramidite synthesized above.

After completion of the synthesis and removal of the terminal DMTr group, the resin support was transferred to a small flask and dried by co-evaporation with dry pyridine (2×10 ml), followed by removal of pyridine by co-evaporation with dry toluene (2×10 ml). The resin was treated with 2 ml of the mixture of DBU:tetrahydrofuran:ethanol (14:43:43) under anhydrous conditions with occasional shaking (34,35). After one week at room temperature, the resin was removed by centrifugation and the pellet washed with water (2×0.2 ml). Combined supernatant and washings were immediately extracted with benzene (5×2 ml) to remove DBU. After adjusting the pH to 6.5, the solution was concentrated under reduced pressure and desalted on a Sephadex G-50 column (1×30 cm) with ethanol:water (20:80) as eluting solvents. The fractions containing the site-modified oligomer were concentrated and purified by electrophoresis on a 16% polyacrylamide-8M urea gel. The 21-nucleotide oligomer band was visualized with UV light, excised, crushed and finally eluted in TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) containing 20 mM of NaCl at 37°C for 3 h. The oligomer was recovered by passing the eluent through a Sep-Pak C18 cartridge (Waters Associates) as described by the supplier.

The site-modified oligomer was phosphorylated at the 5'-OH group using T4 polynucleotide kinase and $\gamma$-$^{32}$P ATP, and fully characterized. The homogeneity of the oligomer was checked by electrophoresis on a 20% polyacrylamide-8M urea sequencing gel. The expected DNA sequence was confirmed by sequencing (36); and $O^2$-Et-dT present in the site-modified 21-mer determined by HPLC. For HPLC analysis, the oligonucleotide

(2.0 $A_{260}$) was digested at 37°C for 2 h with snake venom phosphodiesterase (0.5 unit) and bacterial alkaline phosphatase (0.5 unit) in 50 $\mu$l of 20 mM Tris·HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl. The enzymatic digest was mixed with 300 $\mu$l of acetone, chilled at 4°C for 30 min and centrifuged to remove denatured proteins. The acetone solution was evaporated to dryness under reduced pressure, and the residue dissolved in water and analyzed by HPLC on a Beckman Ultrasphere ODS 5 $\mu$m column (10 mm×25 cm) developed with acetonitrile:$H_2O$ (5:95) at a flow rate of 2 ml/min. Under these conditions dC and dG were not separated. Ratios of dA:dT:$O^2$-Et-dT were calculated from the peak areas and the molar absorption coefficients of the nucleosides at 260 nm.

## Preparation of a 36-Nucleotide-long Primed Template Containing $O^2$-Et-dT at a Single Site

The primed template consists of a 36-nucleotide-long site-modified template hybridized to a 17-nucleotide primer. The site-modified template, containing a single $O^2$-Et-dT adduct at position 26 from the 3'-end, was constructed by ligation of the $O^2$-Et-dT-containing 21-mer (125 pmol) to a second $^{32}$P-labeled (<3 Ci/mmol) 15-mer (125 pmol), as previously described (6). The low radioactivity present in the template did not interfere in the following experiments. The site-modified template was annealed with two-fold molar excess of complementary 5'-$^{32}$P-labeled (3,000 Ci/mmol) 17-nucleotide primer (6). $O^2$-Et-dT was stable under the conditions of ligation and annealing used to prepare the primed template. Formation of the primed template was analyzed by electrophoresis on a non-denaturing 12% polyacrylamide gel. A single batch of primed template was used in all DNA replication studies of $O^2$-Et-dT.

A control primed template, containing dT in place of $O^2$-Et-dT, was also prepared in a similar manner.

## DNA Polymerase Reaction

The polymerase reactions were conducted on 0.05 to 0.1 pmol of the primed template in 10 mM Hepes buffer, pH 7.5, containing 1 mM dithiothreitol and 5 mM $Mg^{++}$ (total volume of 8 $\mu$l) at 37°C. The polymerization was started by the addition of 0.2 unit of T7 DNA polymerase. The concentration of dNTPs was varied depending upon the type of study being conducted. In some reactions 10 mM dAMP was used to inhibit the 3'→5'-exonuclease activity associated with T7 DNA polymerase (37). The exact conditions used are documented in the figure legends. DNA synthesis products were analyzed as previously described (6,7).

