In Vitro Mispairing Specificity of O^2-Ethylthymidine†

Peter C. Grevatt,‡ Jerome J. Solomon,‡ and Opinder S. Bhanot*,‡,§

Department of Environmental Medicine, New York University Medical Center, 550 First Avenue, New York, New York 10016, and Department of Microbiology and Molecular Genetics, UMDNJ-New Jersey Medical School, Newark, New Jersey 07103

Received August 21, 1991; Revised Manuscript Received January 28, 1992

ABSTRACT: The O^2-position of thymine is a major site of base alkylation by N-nitroso-alkylating agents, and its biological relevance remains obscure. The potential significance of this DNA damage was ascertained by studying in vitro DNA replication properties of O^2-ethylthymidine (O^2-Et-dT) site-specifically incorporated into a 36-nucleotide template. DNA replication was initiated eight nucleotides away from the O^2-Et-dT lesion by *Escherichia coli* polymerase I (Klenow fragment) using a 17-nucleotide primer. In the presence of 10 μM dNTP and Mg^{2+}, O^2-Et-dT blocked DNA replication predominantly (94%) 3′ to O^2-Et-dT, with the remainder (5%) blocked after incorporation of a nucleotide opposite O^2-Et-dT (incorporation-dependent blocked product). Postlesion synthesis was negligible (<1%). Nucleotide incorporation opposite O^2-Et-dT increased to 23% at 200 μM dNTP. Postlesion synthesis remained negligible (<2%). DNA sequencing revealed dA present opposite O^2-Et-dT in the incorporation-dependent blocked product. Negligible postlesion synthesis suggests that incorporation of dA opposite O^2-Et-dT inhibits in vitro DNA synthesis. The O^2-Et-dT·dA base pair may also impede DNA synthesis in vivo, contributing to the cytotoxicity of the ethylating agents. Substitution of Mn^{2+} for Mg^{2+} enhanced nucleotide incorporation opposite O^2-Et-dT and produced postlesion synthesis (16%) at 10 μM dNTP, which increased to 39% at 200 μM dNTP. DNA sequence analysis showed that while dA was present opposite O^2-Et-dT in the incorporation-dependent blocked product, both dA and dT were present opposite this lesion in the postlesion synthesis product. The results indicate that when dT is incorporated opposite O^2-Et-dT, the nascent chains containing the O^2-Et-dT·dT base pair at the 3′-terminus are efficiently extended. The studies suggest a role for O^2-Et-dT in A·T → T·A transversion mutagenesis by ethylating agents.

Most nitroso-alkylating agents are potent mutagens and carcinogens. They are electrophilic reagents either inherently or after metabolic activation, and they mediate their biological activity in part by interacting with genomic DNA to form covalent adducts (Miller, 1978; Singer & Grunberger, 1983). During replication and/or repair, these adducts may produce mutations (Singer & Kusmierek, 1982; Saffhill et al., 1985; Basu & Essigmann, 1988), which in turn may contribute to the neoplastic transformation of target cells. Isolation of oncogenes from experimental tumors, which contain activating mutations consistent with the mutational specificity of the agent that induced the tumors, supports this hypothesis (Varmus, 1984; Barabacid, 1987; Balmain & Brown, 1988).

The alkylating agent *N*-ethyl-*N*-nitrosourea (ENU)[1] is capable of inducing a variety of tumor types in a broad range of animal species (Magee et al., 1976; Bogovski & Bogovski, 1982) and in some human cancers (Bartsch & Montesano, 1984). The reactivity of ENU allows it to form a diverse set of DNA adducts both in vitro and in vivo (Singer & Grunberger, 1983). DNA ethylation occurs at nucleophilic oxygen sites, such as the O^6-position of dG, the O^4- and O^2-positions of dT, and phosphodiester groups, and at the nucleophilic ring-nitrogen sites. Alkylation of the DNA base may alter its base-pairing pattern to form a miscoding lesion such as O^6-alkyldeoxyguanosine or O^4-alkylthymidine. Alternatively, alkylation may compromise the ability of a base to serve as a template during DNA replication, producing a noncoding lesion (Saffhill et al., 1985; Basu & Essigmann, 1988; Bhanot et al., 1990).

In *Escherichia coli* (*E. coli*), ENU induces mainly G·C → A·T and A·T → G·C transition mutations (Richardson et al., 1987). Presumably, these mutations result from the unrepaired miscoding lesions O^6-ethyldeoxyguanosine (O^6-Et-dG) (Loechler et al., 1984; Bhanot & Ray, 1986) and O^4-ethylthymidine (O^4-Et-dT) (Preston et al., 1986), respectively, as a consequence of their capacity to mispair during DNA replication. In the human cell, ENU induces a significant number of transversion mutations at A·T base pairs (Eckert et al., 1988), in addition to the same G·C → A·T and A·T → G·C transition mutations observed in *E. coli*. The two transition mutations induced in human cells are consistent with the predicted mispairing of the DNA lesions O^6-Et-dG and O^4-Et-dT. The ENU-induced lesions responsible for transversion mutations at A·T base pairs are not known.

Animal studies have revealed the biological importance of A·T transversion mutations. The A·T → T·A transversion event has been proposed to account for two mutations of the mouse α- and β-globin genes arising in the progeny of ENU-treated female mice (Popp et al., 1983; Lewis et al., 1985). Neuroblastomas, induced by transplacental treatment of rats with ENU, contained the *neu* oncogene activated by an A·T → T·A transversion mutation (Bargmann et al., 1986; Per-

† This work was supported in part by National Science Foundation Grant DMB-8607556; National Institute of Environmental Health Sciences Grants ES 00260 and ES 05694; a Special Institutional Grant SIG 9A from the American Cancer Society; and National Cancer Institute Grant CA 13343. At the initial stage of this work, P.C.G. was supported by the National Institute of Environmental Health Sciences Training Grant 2T 32 ES 07081 and is a recipient of a Helen Q. Woodard Graduate Scholar Award.

* To whom correspondence should be addressed at New York University Medical Center.

[1] Abbreviations: O^2-Et-dT, O^2-ethylthymidine; dT, thymidine; dAMP, deoxyadenosine 5′-monophosphate; DMTr-dT, 5′-*O*-(4,4′-dimethoxytrityl)thymidine; ATP, adenosine 5′-triphosphate; ENU, *N*-ethyl-*N*-nitrosourea; *E. coli*, *Escherichia coli*; O^6-Et-dG, O^6-ethyldeoxyguanosine; O^4-Et-dT, O^4-ethylthymidine; AP, apurinic/apyrimidinic; *N*3-Et-dT, *N*3-ethylthymidine; O^2-alkyl-dT, O^2-alkylthymidine; Kf Pol I, Klenow fragment of *E. coli* polymerase I; DTT, dithiothreitol.

0006-2960/92/0431-4181$03.00/0

antoni et al., 1987). An A·T → T·A activating mutation was also observed in c-Ha-*ras* genes of liver tumors induced by treating mice with diethylnitrosamine (Stowers et al., 1988), which produces the same pattern of base alkylation as ENU (Singer & Grunberger, 1983). These animal studies together with mutagenesis studies in human cells (Eckert et al., 1988; Moriya et al., 1988) emphasize the existence of an important, but as yet uncharacterized (Brent et al., 1988), pathway for the fixation of ENU-induced DNA lesions to yield transversions at A·T base pairs in mammalian cells.

