The Role of Mutagenic Metal Ions in Mediating *In Vitro* Mispairing by Alkylpyrimidines

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A variety of alkylating mutagens and carcinogens produce pyrimidine adducts in DNA that block DNA synthesis *in vitro*. Since DNA synthesis past the lesion is a necessary step to produce mutations, we investigated the role of the mutagenic metal ion Mn^{++} in facilitating DNA synthesis past alkylpyrimidines. In the presence of the natural metal activator Mg^{++}, *N*3-ethyldeoxythymidine (*N*3-Et-dT) and O^2-ethyldeoxythymidine (O^2-Et-dT), present at a single site in DNA, blocked *in vitro* DNA synthesis 3' to the lesion and after incorporating dA opposite each lesion. The presence of Mn^{++} permitted postlesion synthesis with dT misincorporated opposite *N*3-Et-dT and O^2-Et-dT, implicating these lesions in A•T→T•A transversion mutagenesis. The DNA synthesis block by O^4-ethyldeoxythymidine (O^4-Et-dT) in the presence of Mg^{++} was partial and was also removed by Mn^{++}. Consistent with *in vivo* studies, dG was incorporated opposite O^4-Et-dT during postlesion synthesis, leading to A•T→G•C transition mutagenesis. We also have discovered a new class of DNA adducts, *N*3-hydroxyalkyldeoxyuridine (3-HA-dU) lesions, which are produced by mutagenic and carcinogenic aliphatic epoxides. 3-HA-dU is formed after initial alkylation at the *N*3 position of dC followed by a rapid hydrolytic deamination. As observed with the analogous mutagenic *N*3-Et-dT, the ethylene oxide-induced 3-hydroxyethyldeoxyuridine (3-HE-dU) blocked *in vitro* DNA synthesis, which could be bypassed in the presence of Mn^{++}. The nucleotide incorporated opposite 3-HE-dU during postlesion synthesis is being identified. These studies suggest a role for Mn^{++} in mediating mutagenic and carcinogenic effects of environmentally important ethylating agents and aliphatic epoxides. — Environ Health Perspect 102(Suppl 3):81-90 (1994).

Key words: alkylating agents, alkylpyrimidines, DNA adducts, divalent metal activator, misincorporation, DNA synthesis, mutation, nitroso compounds, DNA polymerase

Introduction

Alkylating agents have been used extensively in studying the mechanisms of mutagenicity and carcinogenicity (*1–8*), because of their ability to react with DNA either *in vitro* or *in vivo*. Of the best characterized alkylating agents are the *N*-nitroso compounds. Their occurrence is widespread in the environment, and human exposure from natural and pollutant sources is universal (*9*). These agents induce tumors in a wide variety of tissues of different animal species (*5,10–14*) and probably humans (*9,15*).

Most *N*-nitroso-alkylating agents mediate their biological activities in part by interacting with genomic DNA, forming covalent adducts (*4*). Alkylation at thymine occurs at nucleophilic oxygen sites, such as the O^4- and O^2positions of the base and at the *N*3 position (*4,16*). There are many factors determining the sensitivity to the toxic, mutagenic, and carcinogenic potential of *N*-nitroso alkylating agents. One of these factors is the capacity to repair alkylated DNA. A substantial body of experimental evidence has indicated that it is not the initial level of alkylation but the persistence (lack of repair) of premutagenic alkyl adducts in tissues which is of major importance in mutagenesis and malignancy in specific organs. This has been demonstrated for O^6-alkyldeoxyguanosine (O^6-alkyl-dG) and O^4-alkyldeoxythymidine (O^4-alkyl-dT), where the persistence of these lesions did correlate with organotropic malignancy (*17–20*). A wide range of independent studies has indicated that the repair of ethyldeoxythymidine (Et-dT) adducts in mammalian cells is very slow (*21*). These adducts are among the highly persistent DNA alkylation products in both cultured mammalian cells and animal tissues (*19,22*). The persistence of premutagenic Et-dT adducts would increase the probability of mutation at A•T base pairs relative to G•C. This is consistent with the prevalence of transversion and transition mutations at A•T base pairs following *in vivo* exposure of mice to *N*-ethyl-*N*-nitrosourea (ENU) (*23*).

The mutational spectra of ENU has been reported in a variety of systems. In *E. coli*, ENU induced mainly G•C→A•T and A•T→G•C transition mutations (*24*). Presumably, these mutations resulted from the unrepaired O^6-Et-dG (*2,25,26*) and O^4-Et-dT (*2,5,27*) lesions, respectively, as a consequence of their capacity to mispair with dT and dG, respectively, during DNA replication. Under SOS-induction, ENU generated a large fraction (46%) of transversion mutations at A•T base pairs in *E. coli* (*28*). In human cells, ENU produced a significant number (29% or more) of transversion mutations at the A•T base pairs (*8,29*) in addition to the same G•C→A•T and A•T→G•C transitions observed in *E. coli*. *In vivo* exposure of mice to ENU predominantly (94%) induced transversion and transition mutations at A•T base pairs (*23*). A study of mutational spectra in Salmonella attributed transversions at A•T base pairs to Et-dT adducts (*6*). Transitions at A•T base pairs can result from O^4-Et-dT. The Et-dT lesions responsible for transversion mutations at A•T base pairs are not known.

The biological importance of A•T transversion mutations has been demonstrated in mammalian systems. The A•T→T•A transversion event has been proposed to account for two mutations of

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the mouse α- and β-globin genes arising in the progeny of ENU-treated female mice (*31,32*). Tumors of the nervous system induced by transplacental treatment of rats with ENU contained the *neu* oncogene activated by an A•T→T•A transversion mutation (*13,14*). The two activating mutations, observed in c-Ha-*ras* genes of liver tumors induced by treating mice with diethylnitrosamine (*33*), were the A•T→T•A and A•T→G•C that occurred at codon 61.

We also have discovered a new class of DNA adducts, 3-HA-dU, which are produced by the mutagenic and carcinogenic epoxides ethylene oxide (EO), propylene oxide (PO), glycidol, epichlorohydrin (ECH), and the epoxide of acrylonitrile (*34–38*). 3-HA-dU is formed after initial alkylation at the *N*3 position of dC, followed by rapid hydrolytic deamination to 3-HA-dU (*34,37,38*). The hydroxyalkyl group of 3-HA-dU occupies a central Watson-Crick hydrogen-bonding position and is likely to disrupt normal base pairing. 3-HA-dU is stable in DNA *in vitro* and may be the critical promutagenic lesion produced by aliphatic epoxides *in vivo*. The role of 3-HA-dU in mutagenesis by aliphatic epoxides is unknown.

To ascertain the mutagenic potential of pyrimidine alkylation in DNA, we initiated *in vitro* DNA replication studies on Et-dT (*39–43*) and 3-HA-dU lesions (*44,45*), site-specifically placed in the same DNA template sequence. *In vitro* replication studies cannot precisely mimic the actual conditions of *in vivo* DNA synthesis, but they have proven to be a powerful means to understand the mechanisms responsible for the fine nucleotide selection exhibited by DNA polymerases. The site-modified template used in the *in vitro* DNA replication studies corresponds to a portion of the bacteriophage ϕX174 genome in the gene G region (*46*). In addition to replication studies, the same DNA sequence can be used in separate *in vivo* site-specific mutagenesis studies, using the ϕX174-based mutagenesis system (*25*) to facilitate comparison between *in vitro* and *in vivo* mutagenesis studies.

In the presence of the natural metal activator Mg^{++}, Et-dT and 3-HA-dU adducts interfered with DNA replication by the Klenow fragment of *E. coli* DNA polymerase I (Kf Pol I) (*39,41,44*). The block by O^4-Et-dT was partial, while O^2-Et-dT, *N*3-Et-dT and 3-HA-dU lesions presented a complete block to DNA replication. Since DNA replication past a lesion is required to produce mutations, we investigated the role of mutagenic metal ions, such as Mn^{++}, in mediating DNA synthesis past Et-dT and 3-HA-dU lesions. Mn^{++} facilitated mispairing by Et-dT (*40,41,43*) and 3-HA-dU (*44*) adducts and subsequent extension of the resulting mispair. Postlesion synthesis implicated Et-dT adducts in A•T→T•A and A•T→G•C mutations. The epoxide-induced 3-HA-dU lesion may be involved in G•C→A•T transitions. A mechanistic explanation for Mn^{++}-mediated mispairing by Et-dT and 3-HA-dU is not known. Mn^{++} binds to nucleotides (*47–51*) and hence may affect either template or substrate molecules so as to alter their base-pairing properties. Alternatively, Mn^{++} may interact with DNA polymerase (*50*), either reducing the accuracy of base selection prior to insertion (*52*) or modifying an exonucleaselike proofreading function. Mn^{++} is known to modify fidelity of DNA replication by DNA polymerases (*53–56*) and facilitate DNA synthesis past DNA lesions (*5,57–59*). Mn^{++}, which is a weak mutagen, has been shown to exert strong comutagenic effects with UV (*60*). In this paper we summarize the DNA replication properties of ethylating agent-induced Et-dT adducts (O^4-, O^2- and *N*3-Et-dT), epoxide-induced 3-hydroxyalkyl-dU [3-HE-dU from EO and 3-hydroxypropyldeoxyuridine (3-HP-dU) from PO] and the role of Mn^{++} in mediating mutagenesis by these lesions.