## Identification of Nucleotide Incorporated Opposite $O^2$-Et-dT

The identity of the nucleotide incorporated opposite $O^2$-Et-dT was established by sequencing (36) the DNA products isolated from large-scale ($\approx$5−10 pmol of the primed template) polymerization reactions. Each product was sequenced three times.

## RESULTS

### Synthesis and Characterization of the 21-Nucleotide Oligomer Containing $O^2$-Et-dT at a Single Site

The $O^2$-Et-dT moiety was incorporated at the desired site (position 11) of the 21-nucleotide oligomer using the synthesized DMTr($O^2$-Et)dT-3'-phosphoramidite. DMTr-dT was chosen as the starting material over dT for the synthesis of

DMTr($O^2$-Et)dT-3′-phosphoramidite for the following reasons. First, an acid labile group (such as the DMTr group) at the 5′-OH of the nucleoside-3′-phosphoramidite molecule (which after addition of the amidite molecule to the synthesizing chain resides at the 5′-end) is a prerequisite in oligonucleotide synthesis with the automated phosphite triester method (33). Second, DMTr-dT is highly soluble in the diazoethylation reaction medium as compared with dT, facilitating ethylation of DMTr-dT. Finally, the presence of the DMTr group facilitated the separation of the products formed in the ethylated reaction mixture. To avoid formation of di-ethylated derivatives of DMTr-dT, diazoethylation was terminated when ≈10% of the starting DMTr-dT remained. DMTr($O^2$-Et)dT (Fig. 1), after separation from the N3- and $O^4$-ethyl derivatives by two successive silica gel chromatographic steps, moved as a single sharp spot on TLC.

The DMTr($O^2$-Et)dT was analyzed by chemical ionization (CI) mass spectrometry (MS). The analysis confirmed the expected molecular weight of 572 with ethylation occurring on the base. The structure of DMTr($O^2$-Et)dT was confirmed by NMR. All NMR assignments, based on one- and two-dimensional proton homonuclear correlation spectra, were consistent with the expected structure of DMTr($O^2$-Et)dT (Fig. 1). These assignments were additionally supported by absence of the N3 proton (observed at δ 11.35 in DMTr-dT) and loss of 3′-OH at δ 5.38 upon addition of $D_2O$.

The DMTr($O^2$-Et)dT derivative was also characterized by removing the DMTr group from the 5′-end and comparing the resulting $O^2$-Et-dT product with the authentic sample. Both samples comigrated as a single spot on the TLC in solvent system II (RF = 0.06). They also coeluted (retention time = 37.4 min) on HPLC using a Beckman Ultrasphere ODS 5 μm column (10 mm×25 cm) developed in 5% acetonitrile at a flow rate of 2 ml/min. UV spectra of $O^2$-Et-dT was essentially identical ($\lambda_{max}$ 257) to the spectra previously reported for $O^2$-Et-dT (2) and the spectra obtained from the authentic sample prepared in our laboratory. Electron impact (EI) MS for $O^2$-Et-dT revealed a molecular weight of 270 with ethylation occurring on the base (b). The assignments were confirmed by an accurate mass
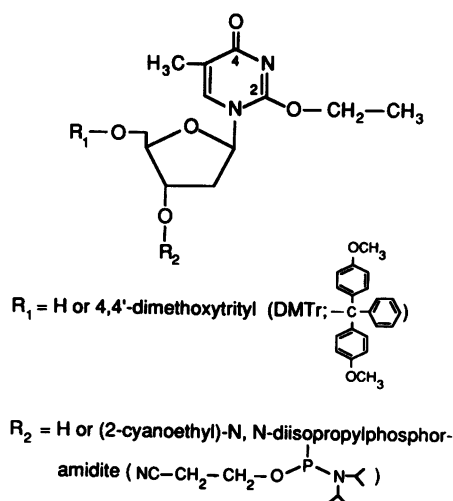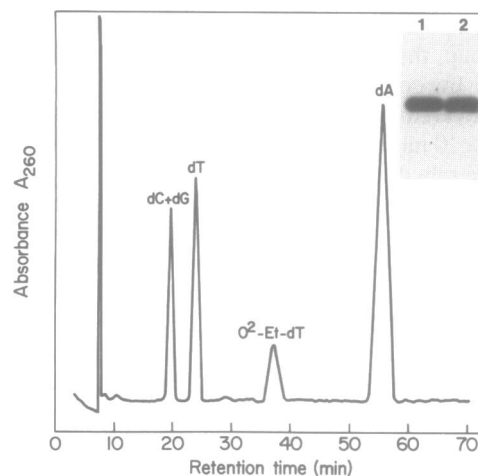
measurement technique (30), where diagnostic ethylated base containing ions could be measured. Accurate measurements were made on the most intense base-containing ion at m/z 154 (bH) in the EI spectra of $O^2$-Et-dT. The calculated mass for this ion with the elemental composition of $C_7H_{10}N_2O_2$ is 154.0742. The measured mass for bH was 154.0744 with a root mean square error of 0.4 millimass unit (N = 3). These results established conclusively that the DMTr($O^2$-Et)dT used in synthesizing the site-modified primer had the correct structure.

