Translesional Synthesis on DNA Templates Containing 8-Oxo-7,8-dihydrodeoxyadenosine†

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ABSTRACT: This study was designed to establish the miscoding potential of 8-oxo-7,8-dihydrodeoxyadenosine (8-oxo-dA). Oligodeoxynucleotides modified site-specifically with 8-oxo-dA were used as templates in primer extension reactions catalyzed by DNA polymerase I (Klenow fragment), DNA polymerase α (pol α), or DNA polymerase β (pol β). dTMP or dGMP is incorporated opposite 8-oxo-dA when either of these dNTPs is provided as substrate for DNA polymerase. dTMP is incorporated exclusively opposite 8-oxo-dA when all four dNTPs are present in the reaction mixture at equimolar concentrations. Chain extension is catalyzed efficiently by Klenow fragment and pol β under conditions where 8-oxo-dA is paired with dT at the 3′ terminus of the primed DNA template. Chain extension catalyzed by pol α proceeds more slowly. As shown by steady-state kinetic experiments, incorporation of dGMP is higher in reactions catalyzed by pol β than by Klenow fragment or pol α. The dG·8-oxo-dA pair is extended efficiently from the 3′ terminus in the absence of dTTP. We conclude that DNA containing 8-oxo-dA is capable of miscoding; however, unlike 8-oxo-dG, the mutagenic potential of this lesion is limited.

Reactive oxygen species generated by ionizing radiation or endogenous oxidative processes react with DNA, modifying base and sugar moieties and generating interstrand cross links (Imlay & Linn, 1988; Steenken, 1989; Hutchinson, 1985). Base damage creates miscoding lesions that are potentially mutagenic (Levin et al., 1982). For example, DNA containing 8-oxo-dG[1] promotes misincorporation of dAMP opposite the lesion (Shibutani et al., 1991), the relative degree depending on the DNA polymerase involved (Shibutani et al., 1991). 8-Oxo-dG has been shown to be mutagenic in *Escherichia coli* (Wood et al., 1990; Moriya et al., 1991; Cheng et al., 1993) and mammalian cells (Moriya, 1993).

8-Oxo-7,8-dihydro-2′-deoxyadenine (8-oxo-dA) is generated when deoxyadenine (Conlay, 1963; VanHemmen & Bleichrodt, 1971) and DNA (Bonicel et al., 1980) are exposed to ionizing radiation. 8-Oxo-dA is found in genomic DNA (malins & Haimanot, 1990; Stillwell et al., 1989), presumably arising from oxidative processes. The number of 8-oxo-dA lesions in DNA increases following irradiation (Gajewski et al., 1990) or after treatment with agents that generate oxygen free radicals (Arouma et al., 1989a,b).

This paper explores the miscoding properties of 8-oxo-dA, providing insight into the mutagenic potential of oxidized purines in DNA. We have established kinetic parameters for the primary enzymatic reactions by which dNMPs are incorporated opposite 8-oxo-dA and the 3′-terminal pair containing the lesion is extended. Our results indicate that 8-oxo-dA promotes incorporation of dGMP under certain experimental conditions; thus, this lesion is capable of miscoding. However, on the basis of quantitative data obtained in this study, 8-oxo-dA may play only a minor role in cellular mutagenesis resulting from oxidative DNA damage.

EXPERIMENTAL PROCEDURES

Materials and Methods. Organic chemicals used for synthesis of oligodeoxynucleotides were supplied by Aldrich Chemical Co. Acetonitrile, triethylamine, and distilled water, all HPLC-grade, were purchased from Fisher Chemical Co. [γ-^{32}P]ATP (specific activity 5000 Ci/mmol) was obtained from Amersham Corp. Cloned Klenow fragment of *E. coli* DNA polymerase I and T4 polynucleotide kinase were purchased from International Biotechnologies, Inc.; calf thymus DNA polymerase α was from Molecular Biology Resources Inc.; DNA polymerase I and deoxynucleotide triphosphates were from Pharmacia; and nuclease P1 and alkaline phosphatase, type III-S, were from Boehringer Mannheim Biochemicals and Sigma Chemical Co., respectively. DNA polymerase β, provided by Samuel H. Wilson, was purified as described previously (Abbots et al., 1988). A Waters 990 HPLC instrument, equipped with a photodiode array detector, was used for the separation and analysis of modified and unmodified oligodeoxynucleotides.

