Translesional Synthesis on DNA Templates Containing an Estrogen Quinone-Derived Adduct: N^2-(2-Hydroxyestron-6-yl)-2′-deoxyguanosine and N^6-(2-Hydroxyestron-6-yl)-2′-deoxyadenosine†

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ABSTRACT: Miscoding properties induced by estrogen quinone-derived DNA adducts were analyzed using an in vitro experimental system to quantify base substitutions and deletions. Site-specifically modified oligodeoxynucleotides containing a single N^2-(2-hydroxyestron-6-yl)-2′-deoxyguanosine (2-OHE$_1$-N^2-dG) or N^6-(2-hydroxyestron-6-yl)-2′-deoxyadenosine (2-OHE$_1$-N^6-dA) were prepared postsynthetically and used as templates in primer extension reactions catalyzed by mammalian DNA polymerases (pol) α, β, and δ. The 2-OHE$_1$-N^2-dG adduct blocked primer extension reactions more strongly than 2-OHE$_1$-N^6-dA. Using pol α and δ, 2-OHE$_1$-N^2-dG promoted incorporation of dCMP (6.3 and 3.1%, respectively), the correct base, opposite the lesion: when pol δ was used, misincorporation of dTMP (0.52%) was detected. 2-OHE$_1$-N^6-dA also promoted incorporation of dTMP, the correct base, opposite the lesion, accompanied by misincorporation of dCTP (0.54% for pol α and 3.2% for pol δ) and one-base deletion (0.3−0.5%). Using pol β, no miscoding was detected. The miscoding occurred only when replicative DNA polymerases were used. Kinetic data were consistent with those obtained from the analysis of fully extended products formed by pol α or pol β. These results indicate that endogenous estrogen quinone-derived DNA adducts have miscoding potential: G → A and A → G transitions and deletions are predicted in mammalian cells.

Endogeneous and synthetic estrogens have been suspected to be involved in the development of breast and endometrial cancers (*1*, *2*). Exposure to estrogens induces several different tumors including mammary and uterine tumors in laboratory animals (*3*−*5*). Estrogens act as stimulatory hormones, increasing the frequency of mitotic activity in target organs. As a rare consequence of this estrogen-induced proliferation, malignant phenotypes may occur due to errors during cell division such as DNA replication errors and chromosomal translocations errors (*1*, *6*) and may develop cancers. However, in some cases, the hormonal potencies did not correlate with the tumor incidence (*3*, *7*−*9*). Mechanisms other than hormonal activity may be involved in estrogen-induced cancer. DNA damage is an initiating event in human cancer and may lead to mutation (*10*). Treatment with estrogens has been known to form DNA adducts in tissues of animals (*5*, *11*). Thus, estrogen metabolites have been considered to have some role in hormonal carcinogenesis (*12*, *13*).

Estrogens are metabolized by cytochrome P450 enzymes to form 2- or 4-hydroxylestrogen (*14*, *15*). Exposure of 2-hydroxyestradiol or 4-hydroxyestradiol induced DNA adducts in cultured mammalian cells (*16*). These catecholestrogens are further oxidized to form corresponding quinones by P450, peroxidases, or autoxidization (*15*, *17*, *18*). The 2,3- and 3,4-quinones of estrogens react with DNA to form DNA adducts (*18*). Recently, Stack et al. (*19*) found that 2-hydroxyestrone quinone (2-OHE$_1$ quinone)[1] directly reacts with 2′-deoxyguanosine (dG) and 2′-deoxyadenosine (dA) to form N^2-(2-hydroxyestron-6-yl)-2′-deoxyguanosine (2-OHE$_1$-N^2-dG) and N^6-(2-hydroxyestron-6-yl)-2′-deoxyadenosine (2-OHE$_1$-N^6-dA) (the structures in Figure 1), respectively. Reaction of 4-hydroxyestrone quinone with dG produced N^7-(4-hydroxyestron-1-yl)guanine, with loss of deoxyribose (*19*).

To explore the miscoding properties of estrogen quinone-derived DNA adducts, site-specifically modified oligonucleotides containing a single 2-OHE$_1$-N^2-dG or 2-OHE$_1$-N^6-dA were prepared postsynthetically and used as DNA templates in primer extension reactions catalyzed by mammalian replicative DNA polymerases. Miscoding specificities of 2-OHE$_1$-N^2-dG and 2-OHE$_1$-N^6-dA adducts were investi-

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[1] Abbreviations: E$_1$, estrone; 2-OHE$_1$, 2-hydroxyestrone; E$_1$-2,3-Q, estrone 2,3-quinone; E$_1$-3,4-Q, estrone 3,4-quinone; 2-OHE$_1$-N^2-dG, N^2-(2-hydroxyestron-6-yl)-2′-deoxyguanosine; 2-OHE$_1$-N^6-dA, N^6-(2-hydroxyestron-6-yl)-2′-deoxyadenosine; dG, 2′-deoxyguanosine; dA, 2′-deoxyadenosine; dNTP, 2′-deoxynucleoside triphosphate; K_m, Michaelis constant; V_{max}, maximum rate of reaction; F_{ins}, frequency of insertion; F_{ext}, frequency of extension; pol α, DNA polymerase α; pol β, DNA polymerase β; pol δ, DNA polymerase δ; PCNA, proliferating cell nuclear antigen; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; t_R, retention time; Δ, deletion.

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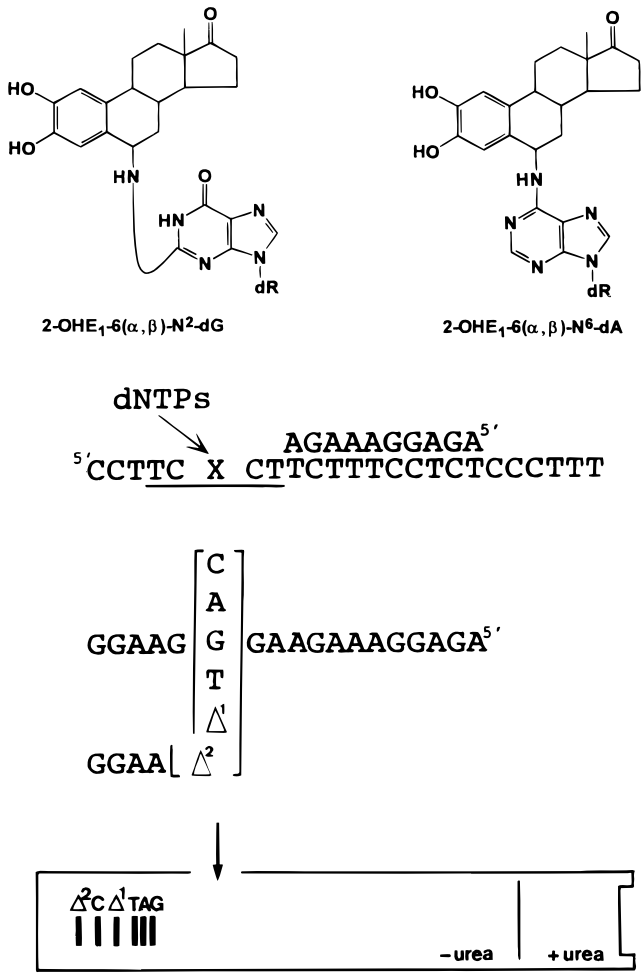

FIGURE 1: Structures of estrogen quinone-derived DNA adducts and diagram of the primer extension methods and analysis of reaction products.

gated, using an in vitro experimental system that can quantify base substitutions and deletions (*20*) and steady-state kinetic analysis. Both estrogen−DNA adducts were miscoding lesions; G → A and A → G transitions and deletions are predicted in mammalian cells.