Conversion of DMTr($O^2$-Et)dT to the corresponding DMTr($O^2$-Et)dT-3′-phosphoramidite gave a homogeneous product as judged by a single spot on TLC (solvent system III). The RF was 0.55, which is much higher (>50%) than the starting material DMTr($O^2$-Et)dT. Incorporation of DMTr($O^2$-Et)dT at the desired site in the primer was achieved using an 'in situ' activation and automated phosphite triester approach (33). The coupling efficiency for DMTr($O^2$-Et)dT-3′-phosphoramidite in the synthesis was >97%, similar to those obtained for the normal nucleoside-phosphoramidites.

Chemical synthesis of oligodeoxyribonucleotides involves treatment with 3% trichloroacetic acid and concentrated $NH_4OH$ (33). $O^2$-Et-dT is stable under these acidic conditions but unstable to $NH_4OH$ (34). Deprotection of the synthesized $O^2$-Et-dT-containing oligomer was achieved using non-aqueous conditions of DBU treatment (34,35) in tetrahydrofuran and ethanol for one week. Under these conditions, the deprotection was complete. No detectable side products were obtained as demonstrated by HPLC analysis (Fig. 2). The use of cyanoethyl as the internucleotide phosphate-protecting group was useful since this group was removed during DBU treatment. Others (34) have used methyl as the phosphate-protecting group. They observed



**Fig. 1.** Structure of $O^2$-ethylated dT derivatives. $O^2$-Et-dT: $R_1 = R_2 = H$; DMTr($O^2$-Et)dT: $R_1 = DMTr$, $R_2 = H$; 3′-phosphoramidite of DMTr($O^2$-Et)dT: $R_1 = DMTr$, $R_2 = (2$-cyanoethyl)-N,N-diisopropylphosphoramidite.



**Fig. 2.** Nucleoside analysis of $O^2$-Et-dT-containing 21-mer. The profile of nucleotide separation by HPLC is shown. Nucleosides released by enzymatic digestion (see text) were chromatographed on a Beckman Ultraspore ODS 5 μm column (1×25 cm). The column was eluted with 5% acetonitrile in $H_2O$ at a flow rate of 2 ml/min. The peaks were assigned on the basis of identical retention times as standards. HPLC analysis indicated the presence of $O^2$-Et-dT in essentially all oligomer molecules (see text). The figure inset represents an autoradiogram showing the purity of the $O^2$-Et-dT-containing 21-mer. The ^{32}P-labeled site-modified primer was electrophoresed on a 20% polyacrylamide-8M urea sequencing gel. Lane 1, 21-nucleotide marker containing the same sequences as the site-modified 21-mer except dT in place of $O^2$-Et-dT. Lane 2, $O^2$-Et-dT-containing 21-mer. The presence of a single sharp band in Lane 2 indicates that the $O^2$-Et-dT-containing 21-mer is electrophoretically homogeneous.

the loss of O²-methyl apparently during removal of the internucleotide-protecting group with thiophenol in the presence of triethylamine (34).