Biological Significance of Thymine Ethylation in DNA

The alkylating agent ENU is capable of inducing a variety of tumor types in a broad range of animal species (*10–14*) and humans (*9,15*). The reactivity of ENU allows it to form a diverse set of DNA adducts both *in vitro* and *in vivo* (*4,6,16*). The order of formation of Et-dT adducts is O^2-Et-dT > O^4-Et-dT > *N*3-Et-dT. These lesions are poorly repaired in mammalian systems (*21*) and thus may be more biologically important in these systems. Ethylation of dT may alter its base-pairing pattern to form a miscoding lesion such as O^4-Et-dT (*5,61*). Alternatively, ethylation may compromise the ability of the base to serve as a template during DNA replication, producing noncoding lesions such as *N*3-Et-dT (*39,62*) and O^2-Et-dT (*1,41*). Under conditions of relaxed fidelity of DNA synthesis, the noncoding lesions may mispair to produce mutations.

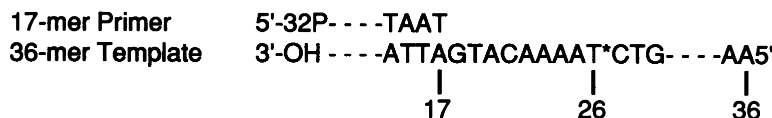

Figure 1. DNA replication system for T* = O^2-Et-dT, O^4-Et-dT or *N*3-Et-dT. For the *N*3-Et-dT-containing template, the base 5' to the lesion is T (*39*).

Transition and transversion mutations at A•T base pairs form an important component of ethylating agent-induced mutagenesis in SOS-induced bacteria (*6,28*), human cells (*8,29*) and animal systems (*23*), suggesting that dA or dT adducts and/or a breakdown product of these adducts are responsible for A•T mutations. A•T→G•C transitions can be derived from O^4-Et-dT by mispairing with dG (*27*). A comparison of ENU-induced mutations with base substitutions produced by other alkylating agents in bacteria and human cells has led to the suggestion that O^2-Et-dT may be a significant premutagenic lesion in mammalian cells capable of inducing A•T→T•A tranversion mutations (*8,29,63*). Indirect support for this hypothesis was derived from mutations observed *in vivo* in ENU-treated mice, where mutations at A•T base pairs accounted for 94% of all mutations (*23*). Among the A•T mutations, 55% were A•T→T•A transversions. To ascertain the mutagenic potential of Et-dT lesions, we studied *in vitro* DNA replication properties of each Et-dT lesion site-specifically incorporated into the same DNA template. The replication studies utilized the primed template system shown in Figure 1.

The replication system contains a 36-nucleotide site-modified DNA template hybridized to a ^{32}P-labeled 17-nucleotide complementary primer. The construction of site-modified templates has been described (*39,42*). The Et-dT adducts and their derivatives, used in the synthesis of site-modified oligomers, were fully characterized by thin-layer chromatography, high pressure liquid chromatography (HPLC), ultraviolet, mass and nuclear magnetic resonance (NMR) spectroscopy. The presence of the Et-dT moiety in the purified oligomer was demonstrated by HPLC analysis of the nucleosides released from the site-modified oligomer following diges-

tion with phosphodiesterase and phosphatase (*39,42*).

In the DNA replication system (Figure 1), the 3'-end of the primer is eight nucleotides away from the thymine modification (T*) present in the template. This system represents a "running start" for DNA replication in that synthesis occurs prior to the polymerase reaching the lesion. The hybridized primer is extended by the polymerase until T* is encountered. The following DNA products, reflecting the influence of template T*, are feasible. First, the progress of the polymerase is blocked 3'to T*. No nucleotide is incorporated opposite the lesion and a 25-nucleotide preincorporation blocked product accumulates. Second, DNA synthesis terminates after incorporating a nucleotide opposite T*, producing a 26-nucleotide incorporation-dependent blocked product. Finally, DNA synthesis proceeds past the lesion yielding a 36-nucleotide postlesion synthesis product. Products of DNA synthesis were analyzed by polyacrylamide gel electrophoresis. Since the ^{32}P-end labeled primer is used to prime DNA synthesis, each product is only labeled once at the 5'-end. This facilitates the quantitation of DNA synthesis products in the polymerization reaction by measuring the radioactivity associated with the individual product bands. The identity of the nucleotide incorporated opposite T* was established by DNA sequencing of the 26-nucleotide blocked and the 36-nucleotide postlesion synthesis products.

DNA Replication Properties of *O*^{2}-Et-dT

The *O*^{2}-position of dT does not participate in Watson-Crick base pairing. However, ethylation of the *O*^{2}-position of dT interferes with normal hydrogen bonding of dT with dA, by fixing the thymine base in the enol tautomer with the loss of a hydrogen atom at the central hydrogen-bonding site (*N*3) of dT (*42*). Disruption of normal base pairing may inhibit DNA synthesis. This is consistent with our DNA replication studies where, in the presence of the natural metal activator Mg^{++}, *O*^{2}-Et-dT blocked DNA replication by Kf Pol I predominantly 3' to the lesion (*41*). DNA synthesis past the lesion was negligible (<1%). Incorporation of dA opposite *O*^{2}-Et-dT occurred with increasing deoxyribonucleoside-5'-triphosphate (dNTP) concentrations (*41*), which was further enhanced by inhibiting the 3'→5' exonuclease proofreading activity of the polymerase with deoxyadenosine-5'-phosphate. The postlesion synthesis remained negligible (*41*). The *O*^{2}-Et-dT•dA base pair may occur with the formation of two hydrogen bonds between the *O*^{4} of *O*^{2}-Et-dT and the *N*^{6} hydrogen atom of dA, and between the *N*3 of *O*^{2}-Et-dT and the protonated *N*1 of dA. A similar hydrogen bonding scheme has been suggested for *O*^{4}-Et-dT•dA by NMR studies (*64*). The *O*^{2}-Et-dT•dA base pair with two hydrogen bonds is expected to be thermodynamically stable. This is consistent with thermal denaturing studies where *O*^{2}-Et-dT, present in the alternating poly[d(A•T)]polymers, did not alter the thermal melting profile (*65*).

Inhibition of DNA synthesis after incorporation of dATP opposite template *O*^{2}-Et-dT (*41*) or *O*^{2}-Et-dT-5'-triphosphate opposite template dA (*66*) suggests that the geometric conformation of the *O*^{2}-Et-dT•dA base pair deviates significantly from that of the normal Watson-Crick pair and may adopt a wobble conformation (*64,67*). In the wobble conformation, phosphodiester links (both 3' and 5' to dA) may have to be distorted to accommodate the *O*^{2}-Et-dT•dA base pair in a DNA helix. This hypothesis is consistent with molecular and computer modeling studies, indicating that the presence of *O*^{2}-alkyl-dT in DNA may cause distortion in the DNA structure (*1*). Additional support for this hypothesis is derived from ^{31}P NMR studies of DNA duplexes, containing the wobble base pairs *O*^{6}-Et-dG•dC and *O*^{4}-Et-dT•dA (*64*). Distortion of the phosphodiester links 3' and 5' to dT and dG, respectively, was observed. The conformational changes associated with phosphodiester bonds during the formation of the *O*^{2}-Et-dT•dA base pair are expected to adversely effect the catalysis of phosphodiester links on both the 3' and 5' sides of the incoming dA opposite *O*^{2}-Et-dT during DNA replication. This would suggest that incorporation of dA opposite *O*^{2}-Et-dT and extension of the resulting *O*^{2}-Et-dT•dA base pair will be inefficient. This is supported by *in vitro* DNA replication studies in the presence of Mg^{++}, where DNA synthesis was terminated predominantly (94%) 3' to *O*^{2}-Et-dT, and postlesion synthesis did not occur (*41*). Similar results were obtained during DNA replication using bacteriophage T7 DNA polymerase (T7 Pol) (*42*). The block by the *O*^{2}-Et-dT•dA base pair appears to be an inherent property of the spatial conformation of this base pair, since it was observed repeatedly during synthesis by Kf Pol I (*41*) or T7 Pol (*42*) in the presence of Mg^{++} or Mn^{++} from running (*41,42*) or standing (*68*) starts. Under normal cellular conditions, extension of the *O*^{2}-Et-dT•dA base pair may either not occur or may occur with low efficiency. Our DNA replication studies in the presence of Mg^{++} (*41*) suggest that *O*^{2}-Et-dT may contribute in part to the cytotoxicity of ethylating agents.