A general mechanism for induction of transversion mutations is misincorporation of nucleotides opposite DNA lesions that block DNA replication. The lesions produced by ENU that impede the progress of in vitro DNA replication include AP (apurinic/apyrimidinic) sites (Strauss et al., 1982; Loeb & Preston, 1986; Takeshita et al., 1987), *N*3-ethylthymidine (*N*3-Et-dT) (Huff & Topal, 1987; Bhanot et al., 1990; Grevatt et al., 1991), and O^2-Et-dT (Saffhill et al., 1985; Singer, 1986). Alkylated DNA lesions which normally block DNA replication are thought to induce SOS-dependent mutagenesis in *E. coli* (Witkin, 1976; Little & Mount, 1982; Walker, 1984). Transversion mutations at A·T base pairs form an important component of alkylating agent-induced SOS-dependent mutagenesis in *E. coli* (Foster & Eisenstadt, 1985; Eckert & Drinkwater, 1987; Cuoto et al., 1989; Eckert et al., 1989).

The preference for A·T mutations, induced by alkylating agents in mammalian cells or through the SOS-dependent pathway in bacteria, suggests that the dA or dT adduct and/or breakdown products of these adducts are responsible for transversion mutations at A·T base pairs. On the basis of a comparison of ENU-induced mutations in human cells with the type of base-substitution mutations observed for other alkylating agents, it was suggested (Eckert et al., 1988) that O^2-Et-dT may be a significant premutagenic lesion that is capable of inducing A·T → T·A transversion mutations in vivo. However, the involvement of other DNA replication-blocking lesions produced by ENU at A·T base pairs cannot be ruled out. We recently have shown (Grevatt et al., 1991) that *N*3-ethylthymidine (*N*3-Et-dT) can mispair with dT during in vitro DNA replication and may induce A·T → T·A transversions in vivo.

Studies reported here demonstrate that in the presence of Mg^{2+} and a low concentration of dNTP (10 μM), O^2-Et-dT blocked DNA synthesis by the Klenow fragment of *E. coli* polymerase I (Kf Pol I) 3′ to the lesion. At a higher dNTP concentration (200 μM), dA was incorporated opposite O^2-Et-dT. Postlesion synthesis was negligible, indicating that formation of an O^2-Et-dT·dA base pair at the growing end of the chain inhibits DNA synthesis. These results suggest a role for the O^2-Et-dT lesion in cytotoxicity of the ethylating agents. In the presence of the mutagenic metal ion Mn^{2+} (Beckman et al., 1985), postlesion synthesis occurred. Both dA and dT were incorporated opposite O^2-Et-dT. Incorporation of dT opposite O^2-Et-dT during in vitro DNA synthesis implicates O^2-Et-dT in A·T → T·A transversion mutagenesis. These studies provide insight into the molecular mechanisms by which ethylating agents may induce transversion mutations at A·T base pairs in human cells (Eckert et al., 1988) and activate cellular protooncogenes by an A·T → T·A transversion event in tumors induced by ethylating agents (Bargmann et al., 1986; Perantoni et al., 1987; Stowers et al., 1988).

Materials and Methods

Ultrapure grade dNTP, thymidine (dT), and deoxyadenosine 5′-monophosphate (dAMP) were purchased from Pharmacia P-L Biochemicals. γ-^{32}P-Labeled adenosine 5′-triphosphate (ATP) was obtained from Du Pont–New England Nuclear. Kf Pol I and T4 DNA ligase were purchased from Boehringer Mannheim. One unit of Kf Pol I (as reported by the supplier) incorporates 10 nmol of total nucleotides into acid-precipitable material in 30 min at 37 °C, using poly[d-(A-T)] as the primed template. T4 polynucleotide kinase was obtained from New England Biolabs. The ultrapure electrophoresis reagents were purchased from Bio-Rad. All other chemicals were of high-grade quality and were purchased from different sources.

Synthesis of the Site-Modified Oligodeoxynucleotide. The 21-nucleotide-long oligomer, 5′AATAAAAGTCT*AAAACATGAT (T* = O^2-Et-dT) was synthesized on an Applied Biosystems Model 381A synthesizer using phosphite triester chemistry (Caruthers, 1985). O^2-Et-dT was introduced at the desired site during synthesis using O^2-ethyl-5′-*O*-(4,4′-dimethoxytrityl)-2′-deoxythymidine 3′-(2-cyanoethyl *N*,*N*-diisopropylphosphoramidite) synthesized in our laboratory (Bhanot et al., 1992). After completion of the synthesis, the oligomer was deprotected by treatment with 1,8-diazabicyclo[5.4.0]undec-7-ene (Li et al., 1987; Borowy-Borowski & Chambers, 1989). The oligomer was purified by electrophoresis on a 20% polyacrylamide–8 M urea gel and labeled with ^{32}P at the 5′-terminus. DNA sequencing by the modified Maxam–Gilbert procedure (Maxam & Gilbert, 1980) revealed the expected sequences. The presence of the O^2-Et-dT moiety in the oligomer was demonstrated by HPLC analysis of the nucleosides released from the site-modified oligomer following digestion with phosphodiesterase and phosphatase. Comparison of the observed and calculated ratios of the nucleosides indicated that O^2-Et-dT is present in essentially all oligomer molecules. Details of the synthesis of the O^2-Et-dT-containing oligomer have been described elsewhere (Bhanot et al., 1992).

The O^2-Et-dT-containing oligomer is complementary to φX174 sequences from position 2392–2412 (Sanger et al., 1978) and contains the O^2-Et-dT lesion at position 2402. This position corresponds to the second nucleotide in the third codon of φX gene G. In a separate investigation, this site-modified oligomer will be used to study the in vivo mutagenic properties of O^2-Et-dT using a φX174-based site-specific mutagenesis system (Bhanot & Ray, 1986).

Construction of a 36-Nucleotide-Long Template Containing O^2-Et-dT at a Single Site. A 36-nucleotide-long template containing a single O^2-Et-dT adduct at position 26 from the 3′-end was constructed by ligating the site-modified O^2-Et-dT-containing 21-mer (125 pmol) to a second ^{32}P-labeled (<3 Ci/mmol) 15-mer (^{32}pTAAACTCCTAAGCAG) (125 pmol) in the presence of an 18-nucleotide (5′HOTTAGGAGTTTAATCATGT) complementary oligomer (75 pmol) that held the two ligating primers together. The three oligomers, in 50 μL of a solution of 25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, and 5 mM dithiothreitol (DTT), were annealed by heating at 55 °C for 15 min with subsequent slow cooling to 4 °C (4 h). ATP was added to the final concentration of 1 mM, and the reaction was started by the addition of T_4 DNA ligase (50 units as reported by the supplier) and incubation at 16 °C. After 2 h, the reaction was stopped by the addition of EDTA to 12 mM. The ligated complex was denatured by the addition of an equal volume of formamide and heating at 55 °C for 3 min. The 36-nucleotide ligated product was isolated as described (Bhanot et al., 1990). The low radioactivity present in the template did not interfere in the following experiments.