The ³²P-labeled oligomer was electrophoretically homogeneous on a 20% polyacrylamide-8M urea sequencing gel (Fig. 2 inset) and had the same mobility as a 21-nucleotide standard containing the same sequence except that dT is present in place of O²-Et-dT. DNA sequencing revealed the expected sequence (Fig. 3) except that the O²-Et-dT (T*) lesion was not differentiated by the T and C spectific reactions used in the Maxam-Gilbert procedure. Bands in both the T and C lanes were obtained at T* (Fig. 3). Repeated sequencing gave identical results. The O²-Et-dT-containing oligomer was stable to piperidine treatment, indicating the absence of an AP site at the O²-Et-dT or any other position in the oligomer. HPLC analysis (Fig. 2) of the nucleosides released from the site-modified oligomer following enzymatic digestion revealed a ratio of 11.64:5.1:1.05 between dA:dT:O²-Et-dT, as compared with the predicted ratio of 12:5:1. The results indicate that O²-Et-dT is present essentially in all oligomer molecules. Since dG and dC were eluted together (Fig. 2), we were unable to calculate their observed ratios. Their presence at the desired sites was confirmed by sequencing the site-modified 21-mer (Fig. 3).

The O²-Et-dT-containing oligomer is complementary to φX174 DNA from position 2392 to 2412 (38) and contains the O²-Et-dT lesion at position 2402. This position corresponds to the second nucleotide in the third codon of φX gene G. In a separate investigation, this site-modified oligomer will be used to study the *in vivo* mutagenic properties of O²-Et-dT using a φX174-based site-specific mutagenesis system (23).

### In Vitro DNA Replication System

*In vitro* DNA replication studies were initiated on the primed template shown in Figure 4. The primed template includes a
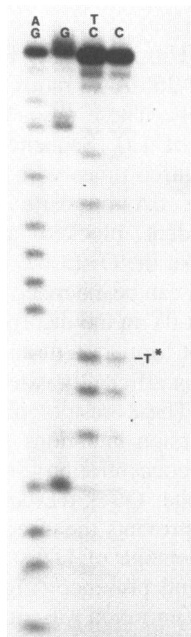
complementary 5'-³²P-labeled 17-nucleotide primer annealed to the O²-Et-dT-containing 36-nucleotide template. Formation of the site-modified primed template was >80% as demonstrated by polyacrylamide gel electrophoresis. In this system, the 3'-terminus of the primer is eight nucleotides away from the O²-Et-dT lesion present in the template, representing a 'running start' for O²-Et-dT in DNA replication (39), in that synthesis occurs prior to the polymerase reaching the O²-Et-dT lesion.

*In vitro* DNA replication was catalyzed by Mg⁺⁺-activated T7 DNA polymerase with varying concentrations of dNTP at 37°C for 30 min. DNA synthesis products were analyzed by polyacrylamide gel electrophoresis (6,7). Effect of 3'→5' exonuclease-proofreading activity of the polymerase on DNA replication past O²-Et-dT was studied by inhibiting this activity by adding 10 mM dAMP to some experiments (indicated in the text and figure legends).

The hybridized primer (Fig. 4) was extended by the DNA polymerase until the O²-Et-dT lesion was encountered. The following products, reflecting the influence of template O²-Et-dT, are feasible. First, the progress of the polymerase is blocked 3' to O²-Et-dT. No nucleotide is incorporated opposite the lesion and a 25-nucleotide 'pre-incorporation blocked product' accumulates. Variable amounts of the 22–24-nucleotide pre-incorporation blocked product were also obtained, probably due to distortion caused by O²-Et-dT. These products were included in the estimation of the pre-incorporation blocked product. Second, DNA synthesis terminates after incorporating a nucleotide opposite the O²-Et-dT lesion, producing a 26-nucleotide 'incorporation-dependent blocked product'. Finally, the adduct does not present a block to DNA replication and the synthesis proceeds past the lesion yielding a 'postlesion synthesis product'. The efficiency of DNA synthesis past O²-Et-dT may be reduced due to the distortion caused by this lesion in the template producing products ranging from 27 to 36 nucleotides. Alternatively, nucleotide addition at the blunt end of the synthesized 36-nucleotide duplex may produce a 37-nucleotide product (40). Postlesion synthesis included 27 to 37 nucleotide products. For DNA sequencing of postlesion synthesis products, only 35 to 37 nucleotide-long products were used.