Since DNA replication past the lesion is a necessary step in the production of mutations, we investigated the role of the mutagenic metal ion Mn^{++} in mediating DNA synthesis past the *O*^{2}-Et-dT adduct. When Mn^{++} was substituted for Mg^{++} in the polymerization reaction, incorporation of a nucleotide opposite *O*^{2}-Et-dT and subsequent postlesion synthesis were enhanced (*41*). Increasing the dNTP concentration and inhibiting the proofreading activity of Kf Pol I increased postlesion synthesis (which reached 66% at 200 μM dNTP) (*41*). DNA sequencing of the blocked and postlesion synthesis products revealed that while dA was present opposite *O*^{2}-Et-dT in the blocked product, both dA and dT were present opposite the lesion in the postlesion synthesis product (*41*). The presence of dA opposite *O*^{2}-Et-dT in both the blocked and postlesion synthesis products indicates that, in the presence of Mn^{++}, the *O*^{2}-Et-dT•dA base pair at the 3'-end of the growing chain can be extended but inefficiently. This is in contrast to DNA replication in the presence of Mg^{++}, where the *O*^{2}-Et-dT•dA base pair was not extended. Absence of dT opposite *O*^{2}-Et-dT in the blocked product suggests that formation of an *O*^{2}-Et-dT•dT base pair at the replication fork is efficiently extended. The results implicate *O*^{2}-Et-dT in transversion mutagenesis at A•T base pairs and suggest a role for Mn^{++} in mediating A•T→T•A transversion mutation by *O*^{2}-Et-dT.

Formation of a pyrimidine-pyrimidine base pair is rare. The *O*^{2}-Et-dT•dT base pair probably has one hydrogen bond forming between the *N*3 nitrogen atoms. The pairing of two pyrimidines would allow a long hydrogen bond, which would decrease steric hindrance between the ethyl group of *O*^{2}-Et-dT and the carboxyl group at C2 of dT. This hydrogen-bonding scheme could result in a normal sugar-phosphate backbone with the *O*^{2}-Et-dT base pair retaining the Watson-Crick alignment. The normal Watson-Crick alignment of the *O*^{2}-Et-dT•dT mispair would facilitate formation of phosphodiester bonds on both the 3' and 5' sides of dT. This is consistent with our DNA replication studies (*41*), where incorporation of dT opposite *O*^{2}-Et-dT was effi-

ciently extended. Other mispairs, including O^6-Et-dG•dT and O^4-Et-dT•dG, which contain one hydrogen bond and retain normal Watson-Crick alignment, have been shown to be efficiently extended *in vitro* (*61,69*). These studies suggest that correct alignment of the backbone is crucial in DNA replication (*64,67*). The strength of hydrogen bonding 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 hypothesis.

The kinetic mechanisms by which O^2-Et-dT impedes DNA synthesis and miscodes were studied (*70*). The kinetic parameters, K_m and V_{max}, for dA and dT insertion opposite and extension past O^2-Et-dT by Kf Pol I in the presence of Mg^{++} and Mg^{++}, were determined using a polyacrylamide gel assay (*71–73*). Insertion and extension frequencies of O^2-Et-dT•dA and O^2-Et-dT•dT base pairs, relative to the right base pair (dT•dA), were estimated from V_{max}/K_m ratios. The preliminary results revealed (*70*) that, in the presence of Mn^{++}, O^2-Et-dT inhibited insertion and extension of dA and dT at this lesion with efficiencies of 10^{-4} or lower. As compared to Mg^{++}, Mn^{++} increased insertion frequencies of dA and dT opposite O^2-Et-dT by 10-fold or more. The insertion was enhanced primarily through K_m discrimination. The extension frequency at the O^2-Et-dT•dT base pair was enhanced 6-fold when Mn^{++} was substituted for Mg^{++} in the polymerization reaction. Mn^{++} had little effect on extension of the O^2-Et-dT•dA base pair. As compared to O^2-Et-dT•dA, the O^2-Et-dT•dT mispair was extended 30 times more efficiently (*70*). A higher extension frequency of the O^2-Et-dT•dT mispair was primarily achieved through an increase in V_{max}. The results suggest that, as compared to Mg^{++}, Mn^{++} may increase the residence time of the O^2-Et-dT•dT mispair at the catalytic site of the polymerase. These kinetic studies are consistent with *in vitro* DNA replication studies of O^2-Et-dT discussed above (*41,42*). They suggest that the O^2-Et-dT•dA base pair plays a central role in the inhibition of DNA synthesis by O^2-Et-dT and is consistent with the notion that this base pair may cause distortion in the DNA structure (*1*). Efficient insertion and extension of dT at O^2-Et-dT suggest a role for Mn^{++} in mediating A•T→T•A transversion mutagenesis by O^2-alkyl-dT lesions.

DNA Replication Properties of O^4-alkyl-dT

The established role of O^6-alkyl-dG in the mutagenesis (*2,25,26*) and initiation of carcinogenesis (*12,17*) by alkylating agents, obscured the possible similar role of other alkyl DNA adducts. Recently, increased attention has been focused on *O*-alkylpyrimidines. Several studies have correlated the persistence of O^4-alkyl-dT in target tissue with organ specificity of tumors resulting from *N*-nitrosoalkylating agents (*18–20*). 5'-Triphosphates of O^4-alkyl-dT were able to substitute for dTTP in poly[d(A•T)] synthesis by *E. coli* DNA polymerase (*65,74*). The resulting polymer, poly[d(A•T,O^4-alkyl-dT)] supported the incorporation of dG during *in vitro* DNA replication by the same polymerase. The results suggest the likely pairing of O^4-alkyl-dT with dG as well as with dA. The role of O^4-alkyl-dT in A•T→G•C transition was unambiguously established through site-specific mutagenesis studies on O^4-Me-dT (*27*). The alkyl group of O^4-alkyl-dT is located within the Watson-Crick base pairing region and may interfere with normal hydrogen bonding of dT with dA. The O^4-alkyl-dT lesion may behave as a miscoding and/or noncoding lesion during DNA replication. The noncoding lesions usually inhibit DNA replication by distorting the DNA structure. Miscoding lesions alter the precision of base pairing during DNA synthesis leading to mutation. The role of O^4-alkyl-dT in blocking DNA synthesis and the reaction conditions allowing DNA synthesis past the lesion were studied through *in vitro* DNA replication studies of O^4-Et-dT, site-specifically incorporated into a DNA template. The replication studies utilized the primed-template shown in Figure 1.

In the presence of the natural metal activator Mg^{++}, O^4-Et-dT presented a partial (54%) block to DNA replication by Kf Pol I, with replication mainly (48%) interrupted 3' to O^4-Et-dT. DNA synthesis past the O^4-Et-dT lesion was 46%. Accumulation of the blocked product, obtained after incorporation of a nucleotide opposite O^4-Et-dT, was low (6%) and remained constant over a wide range of dNTP concentrations (10–200 µM). The results suggest that, during DNA replication past O^4-Et-dT in the presence of Mg^{++}, insertion of a nucleotide opposite this lesion may be the rate-limiting step. The replication block by O^4-Et-dT was removed when Mn^{++} was substituted for Mg^{++} and a postlesion synthesis product was obtained in high yield (>90%). DNA sequencing revealed that predominantly dG was incorporated opposite O^4-Et-dT (Figure 2) during DNA synthesis past the lesion. The results implicate Mn^{++} in facilitating the insertion of dG opposite O^4-Et-dT and extension of the resulting base pair.

Kinetics of insertion opposite template O^4-Me-dT have been described (*61*). From V_{max}/K_m ratios, the pairing of O^4-Me-dT•dG was preferred 10-fold over that of O^4-Me-dT•dA. The relative incorporation efficiencies of dA and dG opposite O^4-Me-dT appears to reflect the relative rates of base pair formation

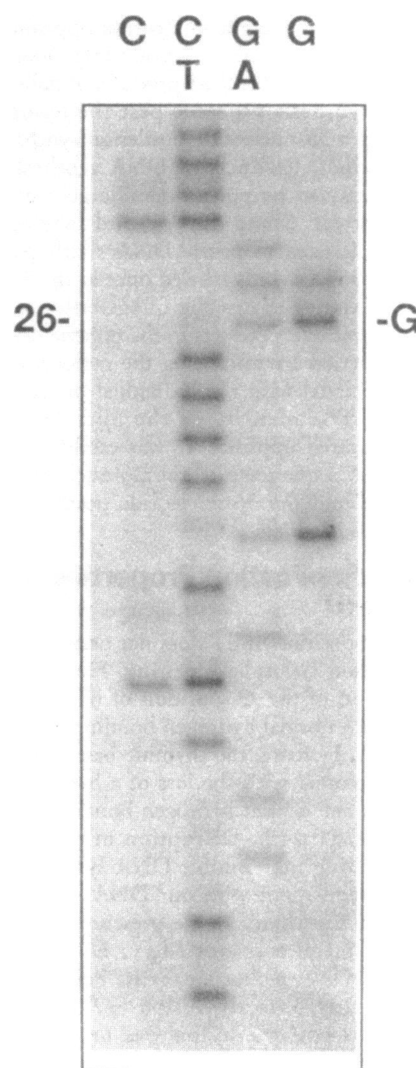

Figure 2. DNA sequence analysis of the postlesion synthesis product synthesized on an O^4-Et-dT-containing template by Kf Pol I in the presence of Mn^{++}. A Maxam-Gilbert sequencing gel is shown. The presence of bands in dG-specific lanes at position 26 indicates incorporation of dG opposite O^4-Et-dT during postlesion synthesis.