Formation of the Primed Template. The primed template, with the 3′-terminus of the primer eight nucleotides away from

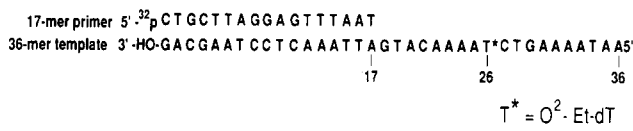

FIGURE 1: "Running start" primed template. The 36-nucleotide template contains a single O^2-Et-dT adduct at template position 26 from the 3'-end. The adduct is eight nucleotides away from the 3'-end of the hybridized 17-nucleotide primer.

the O^2-Et-dT lesion in the template, was prepared by annealing about a 2-fold molar excess of complementary 5'-^{32}P-labeled (3000 Ci/mmol) 17-nucleotide primer to the site-modified template in 30 mM Hepes buffer, pH 7.5, 50 mM NaCl. The annealing mixture (10 μL/pmol of the template) was heated at 55 °C for 15 min followed by slow cooling to 4 °C (4 h). Formation of the primed template was checked by electrophoresis on a nondenaturing 12% polyacrylamide gel. A single batch of primed template was used in all DNA replication studies with O^2-Et-dT.

A control primed template, containing dT in place of O^2-Et-dT, was prepared in a similar manner except that the annealing mixture contained equivalent amounts of primer and template.

DNA Polymerase Reaction. Polymerase reactions were conducted on 0.05–0.1 pmol of primed template in 10 mM Hepes buffer, pH 7.5, containing 1 mM DTT (total volume of 8 μL). Polymerization was started by adding 0.2 unit of Kf Pol I. The concentration of dNTP and Mg^{2+} or Mn^{2+}, incubation time, and temperature were varied depending upon the type of study being conducted. In some reactions, 10 mM dAMP was used to inhibit the 3'→5' exonuclease activity associated with Kf Pol I (Que et al., 1978). The exact conditions used are documented in the figure legends.

The polymerization reaction was terminated by the addition of EDTA (to a final concentration higher than the divalent cation used) and an equal volume of formamide and subsequent heating at 100 °C for 3 min. DNA synthesis products were separated by electrophoresis on 16% polyacrylamide–8 M urea sequencing gels. Product bands were visualized by autoradiography and excised, and the radioactivity was counted after suspension in 5 mL of scintillation cocktail (aqueous-based). The radioactivity present in each product band was used to calculate the abundance of each of the DNA synthesis products in the polymerization reaction.

To prepare various DNA synthesis products in amounts sufficient for DNA sequencing, the polymerase reaction was performed at 37 °C using 5–10 pmol of primed template in the presence of the desired concentration of dNTP and Mg^{2+} or Mn^{2+}. Bands were separated by electrophoresis as described above except that a 1-mm-thick gel was used. Product bands were visualized by autoradiography, excised, crushed, and extracted with 4 × 10 mL of methanol to remove the urea. The products were then eluted with a minimum volume of 10 mM NaCl in 2.5 mM Tris-HCl, pH 7.5, 0.25 mM EDTA.

Identification of the Nucleotide Incorporated Opposite O^2-Et-dT. The identity of the nucleotide incorporated opposite O^2-Et-dT was established by sequencing (Maxam & Gilbert, 1980) the DNA products synthesized by Kf Pol I on an O^2-Et-dT-containing template.

Results

In Vitro DNA Replication System. In vitro DNA replication studies were initiated on the primed template shown in Figure 1. In this system, the 3'-terminus of the primer is eight nucleotides away from the O^2-Et-dT lesion present in the 36-nucleotide template. This system represents a "running start" for O^2-Et-dT in DNA replication (Mendelman et al., 1989), in that synthesis occurs prior to the polymerase reaching the O^2-Et-dT lesion.

The DNA replication properties of the O^2-Et-dT lesion were studied using Kf Pol I in the presence of Mg^{2+} or Mn^{2+} as the enzyme cofactors and varying concentrations of dNTP at 37 °C for 30 min. The effect of the 3'→5' exonuclease-editing activity of Kf Pol I on DNA replication past O^2-Et-dT was investigated by inhibiting this activity in some experiments by adding 10 mM dAMP.

In the DNA replication system used in these studies, >95% of the annealed primer was extended [calculated as described by Grevatt et al. (1991)] by the DNA polymerase. When encountered by O^2-Et-dT, the following products, reflecting the influence of template O^2-Et-dT, are feasible. First, the progress of the polymerase is blocked 3' to the lesion. No nucleotide is incorporated opposite the lesion and a preincorporation blocked product is accumulated. Variable amounts of the 22–25-nucleotide preincorporation blocked product were obtained, especially in the presence of Mg^{2+}. These products were included in the quantitation of the preincorporation blocked product. Second, DNA synthesis is stopped after incorporation of a nucleotide opposite O^2-Et-dT, producing a 26-nucleotide incorporation-dependent blocked product. Finally, the adduct does not represent a block to DNA replication and the synthesis proceeds past the O^2-Et-dT lesion, yielding a postlesion synthesis product. The efficiency of DNA synthesis past the O^2-Et-dT lesion was reduced by the presence of this lesion in the template, producing products ranging from 27 to 36 nucleotides. Furthermore, a nucleotide addition at the blunt end (Clark et al., 1987) of the synthesized 36-nucleotide duplex produced a 37-nucleotide product. Postlesion synthesis included 27–37-nucleotide products. For DNA sequencing by the Maxam–Gilbert procedure, only 35–37-nucleotide postlesion synthesis products were used. Postlesion synthesis products ranging from 27 to 34 nucleotides comprised less than 10% of the total postlesion synthesis.

The products of DNA synthesis on the site-modified template were characterized by polyacrylamide gel analysis. Since each synthesis product was only labeled once at the 5'-end (5'-^{32}P-end-labeled primer was used to prime the DNA synthesis), the abundance of a particular synthesis product was directly proportional to the radioactivity present in the band after gel electrophoresis. The radioactivity associated with the individual product bands was measured and used to calculate the relative percentage of DNA synthesis products in the polymerization reaction.