Synthesis and Purification of Oligodeoxynucleotides. The DMT-phosphoramidite of 8-oxo-dA, required for DNA oligomer synthesis, was synthesized by a five-step procedure, similar to that used to prepare 8-oxo-dG. Treatment of 8-bromo-2′-deoxyadenosine with sodium benzylate in benzyl alcohol afforded 8-benzyloxy-2-deoxyadenosine in 82% yield. Transient hydroxyl protection of the latter material with hexamethyldisilazane, followed by addition of benzoyl chloride, led to N^6-benzoyl-8-benzyloxy-2′-deoxyadenosine in high yield. Hydrogenation of N^6-benzoyl-8-benzyloxy-2′-deoxyadenosine over a palladium-on-carbon catalyst in MeOH–$CH_3COOC_2H_5$ gave N^6-benzoyl-8-oxo-7,8-dihydro-2′-deoxyadenosine, which was converted under standard conditions to its 4,4′-dimethoxytrityl derivative in 91% yield. The required phosphoramidite was obtained in quantitative yield as a diastereomeric pair, using the anhydrous procedure described in an earlier publication (Bodepudi et al., 1991).

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[1] Abbreviations: 8-oxo-dA, 8-oxo-7,8-dihydrodeoxyadenosine; 8-oxo-dG, 8-oxo-7,8-dihydrodeoxyguanosine; pol I, DNA polymerase I; pol α, DNA polymerase α; pol β, DNA polymerase β; F_{ins}, frequency of insertion; F_{ext}, frequency of extension; dNTP, 2-deoxynucleotide triphosphate; PAGE, polyacrylamide gel electrophoresis; RT, retention time; DMT, dimethyltrityl.

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Oligodeoxynucleotides (Takeshita et al., 1987) and 8-oxo-dA-modified oligodeoxynucleotides were prepared with a Dupont Coder 300 automated DNA synthesizer. Oligomers were removed from the resin support by treating with a solution of concentrated ammonia containing 0.1 M 2-mercaptoethanol at 55 °C for 16 h. Oligodeoxynucleotides were isolated on a reverse-phase column, μBondapak C_{18} (0.39 × 30 cm, Waters), eluted over 60 min at a flow rate of 1.0 mL/min with a linear gradient of 0.05 M triethylamine acetate, pH 7.0, containing 10–15% acetonitrile. Oligodeoxynucleotides were further purified on a 20% polyacrylamide gel (15 × 72 × 0.2 cm) in the presence of 7 M urea. Bands located under ultraviolet light were extracted with 2.0 mL of distilled water overnight at 4 °C and then concentrated on a Centricon 3 filter (Amicon) by centrifugation at 5000 rpm for 2 h at 15 °C. Samples were subjected to HPLC, as described above, to remove urea. Oligodeoxynucleotides were labeled at the 5′ terminus by treating with bacteriophage T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP (Maniatis et al., 1982). The product was applied to a 20% polyacrylamide gel (35 × 42 × 0.04 cm) in the presence of 7 M urea to check homogeneity. Following electrophoresis, the positions of the oligomers were established by autoradiography, using Kodak X-Omat XAR film. Oligodeoxynucleotides (18-mers), modified with 8-oxo-dA (sequence 2, RT = 30.4 min), were separated completely from their unmodified counterpart (sequence 1, RT = 28.5 min) by HPLC. Using a modification of the method of Gehrke (1984), modified oligodeoxynucleotides (sequence 2, 3.0 μg) were digested for 2 h at 37 °C with nuclease P1 (2 units) and alkaline phosphatase (3 units) in 100 μL of a 30 mM sodium acetate buffer (pH 5.3) containing 10 mM 2-mercaptoethanol and 5 μL of 20 mM zinc sulfate. The pH was adjusted by adding 20 μL of 0.5 M Tris-HCl, pH 8.5; the reaction mixture was further incubated for 2 h at 37 °C. The sample was immersed in boiling water for 3 min, evaporated to dryness in a vacuum desiccator, and extracted twice with 350 μL of methanol. Methanol extracts were evaporated to dryness, and the product was dissolved in distilled water and analyzed by HPLC, using a Waters reverse-phase μBondapak C_{18} column (0.39 × 30 cm). The column was eluted with an isocratic system consisting of distilled water (pH 5.85) for 20 min, followed by a continuous linear gradient of distilled water containing 0–10% acetonitrile, over 30 min, with a flow rate of 1.0 mL/min. The ratio of nucleosides (dC:dT:8-oxo-dA:dA) recovered from enzymatic digestion of the purified 8-oxo-dA-18-mer (sequence 2) was 8:8:1:1. The UV spectrum and retention time (RT = 40.5 min) of 8-oxo-dA was consistent with that of an authentic standard. Absorption maxima were observed at 208 and 276 nm.