EXPERIMENTAL PROCEDURES

Materials and Methods. [γ-^{32}P]ATP (specific activity, >6000 Ci/mmol) was obtained from Amersham Corp. Calf thymus DNA pol α (30 000 units/mg) and human pol β (100 000 units/mg) were from Molecular Biology Resources, Inc. Proliferating cell nuclear antigen (PCNA) was provided by Dr. P. A. Fisher (*21*). 2-Hydroxyestrone (2-OHE$_1$) was purchased from Sigma Chemical Co. A Waters 990 HPLC instrument, equipped with a photodiode array detector, was used for the separation and purification of the oligodeoxynucleotides.

Synthesis of Oligodeoxynucleotides. DNA template, primer, and standard markers, listed in Table 1, were prepared by solid-state synthesis on an automated DNA synthesizer (*22*). A solution containing 2-OHE$_1$ quinone was prepared by reacting 0.5 mg of 2-OHE$_1$ with 1 mg of MnO_2 in 0.5 mL of acetonitrile at −40 °C for 10 min. The solution of 2-OHE$_1$ quinone was filtered to remove MnO_2. 2-OHE$_1$-N^2-dG and 2-OHE$_1$-N^6-dA were synthesized by reacting dG or dA with 2-OHE$_1$ quinone as reported previously (*19*). DNA template

Table 1: Sequence of Oligodeoxynucleotides[a]

Number	Sequence
	5′ 3′
1	CCTTCGCTTCTTTCCTCTCCCTTT
2	CCTTCACTTCTTTCCTCTCCCTTT
3	CCTTCXCTTCTTTCCTCTCCCTTT
4	CCTTCCTTCTTTCCTCTCCCTTT
5	AGAGGAAAGA
6	AGAGGAAAGAAG
7	AGAGGAAAGAAGN
8	AGAGGAAAGAAGNGAAGG
9	AGAGGAAAGAAGGAAGG
10	AGAGGAAAGAAGAAGG

[a] Sequence of templates, primers, and standard markers. X = 2-OHE$_1$-N^2-dG or 2-OHE$_1$-N^6-dA; N = dC, dA, dG, or dT.

containing a single 2-OHE$_1$-N^2-dG or 2-OHE$_1$-N^6-dA was also prepared by reacting unmodified 24-mer (150 μg, 5′CCTTCGCTTCTTTCCTCTCCCTTT or 5′CCTTCACTTCTTTCCTCTCCCTTT) in 1 mL of CH_3COOH/H_2O (1:1) solution with 2-OHE$_1$ quinone for 2.5 h at room temperature. Similarly, 5′TCGCT and 5′TCACT, underlined in the 24-mer sequence, were reacted with 2-OHE$_1$ quinone. The reaction mixture was evaporated to dryness, dissolved in 100 μL of distilled water, and subjected to HPLC. The 2-OHE$_1$-N^2-dG- or 2-OHE$_1$-N^6-dA-modified oligodeoxynucleotides were isolated on a Waters reversed-phase μBondapak C_{18} (0.39 × 30 cm), using a linear gradient of 0.05 M triethylammonium acetate, pH 7.0, containing 10 → 20% acetonitrile with an elution time of 60 min and a flow rate of 1.0 mL/min as described elsewhere (*23*). These modified oligodeoxynucleotides were further purified by HPLC twice and by a 20% polyacrylamide denaturing gel electrophoresis. The molecular weight of the modified oligodeoxynucleotides was measured using a negative ion electrospray mass spectrometry (TRIO-2000, Micromass).

Primer Extension Reactions. Primer (5′AGAGGAAAGA) was labeled at the 5′-terminus by treating with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP (*24*). Using the ^{32}P-labeled 10-mer (0.5 pmol) primed with a 24-mer template (0.75 pmol, 5′CCTTCXCTTCTTTCCTCTCCCTTT, X = dG, dA, 2-OHE$_1$-dG, or 2-OHE$_1$-dA), primer extension reactions catalyzed by pol α, pol β, or pol δ were carried out at 25 °C in 10 μL of a buffer containing all four dNTPs (100 μM each) (*25*). The reaction buffer for pol α or pol β consisted of 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), and BSA (0.5 μg/μL). Calf thymus DNA pol δ was prepared as described previously (*26*). The buffer for pol δ consisted of 50 mM Tris-HCl, pH 6.5, 10 mM KCl, 6 mM $MgCl_2$, 2 mM DTT, BSA (0.04 μg/μL), and PCNA (6 ng/μL). Reactions were stopped by adding

