Ethylene oxide, a direct-acting mutagen and carcinogen, produces 3-hydroxyethyldeoxyuridine (3-HE-dU) after initial alkylation at N3 of dC, followed by rapid hydrolytic deamination. The significance of formation of 3-HE-dU in DNA was investigated by in vitro DNA replication of 3-HE-dU. A 55-nucleotide DNA template, containing 3-HE-dU at a single site, was constructed. DNA products, synthesized on the site-modified template, were analyzed and mutagenic bypass at 3-HE-dU estimated. The 3-HE-dU lesion blocked DNA replication by the Klenow fragment of Escherichia coli polymerase I (Kf Pol I) and bacteriophage T7 polymerase (T7 Pol) 3'- to 3-HE-dU and after incorporating a nucleotide opposite 3-HE-dU. DNA synthesis past 3-HE-dU was negligibly (~3%). Substitution of Kf Pol I (exo-) and T7 Pol (exo-), polymerases lacking 3' → 5' exonuclease proof-reading activity, for Kf Pol I and T7 Pol, respectively, facilitated DNA synthesis past 3-HE-dU. The bypass synthesis by Kf Pol I (exo-) was 90% and 90% by T7 Pol (exo-). These results suggest that the 3-HE-dU lesion could be bypassed, but that the extension at 3-HE-dU is rate-limiting. In the absence of proofreading, the nucleotide incorporated opposite 3-HE-dU is not excised and remains in position long enough for extension to occur. During post-lesion synthesis, both dA and dT were incorporated opposite 3-HE-dU. Since 3-HE-dU is derived from dC alkylation by ethylene oxide, incorporation of dA and dT opposite 3-HE-dU implicates this lesion in G-C → A-T and G-C → T-A mutagenesis.

Aliphatic epoxides are a class of highly reactive compounds which are effective mutagens and carcinogens (1, 2). One of these simple and industrially important aliphatic epoxides is ethylene oxide. Ethylene oxide is widely used as an intermediate in the chemical industry and as a fumigant and sterilent of food and medical products. Ethylene oxide is considered to be an environmental carcinogen (3, 4). It is produced metabolically from ethylene present in cigarette smoke and as a pollutant in urban air and by the combustion of organic compounds. Ethylene oxide is a direct-acting alkylating agent. It reacts with DNA by an S2,2 mechanism which favors alkylation at strongly nucleophilic endocyclic nitrogen atoms (1). Ethylene oxide binds to DNA both in vitro and in vivo (19–25) leading mainly (~90% of total alkylation) to the formation of 7-(2-hydroxyethyl)deoxyguanosine (7-HE-dG) (1). Other purine adducts formed are 3-(2-hydroxyethyl)deoxyadenosine (3-HE-dA) and O6-(2-hydroxyethyl)deoxyguanosine (O6-HE-dG). Hydroxyethylation at N1, N6, and N7 of the adenine moiety in DNA have also been reported (21, 23). Evidence exists that ethylene oxide can also react at DNA-phosphate groups (1, 2, 26). The purine adducts at N7 and O6 of dG and N3 of dA are not persistent in vivo and are removed from DNA through depurination and DNA repair (23, 25, 27, 28). It has been suggested that these purine adducts may induce mutations at G-C and A-T base pairs (29, 30). Lack of in vivo persistence of these lesions is not consistent with this suggestion. The specific ethylene oxide-induced DNA adducts involved in mutagenesis by ethylene oxide are not established.

We have recently reported that in vitro reaction of direct-acting aliphatic epoxides including ethylene oxide (21), propylene oxide (31), glyceral (32), and the epoxide of acrylonitrile, 2-cyanoethylene oxide (33) with DNA gave rise to a new DNA lesion, 3-(2-hydroxyalkyl)deoxyuridine (3-HA-dU). This lesion was formed following hydroxyalkylation at N3 of deoxyctydine and rapid hydrolytic deamination of the 3-(2-hydroxyalkyl)deoxyuridine (3-HA-dU) intermediate. Based on the significant increase in the rate of deamination (60 fold) observed for 3-(2-hydroxyethyl)deoxyctydine (3-HE-dC) as compared with 3-ethyldeoxyctydine (21, 34), we hypothesized that the hydroxyl group at the C-2 position on the alkyl side chain of the

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1 The abbreviations used are: 7-HE-dG, 7-(2-hydroxyethyl)deoxyguanosine; 3-HE-dU, 3-(2-hydroxyethyl)deoxyuridine; O6-HE-dG, O6-(2-hydroxyethyl)deoxyguanosine; 3-HE-dA, 3-(2-hydroxyethyl)deoxyadenosine; 3-HE-dT, 3-(2-hydroxyethyl)deoxycytidine; 3-Et-dT, 3-ethyldeoxycytidine; O6-Et-dT, O6-ethyldeoxycytidine; O6-Et-dG, O6-ethyldeoxyguanosine; Kf Pol I, Klenow fragment of E. coli polymerase I; hprt, hypoxanthine-guanine phosphoribosyltransferase; CI-MS, chemical ionization mass spectrometry; DTT, dithiothreitol; TLC, thin layer chromatography; HPLC, high performance liquid chromatography; DMT, 4,4′-dimethoxytrityl.
3-HA-dC intermediate facilitated the hydrolytic deamination and a mechanism was proposed (21, 33, 35). The in vitro persistence of ethylene oxide-induced 3-(2-hydroxyethyl)deoxyuridine (3-HE-dU) is not known. The 3-HE-dU adduct is chemically stable in DNA (21). No repair activity for this lesion is known in prokaryotes and eukaryotes. 3-HE-dU may be a persistent lesion in vivo. The persistence of ethylene oxide-induced 3-HE-dU in DNA may have a significant role in ethylene oxide-induced mutagenesis.