DNA Synthesis. Primer extension was conducted for 1 h at 25 °C in reaction mixtures containing four dNTPs (100 μM), unmodified or 8-oxo-dA-modified templates primed with ^{32}P-labeled 10-mer (0.05 μM) and containing varying amounts of DNA polymerase, buffer, and salts as described in the following paragraph. Products of the reaction were separated by electrophoresis for 24–32 h on 20% polyacrylamide gels containing 7 M urea. Since these conditions create "smiling", synthetic standards were placed in adjacent lanes to confirm the relative position of the reaction products. Using this procedure, full-length reaction products (17-mers) containing dT and dG can be separated from one another and also from a mixture of 17-mers containing dA and dC. The nature of the base misincorporated was confirmed by Maxam–Gilbert sequence analysis; however, this procedure may fail to detect low levels of incorporation.

Table I: Sequence of Oligodeoxynucleotides[a]

no.	sequence (5′ → 3′)
1	CCTTCACTACTTTCCTCT
2	CCTTCA*CTACTTTCCTCT
3	AGAGGAAAGTAG
4	AGAGGAAAGT
5	AGAGGAAAGTAGN
6	AGAGGAAAGTAGNGAAGG
7	GTTGAGTACTTTCCTCT
8	GTTGA*GTACTTTCCTCT
9	AGAGGAAAGTACNGAAC

[a] Sequences of templates and primers. A* = 8-oxo-dA; N = C, A, G, or T.

Insertion and Extension Kinetics. For experiments using the Klenow fragment of *E. coli* DNA polymerase I, incorporation of dNMP opposite the lesion and 3′ extension of the nascent chain were measured in reactions (10 μL) containing oligonucleotide templates annealed with primer (0.05 μM), dNTP (0.4–500 μM), 0.005–0.05 unit of enzyme, 50 mM Tris-HCl, pH 8.0, 8 mM $MgCl_2$, and 5 mM 2-mercaptoethanol. Incubations were at 20 °C for 1–3 min. Studies with DNA polymerase α (1.0 unit) were conducted for 5–30 min at 25 °C in 10 μL of a reaction mixture containing templates annealed with primer (0.05 μM), dNTP (0.4–10,000 μM), 50 mM Tris-HCl (pH 8.0), 10 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$, 2 mM dithiothreitol, and bovine serum albumin (100 μg/μL). Experiments with HeLa DNA polymerase β (0.67–6.7 units) were carried out at 20 °C for 1.5–6 min under similar conditions, omitting $(NH_4)_2SO_4$ (Abbots et al., 1988). Primer was shown to be fully annealed to the template and the enzyme was saturated with primer–template.

Samples were heated at 95 °C for 3 min in the presence of formamide dye, then applied to 20% polyacrylamide gels (35 × 42 × 0.04 cm) in the presence of 7 M urea, and subjected to electrophoresis for 15 h at 800 V. Bands were identified by autoradiography. Radioactivity was measured in a Packard scintillation counter using Liquiscint (National Diagnostics). The Michaelis constant (K_m) and the maximum rate of the reaction (V_{max}) were obtained from Hanes–Woolf plots of the kinetic data. Insertion (F_{ins}) and extension (F_{ext}) frequencies was determined relative to dT·dA according to equations developed by Mandelman et al. (1989, 1990), where $F = (V_{max}/K_m)$[wrong pair]$/(V_{max}/K_m)$[right pair] with "wrong pair" defined as a base mismatch or any base pair containing 8-oxo-dA. All reaction rates were linear over the course of the experiment; data reported represent the average of 2–4 separate experiments in which less than 20% of the primer is extended (Dosanjh et al., 1991).

RESULTS

Incorporation of Single dNMP opposite 8-Oxo-dA. Oligodeoxynucleotide sequences used in these experiments are listed in Table I. The relative incorporation of dNMP opposite dA or 8-oxo-dA when a single dNTP is present in the reaction mixture is shown in Figure 1. dTMP is incorporated opposite 8-oxo-dA in reactions catalyzed by the three DNA polymerases tested (lanes 8, 12, and 16). The Klenow fragment also catalyzes incorporation of small amounts of dAMP and dGMP (lanes 14 and 15). Pol α and pol β catalyze incorporation of dGMP (see arrow, lanes 7 and 11). This experiment was conducted with 10 μM dNTP to accentuate differences between incorporation of dGMP and dTMP. Qualitatively similar results were obtained when 100 μM concentrations of

