Miscoding Properties of 6α- and 6β-Diastereoisomers of the N^2-(Estradiol-6-yl)-2′-deoxyguanosine DNA Adduct by Y-Family Human DNA Polymerases[†]

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ABSTRACT: Treatment with estrogen increases the risk of breast, ovary, and endometrial cancers in women. DNA damage induced by estrogen is thought to be involved in estrogen carcinogenesis. In fact, Y-family human DNA polymerases (pol) η and κ, which are highly expressed in the reproductive organs, miscode model estrogen-derived DNA adducts during DNA synthesis. Since the estrogen−DNA adducts are a mixture of 6α- and 6β-diastereoisomers of dG-N^2-6-estrogen or dA-N^6-6-estrogen, the stereochemistry of each isomeric adduct on translesion synthesis catalyzed by DNA pols has not been investigated. We have recently established a phosphoramidite chemical procedure to insert 6α- or 6β-isomeric N^2-(estradiol-6-yl)-2′-deoxyguanosine (dG-N^2-6-E_2) into oligodeoxynucleotides. Using such site-specific modified oligomer as a template, the specificity and frequency of miscoding by dG-N^2-6α-E_2 or dG-N^2-6β-E_2 were explored using pol η and a truncated form of pol κ (pol κΔC). Translesion synthesis catalyzed by pol η bypassed both the 6α- and 6β-isomers of dG-N^2-6-E_2, with a weak blockage at the adduct site, while translesion synthesis catalyzed by pol κΔC readily bypassed both isomeric adducts. Quantitative analysis of base substitutions and deletions occurring at the adduct site showed that pol κΔC was more efficient than pol η by incorporating dCMP opposite both 6α- and 6β-isomeric dG-N^2-6-E_2 adducts. The miscoding events occurred more frequently with pol η, but not with pol κΔC. Pol η promoted incorporation of dAMP and dTMP at both the 6α- and 6β-isomeric adducts, generating G → T transversions and G → A transitions. One- and two-base deletions were also formed. The 6α-isomeric adduct promoted slightly lower frequency of dCMP incorporation and higher frequency of dTMP incorporation and one-base deletions, compared with the 6β-isomeric adduct. These observations were supported by steady-state kinetic studies. Taken together, the miscoding property of the 6α-isomeric dG-N^2-6-E_2 is likely to be similar to that of the 6β-isomeric adduct.

Prolonged exposure to endogenous and/or exogenous estrogens is associated with development of breast, ovary, and endometrial cancers (*1*). The mechanism of estrogen carcinogenicity may be related to the initiation and/or promotion process affected by estrogen metabolites (*2, 3*). Hydroxylation at the C2- or C4-position of estrogens by cytochrome P450 enzymes produces catecholestrogens, which are further oxidized by the enzymes to form corresponding semiquinones and quinones; both can directly or indirectly cause genotoxic effects through redox cyclic reactions (*4*). Reaction of estrogen 2,3-quinone with 2′-deoxyguanosine (dG) and 2′-deoxyadenosine (dA) residues in DNA produces N^2-(2-hydroxyestrogen-6-yl)-2′-deoxyguanosine (dG-N^2-2-OHE_2; structure in Figure 1) and N^6-(2-hydroxyestrogen-6-yl)-2′-deoxyadenosine, respectively (*5*), both of which are mutagenic, generating primarily G → T and A → T mutations in mammalian cells (*6*). Estrone 3,4-quinone reacts with dG and dA to form N^7-(4-hydroxyestrone-1-yl)guanine and N^3-(4-hydroxyestrone-1-yl)adenine, respectively, with loss of 2-deoxyribose (*5*).

6α- and/or 6β-OH-estrogens were detected in ovarian follicles (*7*) and urine of pregnant women (*8*). Formation of 6α-OHE_2 from E_1[1] or E_2 was observed in human placental perfusate (*9*), in reaction mixture with human liver microsome (*10*), and in cultured human breast cancer MCF-7 cells exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (*11*). Both 6α- and 6β-hydroxylation products were also detected *in vitro* upon incubation with human CYP enzymes, with the ratio of 6α- and 6β-isoforms depending on the CYP enzyme used (*12, 13*). We have proposed another genotoxic mechanism based on sulfonation of C6-OH-estrogens (*14*). In fact, a model estrogen 6-sulfate, pyridinium 3-methoxyestra-1,3,5(10)-trien-6(α or β)-yl sulfate [3MeE-6(α or β)-S] was highly reactive with dG and dA, resulting in the

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[1] Abbreviations: E_1, estrone; E_2, estradiol; dG-N^2-6-E_2, N^2-(estradiol-6-yl)-2′-deoxyguanosine; dNTP, 2′-deoxyribonucleoside triphosphate; pol η, DNA polymerase η; pol κ, DNA polymerase κ; pol κΔC, a truncated form of pol κ; F_{ins}, frequency of insertion; F_{ext}, frequency of extension; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography.