(nucleotide insertion) rather than the effect of proofreading because the efficiencies using Kf Pol I were similar to those using the *Drosophila melanogaster* polymerase α-primase complex, which does not contain detectable 3'→5' exonuclease proofreading activity (*75*). In the absence of any dNTP, the "weak" 3'→5' exonuclease activity of Kf Pol I did not excise the O^4-Me-dT•dG base pair (*5*). This is consistent with our DNA replication studies where the O^4-Et-dT•dG base pair can be easily extended.

NMR studies on DNA duplexes containing O^4-Me-dT have indicated that the O^4-Me-dT•dA base pair has a wobble conformation with the alkylated base moved towards the major groove of the helix (*64*). A wobble alignment for O^4-Me-dT•dG was ruled out and it was suggested that this base pair retains the normal Watson-Crick alignment (*64*). Due to steric hindrance by the O^4-methyl group, the normal alignment may have only one hydrogen-bond between the 2-amino of dG and the O^2 of O^4-Me-dT. The O^4-Me-dT•dG mispair having less hydrogen bonding than O^4-Me-dT•dA is consistent with the optical melting profiles of duplexes where the duplex containing O^4-Me-dT•dA pairs melted at higher temperatures than the one containing O^4-Me-dT•dG pairs (*76*).

An important factor in miscoding by O^4-Me-dT is that the O^4-Me-dT•dG mispair retains the Watson-Crick alignment, with no distortion of phosphodiester links 3' and 5' to the dG, as revealed by NMR studies (*64*). This is consistent with the greater efficiency of dG incorporation opposite O^4-Me-dT as compared to dA (*61*). Stimulation of dG incorporation opposite O^4-Et-dT in the presence of Mn^{++} in our studies suggests a role for Mn^{++} in stabilizing the O^4-Et-dT•dG mispair in a normal Watson-Crick alignment to facilitate the formation of phosphodiester bonds. Based on NMR studies on nucleotide binding to *E. coli* DNA polymerase I (*77*), it has been suggested that the polymerase-Mn^{++} complex may be less selective of the sugar ring conformation of the nucleotide than the enzyme complexed with Mg^{++}. Binding of the dNTP substrate to the active site of the polymerase in the presence of Mn^{++} may occur in conformations favorable for Watson-Crick base pairing in DNA B form. These types of conformations may not be favored in the presence of Mg^{++}. The DNA replication studies suggest a role for Mn^{++} in stimulating A•T→G•C transition mutagenesis by O^4-alkyl-dT lesions.

DNA Replication Properties of *N*3-Et-dT

*N*3-alkyl-dT is formed, both *in vitro* and *in vivo*, but in relatively small amounts (*4,6,16*). Among the alkylating agents, ENU demonstrates a significant amount of binding to *O*-alkyl and *N*3-alkyl pyrimidines. *N*3-alkyl-dT is stable in DNA *in vitro*. No DNA repair activity has been reported for *N*3-alkyl-dT. This DNA lesion may be persistent *in vivo* and exerts its biological consequences long after exposure has occurred. The alkyl group of *N*3-alkyl-dT occupies the central Watson-Crick hydrogen bonding site (*N*3) of thymine and is likely to interfere with normal hydrogen bonding of dT, probably leading to mispairing and/or inhibition of DNA synthesis. *N*3-alkyl-dT is likely to be a potentially cytotoxic and mutagenic lesion produced by alkylating agents. The biological significance of the *N*3-alkyl-dT lesion was ascertained through *in vitro* DNA replication studies of *N*3-Et-dT (*39,40,43*) present at a single site in the DNA template shown in Figure 1.

In the presence of the natural metal activator Mg^{++} and a low dNTP concentration (10 µM), *N*3-Et-dT blocked DNA synthesis by Kf Pol I predominantly 3' to *N*3-Et-dT. DNA synthesis past the lesion was not observed (*39*). Incorporation of dA opposite *N*3-Et-dT occurred with increasing dNTP concentrations, but no postlesion synthesis was obtained (*39*). Similar results were obtained during DNA replication with T7 Pol, where incorporation of dA opposite *N*3-Et-dT blocked DNA synthesis in the presence of Mg^{++} (*43*). These studies implicate *N*3-Et-dT as a potentially cytotoxic lesion produced by ethylating agents.

No postlesion synthesis suggests that the *N*3-Et-dT•dA base pair, formed at the replication fork, did not retain the Watson-Crick alignment and may cause distortion in the DNA structure. Due to steric hindrance by the lesion, translocation of the polymerase to the nucleotide past the lesion may be extremely slow. Polymerization of the nucleotide past *N*3-Et-dT may also be very slow, owing to the need to extend the distorted terminus formed by the wobble *N*3-Et-dT•dA base pair. During a pause at the lesion, the polymerase may dissociate from the lesion-blocked primer-template complex and cease elongation. Once dissociated, rebinding of the polymerase to the primer-template complex may lead to formation of a defective initiation complex of lower stability. This complex may be stable enough to allow the relatively easy first polymerization step of inserting dA opposite *N*3-Et-dT, but not the next very slow step of extending the *N*3-Et-dT•dA base pair. This notion is consistent with DNA replication studies (*39*), where in the presence of Mg^{++} the blocked product, obtained after incorporation of dA opposite *N*3-Et-dT, was formed but not extended.

When Mn^{++} was substituted for Mg^{++}, incorporation of dA opposite *N*3-Et-dT was increased from 4% in the presence of Mg^{++} to 60% in the presence of Mn^{++} at 10 µM dNTP (*40*). At this dNTP concentration, DNA synthesis past the lesion was not obtained. Postlesion synthesis occurred at higher dNTP concentrations and reached 68% at 200 µM. During postlesion synthesis, dT was incorporated opposite *N*3-Et-dT (*40*), implicating this lesion in transversion mutagenesis at the A•T base pair by ethylating agents. The results suggest a role for Mn^{++} in mediating an A•T→T•A transversion mutation by the *N*3-Et-dT lesion.

The absence of dT opposite *N*3-Et-dT in the blocked product and its presence only in the postlesion synthesis product (*40*) suggest that the *N*3-Et-dT•dT mispair formed at the replication fork is not inhibitory to DNA synthesis. This mispair is efficiently extended, leading to an A•T→T•A mutation. These results are similar to those observed in the case of O^6-Me-dG•dT (*64*), O^4-Me-dT•dG (*64*) and O^2-Et-dT•dT (*41,42*) mispairs, which were efficiently extended and led to mutations. As shown for O^6-Me-dG•dT and O^4-Me-dT•dT mispairs (*64*), the *N*3-Et-dT•dT mispair may also retain the normal Watson-Crick alignment, facilitating extension of this mispair. This is consistent with postlesion synthesis in high yield (68%) observed in our DNA replication studies (*40*).

Since the *N*3-Et-dT•dT mispair is efficiently extended, incorporation of dT opposite *N*3-Et-dT appears to be the rate-limiting step during postlesion synthesis. In contrast to the low dNTP concentration (10 µM), higher dNTP concentrations facilitated insertion of dT opposite *N*3-Et-dT (*40*). Polymerization of the next correct nucleotide following the *N*3-Et-dT•dT mispair protected the mispair from excision by the proofreading activity of the polymerase. Since the polymerase has difficulty extending from the *N*3-Et-dT•dA base pair, this base pair becomes susceptible to

12-mer Primer 5'-32P- - - -TAAT
50-mer Template 3'-OH - - - -ATTAGTACAAAAU*CTGAAAA - - - TCGA-5'
12 21 50

Figure 3. DNA replication system for U* = 3-HE-dU or 3-HP-dU.

proofreading. Excision of dA from *N*3-Et-dT•dA makes the template *N*3-Et-dT lesion available for insertion of dA and dT opposite the lesion with dT insertion efficiently extended. The net result of this cycling is accumulation of the postlesion synthesis product, containing dT opposite *N*3-Et-dT in high yield (68%) (*40*).