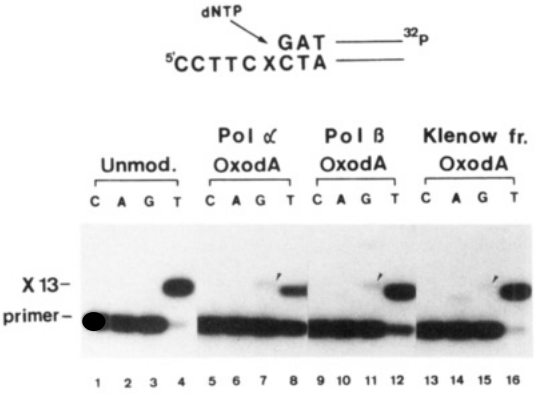

FIGURE 1: Nucleotide incorporation in the presence of a single dNTP. Reaction mixtures contained an unmodified 18-mer (sequence 1) or 8-oxo-dA-modified 18-mer (sequence 2) as template, primed with ^{32}P-labeled 12-mer (sequence 3, 0.05 μM), a single dNTP (10 μM), and either calf thymus DNA pol α (1 unit), HeLa DNA pol β (1 unit), or Klenow fragment (0.02 unit) in a final volume of 10 μL. DNA pol α was used for reactions containing unmodified 18-mers (lanes 1–4). Reactions were incubated for 1 h at 25 °C and then processed as described under Experimental Procedures. Arrows show band representing dGMP incorporation at position 13.

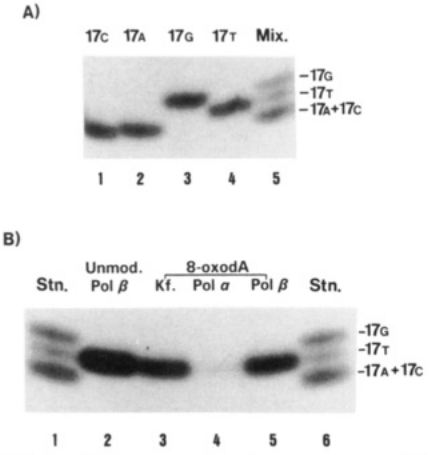

FIGURE 2: Nucleotide incorporation in the presence of four dNTPs. (A) Separation of synthetic standards (sequence 9) containing dC (lane 1), dA (lane 2), dG (lane 3), or dT (lane 4) at position 13 and a mixture of these oligodeoxynucleotides (lane 5). (B) Full-length extended products of reactions with DNA polymerases. Reaction mixtures contained four dNTPs (100 μM each) and an unmodified 17-mer (sequence 7) or 8-oxo-dA-modified 17-mer (sequence 8) as template, primed with ^{32}P-labeled 10-mer (sequence 4, 0.05 μM). Mobilities of the 17-mer reaction products (lanes 2–5) are compared with synthetic standards (lanes 1 and 6). See Experimental Procedures and legend to Figure 1 for further details.

dNTP were used. Addition of KCl (10–100 μM) to the standard reaction mixture did not affect the pattern of incorporation observed.

A primed 8-oxo-dA-modified 17-mer template was used to examine the rate of chain extension from the 3′-terminus. As shown in the experiment depicted in Figure 2, oligodeoxynucleotides containing dT, dG, and dA (or dC) can be distinguished by their relative electrophoretic mobility on 20% polyacrylamide gels (Figure 2A). DNA synthesis on unmodified templates, catalyzed by pol β (Figure 2B, lane 2) or other polymerases (data not shown), led to the expected incorporation of dTMP at position 13 when tested in the presence of all four natural dNTPs (data not shown). Using templates modified with 8-oxo-dA, all DNA polymerases tested catalyzed synthesis of a 17-mer containing dT (lanes 3–5). Lesser amounts of reaction product were synthesized by pol α, compared with the other DNA polymerases.

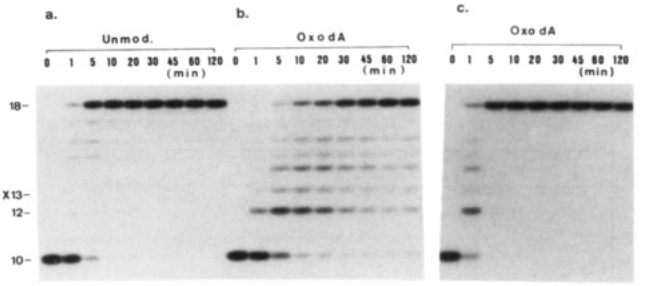

FIGURE 3: Time course of chain extension reaction catalyzed by Klenow fragment. The polymerase reaction was carried out at 20 °C using varying amounts of Klenow fragment (panels a and b, 0.01 unit; panel c, 0.1 unit), an unmodified 18-mer (sequence 1) or 8-oxo-dA-modified 18-mer (sequence 2) template primed with a ^{32}P-labeled 10-mer (sequence 4, 0.05 μM) in the presence of four dNTPs (100 μM each), as described in Experimental Procedures.