The role of a single base modification in mutagenesis can be investigated by site-specific incorporation of the modification into DNA and analysis of the products synthesized by DNA polymerases on the site-modified DNA template. We previously utilized such an in vitro DNA system to study the DNA replication properties of ethylating agent-induced 3-ethylothymidinidyline (3-Et-dT), an analog of ethylene oxide-induced 3-HE-dU (36, 37). We demonstrated that 3-Et-dT, present at a single site in the DNA template, blocked in vitro DNA synthesis 3’ to 3-Et-dT and after incorporating dA opposite the lesion, possibly contributing to the cytotoxicity exhibited in vivo by ethylating agents. Relaxation of the fidelity of replication by the substitution of Mn2+ for the normal cofactor, Mg2+, permitted incorporation of dT opposite 3-Et-dT during synthesis past the lesion, implicating 3-Et-dT in A.T mispairing by ethylene oxide-induced 3-HE-dU (Fig. 1).

The biological importance of A.T → T.A transversion is indicated by the observation of this mutation in the activation of cellular proto-oncogenes in N-ethyl-N-nitrosourea-induced mutational spectra in mamma- lian systems (38–40). The biological importance of A.T → T.A transversion is indicated by the observation of this mutation in the activation of cellular proto-oncogenes in N-ethyl-N-nitrosourea-induced tumors (41–43) and in germ line mutations produced by treatment of mice with N-ethyl-N-nitrosourea (44, 45).

In this paper we report that the 3-HE-dU lesion, present at a single site in the DNA template, blocked DNA synthesis 3’ to 3-HE-dU and after incorporating a nucleotide opposite the lesion. Under conditions of relaxed fidelity, i.e., absence of proofreading, mutagenic bypass with dA and dT incorporation opposite 3-HE-dU occurred. Since 3-HE-dU is derived from ethylene oxide-induced 3-HE-dC, incorporation of dA and dT opposite 3-HE-dU implicates this lesion in G.C → A.T and G.C → T.A mutagenesis.

**EXPERIMENTAL PROCEDURES**

Ultra-pure grade 2’-deoxynucleotide 5’-triphosphates (dNTP) and ATP were purchased from Pharmacia-LKB Biochemicals. 2’-Deoxy-oxynucleosides were from Sigma. [γ-32P]ATP was obtained from DuPont NEN. Bacteriophage T4 polynucleotide kinase was purchased from New England Biolabs. Klenow fragment of E. coli polymerase I (KF Pol I), T4 DNA ligase, calf intestinal alkaline phosphatase, phosphodiesterase from Croatulus durissus, and diithiothreitol (DTT) were obtained from Boehringer Mannheim. Bacteriophage T7 DNA polymerase (T7 Pol), T7 Pol (3’ → 5’ exonuclease-free) sequence version 2.0 and KF Pol I (3’ → 5’ exonuclease-free) were purchased from United States Biochemical Corp (Cleveland, OH). 1-Bromo-2-acetoxyethane, anhydrous dimethyl sulfoxide (Me_2SO), anhydrous pyridine, 4,4-dimethoxytrimethyl (DMTr) chloride, 4-dimethylaminopyridine, N,N-diisopropylamidine, 2-cyan-ethoxy N,N-diisopropylchlorophosphoramidite, silicon gel (grade 60; 70–230 mesh, 60 Å), solvents for nuclear magnetic resonance spectroscopy and reagents for DNA sequencing were obtained from Aldrich and used as received except where noted. Whatman aluminum-backed thin layer chromatography (TLC) plates (Silica Gel G-F_254, 250 μm thickness) were from VWR Scientific. Ultra pure electrophoresis reagents were purchased from Bio-Rad. High pressure liquid chromatography (HPLC) grade solvents were obtained from Fisher Scientific. All other chemicals were of high grade quality and purchased from different sources.

Synthesis and site-specific incorporation of 3-HE-dU into the oligodeoxynucleotide are described below. Reverse-phase HPLC was performed on a Waters Associates system consisting of two pumps (models 600A and 530), a variable wavelength detector (model 490) and a System Interface Module. The system was controlled from a personal computer (Professional 350) computer operating a Waters 840 data and chromatography control station. Proton homonuclear magnetic resonance (H NMR) spectra were recorded on a Bruker AM360 (360 MHz) instrument, and all chemical shifts are assigned relative to tetramethylsilane (0.0). Mass spectra (MS) were obtained on a VG-70SE high resolution mass spectrometer interfaced to a VG 11250 data system (VG Manchester, United Kingdom). TLC plates were developed in the following solvent systems: I, chloroform/ methanol (9:1); II, dichloromethane/methanol/triethylamine (45:11:1); III, dichloromethane/methanol/acetic acid (18:1:1). The reaction mixture was stirred at room temperature for 30 min and subsequently treated with 1.6 g of 4,4'-dimethoxytrityl chloride (DMTrCl) and 0.9 g of 1-bromo-2-acetoxyethane. The reaction mixture was stirred at room temperature for 24 h. TLC analysis (solvent system I) revealed the presence of two UV-absorbing spots (R_f = 0.27 and 0.60) with the more polar spot corresponding to unreacted dU. The reaction mixture was diluted with 20 ml of water and neutralized with dilute acetic acid. The resulting mixture was concentrated under diminished pressure at 40 °C, treated with 50 ml of methanol, 10 g of silica, and again concentrated. The residue was coevaporated with methanol (2 × 50 ml) and dried in vacuo overnight. The dried, impregnated silica was applied to a silica gel column (3.8 cm × 16.5 cm) and eluted with 7% methanol in chloroform. Fractions of 10 ml each were collected and analyzed by TLC (solvent system I). The fractions containing the desired pure product (single spot; R_f = 0.60) were pooled and the solvent removed under reduced pressure to yield 1.98 g (72%) of 3-HE-dU.