In contrast to Kf Pol I, T7 Pol was highly specific in incorporating only dT opposite *N*3-Et-dT in the presence of Mn^{++} during postlesion synthesis (>95%) even at 10 μM dNTP (*43*). The greater ability of T7 Pol to incorporate dT opposite *N*3-Et-dT may reflect the large difference in processivity between T7 Pol and Kf Pol I (*67*). T7 Pol incorporates thousands of nucleotides prior to dissociating from the template (*78*). The high affinity of T7 Pol for the DNA template may increase the likelihood of insertion and extension of dT opposite *N*3-Et-dT by the polymerase. The high yield of postlesion synthesis probably was achieved through protection of the *N*3-Et-dT•dT mispair by extension and excision of the *N*3-Et-dT•dA base pair by the highly potent 3'→5' exonuclease proofreading activity associated with T7 Pol.

Biological Significance of Aliphatic Epoxide-induced 3-HA-dU Lesions

Aliphatic epoxides include a number of very reactive reagents. They contain three-membered rings, which are highly strained. The least substituted carbon, which is sterically more accessible, is the site of attack of most epoxides (S_N2 mechanism) under neutral conditions. These epoxides are a class of direct-acting alkylating agents, which are effective mutagens and animal carcinogens (*79–81*). Because of their usefulness as chemical reagents, the simple volatile epoxides, especially EO, PO, and ECH, are used extensively in industry and result in potential human exposure in the work place. An association between exposure to EO and cancer has led to the suggestion that EO may be involved in the etiology of human cancer. PO and ECH have been shown to be rodent carcinogens.

Alkylation of DNA, followed by the persistence of premutagenic adducts during the period of DNA replication and cell division, seem to be necessary but not sufficient requirements for initiation of carcinogenesis with many alkylating agents. We discovered a new class of DNA adducts (*34–37*), 3-HA-dU, which is produced by mutagenic and carcinogenic epoxides. The 3-HA-dU lesion is stable in DNA *in vitro*. This lesion is formed in DNA after rapid hydrolytic deamination of a 3-hydroxyalkyldeoxycytidine (3-HA-dC) adduct (*34,38*). The initial reaction of S_N2 epoxides occurs at *N*3 of dC. The charge on the protonated 3-HA-dC intermediate (pK≈9) is delocalized over the C4 carbon, which is then attacked by the side chain OH group, resulting in the loss of ammonia (deamination) with retention of the charge at C4. Hydroxyl attack at this carbon results in ring opening and formation of 3-HA-dU (Solomon, unpublished). The halflives of hydrolytic deamination of 3-HE-dU and 3-HP-dU at pH 7.4 and 37°C were 10 hr and 6 hr respectively (*34,37*).

The 3-hydroxyalkyl group of 3-HA-dU occupies a central Watson-Crick hydrogen bonding position and is likely to disrupt normal hydrogen bonding, leading to mispairing and/or inhibition of DNA replication. 3-HA-dU may be the critical premutagenic lesion produced by S_N2 epoxides *in vivo*. In order to understand the role of 3-HA-dU in mutagenesis and cancer induction, we initiated *in vitro* DNA replication studies of 3-HE-dU and 3-HP-dU, produced by EO and PO respectively.

The replication system for 3-HA-dU (Figure 3) is similar to the one used for Et-dT adducts (Figure 1), except that the primer used was a 12-mer and the site-modified DNA template was a 50-mer. Use of the 50-nucleotide template was necessitated by the need to facilitate sequencing of the postlesion synthesis product by Sanger's dideoxy-sequencing protocol, and to incorporate the site-modified 50-mer into a 50-nucleotide gap in ϕX174 replicative form DNA for *in vivo* site-specific mutagenesis studies. To facilitate comparison of the results between Et-dT and 3-HA-dU adducts, U* was located in the same DNA sequence from ϕX174. The site-modified oligomers were fully characterized for their purity, expected DNA sequence and presence of U* in essentially all oligomer molecules. The details of the synthesis of site-modified oligomers and their characterization will be described elsewhere. In this replication system, the expected DNA synthesis products are a 20-nucleotide preincorporation-blocked, a 21-nucleotide incorporation-blocked and a 50-nucleotide postlesion synthesis product.

DNA Replication Properties of 3-HE-dU

As observed with the analogous mutagenic lesion, *N*3-Et-dT, 3-HE-dU blocked DNA synthesis by Kf Pol I (Figure 4). In the presence of the natural metal activator Mg^{++} and at 10 μM dNTP, the majority (70%) of the block was 3' to 3-HE-dU and the remainder (27%) was blocked after incorporating a nucleotide opposite 3-HE-dU (*44*). Synthesis past the lesion was negligible (<3%). Incorporation opposite 3-HE-dU increased with increasing dNTP concentrations, reaching 60% at 200 μM. Postlesion synthesis remained negligible (<3%). DNA sequencing of the 21-nucleotide blocked product revealed that

Figure 4. *In vitro* DNA replication catalyzed by Kf Pol I in the presence of Mg^{++} on a template containing 3-HE-dU and 3-HP-dU at a single site.

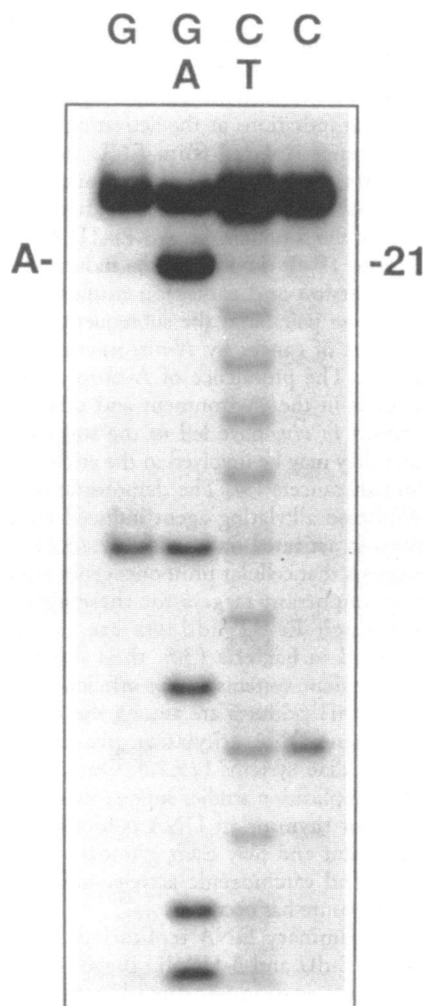

Figure 5. DNA sequence analysis of the 21-nucleotide blocked product synthesized on a 3-HE-dU-containing template by Kf Pol I in the presence of Mg^{++}. A Maxam-Gilbert sequencing gel is shown. Presence of a band in the dA-specific lane at position 21 indicates incorporation of dA opposite 3-HE-dU during the synthesis block.

dA is incorporated opposite 3-HE-dU (Figure 5). Since postlesion synthesis is negligible, the results suggest that the 3-HE-dU•dA present at the growing replication fork is inhibitory to DNA synthesis. The results are similar to the analogous *N*3-Et-dT lesion, where the *N*3-Et-dT•dA base pair was not extended in the presence of Mg^{++} (*39*). Our DNA replication studies implicate 3-HE-dU as a potentially-cytotoxic lesion produced by EO.

During *in vitro* DNA replication, the 3-HE-dU•dA base pair behaved in a manner similar to *N*3-Et-dT•dA, O^2-Et-dT•dA, O^4-Et-dT•dA, and O^6-Me-dG•dC base pairs. As suggested for the O^4-Me-dT•dA and O^6-Me-dG•dC base pairs (*64*), the 3-HE-dU•dA base pair may also exist in a wobble conformation and cause distortion in the DNA structure, making extension of this pair difficult. This increases the exposure time of the 3-HE-dU•dA base pair to the 3'→5' exonuclease activity of the polymerase, making the base pair susceptible to excision by proofreading. This was manifested in our DNA replication studies when Kf Pol I (exo^-) (deficient in 3'→5' exonuclease) was substituted for Kf Pol I (exo^+). As expected, post lesion synthesis was dramataically increased from <3% for Kf Pol I to <50% for Kf Pol I (exo^-).

Substitution of Mn^{++} for Mg^{++} increased incorporation opposite 3-HE-dU and subsequent synthesis past the lesion (Figure 6). At 200 µM dNTP, postlesion synthesis increased from <3% in the presence of Mg^{++} to >85% in the presence of Mn^{++}. The specificity of nucleotide incorporation opposite 3-HE-dU is under investigation.