Frequency of Insertion and Chain Extension. Using an 8-oxo-dA-modified template and a 10-mer primer, chain extension catalyzed by the Klenow fragment is retarded one base before (position 12) and opposite (position 13) 8-oxo-dA (Figure 3b). This inhibition of translesional synthesis can be overcome by adding a 10-fold excess of the enzyme (Figure 3c).

Kinetic parameters of dNTP insertion and extension, catalyzed by the Klenow fragment, were measured during translesional synthesis (Table II). The frequency of insertion (F_{ins}) for dTTP opposite 8-oxo-dA (0.28) is 500–1000 times higher than that for other dNTPs. Frequency of extension (F_{ext}) from dT·8-oxo-dA (0.386) is 630–3000 times higher than from dA·8-oxo-dA or dC·8-oxo-dA and 35 times higher than from dG·8-oxo-dA. F_{ins} for dGTP opposite 8-oxo-dA is similar to F_{ins} for dCTP or dATP.

In reactions catalyzed by pol α (Table III), F_{ins} for dTTP opposite 8-oxo-dA (1.9×10^{-2}) is 10 times higher than for dGTP. F_{ext} for dT·8-oxo-dA and dG·8-oxo-dA is 280–3500 fold lower than for Klenow fragment; thus, chain extension by pol α is retarded strongly opposite the lesion, as confirmed by gel electrophoresis (Figure 4). Sequence analysis of extended primers produced by Klenow fragment and pol α (Maxam & Gilbert, 1980) confirmed predominant incorporation of dTMP opposite the lesion (data not shown).

In reactions catalyzed by pol β, F_{ins} for dTTP (0.289) and F_{ext} for dT·8-oxo-dA (0.526) approach values for the unmodified base (Table IV). Additionally, F_{ins} for dGTP (0.016) and F_{ext} for dG·8-oxo-dA (0.019) are relatively high, compared with reactions catalyzed by Klenow fragment (Table II) or pol α (Table III).

DNA Synthesis. The time course of DNA synthesis on 8-oxo-dA-containing templates in reactions lacking dTTP is shown in Figure 5. Chain extension catalyzed by pol α is blocked opposite the lesion (position 13). A small amount of readthrough was observed in reactions catalyzed by the Klenow fragment. In reactions catalyzed by pol β, 8-oxo-dA is readily bypassed. The electrophoretic migration of fully extended primer in reactions catalyzed by pol β and Klenow fragment was identical to that of oligodeoxynucleotides containing dG opposite the lesion.

The 13-mer containing dG separates from 13-mers containing dA or dC. The upper band in the figure contains dG; the lower band could be a 13-mer containing dA or a mixture of 13-mers containing dA and dC. The latter interpretation appears likely since the kinetic parameters of insertion and extension reactions for dATP and dCTP are similar (Table III).

Table II: Kinetic Parameters of Nucleotide Insertion and Chain Extension Reactions Catalyzed by Klenow Fragment[a]

	dNTP → 5′CCTTCXC—G— 12-mer				dGTP → 5′CCTTCXC—NG— 13-mer		
	K_m (μM)	V_{max} (% min^{-1})	F_{ins}		K_m (μM)	V_{max} (% min^{-1})	F_{ext}
C·A	144	0.500	6.78×10^{-4}	G·CA	13.0	1.36	0.019
A·A	51.7	0.835	3.14×10^{-3}	G·ACA	12.1	0.024	3.52×10^{-4}
G·A	15.3	0.069	8.76×10^{-4}	G·GCA	31.0	0.037	2.14×10^{-4}
T·A	3.66	18.9	1.00	G·TCA	3.51	19.5	1.00
C·A*	28.1	0.041	2.80×10^{-4}	G·CCA*	22.4	0.017	1.31×10^{-4}
A·A*	60.0	0.152	5.09×10^{-4}	G·ACA*	26.2	0.087	6.17×10^{-4}
G·A*	26.1	0.060	5.50×10^{-4}	G·GCA*	21.9	1.31	0.011
T·A*	7.08	10.4	0.28	G·TCA*	7.39	15.9	0.386

[a] Kinetics of insertion were measured in reactions incubated for 1 min (dT·dA and dT·8-oxo-dA pairs), using 0.005 unit of Klenow fragment, and for 3 min (all other pairs), using 0.05 unit of the enzyme, in 10 μL of buffer containing sequence 1 or 2, primed with ^{32}P-labeled 12-mer (0.05 μM, sequence 3), as described in Experimental Procedures. Kinetics of chain extension were determined under similar conditions with template DNA primed with ^{32}P-labeled 13-mer containing dC, dA, dG, or dT (sequence 5, 0.05 μM). Values for K_m and V_{max} are corrected for the amount of enzyme used in the reaction. X = A or 8-oxo-dA.