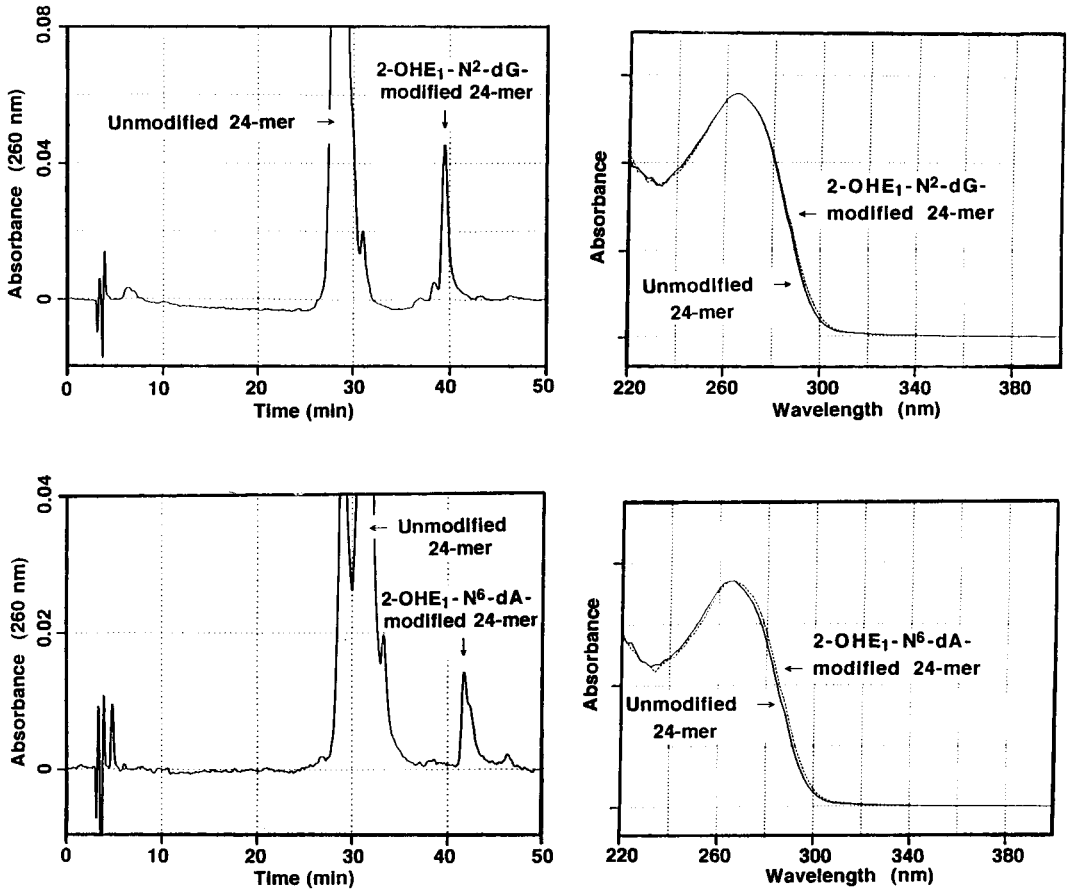

FIGURE 2: HPLC separation of 24-mer oligodeoxynucleotide containing a single modification of 2-OHE_1-N^2-dG or 2-OHE_1-N^6-dA. A 24-mer oligodeoxynucleotide containing a single dG or dA (150 μg) was reacted for 2.5 h at room temperature with a solution containing 2-OHE_1 quinone. The reaction mixture was evaporated to dryness, dissolved with distilled water, and subjected to HPLC as described in Experimental Procedures. UV spectra of 2-OHE_1-N^2-dG- and 2-OHE_1-N^6-dA-modified 24-mers.

formamide dye and heating to 95 °C for 3 min. Samples were subjected to electrophoresis on a 20% polyacrylamide denaturing gel (35 × 42 × 0.04 cm). Bands were identified by autoradiography, using Kodak Xomat XAR film, and the radioactivities were measured by a β-phosphorimager (Molecular Dynamics).

Quantitation of Miscoding Specificity. Using a 24-mer template (0.75 pmol) primed with a ^{32}P-labeled 10-mer (0.5 pmol), primer extension reactions catalyzed by DNA polymerases were conducted at 25 °C in the presence of four dNTPs as described above. As shown in Figure 1, the reaction samples were 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 (*20*). Standards representing products containing dC, dA, dG, or dT opposite the lesion or one- and two-base deletions were completely resolved on the gel, based on their different migration. The detection limit was 0.03% of the starting primer (*20*).

Kinetic Studies of Nucleotide Insertion and Chain Extension. Kinetic parameters associated with nucleotide insertion opposite the lesion and chain extension from the 3′ primer terminus were determined at 25 °C, using the reaction condition containing a single dNTP (*27*, *28*). Reaction mixtures containing 0.1−0.5 units of pol α or 0.005−1.0 units of pol β, 10 μL of Tris-HCl (pH 8.0), and 1.0 pmol of 24-mer template (sequences 1−3), primed with 0.5 pmol of ^{32}P-labeled 12-mer ($^{5'}$AGAGGAAAGAAG) were used to measure nucleotide insertion or, primed with 0.5 pmol of ^{32}P-labeled 13-mer ($^{5'}$AGAGGAAAGAAGN), to study chain extension (*25*, *29*).

Reaction mixtures were subjected to electrophoresis on 20% polyacrylamide gels (35 × 42 × 0.04 cm) in the presence of 7 M urea. The Michaelis constant (K_m) and maximum rate of reaction (V_{max}) were obtained from Hanes−Woolf plots of the kinetic data. Insertion (F_{ins}) and extension (F_{ext}) frequencies were determined relative to the dC:dG or dT:dA base pair according to the equation developed by Mendelman et al. (*27*, *28*), where $F = (V_{max}/K_m)_{(wrong\ pair)}/(V_{max}/K_m)_{(correct\ pair)}$ is defined for any base pair containing a single modified nucleoside. All reactions were linear over the course of the experiment. Data reported represent the average of two to four separate experiments in which less than 20% of the primer was extended (*27*).

RESULTS

Preparation of Oligodeoxynucleotides Containing an Estrogen Quinone-Derived Adduct. We confirmed that 2-OHE_1-derived quinone reacts only with purine bases, forming 2-OHE_1-N^2-dG and 2-OHE_1-N^6-dA (*19*). Therefore, only a single dG (sequence 1 in Table 1) or dA (sequence 2) embedded in a 24-mer oligodeoxynucleotide was reacted with 2-OHE_1-derived quinone. 2-OHE_1-N^2-dG- (t_R = 39.2 min) or 2-OHE_1-N^6-dA- (t_R = 41.5) modified 24-mer was isolated from the corresponding oligomers by HPLC (Figure 2). Since the fraction of 2-OHE_1-N^6-dA-modified 24-mer