The Mn^{++}-mediated synthesis past 3-HE-dU suggests that the role of Mn^{++} in modifying the fidelity of Kf Pol I in DNA replication may be comparable to the SOS-induced functions in bacteria. In bacteria, SOS-induced proteins may alter the fidelity of the DNA replication complex, facilitating the incorporation and subsequent extension at 3-HE-dU. This hypothesis suggests that mutagenesis by aliphatic epoxide-induced 3-HA-dU requires induction of the SOS system in bacteria. This is supported by the production of SOS-dependent mutagenesis by PO at template cytosines (*45*). Involvement of the SOS-like system in mammalian cells is not known. Inside the mammalian cell, DNA polymerase-accessory proteins may facilitate incorporation and subsequent extension at 3-HA-dU. Since 3-HA-dU is derived from deamination of aliphatic epoxide-induced 3-HA-dC, extension of all base pairs (except 3-HA-dU•dG) at 3-HA-dU will produce mutations. Our *in vitro* DNA replication studies have demonstrated formation of a 3-HE-dU•dA base pair. *In vivo* extension of this pair will produce a G•C→A•T transition mutation and implicate the 3-HE-dU lesion in G•C→A•T transition mutagenesis by EO. Support for this hypothesis is derived from our mutagenesis studies with PO, where G•C→A•T transitions represent an important component of PO-induced mutational spectra (*45*).

DNA Replication Properties of 3-HP-dU

PO-induced 3-HP-dU behaved in a similar manner as 3-HE-dU (*44*) and the analogous *N*3-Et-dT lesion during *in vitro* DNA replication (*39,40*). 3-HP-dU blocked DNA synthesis by Kf Pol I in the presence of Mg^{++} 3' to 3-HP-dU and after incorporating a nucleotide opposite the lesion (Figure 4). Postlesion synthesis was negligible. Substitution of Mn^{++} for Mg^{++} mediated DNA synthesis past 3-HP-dU. The specificity of the nucleotide incorporated opposite 3-HP-dU in the blocked and postlesion synthesis products is being investigated.

Role of Mn^{++} in Mediating Mispairing

Manganese is known to be highly mutagenic *in vivo* and to reduce the fidelity of DNA synthesis *in vitro* (*82*). It also shows a strong co-mutagenic effect with UV (*60*). The mechanism of Mn^{++}-induced mutagenesis has been studied using *E. coli* DNA polymerase I (*50*). The role of a free Mn^{++} concentration on Mn^{++}-induced mutagene-

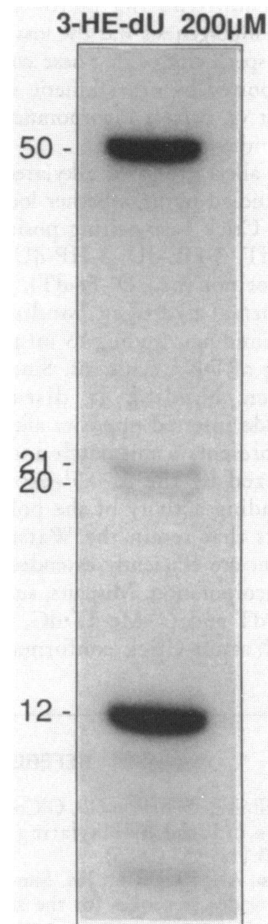

Figure 6. *In vitro* DNA replication catalyzed by Kf Pol I in the presence of Mn^{++} on a template containing 3-HE-dU at a single site.

sis *in vitro* was determined by an analysis of the polymerase error rate and a comparison with dissociation constants of Mn^{++} from the enzyme, template, and dNTP. These studies suggest that, in the presence of physiologically-relevant free Mn^{++} concentrations (10–100 µM), Mn^{++} induced misincorporations by interacting with the DNA template rather than with the polymerase (*50*). At higher Mn^{++} concentrations (0.5–1.5 mM), Mn^{++}-induced mutagenesis is probably due to Mn^{++} association either with single-stranded regions of template DNA or with weak sites in the polymerase. The results with T4 DNA polymerase suggest that the mutagenic action of Mn^{++} can be attributed primarily to a significant differential increase in binding of mispaired relative to correctly paired nucleotides to the polymerase-template complex (*49*). The resulting increase in residence times for mispaired nucleotides on the complex results in their increased frequency of misinsertion. A smaller contributing factor to Mn^{++}-induced mutagenesis was the loss of proofreading specificity (*49*). These conclusions are supported by other kinetic studies of incorrect vs. correct incorporation during DNA synthesis (*51,83*).

The alkyl group of alkylated pyrimidines studied by us, whether located at a Watson-Crick base-pairing position (i.e., *N*3-Et-dT, 3-HE-dU, 3-HP-dU and O^4-Et-dT) or not (i.e., O^2-Et-dT), interferes with normal hydrogen bonding of the alkylpyrimidines, leading to mispairing or blocking of DNA synthesis. Since normal hydrogen bonding is disrupted, a nucleotide inserted opposite alkylpyrimidine represents a mismatch and may be recognized by the 3'→5'-exonuclease proofreading activity of the polymerase. Mispairs that retain the Watson-Crick alignment are efficiently extended, leading to misincorporation. Mispairs, such as O^6-Me-dG•dT and O^4-Me-dT•dG, shown to retain Watson-Crick conformation, are efficiently extended (*64*). Mispairs that adopt a wobble conformation are either not extended or extended inefficiently, owing to the distortion caused by the mispair in the DNA structure. We postulate that the role of Mn^{++} in mediating mispairing by alkylpyrimidines is to increase the probability of inserting a nucleotide opposite the lesion in a conformation that retains the Watson-Crick alignment. In our DNA replication studies, a higher Mn^{++} concentration (500 µM) was used. At this concentration, Mn^{++} binds to DNA polymerase, template DNA, and dNTP substrates (*50*). The normal Watson-Crick alignment of the mispairs formed by the O^2-, O^4- and *N*3-Et-dT lesions may have been achieved through interactions of Mn^{++} with the polymerase-template-dNTP complex. NMR studies of nucleotide binding to *E. coli* DNA polymerase I have shown that binding of the dNTP substrate to the active site of the polymerase in the presence of Mn^{++} may occur in conformations favorable for Watson-Crick base pairing in DNA B form. These types of conformations may not be favored in the presence of Mg^{++} (*77*). This hypothesis is consistent with our DNA replication studies, where O^2-Et-dT•dT, O^4-Et-dT•dG and *N*3-Et-dT•dT mispairs are formed and efficiently extended in the presence of Mn^{++} but not Mg^{++}.

Conclusion

Alkylation of thymine in DNA is toxic, mutagenic, and carcinogenic. Et-dT adducts block *in vitro* DNA synthesis, often after incorporating dA opposite the lesions. *In vivo* extension of the Et-dT•dA base pair is nonmutagenic. Failure to extend the Et-dT•dA base pair implicates Et-dT lesions in cytotoxicity by ethylating agents. Mn^{++}-mediated mispairing and bypass of Et-dT adducts suggest a comutagenic role for Mn^{++} in the mutagenicity of ethylating agents and implicates Et-dT adducts in A•T→T•A and A•T→G•C mutations.

The implication, that ENU-induced Et-dT lesions can produce A•T→T•A and A•T→G•C mutations, and the observation of these mutations at the activating site of oncogenes isolated from ENU-induced tumors, emphasize the existence of an important, but unproved, relationship among the formation of *N*3-Et-dT, O^2-Et-dT and O^4-Et-dT lesions, the induction of transversion and transition mutations at A•T base pairs, and the subsequent development of cancer by *N*-nitrosoethylating agents. The prevalence of *N*-nitroso compounds in the environment and their formation *in vivo* have led to the suggestion that they may be involved in the etiology of human cancers (*9*). The demonstration of *N*-nitroso alkylating agent-induced mutations in activated oncogenes (*11,13,14,32*) suggests that cellular protooncogenes represent important targets for these agents. Although Et-dT adducts are rapidly repaired in bacteria (*30*), their repair in mammalian systems is not efficient (*21*). The Et-dT adducts are among the highly-persistent DNA ethylation products in mammalian systems (*19,22*). Our *in vitro* DNA replication studies suggest that ethylation of thymine in DNA is biologically significant and may exert cytotoxic, mutagenic and carcinogenic activity long after the exposure has occurred.

Preliminary DNA replication studies of 3-HE-dU and 3-HP-dU suggest a dual role for epoxide-induced 3-HA-dU lesions. They block DNA replication *in vitro* and may terminate DNA synthesis *in vivo*, contributing to the cytotoxicity of aliphatic epoxides. Under relaxed polymerase fidelity (Mn^{++}), DNA synthesis past 3-HE-dU and 3-HP-dU occurs, implicating these lesions in mutagenesis at G•C base pairs by epoxides. The studies provide a basis for understanding molecular mechanisms by which environmentally important aliphatic epoxides produce mutations and contribute to the process of carcinogenesis.