Table III: Kinetics of Nucleotide Insertion and Chain Extension Reactions Catalyzed by DNA Polymerase α[a]

	dNTP → 5′CCTTCXC—G— 12-mer				dGTP → 5′CCTTCXC—NG— 13-mer		
	K_m (μM)	V_{max} (% min^{-1})	F_{ins}		K_m (μM)	V_{max} (% min^{-1})	F_{ext}
G·A	1380	0.13	4.27×10^{-5}	G·GCA	4300	0.03	4.29×10^{-6}
T·A	3.24	7.18	1.00	G·TCA	3.52	5.08	1.00
G·A*	384	1.66	1.95×10^{-3}	G·GCA*	1140	0.06	3.89×10^{-5}
T·A*	77.4	3.22	1.90×10^{-2}	G·TCA*	442	0.07	1.10×10^{-4}

[a] Base insertion was measured in reactions incubated for 5 min (dT·dA pair), 10 min (dT·8-oxo-dA), or 15 min (dG·8-oxo-dA and dG·dA), as described in Experimental Procedures. Kinetics of chain extension were determined in reactions conducted for 5 min (dT·dA), 10 min (dT·8-oxo-dA), or 30 min (dG·dA and dG·8-oxo-dA).

DISCUSSION

The presence of a carbonyl group at the C-8 position of deoxyadenosine or deoxyguanosine interferes with rotation around the glycosidic bond. In aqueous solution (Uesugi & Ikehama, 1977) and in DNA (Kouchakdjian et al., 1991), these modified bases relieve steric hindrance by assuming the syn conformation (Uesugi & Ikehama, 1977). Oxidation at C-8 also alters the base-pairing properties of these molecules (Culp et al., 1990; Cho & Evans, 1991). These steric and electronic changes are reflected in the miscoding properties of 8-oxo-dG during DNA synthesis in vitro (Shibutani et al., 1991); surprisingly, this proved not to be the case for 8-oxo-dA.

The frequency (F_{ins}) at which dNTPs are inserted opposite 8-oxo-dA, coupled with the frequency (F_{ext}) at which various base pairs are extended from the 3′ primer terminus, represent kinetic parameters that can be used to predict miscoding by DNA lesions during translesional synthesis (Mendelman et al., 1989, 1990). This approach has been employed to establish the miscoding potential of 8-oxo-dG (Shibutani et al., 1991). Using DNA templates modified with 8-oxo-dA, $F_{ins} \times F_{ext}$ for dTMP in reactions catalyzed by Klenow fragment and pol β was calculated to be 0.108 and 0.152, respectively. $F_{ins} \times F_{ext}$ for reactions involving pol α and dTMP (2.09×10^{-5}) was much lower (Table V). Comparative values for dGMP, the only base other than dTMP to be incorporated by any of the

Table IV: Kinetic Parameters of Nucleotide Insertion and Chain Extension Reactions Catalyzed by DNA Pol β[a]

dNTP ↓ G— 5′CCTTCXC— 12-mer	K_m (μM)	V_{max} (% min^{-1})	F_{ins}	dGTP ↓ NG— 5′CCTTCXC— 13-mer	K_m (μM)	V_{max} (% min^{-1})	F_{ext}
T·A	75.1	6.11	1.00	G·CA	52.2	15.4	1.00
G·A*	251	0.334	0.016	G·CA*	234	1.34	0.019
T·A*	296	6.96	0.289	G·CA*	109	16.9	0.526

[a] Base insertion was determined in reactions incubated for 90 s (dT·dA) or for 6 min (dT·8-oxo-dA and dG·8-oxo-dA), as described in Experimental Procedures. Kinetics of extension were measured in reactions incubated for 90 s (dT·dA), 3 min (dT·8-oxo-dA), or 6 min (dG·8-oxo-dA).