DNA Synthesis Block by O^2-Et-dT. The O^2-Et-dT lesion, present at a single site in the template, blocked DNA synthesis by Kf Pol I in the presence of 10 μM dNTP and 5 mM Mg^{2+} (Figure 2A, lane 1). In the control, containing dT in place of O^2-Et-dT in the template, DNA synthesis proceeded to the 5'-terminus of the template without interruption (Figure 2B, lane 1). Formation of the 36-nucleotide product in the control indicated that O^2-Et-dT was responsible for blocking DNA synthesis in the site-modified template. The observed DNA replication block by O^2-Et-dT is consistent with published reports that the O^2-alkyl-dT lesions present in synthetic polymers impede DNA synthesis by DNA and RNA polymerases (Saffhill et al., 1985; Saffhill, 1985). The results are also consistent with the low efficiency of incorporation of the triphosphates of O^2-Me-dT and O^2-Et-dT by Kf Pol I and *E. coli* polymerase I using synthetic polymers as templates (Singer et al., 1983; 1989). In our studies, the major product (94%) of the DNA synthesis at 10 μM dNTP and 5 mM Mg^{2+} was a preincorporation blocked product, indicating that the ma-

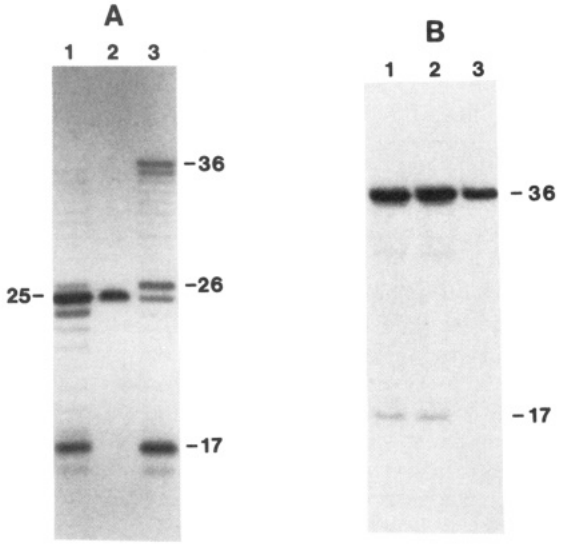

FIGURE 2: Analysis of DNA products synthesized by Kf Pol I on a primed template. The primed template contained a normal nucleoside (dT) or a single O^2-Et-dT adduct at template position 26 from the 3′-end. The primed template was incubated at 37 °C for 30 min with 0.2 unit of Kf Pol I in the presence of Mg^{2+} or Mn^{2+} and different concentrations of dNTP. Products were analyzed by gel electrophoresis and autoradiography. (A) Primed template containing an O^2-Et-dT adduct: lane 1, DNA synthesis in the presence of 10 μM dNTP and 5 mM Mg^{2+}; lane 2, 25-nucleotide marker; lane 3, DNA synthesis in the presence of 200 μM dNTP and 0.5 mM Mn^{2+}. (B) Control primed template containing a normal nucleoside dT: lane 1, DNA synthesis in the presence of 10 μM dNTP and 5 mM Mg^{2+}; lane 2, DNA synthesis in the presence of 200 μM dNTP and 0.5 mM Mn^{2+}; lane 3, 36-nucleotide marker.

jority of DNA synthesis terminated 3′ to O^2-Et-dT. A small amount (5%) of the 26-nucleotide incorporation-dependent blocked product was also obtained, indicating incorporation of a nucleotide opposite O^2-Et-dT with subsequent inhibition of DNA synthesis. A postlesion synthetic product (Figure 2A, lane 1, and Figure 3A, 10 μM dNTP point) was obtained at <1%, suggesting that DNA synthesis past the O^2-Et-dT lesion is not efficient in the presence of 10 μM dNTP and Mg^{2+}. The concentration of divalent cation, Mg^{2+} (1–10 mM), in the polymerization reaction and the incubation temperature (from 16 to 37 °C) had no significant effect on the relative percentages of DNA synthesis products. Unless otherwise stated in this report, all polymerization experiments containing Mg^{2+} were conducted using 5 mM Mg^{2+} at 37 °C for 30 min.

The effect of the dNTP concentration on incorporation of a nucleotide oppsite O^2-Et-dT in the presence of Mg^{2+} was significant. Figure 3 presents the effect of dNTP concentrations on the relative percentage of various DNA synthesis products in the absence (Figure 3A) and presence (Figure 3B) of 10 mM dAMP. When the dNTP concentration was increased from 10 μM to 200 μM, the 26-nucleotide incorporation-dependent blocked product increased from 5% to 23% (Figure 3A). Presumably, the high concentration of dNTP induced the polymerase to incorporate a nucleotide opposite O^2-Et-dT, extending the preincorporation blocked product an additional nucleotide. This was evident from the decrease of the preincorporation blocked product from 94% at 10 μM dNTP to 75% at 200 μM dNTP (Figure 3A). Postlesion synthesis was <2% even at 200 μM dNTP.

Inhibition of the 3′→5′ exonuclease activity associated with Kf Pol I by the addition of 10 mM dAMP (Que et al., 1978) to the polymerization reaction increased the accumulation of the incorporation-dependent blocked product, apparently by eliminating degradation of the nucleotide incorporated opposite O^2-Et-dT in the incorporation-dependent blocked product. The

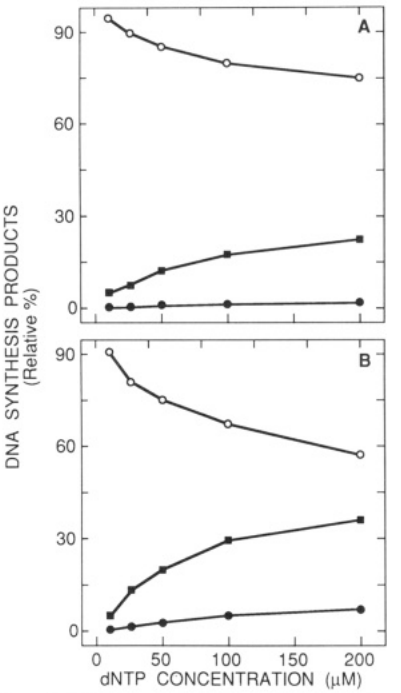

FIGURE 3: Effect of dNTP concentration on DNA products synthesized by Kf Pol I on the primed template containing a single O^2-Et-dT adduct in the presence of 5 mM Mg^{2+}. The primed template (0.05 pmol), containing 5 mM Mg^{2+} and varying concentrations of dNTP, was incubated at 37 °C for 30 min with 0.2 unit of Kf Pol I in the presence or absence of dAMP. The products were analyzed as described in the text. Each point represents an average of three experiments: ○, preincorporation blocked product; ■, 26-nucleotide incorporation-dependent blocked product; ●, postlesion synthesis product. (A) No dAMP added. Standard deviations ranged from 3.4% to 4.0% for preincorporation blocked products, 0.7% to 2.0% for incorporation-dependent blocked products, and 0.03% to 0.5% for postlesion synthesis products. (B) 10 mM dAMP added. Standard deviations ranged from 3.0% to 4.1% for preincorporation blocked products, 1.2% to 2.0% for incorporation-dependent blocked products, and 0.2% to 1.4% for postlesion synthesis products.

effect of dAMP was more pronounced at higher dNTP concentrations. At 200 μM dNTP, the incorporation-dependent blocked product was increased from 23% to 37% in the presence of dAMP (Figure 3B). The postlesion synthesis was increased only from <2 to 7%. Presumably, distortion of the DNA structure by O^2-Et-dT (Saffhill, 1985; Saffhill et al., 1985) interfered with the formation of the phosphodiester bond 5′ to the paired O^2-Et-dT. Effects of dAMP in facilitating the bypass of lesions by DNA polymerases are known (Larson & Strauss, 1987). In our system, the exonuclease activity was efficiently inhibited, as judged by negligible degradation of the 5′-^{32}P-labeled primer by Kf Pol I in the presence of 10 mM dAMP as compared with the extensive degradation in the absence of dAMP. Attempts were not made to maximize postlesion synthesis using dAMP.