### DNA Synthesis on a Template Containing O²-Et-dT

The O²-Et-dT lesion, present at a single site in the template, blocked DNA synthesis by T7 DNA polymerase in the presence of 10 μM dNTP and 5 mM Mg⁺⁺ (Fig. 5A). In the control (not shown), in which O²-Et-dT was replaced with dT, DNA synthesis proceeded to the 5'-terminus of the template without interruption. Formation of the 36-nucleotide product in the control indicated that O²-Et-dT was responsible for blocking the DNA synthesis when the O²-Et-dT-containing template was used. The observed DNA replication block by O²-Et-dT is consistent with published reports (3,41). A low efficiency of incorporation of



**Fig. 3.** DNA sequence analysis of O²-Et-dT-containing 21-mer by the Maxam-Gilbert procedure. T* = O²-Et-dT. Sequencing gel confirmed the expected DNA sequence, 5'AATAAAAGTCT*AAAACATGAT. Bands in both T + C and C lanes corresponding to T* indicate that the presence of an ethyl group at the O² position in dT did not inhibit the hydrazine reaction (in the presence of high salt) at the available 5,6-double bond.

17-mer primer 5'-³²pCTGCTTAGGAGTTTAAT
36-mer template 3'-HO-GACGAATCCTCAAATTAGTACAAAAT*CTGAAAATAA5'
                                    |          |              |
                                    17         26             36

**Fig. 4.** 'Running start' primed template. The 36-nucleotide template contains a single O²-Et-dT adduct at template position 26 from the 3'-end. The adduct is eight nucleotides away from the 3'-end of the hybridized 17-nucleotide primer.
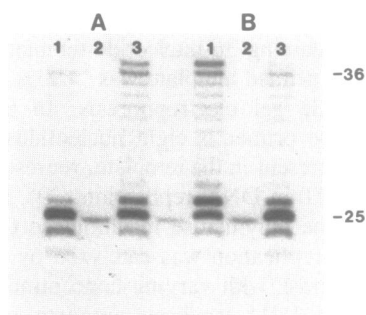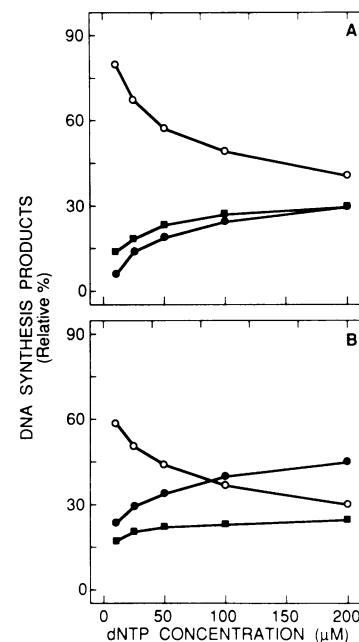
**Fig. 5.** Analysis of DNA products synthesized by T7 DNA polymerase on a primed template. The primed template (0.05 pmol) was incubated at 37°C for 30 min with 0.2 unit of T7 DNA polymerase in the presence of varying concentrations of dNTP. The synthesis products were analyzed by gel electrophoresis and autoradiography (see text). **A)** Primed template containing an $O^2$-Et-dT adduct at template position 26 from the 3'-end. **Lane 1**, DNA synthesis in the presence of 10 $\mu$M dNTP and 5 mM $Mg^{++}$. **Lane 2**, 25-nucleotide marker. **Lane 3**, DNA synthesis in the presence of 200 $\mu$M dNTP and 5 mM $Mg^{++}$. **B)** Same primed template as in (A). **Lane 1**, DNA synthesis in the presence of 200 $\mu$M dNTP, 5 mM $Mg^{++}$ and 10 mM dAMP. **Lane 2**, 25-nucleotide marker. **Lane 3**, DNA synthesis in the presence of and 10 $\mu$M dNTP, 5mM $Mg^{++}$ and 10 mM dAMP.



**Fig. 6.** Effect of dNTP concentration on DNA products synthesized by T7 DNA polymerase on the primed template containing a single $O^2$-Et-dT adduct in the presence of 5 mM $Mg^{++}$. The primed template (0.05 pmol) was incubated at 37°C for 30 min with 0.2 unit of T7 DNA polymerase in the presence of 5 mM $Mg^{++}$ and varying concentrations of dNTP in the presence or absence of dAMP. The products were analyzed as described in the text. Each point represents an average of three experiments. ○, preincorporation blocked product; ■, 26-nucleotide incorporation-dependent blocked product; ●, postlesion synthesis product. **A)** No dAMP added. Standard deviations ranged from 3.8–5.9% for preincorporation blocked products; 1.1–4.6% for incorporation-dependent blocked products; and 0.9–3.1% for postlesion synthesis products. **B)** 10 mM dAMP added. Standard deviations ranged from 3.2–4.8% for preincorporation blocked products; 1.9–4.9% for incorporation-dependent blocked products; and 2.3–3.9% for postlesion synthesis products.