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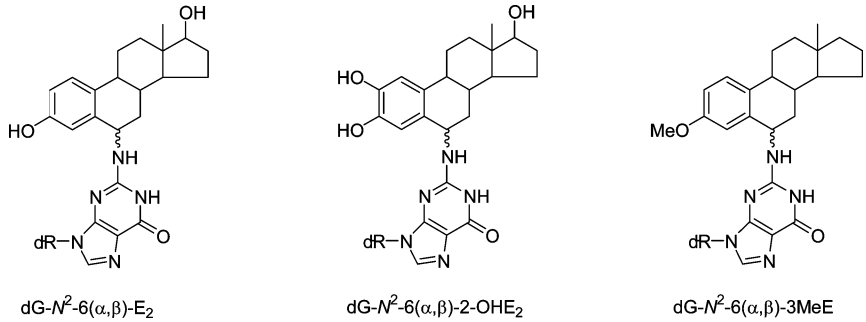

FIGURE 1: Structures of dG-N^2-E_2 and its related adducts.

formation of N^2-[3-methoxyestra-1,3,5(10)-trien-6(α,β)-yl]-2′-deoxyguanosine (dG-N^2-6-3MeE) (Figure 1) and N^6-[3-methoxyestra-1,3,5(10)-trien-6(α,β)-yl]-2′-deoxyadenosine, respectively (*15*). Estrogen 6-sulfate binds dG-N^2 and dA-N^6 at positions similar to that of estrogen 2,3-quinone (*5*). The model estrogen−DNA adducts were found to be mutagenic and miscoding lesions (*16–18*) similar to dG-N^2-2-OHE and dA-N^6-2-OHE (*6, 14*). If our hypothesis is correct, then dG-N^2-E (Figure 1) and dA-N^6-E, lacking a 2-OH moiety present in dG-N^2-2-OHE_2 and dA-N^6-2-OHE_2, respectively, could be formed though sulfonation of C6-OH-estrogens.

Estrogen−DNA adducts prepared by postsynthetic methods (*5, 15*) are a mixture of 6α- and 6β-diastereoisomers at the C6-position. In fact, enzymatic digestion of dG-N^2-6-3MeE-modified oligomer showed that the ratio of 6α- and 6β-isomers of dG-N^2-6-3MeE was 1:5.5 (*17*). A stereoisomeric effect at the C6-position of estrogen−DNA adducts has been proposed using an energy minimization method (*19*). Therefore, it is of interest to know whether the configuration at the C6-position of the steroidal residue may affect the mutagenic properties of estrogen−DNA adducts. We have recently established a phosphoramidite chemical procedure that makes it possible to prepare oligodeoxynucleotides containing either the 6α- or 6β-diastereoisomer of the dG-N^2-6-E_2 adduct (*20*).

Y-family human DNA polymerases (pol), including pol η and pol κ, are associated with translesion synthesis past bulky DNA adducts (*21, 22*). Both enzymes are highly expressed in the human ovary and uterus (*23–27*), which are also major sites of production of steroidal hormones. In the present study, site-specifically modified oligodeoxynucleotides containing a single 6α- or 6β-isomeric dG-N^2-6-E_2 were used as DNA templates for primer extension reactions catalyzed by pol η or a truncated form of human pol κ (pol κΔC). The resulting products, which provide a measure of the polymerase's miscoding properties, were analyzed using an *in vitro* experimental system that quantifies base substitutions and deletions (*28*). Steady-state kinetic studies were also performed to determine the frequency of nucleotide insertion opposite the lesion and chain extension reactions to measure the relative frequency of translesion synthesis past the 6α- or 6β-isomeric dG-N^2-6-E_2 adduct. This is the first demonstration to explore the miscoding properties of the defined 6α- and 6β-isomers of the estrogen−DNA adduct.

MATERIALS AND METHODS

Synthesis of Oligodeoxynucleotides. Unmodified DNA templates (5′CCTTCXCTTCTTTCCTCTCCCTTT and 5′CATGCTGATGAATTCCTTCXCTTCTTTCCTCTCCCTTT, where X is G), primers, and standard markers (Figure 2) were prepared by solid-state synthesis on an automated DNA synthesizer. The 24-mer and 38-mer oligodeoxynucleotides containing a single dG-N^2-6α-E_2 or dG-N^2-6β-E_2 located at the X position were prepared by phosphoramidite chemical synthesis (*20*). The modified and unmodified oligomers were purified using HPLC (*20*). The oligomers were further purified by electrophoresis on a 20% polyacrylamide denaturing gel (PAGE) (35 × 42 × 0.04 cm) (*28, 29*). Oligomers were labeled at the 5′-terminus by treatment with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP (*30*) and subjected to 20% PAGE containing 7 M urea (35 × 42 × 0.04 cm) to confirm homogeneity. The position of the oligomers was established by β-phosphorimager analysis (Molecular Dynamics Inc.). dG-N^2-6α-E_2- and dG-N^2-6β-E_2-modified oligomers used in this study were prepared from the same batch of their phosphoramidites used for synthesis of other oligomers described in our previous paper (*20*). Therefore, the purity and homogeneity of the modified