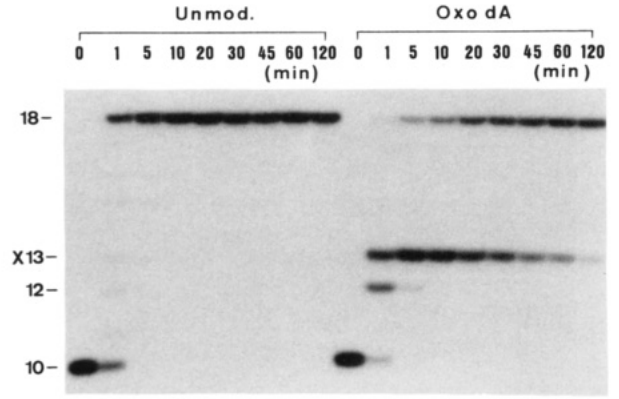

FIGURE 4: Time course of chain extension catalyzed by DNA pol α. The reaction was carried out at 25 °C using 1 unit of calf thymus pol α, as described in the legend to Figure 3.

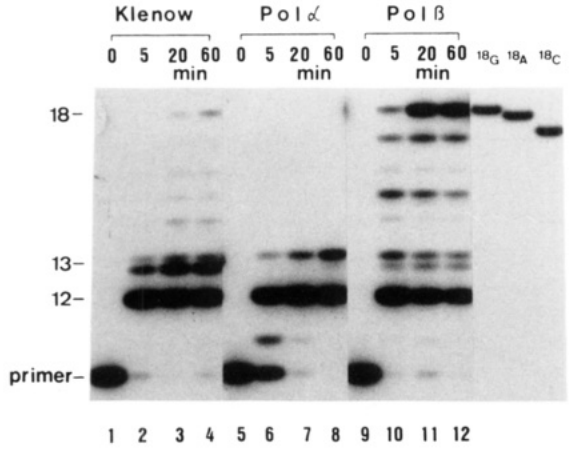

FIGURE 5: Time course of chain extension in the absence of dTTP. Reactions were carried out at 25 °C for 1 h using Klenow fragment (0.1 unit), calf thymus pol α (1 unit), or HeLa pol β (1 unit). Conditions are as described in the legend to Figure 3, omitting dTTP. 18G, 18A, and 18C are synthetic standards (sequence 6) containing the base indicated at position 13.

three polymerases, were 6.05×10^{-6}, 3.04×10^{-4}, and 7.59×10^{-8}, respectively. Thus, translesional synthesis in which dTMP is incorporated opposite 8-oxo-dA is favored by factors of 17 800 and 28 over incorporation of dGMP, a potentially mutagenic event, in reactions catalyzed by Klenow fragment and pol α, respectively. In an analogous study of the same enzymes (Shibutani et al., 1991), incorporation of the potentially mutagenic nucleotide, dAMP, opposite 8-oxo-dG was favored over incorporation of the nonmutagenic species, dCMP, by factors of 2 and 1450, respectively (Table V).

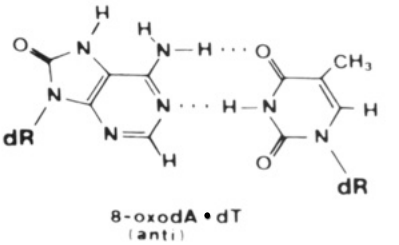

FIGURE 6: Base pairing of 8-oxo-dA with dT.

Table V: Frequency of Insertion and Extension for 8-Oxopurines in Reactions Catalyzed by DNA Polymerases[a]

DNA polymerase		F_{ins}	F_{ext}	$F_{ins} \times F_{ext}$	F_{rel}[a]
Klenow fragment	T·A*	0.28	0.386	0.108	1.0
	G·A*	5.50×10^{-4}	0.011	6.05×10^{-6}	5.60×10^{5}
pol α	T·A*	1.9×10^{-2}	1.10×10^{-4}	2.09×10^{-6}	1.0
	G·A*	1.95×10^{-3}	3.89×10^{-5}	7.59×10^{-8}	0.0363
pol β	T·A*	0.289	0.526	0.152	1.0
	G·A*	0.016	0.019	3.04×10^{-4}	2.00×10^{3}
Klenow fragment	C·G*	0.35	0.033	0.0116	1.0
	A·G*	0.05	0.50	0.025	2.16
pol α	C·G*	0.018	9.45×10^{-4}	1.70×10^{-5}	1.0
	A·G*	0.13	0.19	0.0247	1450

[a] Calculated for $F_{ins} \times F_{ext}$ ("right" base) = 1.0; A*, 8-oxo-dA; G*, 8-oxo-dG. [b] Data for 8-oxo-dA taken from Tables II–IV; data for 8-oxo-dG, from Shibutani et al. (1991).