the triphosphates of $O^2$-Me-dT and $O^2$-Et-dT by Kf Pol I and *E. coli* polymerase I using synthetic poly(dA-dT) as templates has also been reported (42,43). In the studies reported here, the major product (80%) of DNA synthesis was a pre-incorporation-blocked product. Nucleotide incorporation opposite $O^2$-Et-dT and subsequent postlesion synthesis were also obtained but in relatively low yields (14% and 6% respectively). The results indicate that the $O^2$-Et-dT lesion presents a strong block to DNA replication by T7 DNA polymerase in the presence of $Mg^{++}$ and low dNTP concentrations (10 $\mu$M).

Addition of 10 mM dAMP to the polymerization reactions increased the postlesion synthesis from 6% to 23% with a simultaneous decrease in the preincorporation-blocked product from 80% to 59% (Figs. 6A and 6B; 10 $\mu$M dNTP points). dAMP inhibits 3'→5' exonucleases associated with DNA polymerases, such as *E. coli* polymerase I or Klenow fragment (37). The increase in postlesion synthesis is presumably due to inhibition of the 3'→5' exonuclease activity of T7 DNA polymerase by dAMP. No attempt was made to optimize formation of the postlesion synthesis product using dAMP in the polymerization reaction.

Both postlesion synthesis (35–37 nucleotides) and 26-nucleotide incorporation-dependent blocked products, synthesized at 10 $\mu$M dNTP in the absence of 10 mM dAMP, were sequenced by the modified Maxam-Gilbert procedure. The nucleotide incorporated opposite $O^2$-Et-dT is present in the product at position 26 from the 5'-end. The incorporation-dependent blocked product contained dA opposite $O^2$-Et-dT (Fig. 7B). Both dA and dT are incorporated opposite the lesion in the postlesion synthesis product (Fig. 7A). The results indicate that the postlesion product is heterogenous at position 26, with a fraction of the molecules containing dA and the remaining containing dT at this position. As expected, this heterogeneity produced less intense dT (dT-26) and dA (dA-26) bands at position 26 compared to the neighboring dT (dT-25) and dA (dA-28) bands, respectively, in the same lane (Fig. 7A). The autoradiogram, containing these bands (Fig. 7A, lanes A and T),

was scanned using Ultroscan XL. From the peak areas, the dT-26:dT-25 and dA-26:dA-28 ratios were calculated to be 0.75 and 0.33, respectively. These ratios total 1.08 as compared to the expected value of 1.0. Sequences of each product were determined at least three times with similar results.

The presence of dA opposite $O^2$-Et-dT, in both the incorporation-dependent blocked and postlesion synthesis products, suggests that the $O^2$-Et-dT·dA base pair at the 3'-end of the growing chain can be extended but inefficiently. Absence of dT opposite $O^2$-Et-dT in the incorporation-dependent blocked product indicates that formation of an $O^2$-Et-dT·dT base pair at the replication fork is efficiently extended to yield a postlesion synthesis product. These results implicate $O^2$-Et-dT in the induction of transversion mutations at A·T base pairs.

Increasing dNTP concentrations stimulated incorporation of a nucleotide opposite $O^2$-Et-dT and subsequent postlesion synthesis. Figure 6 presents the effect of dNTP concentrations on the relative percentage of DNA synthesis products in the absence (Fig. 6A) and presence (Fig. 6B) of 10 mM dAMP. When the dNTP concentration was increased from 10 $\mu$M to 200 $\mu$M, postlesion synthesis increased from 6% to 30% (Fig. 6A). Postlesion synthesis was further enhanced using 10 mM dAMP in the polymerization reaction (Fig. 6B).