The ultraviolet (UV) spectrum at pH 6 (H_2O) displayed a λ_max at 263 nm which is consistent with alkylation at N3 of dU and excludes any contribution at the O^4' and O^6 positions. With the aid of chemical ionization mass spectrometry (CI-MS), the expected protonated molecular ion (MH) at m/z 315 established the molecular weight as 314. The ion, corresponding to nucleosides plus two hydrogens (bH_2), m/z 199 and the fragment pattern (bH_2)HOA at m/z 159; dU (3-HE-dU) was observed at m/z 230. Each of the adducts of the acetyxyethyl group had occurred on the base. The structure of 3-HE-dU (Fig. 1) was confirmed by 'H NMR. The chemical shift (δ) assignment of the protons of 3-HE-dU in Me_2SO is as follows: 7.93 (d, 1H, J = 8.1 Hz, H_6), 6.17 (t, 1H, J = 6.6 Hz, H_1'), 5.78 (d, 1H, J = 8.1 Hz, H_5), 5.27 (d, 1H, J = 4.3 Hz, 3'OeH), 5.05 (t, 1H, J = 4.3 Hz, H_4), 4.52 (m, 1H, H_3), 4.19 (2H, H_2), 3.81 (1H, -CH(N)=), 3.18 (m, 1H, H_4'), 3.57 (2H, H_5), 2.11 (2H, H_2'), 1.93 (s, 3H, -CO-CH_3). These assignments are based on one- and two-dimensional proton homonuclear correlation spectra. The absence of a signal due to the proton at N3 of dU confirmed that 2-acetyoxyethyl had occurred at this position (Fig. 1).

3-(2-Hydroxyethyl)-2'-deoxyuridine (3-HE-dU, Fig. 1)→ 3-AE-dU (100 mg; 0.32 mmol) was deacetylated by heating with concentrated ammonium hydroxide (2 ml) at 70 °C for 30 min. These conditions were used for deprotection of the synthesized oligomer. To confirm that 3-HE-dU is exclusively formed and is stable following deacetylation of 3-AE-dU, the following experiments were performed. After complete deacetylation, the reaction mixture was treated with gas removal nitrogen gas through the solution. The deacetylated product, 3-HE-dU (Fig. 1), was recovered by lyophilization and fully characterized. The product moved as a single spot on TLC (solvent system I, R_f = 0.31) and coeluted as a single sharp peak (retention time, 26.3 min) with authentic synthetic marker, previously characterized by UV, MS, and NMR (21), during reversed-phase HPLC on a Beckman Ultrasphere ODS semipreparative column (5-μm particle size; 4.6 mm × 25 cm) utilizing a flow rate of 2.4 ml/min and monitoring the UV absorption at 264 nm. Chromatography was performed using water as the mobile phase with 10% methanol at a flow rate of 3.5 ml/min (initially, 10% methanol at a flow rate of 3.5 ml/min) followed by an increase to 25% at 32 min (curve 6) and ultimately to 75% at 38 min (curve 6). CI-MS analysis of 3-HE-dU formed by deacetylation of 3-AE-dU had the identical mass spectral data to authentic 3-HE-dU except for the expected molecular ion at m/z 273, establishing the molecular weight as 272. The presence of the base ions (m/z 273, 259, 245, 231, 217, 203, 189, 175, 161, 147, 133, 119, 105, 91, 77, 63, 51, 39, 25) were observed in the CI-MS spectra of authentic 3-HE-dU. The mass spectrometry of 3-HE-dU was recorded on a VG 702SE high resolution mass spectrometer interfaced to 3-HE-dU system (VG Manchester, United Kingdom).

The pyridine was removed under high vacuum, and the flask was again treated, all at once, with 3.42 g (10.1 mmol) of 4,4'-dimethoxytrityl chloride. The mixture was stirred in an ice-bath for 1 h followed by 2 h
at room temperature. The reaction mixture was diluted with 150 ml of dichloromethane and washed successively with saturated sodium bicarbonate (1 x 200 ml) and brine (1 x 200 ml). The organic layers were dried over anhydrous magnesium sulfate and the solvent removed under reduced pressure at 25 °C. The resulting syrup was dried by coevaporation with 4 ml of anhydrous dichloromethane. 255 mg (1.97 mmol) was obtained from the reaction.

The mixture was stirred at 0 °C for 1.5 h, allowed to warm to room temperature and stirred an additional 1 h. The reaction was diluted with 15 ml of anhydrous ethyl acetate and transferred to a separatory funnel containing 20 ml of ethyl acetate and 50 ml of saturated brine. The organic layers were separated, washed with saturated brine and dried over anhydrous magnesium sulfate. The solvents were removed under reduced pressure at 25 °C. The resulting gum was purified by silica gel column (3.8 cm x 25 cm) chromatography using solvents system II as the eluant. Fractions of 10 ml each were collected and analyzed by TLC (solvent system II). The fractions containing the pure desired product (single spot; Rf = 0.34) were pooled and concentrated in vacuo to yield 5.4 g (66% of DMTM-(3-AE)dU (Fig. 1). CI-MS established the expected molecular weight of 617. The structure was confirmed by ¹H NMR in deuterated chloroform (CDCl₃). The chemical shift assignments are: 5.78 (d, 1H, J = 8.1 Hz, H6), 7.37-7.16 (m, 9H, phenyl ring and meta to -OCH₃ in anisole rings), 6.89 (d, 4H, J = 8.8 Hz, ortho to -OCH₃ in anisole rings), 6.28 (t, 1H, J = 6.1 Hz, H1), 5.39 (d, 1H, J = 8.1 Hz, H5), 4.56 (br, 1H, 3'-OH), 4.53 (m, 1H, H3'), 4.25 (t, 2H, -CH₂-OC₆H₄), 4.02 (m, 1H, H2'-P), 3.94 (m, 2H, H5'), 3.86 (m, 1H, H6), 3.41 (m, 2H, H3'), 2.54 (m, 1H, H2'-P), 2.18 (m, 4H, -CH₂-OC₆H₄), 2.03 (m, 4H, -CH₂-OC₆H₄), 1.98 (s, 3H, -CHO). 3'-O-(4,4'-dimethoxytrityl)-2'-deoxyuridine (3'-O-DMTr-(3-AE)dU) has a retention time of 10 min on a 12% polyacrylamide gel at 4°C. The yield of the 3'-O-DMTr-(3-AE)dU-containing 5'-OH group with T4 polynucleotide kinase and [γ-³²P]ATP and fully phosphorylated, the added advantage of being cleaved in the final enzymatic by the incorporation of acetyl groups for protection of the N3 and conversion of the 3'-OH group to the 3'-phosphoramidite. The reaction mixture for modified nucleosides 3'-O-DMTr-(3-AE)dU-phosphoramidite (Fig. 1). The phosphoramidite was 2-fold molar excess of the complementary 5'-3'P-labeled (3000 Ci/mmole) 17-nucleotide primer annealed to the site-modified 55-nucleotide template as described previously (36). The yield of the resulting primer, designated as authentic standards exhibited 16% polyacrylamide, 8 % urea sequencing gel. The products were visualized by placing the gel on a TLC plate containing a fluorescent indicator. The major band, corresponding to the 3'-HE-dU adduct and conversion of the 3'-OH group to the 3'-phosphoramidite, was excised from the gel (4 x 0.5 cm) at 37 °C to 37 °C to remove the urea. The product was eluted in 10 ml NaCl (70 μl). The yield of the 3'-HE-dU-containing 55-mer template was 65%. The template was characterized by DNA sequencing (data not shown). The site-modified template containing a single 3'-HE-dU adduct after base edition 26 from the 3'-end.