REFERENCES

1. Saffhill R, Margison GP, O'Connor PJ. Mechanisms of carcinogenesis induced by alkylating agents. Biochim Biophys Acta 823:111–135 (1985).
2. Basu AK, Essigmann JM. Site-specifically modified oligodeoxynucleotides as probes for the structural and biological effects of DNA-damaging agents. Chem Res Toxicol 1:1–18 (1988).
3. Horsfall MJ, Gordon AJE, Burns PA, Zielenska M, van der Vliet GME, Glickman BW. Mutational specificity of alkylating agens and the influence of DNA repair. Environ Mol Mutagenesis 15:107–122 (1990).
4. Singer B, Grunberger D. Molecular Biology of Mutagens and Carcinogens. New York:Plenum Press, 1983.
5. Singer B. O-Alkyl pyrimidines in mutagenesis and carcinogenesis: occurrence and significance. Cancer Res 46:4879–4885 (1986).
6. Guttenplan JB. Mutagenesis by *N*-nitroso compounds: relationships to DNA adducts, DNA repair, and mutational efficiencies. Mutat Res 233:177–187 (1990).
7. Maher VM, Domoradzki J, Bhattacharya NP, Tsujimura T, Corner RC, McCormick JJ. Alkylation damage, DNA repair and mutagenesis in human cells. Mutat Res 233:235–245 (1990).

8. Bronstein SM, Cochrane JE, Craft TR, Swenberg JA, Skopek TR. Toxicity, mutagenicity, and mutational spectra of *N*-ethyl-*N*-nitrosourea in human cell lines with different DNA repair phenotypes. Cancer Res 51:5188–5197 (1991).
9. Bartsch H, Ohshima H, Shuker DEG, Pignatelli B, Calmels S. Exposure of humans to endogenous *N*-nitroso compounds: implications in cancer etiology. Mutat Res 238:255–267 (1990).
10. Bogovski P, Bogovski S. Animal species in which *N*-nitroso compounds induce cancer. Int J Cancer 27:471–474 (1982).
11. Zarble H, Sukumar S, Arthur AV, Martin-Zanca D, Barbacid M. Direct mutagenesis of Ha-*ras*-1 oncogenes by *N*-nitroso-*N*-methylurea during initiation of mammary carcinogenesis in rats. Nature (London) 318:382–385 (1985).
12. Pegg AE, Singer B. Is O^6-alkylguanine necessary for initiation of carcinogenesis by alkylating agents? Cancer Invest 2: 221–238 (1984).
13. Bargmann CI, Hung M-C, Weinberg RA. Multiple independent activations of the *neu* oncogene by a point mutation altering the transmembrane domain of p185. Cell 45: 649–657 (1986).
14. Perantoni AO, Rice JM, Reed CD, Watatani M, Wenk ML. Activated *neu* oncogene sequences in primary tumors of the peripheral nervous system induced in rats by transplacental exposure to ethylnitrosourea. Proc Natl Acad Sci USA 84:6317–6321 (1987).
15. Magee PN. The experimental basis for the role of nitroso-compounds in human cancer. Cancer Surv 8:208–239 (1989).
16. Beranek DT. Distribution of methyl and ethyl adducts following alkylation with monofunctional alkylating agents. Mutat Res 231: 11–30 (1990).
17. Goth R, Rajewsky MF. Persistence of O^6-ethylguanine in rat brain DNA. Correlation with nervous system specific carcinogenesis by ethylnitrosourea. Proc Natl Acad Sci USA 71:639–643 (1974).
18. Lewis JG, Swenberg JA. Differential repair of DNA in rat hepatocytes and nonparenchymal cells. Nature (London) 288: 185–187 (1980).
19. Swenberg JA, Dyroff MC, Bedell MA, Popp JA, Huh N. Kirstein U, Rajewsky MF. O^4-ethyldeoxythymidine, but not O^6-ethyldeoxyguanosine, accumulates in hepatocyte DNA of rats exposed continuously to diethylnitrosamine. Proc Natl Acad Sci USA 81:1692–1695 (1984).
20. Huh N, Rajewsky MF. Enzymatic elimination of O^6-ethylguanine from the DNA of ethylnitrosourea-exposed normal and malignant rat brain cells grown under cell culture versus *in vivo* conditions. Int J Cancer 41:762–766 (1988).
21. Brent TP, Dolan ME, Fraenkel-Conrat H, Hall J, Karran P, Laval F, Margison GP, Montesano R., Pegg AE., Potter PM, Singer B, Swenberg JA, Yarosh DB. Repair of O-alkylpyrimidines in mammalian cells: a present consensus. Proc Natl Acad Sci USA 85: 1759–1762 (1988).
22. Den Engelse L, De Graaf A, De Brij R-J, Menkveld G J. O^2- and O^4-ethylthymine and the ethyl phosphotriester dTp(Et)dT are highly persistent DNA modifications in slowly dividing tissues of the ethylnitrosourea treated rats. Carcinogenesis 8:751–757 (1987).
23. Skopek TR, Walker VE, Cochrane JE, Craft TR, Cariello NF. Mutational spectrum at the Hprt locus in splenic T cells of B6C3F1 mice exposed to *N*-ethyl-*N*-nitrosourea. Proc Natl Acad Sci USA 89:7866–7870 (1992).
24. Richardson KK, Richardson FC, Crosby RM, Swenberg JA, Skopek TR. DNA base changes and alkylation following *in vivo* exposure of *Escherichia coli* to *N*-methyl-*N*-nitrosourea or *N*-ethyl-*N*-nitrosourea. Proc Natl Acad Sci USA 84:344–348 (1987).
25. Bhanot OS, Ray A. The *in vivo* mutagenic frequency and specificity of O^6-methylguanine in φX174 RF DNA. Proc Natl Acad Sci USA 83:7348–7352 (1986).
26. Loechler EL, Green GL, Essigmann JM. *In vivo* mutagenesis by O6-methylguanine built into a unique site in a viral genome. Proc Natl Acad Sci USA 81:6271–6275 (1984).
27. Preston BD, Singer B, Loeb LA. Mutagenic potential of O^4-methylthymine in vivo determined by an enzymatic approach to site-specific mutagenesis. Proc Natl Acad Sci USA 83: 8501–8505 (1986).
28. Eckert KA, Ingle CA, Drinkwater NR. *N*-ethyl-*N*-nitrosourea induced A:T to C:G transversion mutations as well as transition mutations in SOS-induced Escherichia coli. Carcinogenesis 10: 2261–2267 (1989).
29. Eckert KA., Ingle CA, Klinedinst D, Drinkwater NR. Molecular analysis of mutations induced in human cels by *N*-ethyl-*N*-nitrosourea. Mol Carcinogenesis 1:50–56 (1988).
30. Lindahl P, Sedgwick B, Sekiguchi M, Nakabeppu Y. Regulation and expression of the adaptive response to alkylating agents. Annu Rev Biochem 57: 133–157 (1988).
31. Popp RA, Baliff EG, Skow LC, Johnson FM, Lewis SE. Analysis of a mouse α-globin gene mutation induced by ethylnitrosourea. Genetics 105:157–167 (1983).
32. Lewis SE, Johnson FM, Skow LC, Popp D, Barnett LB, Popp RA. A mutation in the β-globin gene detected in the progeny of a female mouse treated with ethylnitrosourea. Proc Natl Acad Sci USA 82: 5829–5831 (1985).
33. Stowers SJ, Wiseman RW, Ward JM, Miller EC, Miller JA, Anderson MW, Eva A. Detection of activated proto-oncogenes in *N*-nitrosodiethylamine-induced liver tumors: a comparison between B6C3F1 mice and Fischer 344 rats. Carcinogenesis 9: 271–276 (1988).
34. Li F, Segal A, Solomon JJ. *In vitro* reaction of ethylene oxide with DNA and characterization of adducts. Chem-Biol Interact 84:35–54 (1992).
35. Solomon JJ, Mukai F, Fedyk J, Segal A. Reactions of propylene oxide with 2'-deoxynucleosides and *in vitro* with calf thymus DNA. Chem-Biol Interact 67:275–294 (1988).
36. Segal A, Solomon JJ, Mukai F. *In vitro* reactions of glycidol with pyrimidine bases in calf thymus DNA. Cancer Biochem Biophys 11:59–67 (1990).
37. Solomon JJ, Li F, Mukai F, Segal A. Characterization and quantitation of in vitro DNA adducts of ethylene oxides. Hydroxyethylation of *N*-3 of cytocine results in a rapid hydrolytic deamination to a uracil adduct. Environ Mol Mutagen 19:69–70 (1991).
38. Solomon JJ, Singh U, Segal A. *In vitro* reactions of 2-cyanoethylene oxide with calf thymus DNA. Chem-Biol Interact 88: 115–135 (1993).
39. Bhanot OS, Grevatt PC, Donahue JM, Gabrielides CN, Solomon JJ. Incorporation of dA opposite *N*3-ethyldeoxythymidine terminates *in vitro* DNA synthesis. Biochemistry 29: 10357–10364 (1990).
40. Grevatt PC, Donahue JM, Bhanot OS. The role of *N*3-ethyldeoxythymidine in mutagenesis and cytotoxicity by ethylating agents. J Biol Chem 266:1269–1275 (1991).
41. Grevatt PC, Solomon JJ, Bhanot OS. *In vitro* mispairing of O^2-ethyldeoxythymidine. Biochemistry 31:4181–4188 (1992).
42. Bhanot OS, Grevatt PC, Donahue JM, Gabrielides CN, Solomon JJ. *In vitro* DNA replication implicates O^2-ethyldeoxythymidine in transversion mutagenesis by ethylating agents. Nucleic Acids Res. 20:587–594 (1992).
43. Donahue JM, Bhanot OS. *N*3-ethyldeoxythymidine directs the incorporation of dT by T7 DNA polymerase. Proc Am Assoc Cancer Res 32:101 (1991).
44. Bhanot OS, Singh U, Kher U, Solomon JJ. Ethylene oxide-induced *N*3-hydroxyethyldeoxyuridine (N3-HE-dU) inhibits *in vitro* DNA replication. Proc Am Assoc Cancer Res 34:118 (1993).
45. Snow ET, Singh J, Koenig KL, Solomon JJ. Propylene oxide mutagenesis at template cytosines residues. Environ Mol Mutagen 23:274–280 (1994).
46. Sanger F, Coulson AR, Friedmann T, Air GM, Barrell BG, Brown NL, Fiddes JC, Hutchison CA III, Slocombe PM, Smith M. The nucleotide sequence of bacteriophage φX174. J Mol Biol 125:225–246 (1978).
47. Lai M-D, Beattie KL. Influence of divalent metal activator on the specificity of misincorporation during DNA synthesis catalyzed by DNA polymerase I of *Escherichia coli*. Mutat Res 198: 27–36 (1988).
48. Eichhorn GL, Shin YA. Interaction of metal ions with polynucleotides and related compounds. XII. The relative effect of various metal ions on DNA helicity. J Amer Chem Soc 90: 7323–7328 (1968).
49. Goodman MF, Keener S, Guidotti S, Branscomb EW. On the enzymatic basis for mutagenesis by manganese. J Biol Chem 258: 3469–3475 (1983).
50. Beckman RA, Mildvan AS, Loeb LA. On the fidelity of DNA repli-