The 26-nucleotide incorporation-dependent blocked and postlesion synthesis products synthesized at 200 $\mu$M dNTP in the absence of dAMP were sequenced. As observed with the
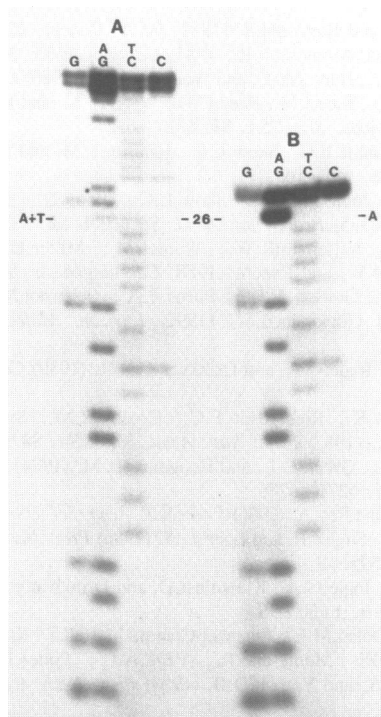
Fig. 7. DNA sequence analysis of incorporation-dependent blocked product and postlesion synthesis product by Maxam-Gilbert procedure. The products were isolated from the same DNA synthesis reaction carried out in the presence of 5 mM Mg$^{++}$ and 10 $\mu$M dNTP at 37°C. The nucleotide incorporated opposite O$^2$-Et-dT is present in the product at position 26 from the 5′-end. A) An autoradiogram of the DNA sequencing gel obtained from the postlesion synthesis product (35- through 37-nucleotides). Presence of bands in the dA and dT-specific lanes at position 26 indicates incorporation of both dA and dT opposite O$^2$-Et-dT. B) An autoradiogram of the DNA sequencing gel obtained from the 26-nucleotide incorporation-dependent blocked product. The presence of a band in the dA-specific lane at position 26 indicates incorporation of dA opposite O$^2$-Et-dT.

corresponding products isolated at 10 $\mu$M dNTP, dA was incorporated opposite O$^2$-Et-dT in the incorporation-dependent blocked product, while both dA and dT were incorporated opposite the lesion in the postlesion synthesis product.

## DISCUSSION

The studies described here demonstrate that O$^2$-Et-dT partially blocks DNA synthesis by T7 DNA polymerase. During bypass of the lesion, both dA and dT are incorporated opposite O$^2$-Et-dT. Incorporation of dA opposite O$^2$-Et-dT impedes DNA synthesis, while dT incorporation opposite the lesion is efficiently extended, leading to an A·T→T·A transversion mutation. Although O$^2$-Et-dT is rapidly repaired in bacteria (44), its repair in mammalian systems is not efficient (45). This lesion is among the highly persistent DNA alkylation products in cultured mammalian cells and animal tissue (27). Our results suggest that formation of the persistent O$^2$-Et-dT lesion in DNA is biologically significant and may contribute to cytotoxic, mutagenic and carcinogenic processes long after the exposure has occurred.

The ethyl group of O$^2$-Et-dT is not located within the Watson-Crick base-pairing region. However, ethylation of the O$^2$-position of dT interferes with normal hydrogen bonding by

fixing the base in the enol tautomer with the loss of a hydrogen atom at the central hydrogen-bonding site (N3) of dT. Molecular and computer models have indicated that the presence of O$^2$-alkyl-dT in DNA may cause distortion in the DNA structure (3) and may inhibit DNA synthesis (41). Our results demonstrate that the presence of O$^2$-Et-dT in a DNA template impedes the progress of DNA synthesis. Even at higher dNTP concentrations (200 $\mu$M), O$^2$-Et-dT inhibited 70% of the DNA synthesis (Fig. 6A).