Specificity of Nucleotide Incorporation Opposite O^2-Et-dT in the Presence of Mg^{2+}. The 26-nucleotide incorporation-dependent blocked product, synthesized in the presence of 200 μM dNTP and Mg^{2+}, was sequenced by the Maxam–Gilbert procedure. Sequencing revealed that dA was incorporated opposite O^2-Et-dT (Figure 4). Negligible postlesion synthesis, at both low and high concentrations of dNTP (Figure 3A), suggests that incorporation of dA opposite O^2-Et-dT does not represent a favorable base pair for further extension and that it impedes DNA synthesis in the presence of Mg^{2+}.

DNA Synthesis Past the O^2-Et-dT Lesion. Relaxation of the polymerase fidelity by substitution of Mg^{2+} with Mn^{2+} (Beckman et al., 1985) increased the incorporation of a nu-

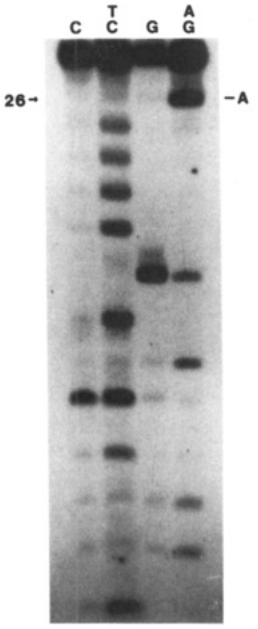

FIGURE 4: DNA sequence analysis of incorporation-dependent blocked product by the Maxam–Gilbert procedure. The product was isolated from the DNA synthesis reaction in the presence of 5 mM Mg^{2+} and 200 μM dNTP. The presence of a band in the dA-specific lane at position 26 indicates incorporation of dA opposite O^2-Et-dT. O^2-Et-dT is present in the template at position 26 from the 3′-end.

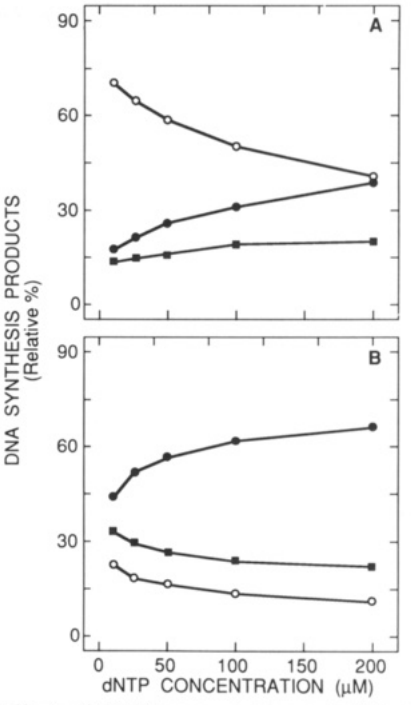

FIGURE 5: Effects of dNTP concentration on DNA products synthesized by Kf Pol I on the primed template containing a single O^2-Et-dT adduct in the presence of 0.5 mM Mn^{2+}. The primed template (0.05 pmol), containing 0.5 mM Mn^{2+} and varying concentrations of dNTP, was incubated at 37 °C for 30 min with 0.2 unit of Kf Pol I in the presence or absence of dAMP. The products were analyzed as described in the text. Each point represents an average of three experiments: ○, preincorporation blocked product; ■, 26-nucleotide incorporation-dependent blocked product; ●, postlesion synthesis product. (A) No dAMP added. Standard deviations ranged from 2.7% to 3.9% for preincorporation blocked products, 1.5% to 3.3% for incorporation-dependent blocked products, and 1.6% to 2.6% for postlesion synthesis products. (B) 10 mM dAMP added. Standard deviations ranged from 1.7% to 2.7% for preincorporation blocked products, 2.2% to 2.9% for incorporation-dependent blocked products, and 4.0% to 4.4% for postlesion synthesis products.

cleotide opposite O^2-Et-dT and produced a postlesion synthesis product (Figure 2A, lane 3). At 10 μM dNTP, the incorporation-dependent blocked product increased from 5% in the presence of Mg^{2+} (Figure 3A) to 14% with Mn^{2+} (Figure 5A), while the postlesion product increased from <1% to 16%. This was associated with a decrease in the preincorporation blocked product from 94% (Figure 3A) to 70% (Figure 5A). The relative percentage of the postlesion synthesis product was increased with the increasing Mn^{2+} concentration; however, the total DNA synthesis was decreased by a factor of 3 at 5 mM Mn^{2+} as compared to synthesis at 0.5 mM Mn^{2+} (data not shown). In this paper, all experiments using Mn^{2+} were performed in the presence of 0.5 mM Mn^{2+}. Whenever EDTA was present in the reaction mixture, the Mn^{2+} concentration was increased to compensate for EDTA. Formation of the postlesion synthesis product in the presence of Mn^{2+} was further enhanced using higher dNTP concentrations. Figure 5A presents the effect of dNTP concentration on the relative percentage of DNA synthesis products synthesized by Kf Pol I in the presence of Mn^{2+}. Postlesion synthesis was increased from 16% at 10 μM to 39% at 200 μM dNTP and was associated with a decrease in the preincorporation blocked product from 70% to 40%. Accumulation of the incorporation-dependent blocked product was not changed significantly (Figure 5A).

Addition of 10 mM dAMP to the polymerization reaction further increased postlesion synthesis (Figure 5B). At 10 μM dNTP, postlesion synthesis increased from 16% in the absence to 44% in the presence of dAMP. A similar effect was observed at 200 μM dNTP, when postlesion synthesis increased from 39% to 66%. This was associated with a decrease in preincorporation and incorporation-dependent blocked products (Figure 5B). The results indicate that inhibition of the 3′→5′ exonuclease proofreading activity of Kf Pol I facilitated DNA synthesis past the lesion. The increase in postlesion synthesis by dAMP was more pronounced in the presence of Mn^{2+} (Figure 5B) compared to the presence of Mg^{2+} (Figure 3B). Attempts were not made to optimize postlesion synthesis in the presence of Mn^{2+} by changing the concentration of dAMP in the polymerization reaction.