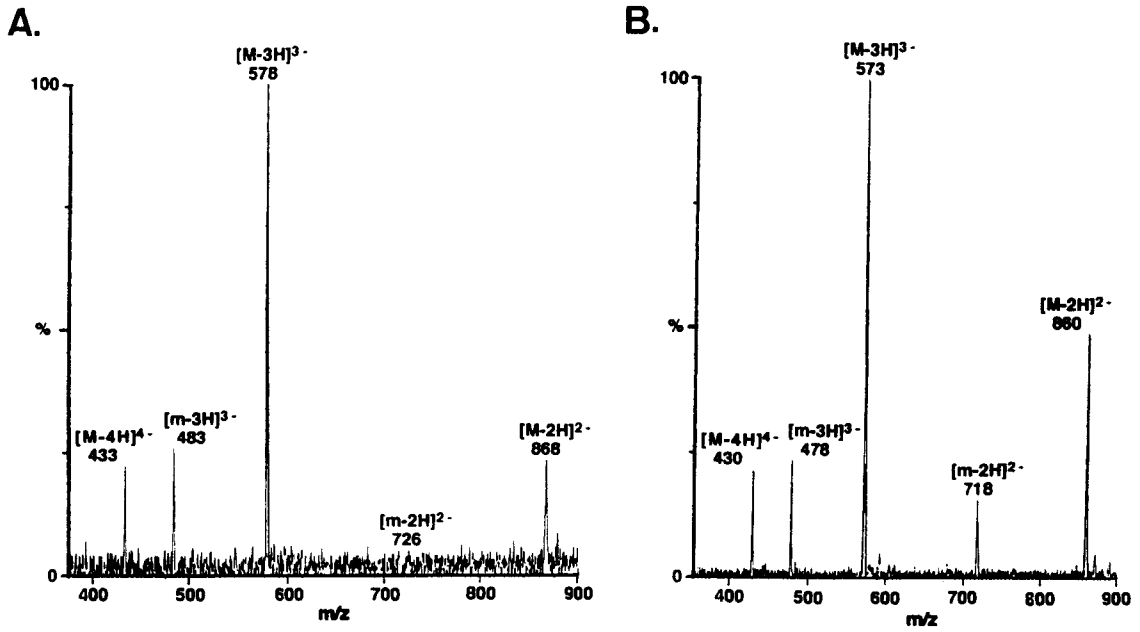

FIGURE 3: Negative ion electrospray ionization mass spectra of 2-OHE$_1$ quinone-modified oligodeoxynucleotides. (A) M: 2-OHE$_1$-N^2-dG-modified d(TpCpG*pCpT) (1738 Da). m: d(TpCpG*pCpT) minus 2-OHE$_1$ (1454 Da). (B) M: 2-OHE$_1$-N^6-dA-modified d(TpCpA*pCpT) (1722 Da). m: d(TpCpA*pCpT) minus 2-OHE$_1$ (1438 Da).

contains two diastereoisomers, 2-OHE$_1$-6α-N^2-dA and 2-OHE$_1$-6β-N^2-dA, the peak may be broad. When a 23-mer without a purine base (sequence 4) was reacted with 2-OHE$_1$-derived quinone, no modified oligomers were detected (data not shown). The yields of 2-OHE$_1$-N^2-dG- and 2-OHE$_1$-N^6-dA-modified 24-mers were 2.8 and 2.3%, respectively. UV spectra of both modified 24-mers were shifted slightly to the long wavelength field as compared with that of the unmodified oligomer (Figure 2). Similarly, ^{5}′TCXCT (X = 2-OHE$_1$-N^2-dG or 2-OHE$_1$-N^6-dA), a part of the modified 24-mer sequence underlined in Figure 1, was prepared and the molecular mass was measured using a ESI mass spectrometry. As shown in Figure 3A, the parent ions of 2-OHE$_1$-N^2-dG-modified 5-mer exhibited at *m*/*z* 868 $[M - 2H]^{2-}$ and *m*/*z* 578 $[M - 3H]^{3-}$, identifying the molecular mass as 1738 Da. A second component is also observed at *m*/*z* 726 $[m - 2H]^{2-}$ and *m*/*z* 483 $[m - 3H]^{3-}$ (1454 Da) representing the 1738 Da minus 2-OHE$_1$ moiety. The parent ions of 2-OHE$_1$-N^6-dA-modified 5-mer exhibited at *m*/*z* 860 $[M - 2H]^{2-}$ and *m*/*z* 573 $[M - 3H]^{3-}$ (Figure 3B), identifying the molecular mass as 1722 Da. A second component is also detected at *m*/*z* 718 $[m - 2H]^{2-}$ and at *m*/*z* 478 $[m - 3H]^{3-}$ (1438 Da) representing the 1722 Da minus 2-OHE$_1$ moiety. Thus, the existence of 2-OHE$_1$-N^2-dG or 2-OHE$_1$-N^6-dA in the modified oligomers was confirmed. The 24-mer modified oligomers were further purified by HPLC twice and by a gel electrophoresis. The migration of ^{32}P-labeled modified 24-mers was slower than that of the unmodified 24-mer on the 20% polyacrylamide denaturing gel (data not shown). When these modified 24-mers were incubated at 25 °C for 1 h in the same buffer used for the primer extension reaction, no degradation was observed (data not shown). These modified oligomers were used for in vitro mutagenesis studies.

Miscoding Properties of Estrogen Quinone-Derived DNA Adducts. Primer extension reactions catalyzed by mammalian DNA polymerases were carried out in the presence of four dNTPs. As shown in Figure 4A, primer extension reaction catalyzed by pol α, a replicative DNA polymerase, occurred rapidly on unmodified template containing dG or dA to form the fully extended products. However, using 2-OHE$_1$-N^2-dG- or 2-OHE$_1$-N^6-dA-modified 24-mer template, primer extension reactions were retarded one-base before the lesion and opposite the lesion (Figure 4). Most of the extended primers blocked at one base before the 2-OHE$_1$-N^2-dG (12-mers) were not extended even after 3 h. In contrast, the 12-mers blocked at one base before the 2-OHE$_1$-N^6-dA could be extended past the lesion to form the fully extended products when longer incubation times or more enzyme was used. Some amounts of 14-mer were observed when the 2-OHE$_1$-N^6-dA-modified template was used. The bulky 2-OHE$_1$-N^6-dA adduct may influence the chain extension reaction beyond the site of the lesion. With the longer incubation, pol α performed nucleotide addition at the blunt end of duplex DNA (*29*, *30*). The formation of the fully extended products past 2-OHE$_1$-N^6-dA (44% of the starting primers) at 3 h was 2.8 times higher than that of 2-OHE$_1$-N^2-dG (16%). 2-OHE$_1$-N^2-dG appears to be a stronger blocking lesion than 2-OHE$_1$-N^6-dA.

To determine what nucleotides are inserted opposite the lesions, the extended products blocked opposite the 2-OHE$_1$-N^2-dG or 2-OHE$_1$-N^6-dA were analyzed by a 20% denaturing gel electrophoresis (15 × 72 × 0.04 cm). The migration of the 13-mer products was compared with standard 13-mer containing dC, dA, dG, or dT positioned at the 3′-terminus of the primer (Figure 5A). The 13-mer standard containing dC (13mer-dC), dT (13-mer-dT), or dG (13-mer-dG) can be resolved on the gel. Since the 13-mer containing dA (13-mer-dA) migrates between 13-mer-dC and 13-mer-dT, 13-mer-dA cannot be separated from 13-mer-dC and 13-mer-dT. Although the migration of 13-mer products blocked opposite the 2-OHE$_1$-N^2-dG or 2-OHE$_1$-N^6-dA was consistent with that of 13-mer-dA, this product may contain some 13-mer-dT and/or 13-mer-dC. Small amounts of 13-mer-dG were also detected opposite the lesions. Thus, the inserted dA and dG opposite the lesions cannot be extended easily.