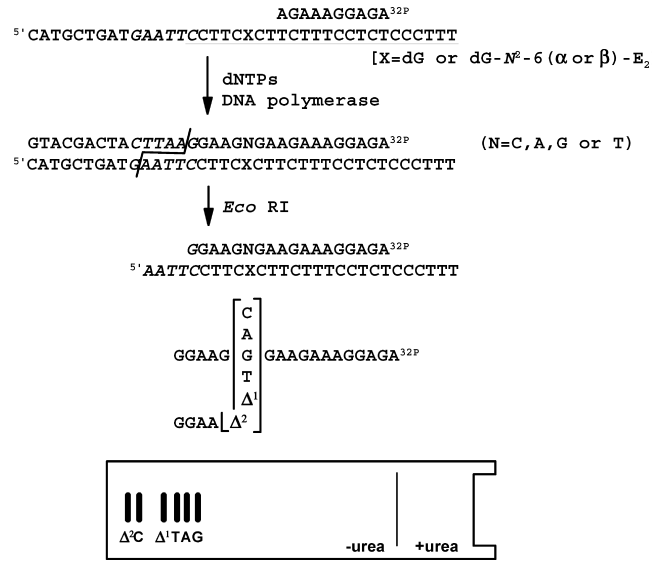

FIGURE 2: Diagram of the method used to determine miscoding specificities. Unmodified or dG-N^2-6(α or β)-E_2-modified 38-mer templates are annealed to a ^{32}P-labeled 10-mer primer. Primer extension reactions catalyzed by pol η or pol κΔC were conducted in the presence of four dNTPs. Fully extended products formed during DNA synthesis are recovered from the polyacrylamide gel (35 × 42 × 0.04 cm), annealed with a complementary 38-mer, cleaved with *Eco*RI, and subjected to a two-phase PAGE (15 × 72 × 0.04 cm), as described in Materials and Methods. To determine miscoding specificity, mobilities of the reaction products are compared with those of 18-mer standards containing dC, dA, dG, or dT opposite the lesion and one- (Δ^1) or two-base (Δ^2) deletions.

oligomers used in this study should be identical to those of other oligomers confirmed by gel electrophoresis and MALDI-TOF mass spectroscopy analyses and by enzymatic digestion analysis (*20*). The absolute configuration of dG-N^2-6α-E$_2$ and dG-N^2-6β-E$_2$ has been described in our previous paper (*20*).

Primer Extension Reactions. pol η and a truncated form of pol κ (pol κΔC) were prepared as described previously (*31, 32*). Although pol κΔC has a lower processivity than the full-length pol κ, the miscoding rate on the undamaged DNA by pol κΔC was similar to that of pol κ (*33*). A 10-mer (5′AGAGGAAAGA) was labeled at the 5′-terminus with T4 polynucleotide kinase and [γ-^{32}P]ATP (*30*). Using modified or unmodified 38-mer oligodeoxynucleotide (200 fmol) primed with a ^{32}P-labeled 10-mer (5′AGAGGAAAGA; 100 fmol), primer extension reactions catalyzed by pol η or pol κΔC (150 ng) were conducted at 25 °C for 30 min in a buffer (10 μL) containing four dNTPs (100 μM each) (Figure 2). The reaction buffer for pol η contains 40 mM Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 10 mM DTT, 250 μg/mL BSA, 60 mM KCl, and 2.5% glycerol. A similar reaction buffer was used for pol κΔC, using 5 mM $MgCl_2$ instead of 1 mM $MgCl_2$. Reactions were stopped with the addition of 5 μL of formamide dye. The samples were subjected to 20% PAGE (35 × 42 × 0.04 cm). The radioactivity of extended products was measured by β-phosphorimager (Molecular Dynamics).

Miscoding Analysis. Using dG-N^2-6α-E$_2$- or dG-N^2-6β-E$_2$-modified or unmodified 38-mer oligodeoxynucleotide primed with a ^{32}P-labeled 10-mer (5′AGAGGAAAGA), primer extension reactions catalyzed by pol η or pol κΔC were conducted at 25 °C for 3.0 or 30 min in a buffer (30 μL) containing four dNTPs (100 μM each) and subjected to 20% PAGE (35 × 42 × 0.04 cm). Extended reaction products (approximately 28−34 bases long) were extracted from the gels. The recovered oligodeoxynucleotides were annealed with an unmodified 38-mer, cleaved with *Eco*RI, and subjected to electrophoresis on two-phase 20% polyacrylamide gels (15 × 72 × 0.04 cm) containing 7 M urea in the upper phase and no urea in the lower phase (two-phase PAGE) (*28*) (Figure 2). To quantify base substitutions and deletions, the mobility of each reaction product was compared with those of 18-mer standards containing dC, dA, dG, or dT opposite the lesion and one- (Δ^1) or two-base (Δ^2) deletions. The detection limit on the gel was 0.03% of the labeled oligomers.

Steady-State Kinetic Studies. Kinetic parameters associated with nucleotide insertion opposite the dG-N^2-6α-E$_2$ or dG-N^2-6β-E$_2$ lesion, and chain extension from the 3′ primer terminus, were determined at 25 °C using varying amounts of single dNTPs (0−500 μM). For insertion kinetics, reaction mixtures containing dNTP (0−500 μM) and either pol η (0.5−5 ng) or pol κΔC (0.5−5 ng) were incubated at 25 °C for 2 min in 10 μL of Tris-HCl buffer (pH 8.0) using a 24-mer template [150 fmol; 5′CCTTCXCTTCTTTCCTCTC-CCTTT, where X is G or dG-N^2-(α or β)-E$_2$] primed with a ^{32}P-labeled 12-mer primer (100 fmol; 5′AGAGGAAA-GAAG). Reaction mixtures containing a template (150 fmol) primed with a ^{32}P-labeled 13-mer primer (100 fmol; 5′AGAG-GAAAGAAGN, where N is C, A, G, or T), with varying amounts of dGTP (0−500 μM), and either pol η (0.5−5 ng) or pol κΔC (0.5−5 ng) were used to measure chain extension. The reaction samples were subjected to 20% denaturing PAGE (35 × 42 × 0.04 cm). The Michaelis constants (K_m) and maximum rates of reaction (V_{max}) were obtained from Hanes−Woolf plots. Frequencies of dNTP insertion (F_{ins}) and chain extension (F_{ext}) were determined relative to the dC·dG base pair according to the equation $F = (V_{max}/K_m)_{wrong\ pair}/(V_{max}/K_m)_{correct\ pair=dC\cdot dG}$ (*34, 35*).

Statistical Analysis. Results are expressed as mean ± standard deviation (SD). Statistical analysis was performed using Student's *t*-test to evaluate the difference. Values of $p \leq 0.05$ were considered statistically significant.