DNA Polymerase Reaction.—The reaction mixture (7 μl) contained 10 μl HEPES buffer, pH 7.5, 5 mM MgCl₂, 2 mM DTT, 0.1 pmol of primed template, and 0.2 unit (as reported by the supplier) of the DNA polymerase. The concentrations of DTNP were varied and included in the figure legends. The incubation was set at 37 °C for 30 min. The reaction was terminated by adding 1 μl of 100 mM EDTA and 1.5 volumes of the formamide dye mix. After heating at 100 °C for 3 min, DNA synthesis products were separated on a 16% polyacrylamide, 8 M urea sequencing gel. DNA synthesis products were analyzed as described previously (37).

Identification of the Nucleotide Incorporated into the 3'-HE-dU Adduct.—The nucleotide incorporated into the 3'-HE-dU adduct was determined by incorporations into a 3'-O-DMTr-(3-AE)dU-containing 5'-OH group with T4 polynucleotide kinase and [γ-³²P]ATP and fully characterized. Homogeneity of the oligomers were checked by electrophoresis on a 20% polyacrylamide, 8 % urea sequencing gel. To quantify the nucleotide content of the oligomers, approximately 1 μg of the oligomers were incubated in 100 mM Tris-HCl buffer, pH 8.2, for 10 min at 37 °C (total reaction volume = 50 μl) followed by treatment with 2 units of alkaline phosphatase for 10 min under the same conditions. Following the 20-min total reaction time, the proteins were denatured by heating at 90 °C for 10 min, the reaction was analyzed by 18% polyacrylamide-4% bis-acrylamide Beckman Ultrasphere ODS semipreparative column (5 μm particle size; 4.6 mm x 25 cm) utilizing a flow rate of 2.4 ml/min and monitoring the UV absorbance at 254 nm. Elution was performed as described for 3'-HE-dU (above). Under those conditions authentic standards exhibited good separation with retention times of dC, 15.8 min; dU, 18.4 min; deoxyinosine (di), 22.3 min; dG, 23.2 min; dT, 25.6 min; 3'-HE-dU, 26.3 min; and dA, 30.8 min. Response factors of the deoxynucleosides (at 254 nm) were established by integrating the peaks areas when equimolar amounts were injected. Deoxythymidine was used as the reference and all other products were normalized to it.

Site-modified Oligonucleotides.—Seventeen-nucleotide long oligomers, 5'-TAAAAGTCUUAAAACATG (U* = 3'-HE-dU and dU as a control), were assembled by Midland Certified Reagent Company (Midland, TX) on an Applied Biosystems model 381A synthesizer using phosphoramidite chemistry (48). Release of the oligomer from the solid support and complete removal of all protecting groups was accomplished in a sealed tube with concentrated ammonium hydroxide at 70 °C for 30 min. The oligomers were purified by anion-exchange HPLC and subsequently desalted utilizing gel-filtration (Midland Certified Reagent Company). The purified oligomers were phosphorylated at the 5'-OH group with T4 polynucleotide kinase and [γ-³²P]ATP and fully characterized. Homogeneity of the oligomers were checked by electrophoresis on a 20% polyacrylamide, 8 % urea sequencing gel. To quantify the nucleotide content of the oligomers, approximately 1 μg of the oligomers were incubated in 100 mM Tris-HCl buffer, pH 8.2, for 10 min at 37 °C (total reaction volume = 50 μl) followed by treatment with 2 units of alkaline phosphatase for 10 min under the same conditions. Following the 20-min total reaction time, the proteins were denatured by heating at 90 °C for 10 min, the reaction was analyzed by 18% polyacrylamide-4% bis-acrylamide Beckman Ultrasphere ODS semipreparative column (5 μm particle size; 4.6 mm x 25 cm) utilizing a flow rate of 2.4 ml/min and monitoring the UV absorbance at 254 nm. Elution was performed as described for 3'-HE-dU (above). Under those conditions authentic standards exhibited good separation with retention times of dC, 15.8 min; dU, 18.4 min; deoxyinosine (di), 22.3 min; dG, 23.2 min; dT, 25.6 min; 3'-HE-dU, 26.3 min; and dA, 30.8 min. Response factors of the deoxynucleosides (at 254 nm) were established by integrating the peaks areas when equimolar amounts were injected. Deoxythymidine was used as the reference and all other products were normalized to it.