The postlesion synthesis product included a 37-nucleotide product containing an additional base (+1) (Figure 2A, lane 3), which was one nucleotide longer than the 36-nucleotide site-modified template. Apparently, the +1 product is formed by a nucleotide addition to the blunt end of the 36-nucleotide duplex formed after postlesion synthesis. Such additions have been reported by others (Clark et al., 1987). The +1 product has sequences identical to those of the 35- and 36-nucleotide postlesion synthesis products. The nucleotide incorporated at the 3′-end of the +1 product was not investigated. Formation of this product was negligible (<1%) at 10 μM dNTP and 5 mM Mg^{2+} (Figure 2A, lane 1), increasing to ≈15% at 200 μM dNTP in the presence of 10 mM dAMP. Substitution of 0.5 mM Mn^{2+} for Mg^{2+} further increased +1 product formation to >50% at 200 μM dNTP (Figure 2A, lane 3). Failure to detect the +1 product in the controls (Figure 2B, lanes 1 and 2) containing dT in place of O^2-Et-dT in the template suggests that formation of the +1 product may be stimulated by the following factors: (a) presence of the O^2-Et-dT lesion in the template, (b) inhibition of 3′→5′ exonuclease activity associated with the polymerase by deoxymononucleotides (Clark et al., 1987; Bhanot & Grevatt, unpublished results), (c) high dNTP concentrations, and (d) presence of Mn^{2+} as the divalent cation in the polymerization reaction.

Specificity of Nucleotide Incorporation Opposite O^2-Et-dT in the Presence of Mn^{2+}. Both postlesion synthesis and in-

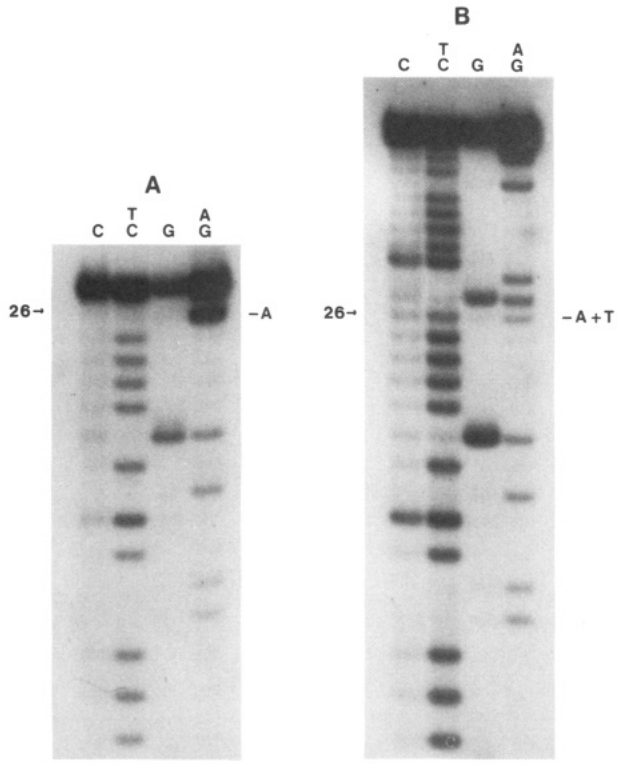

FIGURE 6: DNA sequence analysis of incorporation-dependent blocked product and postlesion synthesis product by the Maxam–Gilbert procedure. The products were isolated from the same DNA synthesis reaction carried out in the presence of 0.5 mM Mn^{2+} and 200 μM dNTP at 37 °C. O^2-Et-dT is present in the template at position 26 from the 3′-end. (A) An autoradiogram of the DNA sequencing gel obtained from the 26-nucleotide incorporation-dependent blocked product. The presence of a band in the dA-specific lane at position 26 indicates incorporation of dA opposite O^2-Et-dT. (B) An autoradiogram of the DNA sequencing gel obtained from the postlesion synthesis product (nucleotides 36–37). The presence of bands in the dA- and dT-specific lanes at position 26 indicates incorporation of both dA and dT opposite O^2-Et-dT.

corporation-dependent blocked products from the same experiment (0.5 mM Mn^{2+} and 200 μM dNTP) were sequenced by the modified Maxam–Gilbert procedure. The incorporation-dependent blocked product contained dA as the nucleotide incorporated opposite the O^2-Et-dT lesion (Figure 6A). Results from the postlesion synthesis product revealed that both dA and dT were incorporated opposite the lesion (Figure 6B). The accumulation of an incorporation-dependent blocked product containing dA opposite O^2-Et-dT (position 26 in Figure 6A) suggests that in the presence of Mn^{2+} the O^2-Et-dT·dA base pair present at the 3′-end of the growing chain can be extended but inefficiently. Absence of accumulation of the incorporation-dependent blocked product containing dT opposite O^2-Et-dT indicates that formation of an O^2-Et-dT·dT base pair at the replication fork can be extended to yield a postlesion synthesis product. The results implicate O^2-Et-dT as a potentially mutagenic lesion capable of inducing A·T → T·A transversion mutations.

The relative incorporation of dT and dA opposite O^2-Et-dT at position 26 in the postlesion synthesis product was estimated by comparing the relative intensity of neighboring bands in the same lane. The autoradiogram (Figure 6B) was scanned at T and A lanes using Ultroscane XL. From the peak areas, the dT-26:dT-25 and dA-26:dA-28 ratios were calculated to be 0.65 and 0.37, respectively. The ratios totaled 1.02 as compared to the expected value of 1.0. The results indicate that in the postlesion synthesis product, synthesized in the presence of Mn^{2+} and 200 μM dNTP, the number of molecules containing dT at position 26 was approximately twice the number of those containing dA at this position.

The autoradiogram (Figure 6B) was also scanned at C and G lanes. The faint bands at position 26 have the same intensities as those of the nearby "background" bands. The data indicate that the incorporation of dC or dG opposite O^2-Et-dT, if any, is negligible.

Discussion

The in vitro DNA replication studies described in this paper demonstrate that the O^2-Et-dT lesion, which is among the highly persistent DNA alkylation products in eukaryotes (Den Engelse et al., 1987), can direct incorporation of both dA and dT when encountered by Kf Pol I. Formation of the O^2-Et-dT·dA base pair greatly decreases the efficiency of DNA synthesis in vitro when it is present at the 3′-end of the growing chain. This base pair may also impede the progress of DNA synthesis in vivo, implicating O^2-Et-dT as a potentially cytotoxic lesion produced by ethylating agents. Formation of the O^2-Et-dT·dT base pair at the replication fork is not inhibitory to DNA synthesis. By pass of the O^2-Et-dT lesion occurs, which could lead to an A·T → T·A transversion mutation. Our results suggest that formation of the persistent O^2-Et-dT lesion in DNA is biologically significant and may contribute to the cytotoxicity, mutagenicity, and carcinogenicity of the ethylating agents long after the original exposure has occurred.

In the presence of Mg^{2+}, DNA synthesis past O^2-Et-dT by Kf Pol I was effectively blocked (Figure 3A), consistent with a published report (Saffhill, 1985). When Mn^{2+} was substituted for Mg^{2+}, incorporation of a nucleotide opposite O^2-Et-dT and subsequent postlesion synthesis were enhanced (compare Figures 3A and 5A). Significant postlesion synthesis in the presence of Mn^{2+} (Figure 5) as compared to in the presence of Mg^{2+} (Figure 3) demonstrates that Mn^{2+} stimulated the formation and subsequent extension of the base pair at O^2-Et-dT. Whether the effects of Mn^{2+} are primarily on the enzyme conformation or are secondary to changes in DNA structure is not known. At low concentrations, Mn^{2+} resulted in decreased specificity of base selection and proofreading by *E. coli* DNA polymerase I (El-Deiry et al., 1988).