cation: manganese mutagenesis *in vitro*. Biochemistry 24:5810–5817 (1985).
51. El-Deiry WS, Downey KM, So AG. Molecular mechanisms of manganese mutagenesis. Proc Natl Acad Sci USA 81: 7378–7382 (1984).
52. Loeb LA, Dube SK, Beckman RA, Koplitz M, Gopinathan KP. On the fidelity of DNA replication, nucleotide monophosphate generation during polymerization. J Biol Chem 256:3978–3987 (1981).
53. Hall ZW, Lehman IR. An *in vitro* transversion by a mutationally altered T4-induced DNA polymerase. J Mol Biol 36: 321–333 (1968).
54. Chang LM S, Bollum FJ. A comparison of associated activities in various deoxyribonucleic acid polymerases. J Biol Chem 248:3398–3404 (1975).
55. Dube SK, Loeb LA. Manganese as a mutagenic agent during in vitro DNA synthesis. Biochem Biophys Res Commun 67:1041–1046 (1975).
56. Sirover MA, Loeb LA. Infidelity of DNA synthesis *in vitro*: screening for potential metal mutagens or carcinogens. Science 194:1434–1436 (1976).
57. Larson K, Strauss BS. Influence of template strandedness on *in vitro* replication of mutagen-damaged DNA. Biochemistry 26:2471–2479 (1987).
58. Rabkin S, Strauss BS. A role for DNA polymerase in the specificity of nucleotide incorporation opposite *N*-acetyl-2-aminofluorene adducts. J Mol Biol 178:569–594 (1984).
59. Michaels ML, Johnson DL, Reid TM, King CM, Romano LJ. Evidence for *in vitro* translesion DNA synthesis past a site-specific aminofluorene adduct. J Biol Chem 262:14648–14654 (1987).
60. Rossman TG, Molina M. The genetic toxicology of metal compounds. II. Enhancement of ultraviolet-induced mutagenesis in *Escherichia coli* WP2. Environ Mutagen 8:263–271 (1986).
61. Dosanjh MK, Essigmann JM, Goodman MF, Singer B. Comparative efficiency of forming m4T•A *versus* m4T•G base pairs at a unique site using *Escherichia coli* DNA polymerase I (Klenow fragment) and Drosophila melanogaster polymerase α-primase complex. Biochemistry 29:4698–4703 (1990).
62. Huff AC,Topal MD. DNA damage at thymine *N*3 abolishes base-pairing capacity during DNA synthesis. J Biol Chem 262:12843–12850 (1987).
63. Zielenska M, Beranek DT, Guttenplan JB. Different mutations induced by *N*-nitroso-*N*-ethylurea: effects of dose, and error-prone DNA repair. Correlation with DNA adducts. Environ Mol Mutagen 11:473–485 (1988).
64. Swan PF. Why do O^6-alkylguanine and O^4-alkylthymine miscode? The relationship between the structure of DNA containing O6-alkylguanine and O^4-alkylthymine and the mutagenic properties of these bases. Mutat Res 233:81–94 (1990).
65. Singer B, Sagi J, Kusmierek JT. Escherichia coli polymerase I can use O^2-methyldeoxythymidine or O^4-methyleoxythymidine in places of deoxythymidine in primed poly(dA•dT)•poly(dA•dT) synthesis. Proc Natl Acad Sci USA 80:4584–4588 (1983).
66. Singer B, Chavez F, Spengler SJ, Kusmierek JT, Mendelman LV, Goodman MF. Comparison of polymerase insertion and extension kinetics of a series of O^2-alkyldeoxythymidine triphosphates and O^4-methyldeoxythymidine triphosphate. Biochemistry 28:1478–1483 (1989).
67. Echols H, Goodman MF. Fidelity mechanisms in DNA replication. Annu Rev Biochem 60:477–511 (1991).
68. Grevatt PC, Bhanot OS. *In vitro* miscoding by O^2-ethyldeoxythymidine in the presence of single or all deoxynucleotide triphosphates. Proc Am Assoc Cancer Res 32:106 (1991).
69. Dosanjh MK, Galeros G, Goodman MF, Singer B. Kinetics of extension of O^6-methylguanine paired with cytosine or thymine in defined nucleotide sequences. Biochemistry 30: 11595–11599 (1991).
70. Grevatt PC, Solomon JJ, Bhanot, OS. Kinetics of base pair formation and extension at O^2-ethyldeoxythymidine (O^2-Et-dT). Proc Am Assoc Cancer Res 34:118 (1993).
71. Boosalis MS, Petruska J, Goodman MF. DNA polymerase insertion fidelity: gel assay for site-specific kinetics. J Biol Chem 262: 14689–14696 (1987).
72. Mendelman LV, Boosalis MS, Petruska J, Goodman MF. Nearest neighbor influences on DNA polymerase insertion fidelity. J Biol Chem 264:14415–14423 (1989).
73. Mendelman LV, Petruska J, Goodman MF. Base mispair extension kinetics: comparison of DNA polymerase α and reverse transcriptase. J Biol Chem 265:2338–2346 (1990).
74. Singer B, Spengler SJ, Fraenkel-Conrat H, Kusmierek JT. O^4-methyl-, ethyl- or isopropyl substituents on thymidine in poly(dA•dT) all lead to transitions upon replication. Proc Natl Acad Sci USA 83:28–32 (1986).
75. Reyland ME, Lehman IR, Loeb LA. Specificity of proofreading by the 3'→5' exonuclease of the DNA polymerase-primase of *Drosophila melanogaster*. J Biol Chem 263:6518–6523 (1988).
76. Li BF, Reese CB, Swan PF. Synthesis and characterization of oligonucleotides containing 4-*O*-methylthymine. Biochemistry 26: 1086–1093 (1987).
77. Sloan DL, Loeb LA, Mildvan AS, Feldmann RJ. Conformation of deoxynucleotide triphosphate substrates on DNA polymerase I from Escherichia coli as determined by nuclear magnetic relexation. J Biol Chem 250:8913–8920 (1975).
78. Tabor S, Richardson CC. Effect of manganese ions on the incorporation of dideoxynucleotides by bacteriophage T7 DNA polymerase and *Escherichia coli* DNA polymerase I. Proc Natl Acad Sci USA 86:4076–4080 (1989).
79. Wade DR, Airy SC, Sinsheimer JE. Mutagenicity of aliphatic epoxides. Mutat Res 58:217–223 (1978).
80. Canter DA, Zeiger E, Haworth S, Lawlor T, Mortelmans K, Speck W. Comparative mutagenicity of aliphatic epoxides in Salmonella. Mutat Res 172:105–138 (1986).
81. Dunkelberg H Carcinogenicity of ethylene oxide and 1,2-propylene oxide upon intragastric administration to rats. Br J Cancer 46:924–933 (1982).
82. Zakour RA, Kunkel TA, Loeb LA. Metal-induced infidelity of DNA synthesis. Environ Health Perspect 40:197–205 (1981).
83. Fersht AR, Shi JP, Tsue W-C. Kinetics of base misinsertion by DNA polymerase I of *Escherichia coli*. J Mol Biol 165: 655–667 (1983).