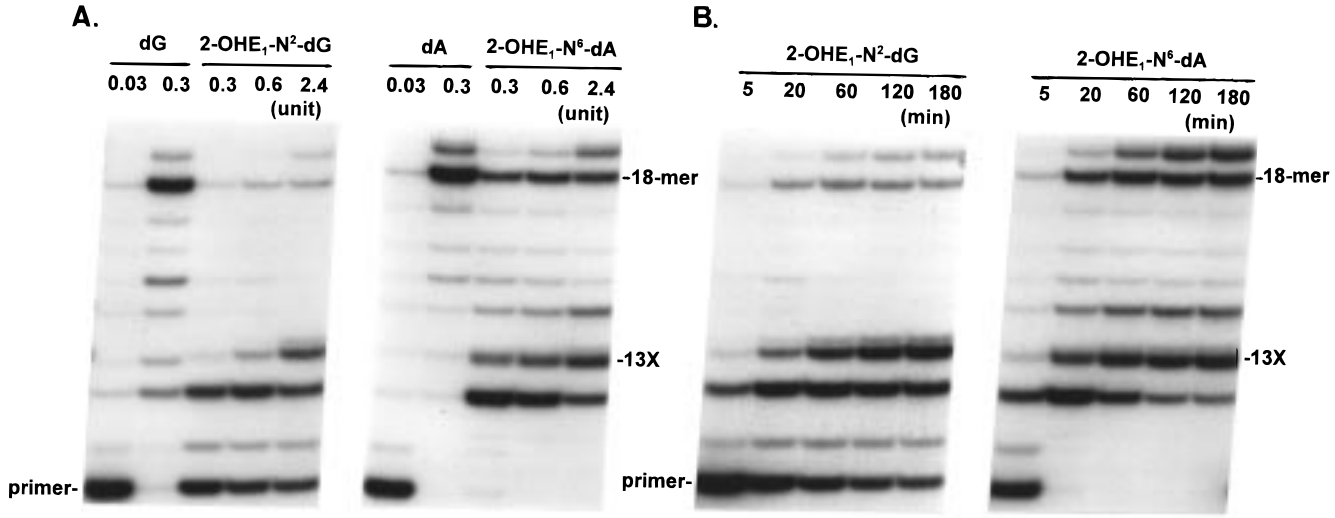

FIGURE 4: Primer extension reactions catalyzed by pol α. Using unmodified, 2-OHE$_1$-N^2-dG- or 2-OHE$_1$-N^6-dA-modified 24-mer templates (sequences 1−3 in Table 1) primed with a ^{32}P-labeled 10-mer (sequence 5), primer extension reactions were conducted at 25 °C, using varying amounts of pol α and 1 h incubation time (A), and using 0.3 units of pol α for unmodified templates and 2.4 units for 2-OHE$_1$-N^2-dG- and 2-OHE$_1$-N^6-dA-modified templates (B) as described in the Experimental Procedures. One-third of the reaction mixture was subjected to the denaturing 20% polyacrylamide gel electrophoresis (35 × 42 × 0.04 cm). 13X shows the location of opposite 2-OHE$_1$-N^2-dG or 2-OHE$_1$-N^6-dA lesion.

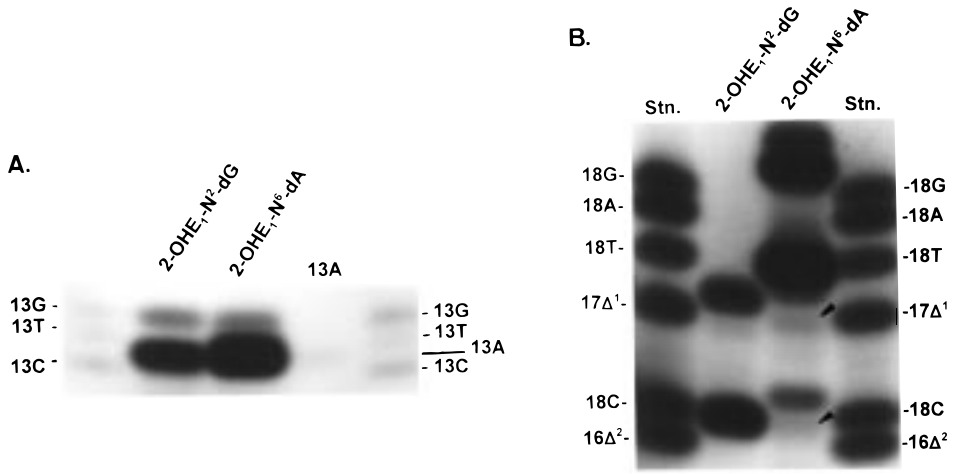

FIGURE 5: Analysis of nucleotide inserted opposite 2-OHE$_1$-N^2-dG or 2-OHE$_1$-N^6-dA lesion and quantitation of miscoding specificities induced by 2-OHE$_1$-N^2-dG or 2-OHE$_1$-N^6-dA. (A) Primer extension samples at 60 min shown in Figure 4B were recovered, and then subjected to a 20% denaturing gel electrophoresis (15 × 72 × 0.04 cm). Mobilities of 13-mer products were compared with those of 13-mer standards (sequence 7) containing dC, dA,dG, or dT at the 3′ terminus. (B) Using a same experimental condition as shown in Figure 4B, primer extension reactions were conducted for 1 h at 25 °C, using 2.4 units of pol α for 2-OHE$_1$-N^2-dG- and 2-OHE$_1$-N^6-dA-modified templates. The reaction samples were subjected to a two-phase 20% polyacrylamide gel electrophoresis (15 × 72 × 0.04 cm) as described in the Experimental Procedures. Mobilities of reaction products were compared with those of 18-mer standards (sequences 8−10) containing dC, dA,dG, or dT opposite the lesion and one-base (Δ^1) or two-base (Δ^2) deletions.

Miscoding specificities were determined quantitatively by using a two-phase PAGE system. When an unmodified 24-mer template was used, the correct bases, dCMP and dTMP, were incorporated opposite dG and dA, respectively (Table 2). 2-OHE$_1$-N^2-dG directed the incorporation of dCMP (6.3%), the correct base, opposite the lesion (Figure 5B). 2-OHE$_1$-N^6-dA generated preferential incorporation of dTMP (22.7%) opposite the lesion, along with small amounts of incorporation of dCMP (0.54%, see arrow) and one-base deletion (0.56%) (Figure 5B).

Primer Extension Reactions Catalyzed by pol β. Using pol β, most of primer extension was blocked at the 2-OHE$_1$-N^2-dG and 2-OHE$_1$-N^6-dA lesions: small amounts of primer extended past the lesions, forming the fully extended products (Figure 6A). When miscoding specificities were analyzed by a two-phase gel electrophoresis, only the correct bases, dCMP (7.0%) and dTMP (23%), were incorporated opposite 2-OHE$_1$-N^2-dG and 2-OHE$_1$-N^6-dA, respectively (Figure 6B and Table 2).