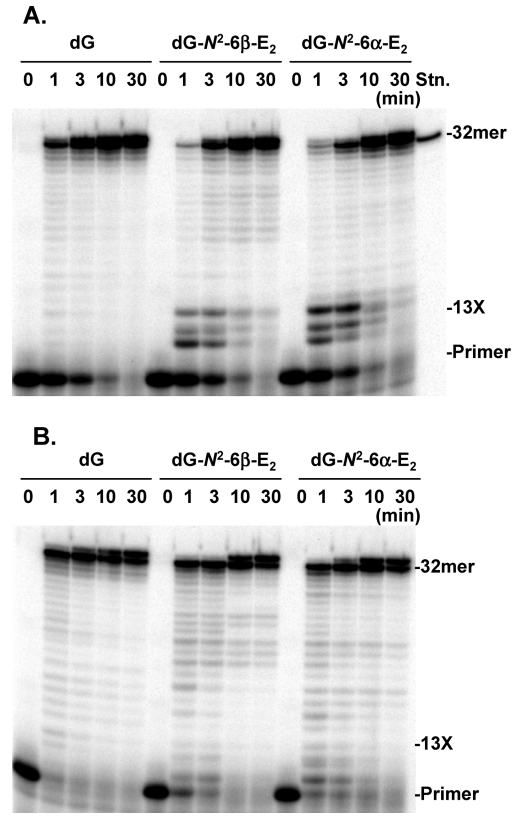

FIGURE 3: Primer extension reactions catalyzed by pol η or pol κΔC on dG-N^2-6(α or β)-E$_2$-modified templates. Using unmodified or dG-N^2-6(α or β)-E$_2$-modified 38-mer templates primed with a ^{32}P-labeled 10-mer (^{32}P-AGAGGAAAGA), primer extension reactions were conducted at 25 °C for 0, 3.0, 10, 15, and 30 min in a buffer containing four dNTPs (100 μM each) and pol η (A) or pol κΔC (B) (50 fmol for the unmodified template; 500 fmol for the modified templates) as described in Materials and Methods. One-third of the reaction mixture was subjected to PAGE (35 × 42 × 0.04 cm). The radioactivity of extended products was measured by β-phosphorimager. 13X shows the location opposite the dG-N^2-6(α or β)-E$_2$ lesion. A 32-mer (5′AGAGGAAAGAAGCGAAGGAAT-TCATCAGCATG) was used as a marker of fully extended product (Stn.).

RESULTS

Translesion Synthesis past 6α- and 6β-Isoforms of dG-N^2-6-E$_2$ DNA Adducts in Reactions Catalyzed by pol η. Primer extension reactions catalyzed by pol η were conducted on unmodified or dG-N^2-6(α or β)-E$_2$-modified 38-mer template in the presence of four dNTPs (Figure 3A). On the templates containing a single unmodified dG, primer extensions catalyzed by this enzyme readily occurred to form the extended products; approximately 30% of the starting primer was fully extended (to 28−34-mers) in 3 min, 58% in 10 min, 77% in 15 min, and 82% in 30 min. With the modified

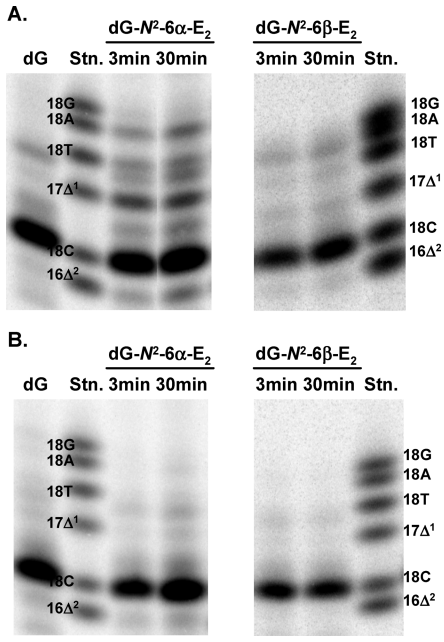

FIGURE 4: Quantitation of miscoding specificities induced by dG-N^2-6(α or β)-E_2 adducts. The fully extended products (>28-mers) generated by primer extension reactions catalyzed for 3.0 or 30 min with pol η (A) or pol $\kappa\Delta$C (B) were recovered from the gel shown in Figure 3. The fully extended products recovered from three independent reaction samples were used for analysis of base substitutions and deletions, as described in the legend of Figure 2.

templates containing a single 6α-isomeric dG-N^2-6-E_2 adduct, primer extensions were retarded one base prior to the adduct, opposite the adduct, and one base past the adduct. In such reactions with pol η, approximately 6.0% of the primer was fully extended in 3 min, 37% in 10 min, 66% in 15 min, and 75% in 30 min. Similar results were obtained using the template containing a 6β-isomeric dG-N^2-6-E_2: 8.0% full extension past the adduct in 3 min, 28% in 10 min, 62% in 15 min, and 75% in 30 min. The rate of forming extended products past both the 6α- and 6β-isomeric adducts was apparently lower than that observed for the unmodified template.