DNA sequencing of the incorporation-dependent blocked product synthesized in the presence of Mg^{2+} revealed that mainly dA was incorporated opposite O^2-Et-dT (Figure 4). The formation of the postlesion synthesis product in insignificant yields suggests that the O^2-Et-dT·dA base pair formed at the replication fork does not represent a favorable base pair for extension. The ethyl group of O^2-Et-dT is not located in the Watson–Crick base-pairing region, but alkylation at the O^2-position fixes the base in the enol tautomer, resulting in the loss of the N3 hydrogen atom, as confirmed by nuclear magnetic resonance (Bhanot et al., 1992). When dA is inserted opposite O^2-Et-dT, two hydrogen bonds may form between O^4 of O^2-Et-dT and the N^6 hydrogen atom of dA and between N3 of O^2-Et-dT and a protonated N1 of dA. NMR studies have suggested similar hydrogen bondings for the O^4-Me-dT·dA base pair (Kalnik et al., 1988a,b). The O^2-Et-dT·dA base pair, with two hydrogen bonds, is expected to be thermodynamically stable. This is supported by the thermal denaturation studies demonstrating that O^2-Me-dT present in alternating poly(dA·dT) polymers does not alter the secondary structure of the DNA helix (Singer et al., 1983). Reduced extension (≈20% efficiency) of the O^2-Me-dT·dA or O^2-Et-dT·dA base pair at the 3′-primer termini as compared with the dT·dA termini extension (Singer et al., 1989) suggests that the O^2-alkyl-dT·dA base pair may have a "wobble" alignment. In the absence of normal Watson–Crick alignment, the O^2-

Et-dT·dA base pair may produce distortion of DNA structure that would present steric difficulties as the newly synthesized DNA passes through the double-stranded DNA-binding cleft of the polymerase, leading to retardation of DNA synthesis. This hypothesis is consistent with the findings reported here that formation of the O^2-Et-dT·dA base pair at the replication fork impeded DNA synthesis in vitro. Under normal cellular conditions, replication past the O^2-Et-dT·dA base pair may either not occur or occur with low efficiency. Our in vitro DNA replication studies in the presence of Mg^{2+} suggest that O^2-Et-dT may contribute in part to the cytotoxicity of the ethylating agents.

DNA sequencing of the postlesion synthesis product obtained in the presence of Mn^{2+} and 200 μM dNTP revealed that both dA and dT are incorporated opposite O^2-Et-dT (Figure 6B). Incorporation of dT opposite O^2-Et-dT is not an artifact of the specific DNA sequence used in the studies reported here. When O^2-Et-dT was replaced with O^4-Et-dT in the same DNA sequence, both dA and dG (but not dT) were incorporated opposite O^4-Et-dT (P. C. Grevatt and O. S. Bhanot, unpublished results), consistent with studies reported earlier (Saffhill, 1985; Singer, 1986; Preston et al., 1986). The incorporation of dT opposite O^2-Et-dT (Figure 6B) occurs when Mn^{2+} is substituted for Mg^{2+} in the polymerization reaction. The results suggest a role for Mn^{2+} in the formation of the O^2-Et-dT·dT base pair, probably through a novel interaction of Mn^{2+} in the replication complex. The nascent chains containing an O^2-Et-dT·dT base pair at the 3′-terminus are efficiently extended, implicating the O^2-Et-dT lesion in A·T → T·A transversion mutagenesis.

Using an alternating poly(dA·dT)polymer, O^2-Me-dT was shown to subsitute as the triphosphate for dTTP and on replication the O^2-Me-dT-containing polymer directed incorporation of dG at low efficiency (Singer et al., 1983). Poly(dT) containing random O^2-Me-dT or O^2-Et-dT adducts directed the incorporation of dG during DNA replication in the presence of Mn^{2+} (Saffhill, 1985). Incorporation of dG was not detected in the presence of Mg^{2+} (Saffhill, 1985). In those studies, the suggested incorporation of dC opposite the O^2-alkyl-dT lesion was not directly established. In our assay, where the identity of the nucleotide incorporated opposite O^2-Et-dT is directly established by sequencing the DNA product synthesized on the O^2-Et-dT-containing template, only the incorporation of dA and dT was detected. If incorporation of dG was below detectable limits in this assay, it may be revealed by the more sensitive in vivo site-specific mutagenesis studies currently in progress.

Formation of the pyrimidine–pyrimidine base pair is rare. The O^2-Et-dT·dT base pair probably has one hydrogen bond forming between the N3 nitrogen atoms. The pairing of two pyrimidines would allow for a long hydrogen bond, which would decrease steric hindrance between the ethyl group of O^2-Et-dT and the carbonyl group at the C2 of dT. This could result in a normal sugar–phosphate backbone with the O^2-Et-dT·dT base pair retaining the Watson–Crick alignment. The normal Watson–Crick alignment of the O^2-Et-dT·dT base pair would facilitate formation of phosphodiester bonds on both the 3′ and 5′ sides of dT. This is consistent with the results reported here. An incorporation-dependent blocked product containing dT opposite O^2-Et-dT was not accumulated. Our results support the hypothesis that normal Watson–Crick alignment (backbone) rather than the strength of the base pairs and the extent of hydrogen bonding between them may be the crucial factor in the miscoding (Swann, 1990).

DNA lesions that impede the progress of in vivo DNA replication require the "error-prone" functions of the SOS response to produce mutations in bacteria (Witkin, 1976; Little & Mount, 1982; Walker, 1984). A significant component of the SOS-dependent mutational spectra induced by alkylating agents in bacteria includes events at A·T base pairs (Foster & Eisenstadt, 1985; Zielenska et al., 1988; Cuoto et al., 1989; Eckert et al., 1989). In vivo, SOS-induced proteins may facilitate incorporation of dT opposite O^2-Et-dT and subsequent extension of the O^2-Et-dT·dT base pair, as mediated by Mn^{2+} in vitro, generating A·T → T·A transversion mutations. The studies reported here provide a basis for understanding the molecular mechanisms by which ethylating agents induce A·T transversions and activate oncogene *neu* by an A·T → T·A transversion event in rat neuroblastomas induced by ENU (Bargmann et al., 1986; Perantoni et al., 1987).

Registry No. O^2-Et-dT, 59495-21-5; dNTP, 56-38-2; Mg^{2+}, 7439-95-4; Mn^{2+}, 7439-96-5.

References

Balmain, A., & Brown, K. (1988) *Adv. Cancer Res. 51*, 147–182.

Barbacid, M. (1987) *Ann. Revu. Biochem. 56*, 779–827.

Bargmann, C. I., Hung, M.-C., & Weinberg, R. A. (1986) *Cell 45*, 649–657.

Bartsch, H., & Montesano, R. (1984) *Carcinogenesis 5*, 1381–1385.

Basu, A. K., & Essigmann, J. M. (1988) *Chem. Res. Toxicol. 1*, 1–18.

Beckman, R. A., Mildvan, A. S., & Loeb, L. A. (1985) *Biochemistry 24*, 5810–5817.