Kinetic parameters were established for DNA polymerase reactions containing a single dNTP. When four dNTPs are present, dTMP was incorporated exclusively opposite 8-oxo-dA. Transient blocking of the enzyme occurs opposite the lesion; this effect was abolished by increasing the concentration of polymerase. The ratio V_{max}/K_m provides a quantitative measure of the efficiency of nucleotide incorporation by DNA polymerase, permitting frequency of dNTP incorporation opposite the lesion to be compared with the frequency of extension from the 3′ primer terminus. Although kinetic analysis can distinguish several major events involved in translesional synthesis, the precise mechanism by which DNA polymerases discriminate between correct and incorrect nucleotides may involve steps not detected by this steady-state approach (Echols & Goodman, 1991). Furthermore, there are functional differences between the several polymerases used in this study. Klenow fragment discriminates primarily by a V_{max} effect (Kuchta et al., 1988) while pol α enhances base selection at the level of substrate binding by a K_m effect (Boosalis et al., 1987). Pol β utilizes both mechanisms (Boosalis et al., 1989). DNA polymerases differ also in their degree of processivity. For pol α and Klenow

fragment, dissociation of the enzyme from the extended primer template is relatively slow; however, pol β is a distributive enzyme and nucleotide incorporation or conformational change may be rate-limiting. Pre-steady-state kinetic analysis has been used to distinguish between these possibilities (Eger & Benkovic, 1992).

8-Oxo-dG and 8-oxo-dA differ significantly with respect to their respective mutagenic potential. The mutagenic properties of 8-oxo-dG (Shibutani et al., 1991) reflect stability of this lesion when paired in the syn conformation with dA (anti) (Kouchakdjian et al., 1991), coupled with the relative resistance of this complex to proofreading exonucleases (Shibutani et al., 1991) and subsequent repair by 8-oxo-dG–DNA glycosylase (Tchou et al., 1991). In contrast, 8-oxo-dA promotes a nonmutagenic event—insertion of dTMP (anti) opposite the lesion, forming a Watson–Crick pair [Figure 8 in Guschulbauer et al. (1991)]. All of the DNA polymerases used in this study incorporate small amounts of dG opposite 8-oxo-dA. The repair polymerases, pol I and pol β, but not the replicative polymerase, pol α, extend primed templates containing dG·8-oxo-dA from the 3′ terminus. Recently, Leonard et al. (1992) described the structure of a dG·8-oxo-dA pair in a duplex oligonucleotide using single-crystal X-ray diffraction techniques. Thus, although dG·8-oxo-dA may effectively block DNA replication, A → C transversions could result from incorporation of dG incorporated during "short patch" repair.

DNA synthesis on primed templates containing 8-oxo-dA has been investigated by Guschlbauer et al. (1991); certain findings reported in our paper disagree with their published results. The dideoxy sequencing procedure used by Guschlbauer et al. (1991), in conjunction with the polymerase chain reaction, will not detect small amounts of bases incorporated opposite the lesion. Further, using a fixed concentration of DNA polymerase, these authors failed to demonstrate significant inhibition of DNA synthesis at the site of the lesion. The rate of DNA synthesis in primer extension reactions depends on the concentration of DNA polymerase. In the present paper, we show that DNA synthesis is blocked transiently by the lesion unless high concentrations of enzyme are present. Kuchino et al. (1987) reported that 8-oxo-dG in the template strand does not inhibit DNA synthesis; again, transient blocking of synthesis can be demonstrated when limiting concentrations of DNA polymerase are used (Shibutani et al., 1991).

Genomic DNA is subject to damage by endogenous and exogenous oxidative processes. Unrepaired DNA damage leads to accumulation of mutations and may contribute to the development of cancer and other diseases associated with cellular aging (Harman, 1981; Ames, 1983). 8-Oxo-dG, a product of oxygen-derived radical damage, is mutagenic (Wood et al., 1990; Moriya et al., 1991; Moriya, 1993; Cheng et al., 1991). In *E. coli*, an elaborate error-avoidance pathway involving several different DNA repair enzymes has evolved to protect this organism from the mutagenic consequences of this lesion (Tchou & Grollman, 1993). To date, enzyme activities have not been detected that specifically repair or otherwise prevent accumulation of 8-oxo-dA in DNA. The apparent lack of repair enzymes and weak miscoding properties of 8-oxo-dA suggest that its potential contribution to cellular mutagenesis and carcinogenesis is limited. This prediction is consistent with recent site-specific mutagenesis studies of 8-oxo-dA (Wood et al., 1993), showing that 8-oxoadenine is at least an order of magnitude less mutagenic than 8-oxoguanine in *E. coli* cells with natural DNA repair capabilities.

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