Accumulation of the incorporation-dependent blocked product, containing dA opposite O$^2$-Et-dT (Fig. 7B), and the presence of dA, opposite the lesion in the postlesion synthesis product (Fig. 7A), indicate that formation of the base pair O$^2$-Et-dT·dA at the replicating fork can be extended but with reduced efficiency. Thermal denaturation studies of O$^2$-Me-dT present in an alternating poly(dA-dT) polymer suggest that O$^2$-Me-dT does not alter the secondary structure of the DNA (42). In a study where E. coli polymerase I or Klenow fragment were used with O$^2$-alkyl-dTTP on a poly(dA-dT) template, the extension of O$^2$-alkyl-dT at the 3′-primer termini was ≈20% of the efficiency of the dT·dA termini (43). These studies indicate that the O$^2$-alkyl-dT·dA base pair is thermodynamically stable but impedes DNA synthesis, possibly by distorting the DNA structure as suggested by molecular model-building studies (3). Due to distortion, even a correctly inserted base may not represent an ideal base pair to the replication complex, leading to retardation of DNA synthesis. Our result, that incorporation of dA opposite O$^2$-Et-dT impedes further DNA synthesis, supports this hypothesis. Under normal cellular conditions, extension of the O$^2$-Et-dT·dA base pair may either not occur or occur with low efficiency. Our in vitro DNA replication studies in the presence of Mg$^{++}$ implicate O$^2$-Et-dT in cytotoxicity by ethylating agents. The presence of dT incorporated opposite O$^2$-Et-dT in the postlesion synthesis product (Fig. 7A) and the absence of dT detection opposite the lesion in the incorporation-dependent blocked product (Fig. 7B) suggest that formation of the O$^2$-Et-dT·dT base pair at the replication fork is efficiently extended to yield postlesion synthesis. The results implicate O$^2$-Et-dT as a potentially promutagenic lesion in vivo capable of producing A·T→T·A transversion mutations.

O$^2$-alkylthymidine directs errors in RNA-synthesizing systems (3). O$^2$-Me-dT, present in poly(dA-dT) templates for E. coli polymerase I, has been implicated in misincorporation of dG with low efficiency (4,42), although this has yet to be demonstrated in vivo (4,46). Using poly(dT, O$^2$-alkyl-dT) templates, no errors were detected during in vitro DNA synthesis by E. coli polymerase I in the presence of Mg$^{++}$ (41). When the polymerase was made error prone using Mn$^{++}$, dG was incorporated into the DNA synthesis product (41). In those studies, incorporation of dG opposite O$^2$-alkyl-dT was not directly established (41,42). Molecular models indicated that the O$^2$-alkyl-dT·dG base pair cannot fit within the confines of the DNA helix due to steric hindrance between the alkyl group attached to the trigonal O$^2$ atom and the sugar phosphate backbone of the polynucleotide (47). In our studies, incorporation of dA and dT opposite O$^2$-Et-dT by T7 DNA polymerase in the presence of Mg$^{++}$ was established by DNA sequencing of the synthesis products.

Incorporation of dT opposite O$^2$-Et-dT is not an artifact of the specific sequence used in the studies reported here. When O$^2$-Et-dT was replaced with O$^4$-Et-dT in the same DNA sequence, both dA and dG (but not dT) were incorporated

opposite O⁴-Et-dT (Grevatt & Bhanot, unpublished results), which is consistent with published reports (3,4,23). If dG incorporates opposite $O^2$-Et-dT below detectable limits in our assay, its presence will be revealed by *in vivo* site-specific mutagenesis studies currently in progress.

Why the $O^2$-Et-dT·dT base pair is efficiently extended remains unclear. The $O^2$-Et-dT·dT base pair probably has one hydrogen bond forming between the N3 nitrogen atoms. The pairing of two pyrimidines would allow for a long hydrogen bond, which would decrease steric hindrance between the ethyl group of $O^2$-Et-dT and the carbonyl group at C2 of dT, and may retain the normal Watson-Crick alignment. The $O^6$-Me-dG·dT and $O^4$-Me-dT·dG mispairs, which retain normal Watson-Crick alignment (reviewed in 48), are efficiently extended *in vitro*. Similar results were obtained with N3-Et-dT, where the N3-Et-dT·dT base pair was efficiently extended during *in vitro* DNA replication (6,7). These results may reflect maintenance of the phosphodiester backbone uniformity by these base pairs for effective recognition of the template-primer complex by the polymerase. The studies suggest that correct alignment of the bases is crucial in DNA replication (48). The hydrogen-bonded strength of the base pairs is of secondary importance. Molecular and computer models together with physicochemical studies on the $O^2$-Et-dT·dT base pair will provide insight into this problem. Our studies clearly demonstrate that $O^2$-Et-dT, which impedes DNA synthesis, can mispair with dT during *in vitro* DNA replication and may induce A·T→T·A transversion mutations *in vivo*. These studies provide a basis for understanding a molecular mechanism by which ethylating agents such as ENU can induce A·T transversion mutagenesis and activate proto-oncogenes by an A·T→T·A transversion event in tumors induced by these agents (13,14,17).

## ACKNOWLEDGMENTS

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