Kinetic Studies of Nucleotide Insertion and Extension. Steady-state kinetic parameters were established for nucleotide insertion opposite 2-OHE$_1$-N^2-dG or 2-OHE$_1$-N^6-dA and for chain extension from 3′-termini containing this lesion. When pol α was used (Table 3), the frequency of dAMP insertion (F_{ins}) opposite 2-OHE$_1$-N^2-dG was 25 times less than that of dCMP, the correct base. Insertion of dGMP or dTMP was not detected. The frequency of chain extension (F_{ext}) from 3′-termini containing dC·2-OHE$_1$-N^2-dG or dT·2-OHE$_1$-N^2-dG pair was only detected. Thus, the relative frequency of translesional synthesis ($F_{ins}F_{ext}$) past dC·2-

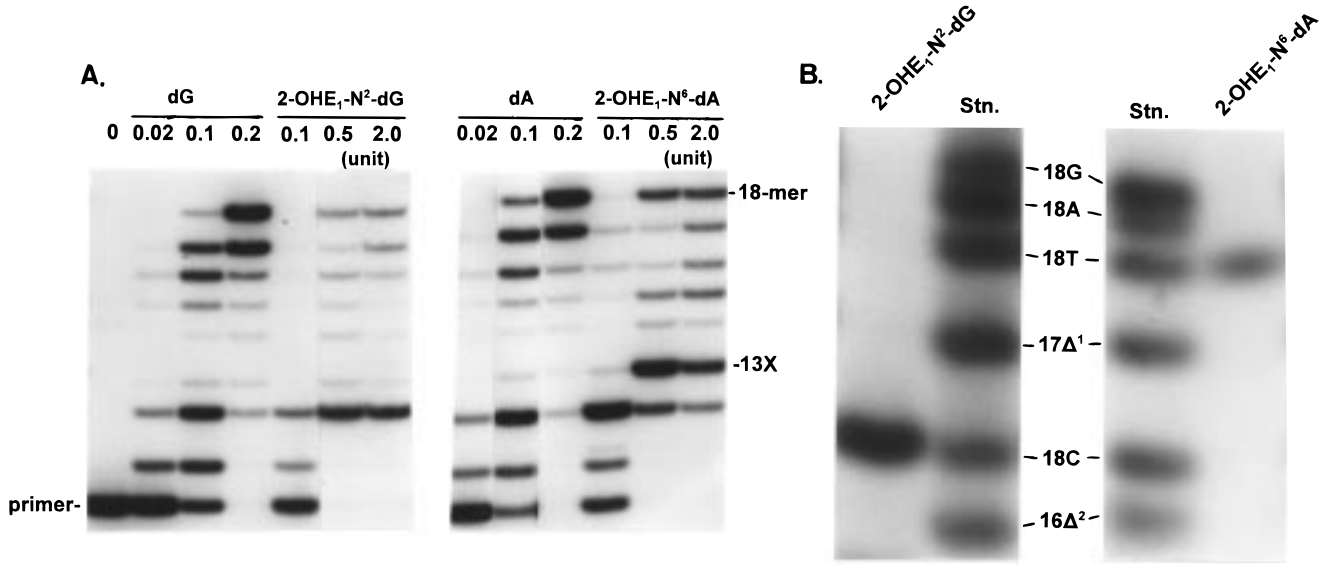

FIGURE 6: Primer extension reactions catalyzed by pol β. (A) Primer extension reactions catalyzed by varying amounts of pol β were conducted for 1 h at 25 °C in the reaction mixture containing four dNTPs as described in the legend of Figure 4. (B) The fully extended products formed by using 2.0 unit of pol β (panel A) were recovered from the gel. A half and one-fourth of fully extended products obtained from 2-OHE_1-N^2-dG- and 2-OHE_1-N^6-dA- modified template, respectively, were subjected to a two-phase 20% polyacrylamide gel electrophoresis (15 × 72 × 0.04 cm) as described in the legend of Figure 5B.

Table 2: Miscoding Specificities of 2-OHE_1 Quinone-Derived Adducts[a]

	enzyme (unit)	C (%)	A (%)	G (%)	T (%)	Δ^1 (%)
pol α						
dG	0.3	57.3				
2-OHE_1-N^2-dG	2.4	6.3				
dA	0.3				79.4	
2-OHE_1-N^6-dA	2.4	0.54			22.7	0.56
pol β						
dG	0.2	83.7				
2-OHE_1-N^2-dG	2.0	7.0				
dA	0.2				86.5	
2-OHE_1-N^6-dA	2.0				23.0	
pol δ						
dG	0.04	63.6				
2-OHE_1-N^2-dG	0.04	3.1			0.52	
dA	0.04				76.0	
2-OHE_1-N^6-dA	0.04	3.2			62.0	0.34

[a] A part of data of pol α, β, and δ was obtained from Figures 5B, 6B, and 8. C, A, G, T, and Δ^1 represent the amount of the fully extended product containing dC, dA, dG, dT, and one-base deletions opposite the lesion.

OHE_1-N^2-dG was only determined. On the other hand, the frequency of dTMP, the correct base, opposite the 2-OHE_1-N^6-dA was 4.5 times higher than that for dGMP and 16 times higher than that for dAMP and dCMP. The chain extension from dT·2-OHE_1-N^6-dA or dC·2-OHE_1-N^6-dA pair only were detected. Although the $F_{ins}F_{ext}$ for dC·2-OHE_1-N^6-dA was 2300 times less than that of dT·2-OHE_1-N^6-dA, a small amount of misincorporation of dCMP was detected during DNA synthesis (Figure 5B). The $F_{ins}F_{ext}$ for dC·2-OHE_1-N^2-dG was 8.1 times lower than that of dT·2-OHE_1-N^6-dA.

Using pol β (Table 4), F_{ins} for dCMP opposite 2-OHE_1-N^2-dG and F_{ins} for dTMP opposite 2-OHE_1-N^6-dA was only determined. Thus, the correct bases inserted only were extended past the lesions, as similarly observed in reactions condition containing four dNTPs (Figure 6B and Table 2). The $F_{ins}F_{ext}$ for dC·2-OHE_1-N^2-dG was 17 times lower than that of dT·2-OHE_1-N^6-dA. This also indicates that 2-OHE_1-N^2-dG tends to block the primer extension reaction, as compared with 2-OHE_1-N^6-dA.

Primer Extension Reactions Catalyzed by pol δ. When pol δ with PCNA, another mammalian replicative enzyme, was used, primer extension reactions were blocked one base before 2-OHE_1-N^2-dG lesion: 3.6% of primers only passed the lesion to form the fully extended products (Figure 7). In contrast, primer extension occurred rapidly at 2-OHE_1-N^6-dA lesion to form 66% of fully extended products. Large amounts (0.3−2.4 unit) of pol α were required to extend the primer past the 2-OHE_1-N^2-dG or 2-OHE_1-N^6-dA lesion (Figure 4A) while only one-tenth the amount (0.04 unit) of pol δ was required. The bypass efficiency may vary depending on the nature of DNA polymerase used.

Fully extended products formed by pol δ were analyzed to determine the miscoding specificities (Figure 8 and Table 2). 2-OHE_1-N^2-dG promoted incorporation of dCMP (3.1%), the correct base, opposite the lesion, accompanied by small amounts of misincorporation of dTMP (0.52%, see arrow). 2-OHE_1-N^6-dA promoted preferential incorporation of dTMP (62%) opposite the lesion, along with small amounts of misincorporation of dCMP (3.2%) and one-base deletions (0.34%).