RESULTS

Chemical Synthesis of Site-modified Oligodeoxynucleotide.—A 17-nucleotide long oligomer, containing a single 3'-HE-dU adduct, was synthesized by the solid-phase phosphite triester method (48). During synthesis, 3'-HE-dU was site specifically incorporated into the oligomer by the use of DMTM-(3-AE)dU-phosphoramidite (Fig. 1). The phosphoramidite was synthesized by reacting 1-bromo-2-acetoxyethane with dU in a bimolecular (S,S) manner under mildly basic conditions, followed by sequential protection of the 5'-OH group of the resulting 3'-HE-dU adduct with 4.4'-dimethoxytrityl (DMTr) group and conversion of the 3'-OH into the 3'-phosphoramidite. The overall yield of the phosphoramidite was >40%. A key feature in the synthesis of DMTM-(3-AE)dU-phosphoramidite (Fig. 1) was the utilization of an acetyl (Ac) group for protection of the N3 side chain hydroxyl moiety. The acetyl group is stable under the acidic conditions employed during oligonucleotide synthesis and afforded the added advantage of being cleaved in the final...
The intermediate product was purified to homogeneity as judged by presence of the bH peptide at 262 nm, which is consistent with alkylation at N3 of dU and a sharp single spot on TLC and absence of the starting material, and fully characterized by mass spectroscopy.

The CI mass spectrum of 3-AE-dU displayed an MH ion at 315 confirming the expected molecular weight of 314. The NMR spectrum of 3-AE-dU at pH 6 (H2O) displayed a peak corresponding to the methylene protons of the side chain. Based on the presence of two correlated resonances (δ 4.04 and 4.18) corresponding to the methylene protons of the side chain, the structure of the phosphoramidite (Fig. 1).

Assignment of the side chain residue in 3-AE-dU was confirmed by the presence of two correlated resonances (δ 4.04 and 4.18) corresponding to the methylene protons of the side chain. Based on the NMR spectrum of 3-(2-acetoxypropyl)dU, the up field resonance (δ 4.04) was tentatively assigned to the methylene protons adjacent to N3 of dU and the down field one (δ 4.18) to the protons adjacent to the acetate group. As demonstrated by MS and co- chromatography with an authentic marker (21) 3-HE-dU was exclusively produced when 3-AE-dU was deblocked by concentrated ammonium hydroxide at 70 °C. The completely deprotected oligomer was purified by coelution with an authentic synthetic marker (21) and the concomitant presence of signals arising from the diisopropyl (δ 1.20) and cyanoethoxy (δ 2.45) residues, established the structure of the phosphoramidite (Fig. 1).

The pure and fully characterized phosphoramidite was used to insert the 3-HE-dU adduct into the oligomer during oligodeoxynucleotide synthesis. Since 3-HE-dU is stable to alkaline conditions, all protecting groups from the synthesized oligomer were fully removed by treatment with concentrated ammonia at 70 °C. The completely deprotected oligomer was purified by anion-exchange HPLC followed by electrophoresis on a 16% polyacrylamide, 8 M urea gel. The 5'-OH in 3-AE-dU was deprotected by 2-acetoxyethyl-N,N-diisopropylphosphoramidite. The profile of nucleoside separation by HPLC is shown. Enzymatic digestion and HPLC conditions are given in the text. A, nucleotide analysis of 3-HE-dU-containing 17-mer. B, nucleotide analysis of dU-containing 17-mer (control). The inset in A represents an autoradiogram showing the purity of the 3-HE-dU-containing 17-nucleotide oligomer. Lane 1, 17-nucleotide marker containing the same sequences as the 3-HE-dU-containing 17-mer except that dU is in place of 3-HE-dU. Lane 2, 3-HE-dU-containing 17-mer.

The pure and fully characterized phosphoramidite was used to insert the 3-HE-dU adduct into the oligomer during oligodeoxynucleotide synthesis. Since 3-HE-dU is stable to alkaline conditions, all protecting groups from the synthesized oligomer were fully removed by treatment with concentrated ammonia at 70 °C. The completely deprotected oligomer was purified by anion-exchange HPLC followed by electrophoresis on a 16% polyacrylamide, 8 M urea gel. The 5'-32P-labeled purified oligomer moved as a sharp band on a 20% polyacrylamide, 8 M urea sequencing gel and had the same mobility as a standard 17-mer marker (Fig. 2B, inset). The 3-HE-dU adduct in the oligomer was quantitated by HPLC analysis (Fig. 2B) of the nucleosides released by enzymatic hydrolysis of the oligomer. The 3-HE-dU peak was unambiguously identified by coelution with an authentic synthetic marker (21) and by UV absorption characteristics. No additional peak, except dI peak (retention time, 22.3 min), was observed (Fig. 2B). The dI peak (retention time, 22.3 min), was observed (Fig. 2B). The dI peak (retention time, 22.3 min), was observed (Fig. 2B).
peak was present in the hydrolysates of both site-modified and control oligomers (Fig. 2). The di was derived from deamination of dA by the contaminating 2'-deoxyadenosinedeoaminationase in the phosphodiesterase preparation used to hydrolyze the oligomers. This was evident by the increase in the di peak (data not shown) with longer incubation periods during enzymatic hydrolysis. For quantitation, the amount of di formed was added to the amount of da obtained. Analysis of the nucleosides released from the site-modified oligomer revealed a ratio of 2.1:1.9:3:0:2.1:1.2 between dC/dG/dT/dA/3-HE-dU, as compared with the predicted ratio of 2:2:3:9:1. The results demonstrate that 3-HE-dU is present in all oligomer molecules. This is consistent with the absence of the dU peak (retention time, 18.4 min) in the analysis of the site-modified oligomer (Fig. 2A) as compared to its presence in the control dU-containing oligomer (Fig. 2B).