The fully extended products (approximately 28−34-mers) generated from both the unmodified and modified templates were recovered, digested with *Eco*RI, and subjected to two-phase PAGE for quantitative analysis of base substitutions and deletions (Figure 2). A standard mixture of six ^{32}P-labeled oligodeoxynucleotides containing dC, dA, dG, or dT opposite the adduct and containing one- and two-base deletions can be resolved by this method (Figure 4A). Preferential incorporation of dCMP, the correct base, opposite the unmodified dG was observed. Small amounts of unknown products were detected; the migration of these products differed from those of the standard markers. Since the amount of the fully extended products past the dG-N^2-6-E_2 adduct was less than that past the unmodified dG, the fully extended products past each adduct were recovered from several reactions and used for the two-phase PAGE analysis (Figure 4A). The amounts of dNMP incorporated opposite each diastereoisomer were normalized for the amounts of their starting primer. 6α-Isomeric dG-N^2-6-E_2 promoted preferential incorporation of dCMP (42.9%) opposite the adduct (Table 1). Small amounts of dAMP (3.6%), dTMP (3.8%), and dGMP (0.5%) incorporation opposite the adduct were observed, in addition to significant amounts of one-base (9.9%) and two-base (2.9%) deletions. With 6β-isomeric dG-N^2-6-E_2, similar miscoding specificity was observed. The frequency of dCMP incorporation opposite the 6β-isomeric adduct was increased 20% ($p < 0.05$) than that observed with the 6α-isomeric adduct, and the frequencies of dTMP incorporation and one-base deletion were decreased by 37% and 66% ($p < 0.05$), respectively, than that observed with the 6α-isomeric adduct (Table 1).

Miscoding Properties of 6α- and 6β-Isoforms of dG-N^2-6-E_2 Adducts in Reactions Catalyzed by pol $\kappa\Delta$C. Primer extension reactions catalyzed by pol $\kappa\Delta$C readily occurred on the templates containing unmodified dG to form the extended products (Figure 3B); 68% of the starting primer was fully extended past dG in 3 min and 79−81% in 10, 15, and 30 min. Unlike the reactions involving pol η, the primer extensions were not retarded at the 6α- or 6β-isomeric dG-N^2-6-E_2 adduct. The rate of forming the fully extended products past these adducts was slightly lower than that observed with the unmodified template; 45−49% and 58−62% of primers were extended past these adducts in 3 and 10 min, respectively, to form the fully extended products. The rate of extending primers on templates containing 6α-isomeric adduct was similar to that observed for the 6β-isomeric adduct.

The products extended past the unmodified and modified adducts were recovered for miscoding analysis using two-phase PAGE. With unmodified template, the expected incorporation of dCMP opposite dG was observed (Figure 4B). Both 6α- and 6β-isomeric dG-N^2-6-E_2 promoted incorporation of dCMP (59.9% for the 6α-form and 53.7% for the 6β-form, respectively) as a primary product (Table 1).

Kinetic Studies on dG-N^2-6-E_2-Modified DNA Templates. The frequency of dNTP incorporation (F_{ins}) opposite dG-N^2-6-E_2 and the frequency of chain extension (F_{ext}) from dN•dG-N^2-6-E_2 pairs were measured within the linear range of the reaction using the same sequence context that was used for the two-phase PAGE assay (Table 2). With pol η, the F_{ins} value for dCTP (1.50×10^{-2}), the correct base, opposite dG-N^2-6α-E_2 was 5.2 times higher than that of dATP (2.91×10^{-3}) and 9.0 and 26 times higher than those of dGTP and dTTP, respectively. Because the F_{ext} for the dC•dG-N^2-6α-E_2 pair was higher than that for the other pairs, the relative bypass frequency ($F_{ins} \times F_{ext}$) past the dC•dG-N^2-6α-E_2 pair was 2 orders of magnitude higher than that for the other dN•dG-N^2-6α-E_2 pairs. Similar results were observed with the 6β-isomeric adduct. The F_{ins} value for dCTP (3.02×10^{-2}) opposite dG-N^2-6α-E_2 was 17 times higher than that of dATP (1.73×10^{-3}) and 12 and 37 times higher than those of dGTP and dTTP, respectively. The F_{ext} for the dC•dG-N^2-6α-E_2 pair was at least 14 times higher than that for the other pairs. Therefore, the $F_{ins} \times F_{ext}$ past the dC•dG-N^2-6β-E_2 pair also was 2 orders of magnitude higher than that for the other pairs. Since the F_{ins} value for dCTP opposite dG-N^2-6β-E_2 was 2 times higher than that opposite the 6α-isomeric adduct, the $F_{ins} \times F_{ext}$ past the dC•dG-N^2-6β-E_2 was significantly ($p < 0.01$) higher than that past dC•dG-N^2-6α-E_2.

When pol $\kappa\Delta$C was used, the F_{ins} for dCTP opposite dG-N^2-6α-E_2 or dG-N^2-6β-E_2 was 6.6 and 4.6 times lower, respectively, than that for unmodified dG and the F_{ext} for

Table 1: Miscoding Events Generated by 6α- and 6α-Isomeric dG-N^2-6-E$_2$ during DNA Synthesis[a]

DNA pol	C (%)	A (%)	G (%)	T (%)	Δ^1 (%)	Δ^2 (%)
pol η						
dG	62.2 ± 0.2[b]					
dG-N^2-6α-E$_2$	42.9 ± 3.6	3.6 ± 0.4	0.5 ± 0.2	3.8 ± 0.5	9.9 ± 3.0	2.9 ± 0.7
dG-N^2-6β-E$_2$	51.4 ± 3.7*	4.3 ± 0.3	1.4 ± 0.5	2.4 ± 0.4*	3.4 ± 0.6*	2.0 ± 0.3
pol κΔC						
dG	64.4 ± 2.0					
dG-N^2-6α-E$_2$	59.9 ± 3.6					
dG-N^2-6β-E$_2$	53.7 ± 2.0					

[a] The fully extended products (>28-mers) produced in the primer extension reactions catalyzed for 30 min by pol α or pol αΔC were recovered from the gel (Figure 3) and used for analysis of base substitutions and deletions. C, A, G, T, Δ^1, and Δ^2 represent the amount of the fully extended product containing dC, dA, dG, dT, and one-base and two-base deletions opposite the adduct. [b] Data expressed as mean ± SD obtained from three independent experiments. *, $p < 0.05$ versus dG-N^2-6α-E$_2$ (Student's *t*-test).