Bhanot, O. S., & Ray, A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 7348–7352.

Bhanot, O. S., Grevatt, P. C., Donahue, J. M., Gabrielides, C. N., & Solomon, J. J. (1990) *Biochemistry 29*, 10357–10364.

Bhanot, O. S., Grevatt, P. C., Donahue, J. M., Gabrielides, C. N., & Solomon, J. J. (1992) *Nucleic Acids Res. 20*, 587–594.

Bogovski, P., & Bogovski, S. (1982) *Int. J. Cancer 27*, 471–474.

Borowy-Borowski, H., & Chambers, R. W. (1989) *Biochemistry 28*, 1471–1477.

Brent, T. P., Dolan, M. E., Fraenkel-Conrat, H., Hall, J., Karran, P., Laval, F., Margison, G. P., Montesano, R., Pegg, A. E., Potter, P. M., Singer, B., Swenberg, J. A., & Yarosh, D. B. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1759–1762.

Caruthers, M. H. (1985) *Science 220*, 281–285.

Clark, J. M., Joyce, C. M., & Beardsley, G. P. (1987) *J. Mol. Biol. 198*, 123–127.

Cuoto, L., Chaudhari, I., Donahue, B., Dempel, B., & Essigmann, J. M. (1989) *J. Bacteriol. 171*, 4170–4177.

Den Engelse, L., De Graaf, A., De Brij, R.-J., & Menkveld, G. J. (1987) *Carcinogenesis 8*, 751–757.

Eckert, K. A., & Drinkwater, N. R. (1987) *Mutat. Res. 178*, 1–10.

Eckert, K. A., Ingle, C. A., Klinedinst, D., & Drinkwater, N. R. (1988) *Mol. Carcinog. 1*, 50–56.

Eckert, K. A., Ingle, C. A., & Drinkwater, N. R. (1989) *Carcinogenesis 10*, 2261–2267.

El-Deiry, W. S., So, A. G., & Downey, K. M. (1988) *Biochemistry 27*, 546–553.

Foster, P. L., & Eisenstadt, E. (1985) *J. Bacteriol. 163*, 213–220.

Grevatt, P. C., Donahue, J. M., & Bhanot, O. S. (1991) *J. Biol. Chem. 266*, 1269–1275.

Hevroni, D., & Livneh, Z. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 5046–5050.
Kalnik, M. W., Kouchakdjian, M., Li, B. F. L., Swann, P. F., & Patel, D. J. (1988a) *Biochemistry 27*, 100–108.
Kalnik, M. W., Kouchakdjian, M., Li, B. F. L., Swann, P. F., & Patel, D. J. (1988b) *Biochemistry 27*, 108–115.
Larson, K. L., & Strauss, B. S. (1987) *Biochemistry 26*, 2471–2479.
Lewis, S. E., Johnson, F. M., Skow, L. C., Popp, D., Barnett, L. B., & Popp, R. A. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5829–5831.
Li, B. F., Reese, C. B., & Swann, P. F. (1987) *Biochemistry 26*, 1086–1093.
Little, J. W., & Mount, D. W. (1982) *Cell 29*, 11–22.
Loeb, L. A., & Kunkel, T. A. (1982) *Annu. Rev. Biochem. 52*, 429–457.
Loeb, L. A., & Preston, B. D. (1986) *Annu. Rev. Genet. 20*, 201–230.
Loechler, E. L., Green, C. L., & Essigmann, J. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6271–6275.
Magee, P. N., Montesano, R., & Preussmann, R. (1976) in *American Chemical Society Monograph No. 173* (Searle, C. E., Ed.) pp 491–625, American Chemical Society, Washington, DC.
Maxam, A. M., & Gilbert, W. (1980) *Methods Enzymol. 65*, 497–560.
Mendelman, L. V., Boosalis, M. S., Petruska, J., & Goodman, M. F. (1989) *J. Biol. Chem. 264*, 14415–14423.
Miller, E. C. (1978) *Cancer Res. 38*, 1479–1496.
Moriya, M. M., Takeshita, F., Johnson, K., Peden, S., Will, S., & Grollman, A. P. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 1586–1589.
Ninio, J. (1975) *Biochimie 57*, 587–595.
Perantoni, A. O., Rice, J. M., Reed, C. D., Watatani, M., & Wenk, M. L. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 6317–6321.
Popp, R. A., Baliff, E. G., Skow, L. C., Johnson, F. M., & Lewis, S. E. (1983) *Genetics 105*, 157–167.
Preston, B. D., Singer, B., & Loeb, L. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8501–8505.
Que, B. G., Downey, K. M., & So, A. G. (1978) *Biochemistry 17*, 1603–1606.
Richardson, K. K., Richardson, F. C., Crosby, R. M., Swenberg, J. A. & Skopek, T. R. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 344–348.
Saffhill, R. (1985) *Chem.-Biol. Interact. 53*, 121–130.
Saffhill, R., & Abbott, P. J. (1978) *Nucleic Acids Res. 5*, 1971–1978.
Saffhill, R., Margison, G. P., & O'Connor, P. J. (1985) *Biochim. Biophys. Acta 823*, 111–135.
Sanger, T., Coulson, A. R., Friedman, T., Air, G. M., Barrell, B. G., Brown, N. L., Fides, J. C., Hutchison, C. A., III, Slocombe, P. M., & Smith, M. (1978) *J. Mol. Biol. 125*, 225–246.
Singer, B. (1986) *Cancer Res. 46*, 4879–4885.
Singer, B., & Kusmierek, J. T. (1982) *Annu. Rev. Biochem. 52*, 655–693.
Singer, B., & Grunberger, D. (1983) *Molecular Biology of Mutagens and Carcinogens*, Plenum Press, New York.
Singer, B., Sagi, J., & Kusmierek, J. T. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4884–4888.
Singer, B., Chavez, F., Spengler, S. J., Kusmierek, J. T., Mendelman, L., & Goodman, M. F. (1989) *Biochemistry, 28*, 1478–1483.
Stowers, S. J., Wiseman, R. W., Ward, J. M., Miller, E. C., Miller, J. A., Anderson, M. W., & Eva, A. (1988) *Carcinogenesis 9*, 271–276.
Strauss, B., Rabkin, S., Sagher, D., & Moore, P. D. (1982) *Biochimie 64*, 829–838.
Swann, P. F. (1990) *Mutat. Res. 233*, 81–94.
Takeshita, M., Chang, C.-N., Johnson, F., Will, S., & Grollman, A. P. (1987) *J. Biol. Chem. 262*, 10171–10179.
Varmus, H. E. (1984) *Annu. Rev. Genet. 18*, 553–612.
Walker, G. (1984) *Microbiol. Rev. 48*, 60–93.
Wilson, M. H., & McCloskey, J. A. (1979) *J. Am. Chem. Soc. 97*, 3436–3444.
Witkin, E. M. (1976) *Bacteriol. Rev. 40*, 869–907.
Zielenska, M., Beranek, D., & Guttenplan, J. (1988) *Environ. Mol. Mutagen. 11*, 473–485.