**In Vitro DNA Replication System**—The in vitro DNA replication system (Fig. 3) was used to study the DNA replication properties of 3-HE-dU, present at a single site in the DNA template. The system utilizes a primed template, similar to the system used in our laboratory for DNA replication studies of the ethylating agent-induced 3-Et-dT (36, 37, 53) and O'-Et-dT (47, 52, 53), except that the length of the site-modified template was increased from 36 to 55 nucleotides without changing the location of the lesion in the system. The increase in the length of the site-modified template was designed to facilitate DNA sequencing of the lesion-bypass products by the Sanger dideoxynucleotide method. The 3-HE-dU lesion was present at template position 26 from the 3'-end and was eight nucleotides away from the 3' terminus of the hybridized primer. This system represents a “running start” for 3-HE-dU in DNA replication in that synthesis occurs prior to the polymerase reaching the lesion. When the polymerase encounters 3-HE-dU, the lesion may block DNA replication producing a 25-nucleotide “preblocked” product, terminating 3' to 3-HE-dU, without incorporation of a nucleotide opposite the lesion, and a 26-nucleotide “blocked” product, terminating after incorporating a nucleotide opposite 3-HE-dU. The 3-HE-dU lesion may allow DNA synthesis past the lesion producing a 55-nucleotide “bypassed” product. The representative DNA products produced during DNA synthesis past 3-HE-dU are shown in Fig. 4. During DNA replication using a 3'→5' exonuclease-deficient polymerase, a 56-nucleotide bypassed product, due to blunt-end addition (54) at the synthesized 55-nucleotide duplex, may also be obtained. In that case, the 55- and 56-nucleotide products were combined for analysis.

**DNA Replication Block by 3-HE-dU**—The 3-HE-dU lesion, present at a single site in a DNA template, blocked DNA synthesis by Kf Pol I. In the control, containing dU in place of 3-HE-dU, DNA synthesis was not interrupted and proceeded to the 5'-end of the template yielding a full-length (55-nucleotide) product (data not shown). The results indicated that in the site-modified template, 3-HE-dU was responsible for blocking DNA replication. In the presence of 5 mM Mg2+ and 10 µM dNTP, the major DNA synthesis product was a 25-nucleotide preblocked product (72%) obtained by DNA replication intcrupted 3'→3-HE-dU (Fig. 5A). Kf Pol I was able to incorporate a nucleotide opposite 3-HE-dU, but DNA synthesis was blocked after incorporation resulting in the accumulation of a 26-nucleotide blocked product (25%). Since DNA synthesis past 3-HE-dU was not observed, the lesion represented a complete block to DNA replication by Kf Pol I.

The effect of dNTP concentration on nucleotide incorporation opposite 3-HE-dU was significant. Fig. 5A presents the effect of dNTP concentration on the relative formation of various DNA products synthesized by Kf Pol I. When dNTP concentration was increased from 10 to 200 µM, the incorporation opposite 3-HE-dU was increased from 25 to 60%. This increase was accompanied by a corresponding decrease in the preblocked product. The accumulation of the 26-nucleotide blocked product represents an equilibrium between nucleotide insertion opposite 3-HE-dU by the polymerase activity and removal of the inserted nucleotide by the 3'→5' exonuclease proofreading activity of Kf Pol I. Since DNA synthesis past 3-HE-dU was not observed (Fig. 5A), even at high dNTP concentration (200 µM), the results suggest that Kf Pol I could incorporate a nucleotide opposite 3-HE-dU, but the resulting base pair was not a suitable substrate for extension. These results are consistent with the analogous 3-Et-dT lesion, where 3-Et-dT presented a complete block to DNA replication by Kf Pol I in the presence of Mg2+ (36).

Kf Pol I, a “repair” polymerase used in the above experiments has low processivity (dissociating before the incorporation of an average 10 nucleotides) and a weak 3'→5' exonuclease proofreading activity (55). In order to investigate the effects of polymerase processivity and efficient proofreading on bypass replication of 3-HE-dU, DNA replication was catalyzed by a “replicative” polymerase, T7 Pol. The in vitro DNA replication system used in these studies was the same as used for Kf Pol I. T7 Pol is a highly processive enzyme (incorporating thousands of nucleotides on the same template before dissociating) and
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posesses a potent (100-fold) 3' → 5' exonuclease activity (56), as compared with Kf Pol I. At 10 μM dNTP and 5 mM Mg²⁺, 3-HE-dU blocked DNA synthesis (92%) by T7 Pol 3' to 3-HE-dU (preblocked product), and 6% after incorporating a nucleotide opposite the lesion (blocked product). DNA synthesis past 3-HE-dU was negligible (<2%; Fig. 6A). These results are similar to those obtained with Kf Pol I except that the blocked product terminating after incorporation opposite 3-HE-dU was higher (25%) with Kf Pol I. The results suggest that the nucleotide incorporated opposite 3-HE-dU was efficiently removed by the potent exonuclease activity of T7 Pol. As shown in Fig. 6A, nucleotide incorporation opposite 3-HE-dU increased with increasing dNTP concentration, reaching 39% at 200 μM, compared to 60% with Kf Pol I (Fig. 5A). Absence of post-lesion synthesis, even at higher dNTP concentration (200 μM), suggests that 3-HE-dU presents a strong block to DNA replication by T7 Pol. Synthesis of the 26-nucleotide blocked products (Figs. 5A and 6A) indicated that T7 Pol and Kf Pol I could incorporate a nucleotide opposite 3-HE-dU, but that the resulting base pair, present at the growing 3'-end of the primer, was difficult to elongate.

To identify the nucleotide incorporated opposite 3-HE-dU during the replication block, the 26-nucleotide blocked products, synthesized by Kf Pol I and T7 Pol, were sequenced by the modified Maxam-Gilbert procedure. The sequencing results, obtained from the 26-nucleotide blocked product synthesized by Kf Pol I, are shown in Fig. 7. An identical sequencing pattern (not shown) was obtained from the blocked product synthesized by T7 Pol. The results indicate that both Kf Pol I and T7 Pol preferentially incorporated dA opposite 3-HE-dU. Incorporation ofdT or other nucleotides at low levels may not be detected by this sequencing procedure. The results suggest that although specificity of nucleotide incorporation opposite 3-HE-dU is the same for T7 Pol and Kf Pol I, T7 Pol with a more potent proofreading activity than Kf Pol I was more efficient in excising dA from the 3-HE-dU-dA base pair formed at the replication fork making elongation at 3-HE-dU difficult.