Table 2: Kinetic Parameters for Nucleotide Insertion and Chain Extension Reactions Catalyzed by DNA pol η and pol κ[a]

Insertion dNTP ↓GAAGAAAGGAGA^{32P} 5'CCTTCXCTTCTTTCCTCTCCCTTT

Extension dGTP ↓NGAAGAAAGGAGA^{32P} 5'CCTTCXCTTCTTTCCTCTCCCTTT

N:X	K_m (μM)	V_{max}(%min^{-1})	F_{ins}	K_m (μM)	V_{max}(%min^{-1})	F_{ext}	F_{ins}xF_{ext}
Pol η							
X = dG							
C:G	0.89± 0.44[b]	31.7 ± 1.7[b]	**1.0**	0.92± 0.25[b]	50.5 ± 5.0[b]	**1.0**	**1.0**
X = dG-N^2-6α-E$_2$							
C:X	19.8 ± 6.9	10.3 ± 2.4	**1.50 ± 0.19 x 10^{-2}**	4.04 ± 0.7	7.00 ± 2.39	**3.09 ± 0.55 x 10^{-2}**	**4.69 ± 1.34 x 10^{-4}**
A:X	31.2 ± 1.5	3.20 ± 0.43	**2.91 ± 0.35 x 10^{-3}**	21.7 ± 5.3	1.99 ± 0.66	**1.65 ± 0.17 x 10^{-3}**	**4.77 ± 0.28 x 10^{-6}**
G:X	31.1 ± 8.6	1.77 ± 0.18	**1.67 ± 0.38 x 10^{-3}**	21.8 ± 10	0.87 ± 0.37	**7.58 ± 1.13 x 10^{-4}**	**1.24 ± 0.18 x 10^{-6}**
T:X	120 ± 16	2.40 ± 0.11	**5.67 ± 0.49 x 10^{-4}**	10.8 ± 4.2	5.07 ± 3.36	**8.83 ± 5.06 x 10^{-3}**	**4.98 ± 2.96 x 10^{-6}**
X = dG-N^2-6β-E$_2$							
C:X	13.6 ± 1.0	14.6 ± 1.0	**3.02 ± 0.22 x 10^{-2}**	6.55 ± 0.7	11.9 ± 0.80	**3.33 ± 0.14 x 10^{-2}**	**1.01 ± 0.03 x 10^{-3}****
A:X	42.3 ± 8.0	2.60 ± 0.40	**1.73 ± 0.07 x 10^{-3}**	17.4 ± 4.2	2.31 ± 0.75	**2.38 ± 0.22 x 10^{-3}**	**4.10 ± 0.33 x 10^{-6}**
G:X	14.6 ± 3.8	1.35 ± 0.81	**2.49 ± 1.25 x 10^{-3}**	32.9 ± 8.4	1.19 ± 0.27	**6.62 ± 0.56 x 10^{-4}**	**1.69 ± 0.98 x 10^{-6}**
T:X	32.2 ± 5.1	0.98 ± 0.66	**8.08 ± 1.43 x 10^{-4}**	12.6 ± 3.1	1.40 ± 0.10	**2.10 ± 0.49 x 10^{-3}**	**1.66 ± 0.83 x 10^{-6}**
Pol κΔC							
X = dG							
C:G	4.00 ± 0.8[b]	22.6 ± 11.9[b]	**1.0**	1.49 ± 1.0[b]	111 ± 47[b]	**1.0**	**1.0**
X = dG-N^2-6α-E$_2$							
C:X	19.7 ± 1.4	16.8 ± 1.7	**1.51 ± 0.08 x 10^{-1}**	3.41 ± 0.5	38.5 ± 10.3	**1.50 ± 0.18 x 10^{-1}**	**2.27 ± 0.26 x 10^{-2}**
A:X	28.4 ± 1.1	0.10 ± 0.05	**6.17 ± 2.97 x 10^{-4}**	36.8 ± 3.1	30.9 ± 9.9	**1.12 ± 0.28 x 10^{-2}**	**7.30 ± 5.08 x 10^{-6}**
G:X	61.6 ± 8.7	0.12 ± 0.06	**3.34 ± 1.10 x 10^{-4}**	26.2 ± 9.8	10.2 ± 3.4	**5.31 ± 0.33 x 10^{-3}**	**1.75 ± 0.49 x 10^{-6}**
T:X	68.3 ± 4.1	0.32 ± 0.13	**8.21 ± 2.75 x 10^{-4}**	107 ± 36	13.8 ± 1.7	**1.84 ± 0.49 x 10^{-3}**	**1.55 ± 0.69 x 10^{-6}**
X = dG-N^2-6β-E$_2$							
C:X	9.98 ± 3.1	12.3 ± 5.9	**2.18 ± 0.88 x 10^{-1}**	3.04 ± 0.6	30.6 ± 3.5	**1.41 ± 0.46 x 10^{-1}**	**3.32 ± 2.41 x 10^{-2}**
A:X	29.1 ± 6.9	0.07 ± 0.02	**4.03 ± 0.40 x 10^{-4}**	30.3 ± 3.1	9.35 ± 2.8	**4.14 ± 1.13 x 10^{-3}**	**1.69 ± 0.58 x 10^{-6}**
G:X	33.2 ± 0.1	0.27 ± 0.04	**1.46 ± 0.21 x 10^{-3}**	30.6 ± 6.9	13.0 ± 0.9	**5.97 ± 1.52 x 10^{-3}**	**8.52 ± 1.15 x 10^{-6}*****
T:X	52.8 ± 4.6	0.49 ± 0.09	**1.65 ± 0.31 x 10^{-3}**	23.2 ± 3.8	33.1 ± 0.7	**1.95 ± 0.31 x 10^{-2}**	**3.25 ± 0.98 x 10^{-5}****