DISCUSSION

In human breast cancer, G → A (*31*, *32*) and A → G transitions (*33*, *34*) were detected mainly, along with some G → T transversions (*30*, *35*, *36*) and deletions (*37*, *38*). When estrogen quinones are reduced to catecholestrogens during redox cycling, reactive oxygen species are produced in cells and form oxidative damage including 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in DNA (*39*). 8-OxodG was detected in mammary DNA obtained from breast cancer patients (*40*). This lesion is mutagenic, primarily generating G → T transversions (*25*, *41*, *42*). However, G → T transversion is a minor mutation in breast cancer.

DNA pol α and δ are thought to be responsible for chromosomal replication, and pol β is associated with repair

Table 3: Kinetic Parameters for Nucleotide Insertion and Chain Extension Reactions Catalyzed by pol α[a]

	insertion dNTP ↓GAAGAAAGGAGA^{32}P $^{5'}$CCTTCXCTTC TTT CCTCTCCCTTT			extension dGTP ↓NGAAGAAAGGAGA^{32}P $^{5'}$CCTTCXCTTC TTT CCTCTCCCTTT			
N:X	K_m (μM)	V_{max} (% min^{-1})	F_{ins}	K_m (μM)	V_{max} (% min^{-1})	F_{ext}	$F_{ins}F_{ext}$
C:G	4.4 ± 2.3[b]	55 ± 15	1.0	4.8 ± 1.1	96 ± 2.5	1.0	1.0
C:G^{2OHE1}	8.5 ± 0.8	0.87 ± 0.01	8.08×10^{-3}	5.5 ± 0.1	0.57 ± 0.03	5.28×10^{-3}	4.27×10^{-5}
A:G^{2OHE1}	69 ± 3.0	0.28 ± 0.01	3.29×10^{-4}	ND	ND	ND	ND
G:G^{2OHE1}	ND	ND	ND	ND	ND	ND	ND
T:G^{2OHE1}	ND	ND	ND	30 ± 2.9	0.19 ± 0.01	3.24×10^{-4}	ND
T:A	11 ± 1.0[b]	56 ± 0.5	1.0	10 ± 3.4	68 ± 21	1.0	1.0
C:A^{2OHE1}	53 ± 6.0	0.23 ± 0.05	8.62×10^{-4}	41 ± 1.6	0.047 ± 0.01	1.71×10^{-4}	1.47×10^{-7}
A:A^{2OHE1}	360 ± 110	1.6 ± 0.42	8.78×10^{-4}	ND	ND	ND	ND
G:A^{2OHE1}	190 ± 53	3.0 ± 0.46	3.21×10^{-3}	ND	ND	ND	ND
T:A^{2OHE1}	22 ± 0.2	1.6 ± 0.02	1.45×10^{-2}	10 ± 1.7	1.6 ± 0.06	2.37×10^{-2}	3.44×10^{-4}

[a] Kinetics of nucleotide insertion and chain extension reactions were determined as described in the Experimental Procedures. Frequencies of nucleotide insertion (F_{ins}) and chain extension (F_{ext}) were estimated by the equation $F = (V_{max}/K_m)_{(wrong\ pair)}/(V_{max}/K_m)_{(correct\ pair)}$. X = dG, 2-OHE$_1$-N^2-dG, dA, or 2-OHE$_1$-N^6-dA. [b] Data were expressed as means ± SD.

Table 4: Kinetic Parameters for Nucleotide Insertion and Chain Extension Reactions Catalyzed by pol β[a]

	insertion dNTP ↓GAAGAAAGGAGA^{32}P $^{5'}$CCTTCXCTTCTTTCCTCTCCCTTT			extension dGTP ↓NGAAGAAAGGAGA^{32}P $^{5'}$CCTTCXCTTCTTTCCTCTCCCTTT			
N:X	K_m (μM)	V_{max} (% min^{-1})	F_{ins}	K_m (μM)	V_{max} (% min^{-1})	F_{ext}	$F_{ins}F_{ext}$
C:G	51 ± 9.2[b]	2000 ± 310	1.0	17 ± 0.0	7300 ± 100	1.0	1.0
C:G^{2OHE1}	57 ± 8.3	11 ± 0.44	5.05×10^{-3}	18 ± 6.5	0.50 ± 0.03	6.22×10^{-5}	3.14×10^{-7}
A:G^{2OHE1}	ND	ND	ND	190 ± 9.0	0.25 ± 0.04	2.94×10^{-6}	ND
G:G^{2OHE1}	ND	ND	ND	200 ± 8.0	2.2 ± 0.38	2.41×10^{-5}	ND
T:G^{2OHE1}	ND	ND	ND	32 ± 7.9	1.1 ± 0.04	8.22×10^{-5}	ND
T:A	71 ± 13[b]	1300 ± 250	1.0	32 ± 14	9200 ± 760	1.0	1.0
C:A^{2OHE1}	ND	ND	ND	77 ± 3.0	0.41 ± 0.04	1.82×10^{-5}	ND
A:A^{2OHE1}	ND	ND	ND	28 ± 8.3	0.14 ± 0.01	1.68×10^{-5}	ND
G:A^{2OHE1}	ND	ND	ND	120 ± 27	0.55 ± 0.14	1.60×10^{-5}	ND
T:A^{2OHE1}	180 ± 45	280 ± 32	8.28×10^{-2}	11 ± 1.9	0.21 ± 0.01	6.44×10^{-5}	5.34×10^{-6}

[a] Kinetics of nucleotide insertion and chain extension reactions were determined as described in the Experimental Procedures. Frequencies of nucleotide insertion (F_{ins}) and chain extension (F_{ext}) were estimated by the equation $F = (V_{max}/K_m)_{(wrong\ pair)}/(V_{max}/K_m)_{(correct\ pair)}$. X = dG, 2-OHE$_1$-N^2-dG, dA, or 2-OHE$_1$-N^6-dA. [b] Data were expressed as means ± SD.

in mammalian cells (*43*). With pol α and pol δ, 2-OHE$_1$-N^6-dA promoted misincorporation of dCMP opposite the lesion and deletions. With pol δ, 2-OHE$_1$-N^2-dG promoted misincorporation of dTMP opposite the lesion. However, with pol β, both 2-OHE$_1$-N^2-dG and 2-OHE$_1$-N^6-dA directed the incorporation only of the correct base opposite the lesions. Thus, the miscoding occurred during DNA synthesis of replicative DNA polymerases. G → A and A → G transitions and deletions are predicted to be formed in mammalian cells. These miscoding spectra were quite consistent with that detected in human breast cancer (*31*−*34*). Although 2-OHE$_1$-N^2-dG and 2-OHE$_1$-N^6-dA adducts so far have not been identified in vivo (*5*, *11*, *16*), 2-OHE$_1$-N^2-dG and 2-OHE$_1$-N^6-dA adducts may initiate development of breast cancer.