DNA Synthesis Past 3-HE-dU—In general, DNA lesion bypass is strongly inhibited in the presence of 3' → 5' exonuclease proofreading activity of the polymerase (57). Since DNA synthesis past 3-HE-dU is a necessary step to produce mutation, the effect of the polymerase proofreading activity in mediating DNA synthesis past 3-HE-dU was investigated. When Kf Pol I (exo-), a polymerase lacking 3' → 5' exonuclease activity was substituted for Kf Pol I, DNA synthesis past 3-HE-dU occurred (Fig. 4A). At 10 μM dNTP, the bypass synthesis was 32% which increased with increasing dNTP concentration reaching 58% at 200 μM (Fig. 5B). This is in contrast to post-lesion synthesis by Kf Pol I, where DNA synthesis past 3-HE-dU was negligible (<3%; Fig. 5A). At 200 μM dNTP, only 4% of DNA synthesis by Kf Pol I (exo-) was blocked 3' to 3-HE-dU indicating that incorporation of a nucleotide opposite 3-HE-dU, to produce a 26-nucleotide blocked product (38%) and a bypassed product (58%), was 96%. In the absence of proofreading the nucleotide incorporated opposite 3-HE-dU in the blocked product was not cleaved, allowing the nucleotide incorporated opposite 3-HE-dU to remain in position long enough for elongation past 3-HE-dU to occur (58%). Accumulation (38%), even at high dNTP concentration (200 μM), of the 26-nucleotide blocked product indicates that the 3-HE-dU-containing base pair could be extended, but with low efficiency.

Similar results were obtained when T7 Pol was substituted with T7 Pol (exo-), a polymerase lacking proofreading (Fig. 4B). At 10 μM dNTP, DNA synthesis past 3-HE-dU was 55% (Fig. 6B). The 25-nucleotide preblocked product, terminating 3' to 3-HE-dU, was 10% suggesting that incorporation opposite
3-HE-dU, to produce a 26-nucleotide blocked product (35%) and a bypassed product (55%), was 90%. The extension of the blocked product was increased with increasing dNTP concentration reaching 90% at 200 μM dNTP (Fig. 6B). This is in contrast to T7 Pol where DNA synthesis past 3-HE-dU was negligible (<2%; Fig. 6A). The formation of a 25-nucleotide preblocked product in higher yields (10–12-fold) during DNA synthesis by T7 Pol (Fig. 6A) as compared to T7 Pol (exo-)(Fig. 6B) is consistent with exonucleolytic cleavage of the incorporated nucleotide opposite 3-HE-dU in the 26-nucleotide blocked product.

In vitro DNA replication studies of 3-HE-dU with Kf Pol I (exo-) and T7 Pol (exo-) indicate that, in the absence of proofreading, the 3-HE-dU lesion could be bypassed. T7 Pol (exo-) was more efficient in elongation at 3-HE-dU than Kf Pol I (exo-). At 200 μM dNTP, while bypass synthesis by Kf Pol I (exo-) was <60% (Fig. 5B), the extension by T7 Pol (exo-) was >90% (Fig. 6B). Efficient extension by T7 Pol (exo-) is consistent with the high processivity of T7 polymerase as compared to the low processivity of Kf Pol I. In general, processive polymerases translocate along DNA faster than they dissociate from primer template termini (57).

To identify the nucleotide incorporated opposite 3-HE-dU during post-lesion synthesis by Kf Pol I (exo-) and T7 Pol (exo-), the 55-nucleotide bypassed products synthesized at 200 μM dNTP were isolated and sequenced by Sanger’s dideoxy sequencing method. The sequencing results are shown in Fig. 8. Both dA and dT were incorporated opposite 3-HE-dU during bypass synthesis by Kf Pol I (exo-) or T7 Pol (exo-). Incorporation of dA and dT opposite 3-HE-dU is not an artifact of the specific sequence used in DNA replication studies reported here. When 3-HE-dU was replaced with O’-ethyldeoxothymidine (O’-Et-dT) in the same DNA sequence, both dA and dG (but not dT) were incorporated opposite O’-Et-dT (53), which is consistent with published reports (58, 59). Since 3-HE-dU is induced at a dC moiety, incorporation of dA and dT opposite 3-HE-dU implicates this lesion in transversion and transition mutagenesis at G-C base pairs.

**DISCUSSION**

The in vitro DNA replication studies described here demonstrate that under conditions of relaxed fidelity, i.e. absence of proofreading, dA and dT are incorporated opposite 3-HE-dU. Since 3-HE-dU is derived from ethylene oxide-induced 3-HE-dC, incorporation of dA and dT opposite 3-HE-dU replicates this lesion in G-C → A-T and G-C → T-A mutagenesis. Our studies suggest that 3-HE-dU may be a critical premutagenic lesion produced by the mutagenic and carcinogenic ethylene oxide *in vitro* and epoxide-induced 3-hydroxyalkyldeoxyuridine (3-HA-dU) lesions may contribute to mutagenesis and initiation of cancer by other environmentally important epoxides.