[a] Kinetics of nucleotide insertion and chain extension reactions were determined as described in Materials and Methods. Frequencies of nucleotide insertion (F_{ins}) and chain extension (F_{ext}) were estimated by the equation $F = (V_{max}/K_m)_{wrong\ pair}/(V_{max}/K_m)_{correct\ pair=dC\cdot dG}$. X = dG or dG-N^2-6-E$_2$ lesion. [b] Data expressed as mean ± SD obtained from three independent experiments. **, $p < 0.01$ versus dG-N^2-6α-E$_2$. ***, $p < 0.001$ versus dG-N^2-6α-E$_2$ (Student's *t*-test).

dC•dG-N^2-6α-E$_2$ and dC•dG-N^2-6β-E$_2$ were only 6.7 and 7.1 times lower than that for unmodified dC•dG (Table 2). Therefore, the $F_{ins} \times F_{ext}$ for both the dC•dG-N^2-6α-E$_2$ and dC•dG-N^2-6β-E$_2$ pairs was only 44 and 30 times lower than that of the dC•dG pair. The F_{ins} for dCTP opposite dG-N^2-6α-E$_2$ or dG-N^2-6β-E$_2$ was 2 orders of magnitude higher than that of the other dNTPs. The F_{ext} for dC•dG-N^2-6α-E$_2$ or dC•dG-N^2-6β-E$_2$ was also 1 or 2 orders of magnitude higher than those of the other pairs. Therefore, the $F_{ins} \times F_{ext}$ for the dC•dG-N^2-6α-E$_2$ or dC•dG-N^2-6β-E$_2$ pair was 3−5 orders of magnitude higher than that for the other base pairs, indicating that dCMP is primarily inserted opposite both adducts and extended readily. The only different behavior between 6α- and 6β-isomeric adducts was that the $F_{ins} \times F_{ext}$ past the dG•dG-N^2-6β-E$_2$ and dT•dG-N^2-6β-E$_2$ pairs was 4.9 and 21 times, respectively, higher than that for the corresponding bases paired with 6α-isomeric adduct.

DISCUSSION

Miscoding Properties of dG-N^2-6-E$_2$−DNA Adducts. 6α- and/or 6α-OH-estrogens have been detected in humans (*7, 8*) and produced both in cultured breast cancer cells (*11*) and in reactions catalyzed by several CYP enzymes (*12, 13*) or human liver microsomes (*10*). As demonstrated with 3MeE-6-S (*15*), if the 6-OH-estrogens are sulfonated and reacted with dG or dA residues of cellular DNA, dG-N^2-6-E or dA-N^6-6-E adducts can be produced. Since estrogen−-DNA adducts are a mixture of 6α- and 6β-diastereoisomers (*5, 17*), their miscoding and mutagenic properties may be attributable to both isomeric DNA adducts. Our phosphoramidite chemical procedure (*20*) made it possible to prepare the dG-N^2-6-E$_2$-modified oligomer containing either the 6α- or 6β-isomer. Using the modified oligomers as a DNA template, properties of translesion synthesis past each 6α- and 6β-

isomer of dG-N^2-6-E$_2$ and their miscoding specificity and frequency were investigated. The translesion synthesis catalyzed by pol η was slightly retarded at the dG-N^2-6α-E$_2$ or dG-N^2-6β-E$_2$ adduct site. Both isomeric adducts incorporated dCMP, the correct base, primarily opposite the adduct; small amounts of dAMP and dTMP incorporation occurred, indicating that low levels of G → T transversions and G → A transitions were possibly generated. The miscoding specificities observed with this enzyme are likely to be consistent with the mutagenic events induced by dG-N^2-6(α,β)-2-OHE$_2$ or dG-N^2-6(α,β)-3MeE in a single-strand vector propagated in simian kidney cells (*6, 16*). In contrast, the translesion synthesis catalyzed by pol κ was not blocked at the adduct site. The resulting extended products had primarily dCMP, the correct base, incorporated opposite both the 6α- and 6β-isomeric adducts; therefore, incorporation of wrong bases opposite the adduct was not detected. Pol η is likely to be error-prone for both dG-N^2-6α-E$_2$ and dG-N^2-6β-E$_2$ while pol κ is not. This observation is supported by steady-state kinetic studies. The behavior of the 6α-isomeric adduct with respect to pols η and κ was similar to that for the 6β-isomer.