Analysis of full-length reaction products should reflect relative rates of nucleotide insertion opposite the lesion. This assumption was confirmed by steady-state kinetic analysis, in which the frequency of nucleotide insertion opposite 2-OHE$_1$-N^2-dG or 2-OHE$_1$-N^6-dA and chain extension from the 3′ primer terminus are measured in the presence of a single dNTP. Although with 2-OHE$_1$-N^6-dA and pol α, the frequency of translesional synthesis ($F_{ins}F_{ext}$) for dCMP was lower than that for dTMP, the kinetic data also indicate that dCMP can be misincorporated opposite the lesion during DNA synthesis.

When pol α and pol δ were used, one-base deletions were detected at the site of 2-OHE$_1$-N^6-dA. On the basis of our proposed general mechanism for frameshift deletions (*44*), when primer extension is blocked at the lesion site, the newly inserted nucleotide(s) opposite the lesion can pair with base 5′ to the lesion on the template strand. The propensity for template misalignment was shown to depend on the (a) nature of the base inserted opposite the lesion, (b) sequence context to the lesion, and (c) frequency of translesional synthesis (*44*). On the basis of kinetic analysis with pol α, dGMP was observed to be inserted opposite 2-OHE$_1$-N^6-dA, while the chain extension of dG•2-OHE$_1$-N^6-dA pair was blocked (Table 3). Thus, the newly inserted dGMP could be paired with dC 5′ to the lesion on the template to form one-base deletion.

N^2-[3-methoxyestra-1,3,5(10)-trien-6-yl]-dG (dG-N^2-3MeE) and *N*^{6}-[3-methoxyestra-1,3,5(10)-trien-6-yl]-dA (dA-N^6-3MeE) have been used as model estrogen−DNA adducts (*45*). The binding positions, N^2 and N^6, of model estrogen to dG and dA were the same as that induced by estrone 2,3-quinone. dG-N^2-3MeE behaved as a blocking lesion while dA-N^6-3MeE allowed the translesional synthesis past the

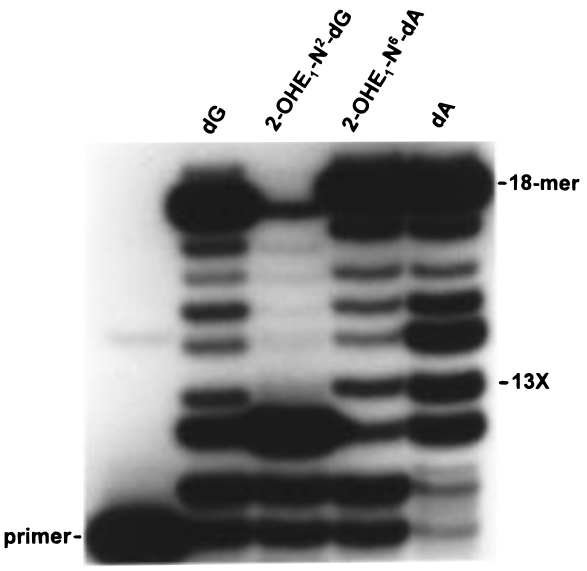

FIGURE 7: Primer extension reactions catalyzed by pol δ. Primer extension reactions catalyzed by 0.04 units of pol δ were carried out for 30 min at 25 °C in the reaction mixture containing four dNTPs and 6 ng of PCNA as described under Experimental Procedures.

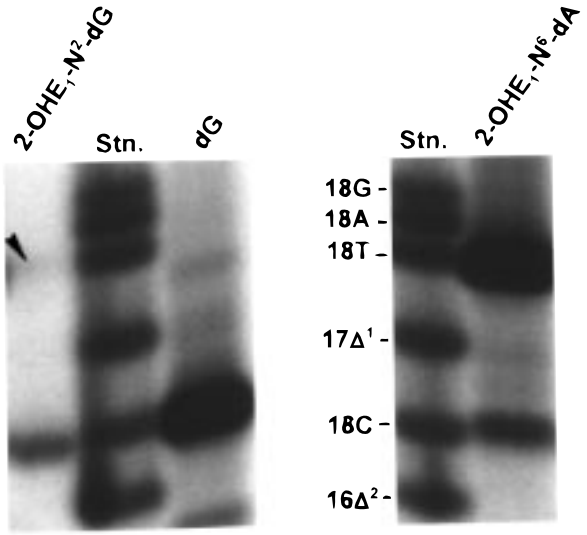

FIGURE 8: Quantitation of the miscoding specificities. Samples shown in Figure 7 were subjected to a two-phase gel (15 × 72 × 0.04 cm) as described in the legend of Figure 5B.

lesion. Similar tendency was observed when 2-OHE$_1$-N^2-dG and 2-OHE$_1$-N^6-dA were used. Using same amounts of pol α, the amounts of fully extended products past 2-OHE$_1$-N^2-dG and 2-OHE$_1$-N^6-dA were 160 and 30 times higher, respectively, than that of model estrogen adducts. In addition, primer extension reactions catalyzed by pol δ were completely blocked opposite model estrogen adducts (*45*) while 2-OHE$_1$-N^2-dG and 2-OHE$_1$-N^6-dA allowed the translesional synthesis past the lesions. Thus, amounts of extended products past estrogen quinone-derived adducts were much higher than that of model estrogen adducts. With pol α, dA-N^6-3MeE miscoded dCMP (*45*), but the frequency was much lower than that observed by 2-OHE$_1$-N^6-dA. Structure of 3-methoxyestra-1,3,5(10)-trien (3MeE) is slightly different from 2-OHE$_1$: hydroxy and keto groups are lacking at C-2 and C-17, respectively, and the methoxy group is positioned at C-3. These small moieties may influence the mutational frequencies and spectra.

4-Hydroxyestrogens have been shown to be carcinogenic in the kidneys of male Syrian hamsters while 2-hydroxyestrogens were not (*46*, *47*). Estrogen-3,4-quinone, a metabolite of 4-hydroxyestrone, reacts with dG residue, resulting in N^7-(4-hydroxyestron-1-yl)guanine and an apurinic site in DNA (*19*, *48*). Apurinic sites have been shown to generate mutations in mammalian cells (*49*−*51*). In our in vitro studies using pol α and pol δ, apurinic sites miscode dAMP during DNA synthesis (*52*, *53*). Thus, depurinating sites induced by estrogen-3,4-quinone may initiate the development of kidney tumors. However, 2- and 4-hydroxyestrogens and their major metabolites, estradiol-2,3-quinone and estradiol-3,4-quinone, did not show any significant tumorigenic potency in liver of B6C3F$_1$ male mice (*48*). Carcinogenicity of estrogen may vary depending on the organs examined.

No carcinogenic studies of estrogen metabolites, including hydroxyestrogen and estrogen quinone, have been investigated in mammary and uterine tissues of animals. In our studies, estrogen quinone-DNA adducts, 2-OHE$_1$-N^2-dG and 2-OHE$_1$-N^6-dA, induced by 2-hydroxyestrogen have been shown to have miscoding potential. The miscoding spectra were similar to that observed in human breast cancer (*31*−*34*). Thus, these estrogen−DNA adducts may also contribute to the development of breast and endometrial cancers.

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