DNA synthesis past a lesion is a necessary step to produce mutation by the lesion. In general, post-lesion synthesis results from three independent reactions (60): nucleotide insertion opposite the lesion, excision by proofreading of the inserted nucleotide before extension, and extension past the lesion. Proofreading by exonuclease becomes more proficient when the extension step is slow. In studies reported here significant accumulation of the 26-nucleotide blocked product (Figs. 5A and 6A), terminating after incorporation of a nucleotide opposite 3-HE-dU during polymerization by Kf Pol I and T7 Pol, suggests that addition opposite 3-HE-dU is a relatively easy step. Negligible post lesion synthesis (<3%) indicates that extension at 3-HE-dU is rate-limiting. The 3’ → 5’ exonuclease proofreading activity, associated with the polymerases, made the extension at 3-HE-dU impossible by excising the incorporated nucleotide opposite 3-HE-dU before extension could occur. The potent exonuclease activity of T7 Pol (56), as compared to the weak activity of Kf Pol I (55), is manifested in the accumulation of the 26-nucleotide blocked product in lower yields during DNA replication by T7 Pol (Fig. 6A) compared to Kf Pol I (Fig. 5A). Use of Kf Pol I (exo-) and T7 Pol (exo-), polymerases lacking proofreading activity, facilitated DNA synthesis past 3-HE-dU (Figs. 5B and 6B). The results are consistent with the suggestion that the elongation step at 3-HE-dU is rate-limiting. The absence of proofreading facilitated bypass at 3-HE-dU by allowing the nucleotide incorporated opposite 3-HE-dU to remain in position long enough for elongation to occur.
Base substitution mutations constitute an important component of the mutational spectra induced by ethylene oxide in vivo at the hypoxanthine-guanine phosphoribosyltransferase (hprt) locus in different systems. In T-lymphocytes of ethylene oxide-exposed B6C3F1 mice (29), A-T → T-A, A-T → G-C, G-C → T-A and G-C → A-T mutations were induced with almost equal frequency. In human diploid fibroblasts (30), ethylene oxide induced both G-C → T-A transversions (30%) and G-C → A-T transitions (30%) at G-C base pairs, but only A-T → T-A transversions (40%) were produced at A-T base pairs. In peripheral lymphocytes of workers exposed to ethylene oxide (18), a hot spot for G-C → A-T transition mutation was observed at the hprt locus. Mutagenicity studies using S. typhimurium test strains have emphasized the importance of G-C and G-C → T-A transversion mutations at 30%. The mutational spectra induced by ethylene oxide in vivo and in vitro have been shown to induce mainly G-C and G-C → A-T mutations (66). A small component of the mutational spectra induced by ethylene oxide exposure in vivo may contribute to A-T mutations (66-68).

We have recently reported in vivo formation of 3-(2-hydroxyethyl)dideoxythymidine (3-HE-dT) in DNA after reaction with ethylene oxide (21). This lesion, which is formed in a relatively small amount (0.5%), is chemically stable in DNA (21). In the absence of a known DNA repair activity for 3-HE-dT, this lesion may be persistent in vivo. The 3-HE-dT lesion may contribute to A-T → T-A transversion mutations by mispairing with dA or through AP site formation in DNA replication (66, 67). The 3-HE-dT lesion is not known to be repaired rapidly and without error (66). Other than the 3-HE-dA adduct judged to be mutagenic by ethylene oxide at A-T base pairs, are not known. 7-HE-dG is the major adduct of dG produced by ethylene oxide in vivo and in vitro (23, 25), where during post-lesion repair, the 7-HE-dG lesion undergoes rapid hydroxylation and that the 3-HE-dC adduct undergoes rapid hydroxylation and glycosylase-mediated removal of 3-HE-dA. Since 3-HE-dA is rapidly removed from DNA both in vivo and in vitro (23, 25), the involvement of this lesion in mutagenesis by ethylene oxide at A-T base pairs is uncertain. A substantial body of experimental evidence has indicated that it is not the initial level of DNA adduction but the in vivo persistence (lack of repair) and mutagenic bypass of premutagenic DNA adducts which is of major importance in mutagenesis and carcinogenesis (62-65). Other adducts that can contribute to A-T → T-A mutagenesis either by mispairing with dA or through AP site formation include N2-, N4-, N7-hydroxyethyldeoxycytosine.

We have recently reported the in vitro formation of 3-(2-hydroxyethyl)dideoxythymidine (3-HE-dT) in DNA after reaction with ethylene oxide (21). This lesion, which is formed in a relatively small amount (0.5%), is chemically stable in DNA (21). In the absence of a known DNA repair activity for 3-HE-dT, this lesion may be persistent in vivo. The 3-HE-dT lesion may contribute to A-T → T-A transversion mutations by mispairing with dA or through AP site formation in DNA replication (66). Support for this hypothesis is derived from our in vitro DNA replication studies of the analogous 3-Et-dT lesion (37) that during post-lesion synthesis, dT was incorporated opposite 3-HE-dT leading to A-T → T-A transversion mutagenesis.

The ethylene oxide-induced modifications of dG and dC, responsible for transversion and transition mutations at G-C base pairs, are not known. 7-HE-dG is the major adduct of dG produced by ethylene oxide both in vivo and in vitro (21, 24, 25). 7-HE-dG may not be premutagenic, but the lesion may contribute to mutagenesis through depurination or imidazole ring opening. AP sites have been shown to induce mainly G-C → T-A and G-C → A-T mutations (66-68). AP sites produced in vivo are usually repaired rapidly and without error (66). A small amount of O6-HE-dG is also induced in DNA by ethylene oxide (24, 25). O6-AlkyldG is of major importance in mutagenesis by alkylating agents (69) giving rise to G-C → A-T transition mutations (70, 71). Unrepaired O6-HE-dG may contribute to G-C → A-T mutagenesis by ethylene oxide. However, O6-HE-dG is efficiently removed from DNA of most tissues, except brain, presumably by O6-alkylguanine-DNA alkyltransferase and excision repair (27, 28). Persistence studies have indicated that the in vivo half-life of 7-HE-dG is significantly greater than O6-HE-dG (25). Based on the formation of 7-HE-dG as the major ethylene oxide-induced DNA adduct and induction of the same G-C → A-T and G-C → T-A mutations by ethylene oxide and AP sites, depurination of 7-HE-dG has been suggested to contribute to ethylene oxide mutagenesis at G-C base pairs (29, 30). However, lack of in vivo persistence of the lesion (25) is not consistent with this suggestion.

We have shown that ethylene oxide reacts with dC at the N3 position and that the 3-HE-dC adduct undergoes rapid hydrolytic deamination to produce a potentially mutagenic lesion, 3-HE-dU (21, 34). This lesion is chemically stable in DNA (21), and no repair activity has been reported in prokaryotes or eukaryotes. In vivo persistence of 3-HE-dU may contribute to mutagenesis at G-C base pairs by ethylene oxide. Our DNA replication studies have implicated 3-HE-dU in G-C → A-T and G-C → T-A mutagenesis. The studies described here suggest that 3-HE-dU may be a critical mutagenic lesion produced by ethylene oxide in vivo and that 3-HE-dU may contribute to transition and transversion mutagenesis at G-C base pairs by other epoxides.

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