Configurational Effects. Several DNA adducts including stereoisomers of dG-N^2-benzo[*a*]pyrene diol epoxide (dG-N^2-BPDE) (*36, 37*) have shown stereochemical effect on translesion synthesis and miscoding properties, and their results were connected with the experimental NMR studies (*38*). Conformational searches with energy minimization revealed that the 2-OHE$_1$-N^2-6-dG adduct prefers to reside in the minor groove of the B-DNA duplex; the nonplanar 2-OHE1 moiety may prefer to reside at the DNA helix exterior (*19*). The 6β-isomeric 2-OHE$_1$-N^2-6-dG is more poorly accommodated than the 6α-isomer within the pol α family of polymerases, indicating that the 6β-isomer would be more prone to cause blockage of translesion synthesis (*19*). However, in the present studies, the behavior of translesion synthesis past the 6β-isomeric dG-N^2-6-E$_2$ in reactions catalyzed by pol η or $\kappa\Delta$C was not significantly different from that for the 6α-isomer (Figure 3). The confined active sites of high fidelity polymerases like pol α compared to the more spacious active sites of low fidelity polymerases like pol η and κ may make comparisons of stereoisomer effects between the two enzyme classes problematic (*39*). The miscoding specificity of the 6α-isomer was similar to that of the 6β-isomer although the 6α-isomer showed slightly lower frequency of dCMP incorporation and higher frequency of dTMP incorporation and one-base deletions, compared with the 6β-isomer (Figure 4). This indicated that stereochemistry affects weakly the frequency of miscoding. Therefore, unlike dG-N^2-BPDE adducts, stereochemistry-dependent differences were not strongly observed with dG-N^2-6-E$_2$ adducts. Solution NMR studies with 6α- and 6β-isomeric dG-N^2-6-E$_2$ adducts are required to explore the discrepancy between dG-N^2-6-E$_2$ and dG-N^2-BPDE.

Mechanism of Deletions. Frame-shift deletions were generated by both dG-N^2-6α-E$_2$ and dG-N^2-6β-E$_2$ adducts during DNA synthesis catalyzed by pol η, but not with pol κ (Figure 4). Although the F_{ins} for dGTP (1.67×10^{-3} for dG-N^2-6α-E$_2$ and 2.49×10^{-3} for dG-N^2-6β-E$_2$) was not much different from that for dATP (2.91×10^{-3} for dG-N^2-6α-E$_2$ and 1.73×10^{-3} for dG-N^2-6β-E$_2$), fully extended products containing dAMP opposite the adduct were detected 7.2 times higher for dG-N^2-6α-E$_2$ and 3.1 times higher dG-N^2-6β-E$_2$ than that of the corresponding products containing dGMP opposite the adduct (Table 1). As demonstrated previously for the mechanism of frame-shift deletion (*40*), a fraction of dAMP inserted opposite the adduct could be paired with dT two bases 5′ to the adduct to form a two-base deletion (Δ^2); another fraction of dAMP inserted opposite the adduct could be extended to form the full-length product. On the other hand, since the chain extension (F_{ext}) of dGMP that paired with dG-N^2-6α-E$_2$ or dG-N^2-6β-E$_2$ was blocked 2.2 and 3.6 times more than that of dA·dG-N^2-6α-E$_2$ or dA·dG-N^2-6β-E$_2$, the newly inserted dGMP opposite the adduct could be paired with dC 5′ to the adduct to form a one-base deletion (Δ^1) showing higher amounts than Δ^2.

Table 3: $F_{ins} \times F_{ext}$ past Estrogen−DNA Adducts by pol η or pol $\kappa\Delta$C

		dG-N^2-6α-E$_2$	dG-N^2-6β-E$_2$	dG-N^2-6(α,β)-3MeE[a]
pol η	C·X	4.69×10^{-4}	1.01×10^{-3}	9.50×10^{-4}
	A·X	4.77×10^{-6}	4.10×10^{-6}	3.06×10^{-6}
	G·X	1.24×10^{-6}	1.69×10^{-6}	2.50×10^{-6}
	T·X	4.98×10^{-6}	1.66×10^{-6}	3.99×10^{-6}
pol $\kappa\Delta$C	C·X	2.27×10^{-2}	3.32×10^{-2}	1.33×10^{-1}
	A·X	7.30×10^{-6}	1.69×10^{-6}	1.12×10^{-6}
	G·X	1.75×10^{-6}	8.52×10^{-6}	4.30×10^{-7}
	T·X	1.55×10^{-6}	3.25×10^{-5}	2.48×10^{-5}

[a] Data are taken from ref *18*.

Miscoding Properties of Model Estrogen−DNA Adducts. dG-N^2-6(α,β)-3MeE containing a mixture of 6α- and 6β-diastereoisomers has previously been used as a model estrogen−DNA adduct (*18*). With pol η, dG-N^2-6(α,β)-3MeE promoted incorporation of dCMP as the primary product, accompanied by small amounts of incorporation of dAMP and dTMP as well as deletions. With pol κ, this adduct promoted incorporation of dCMP as the primary product; no significant miscoding was observed at the lesion. The miscoding specificity and frequency generated by dG-N^2-6(α,β)-3MeE was similar to that observed with dG-N^2-6α-E$_2$ or dG-N^2-6β-E$_2$. The relative bypass frequencies ($F_{ins} \times F_{ext}$) past dG-N^2-6(α,β)-3MeE, dG-N^2-6α-E$_2$, and dG-N^2-6β-E$_2$ adducts obtained from the steady-state kinetic studies with pol η or κ are summarized in Table 3. For pol η, the $F_{ins} \times F_{ext}$ past dC·dG-N^2-6(α,β)-3MeE was at least 2 orders of magnitude higher than those of other bases paired with dG-N^2-6(α,β)-3MeE; for pol κ, the $F_{ins} \times F_{ext}$ past dC·dG-N^2-6(α,β)-3MeE was 3−5 orders of magnitude higher than those of other bases paired with dG-N^2-6(α,β)-3MeE. Our results indicate that the miscoding specificity and frequency of the 6α- and 6β-isomeric dG-N^2-E adducts are similar to those of the model dG-N^2-6(α,β)-3MeE adduct.

In conclusion, both 6α- and 6β-isomeric dG-N^2-6-E$_2$ adducts have similar miscoding potentials, indicating that the configurational effect was minimal. Mutagenic events generated by Y-family DNA polymerases may be involved in initiating reproductive cancers.

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