Translesion Synthesis past Equine Estrogen-Derived 2′-Deoxycytidine DNA Adducts by Human DNA Polymerases η and κ[†]

Naomi Suzuki,[‡] Manabu Yasui,[‡] Y. R. Santosh Laxmi,[‡] Haruo Ohmori,[⊥] Fumio Hanaoka,[∥] and Shinya Shibutani*[,‡]

Laboratory of Chemical Biology, Department of Pharmacological Sciences, State University of New York at Stony Brook, Stony Brook, New York 11794-8651, Department of Genetics and Molecular Biology, Institute for Virus Research, Kyoto University, Kyoto 606-8507, Japan, Graduate School of Frontier Biosciences, Osaka University, Suita, Osaka 565-0871, Japan and Discovery Research Institute, RIKEN, and CREST, JST, Wako, Saitama 351-0198, Japan

Received April 12, 2004; Revised Manuscript Received June 30, 2004

ABSTRACT: Estrogen replacement therapy (ERT), composed of equilenin, is associated with increased risk of breast, ovarian, and endometrial cancers. Several diastereoisomers of unique dC and dA DNA adducts were derived from 4-hydroxyequilenin (4-OHEN), a metabolite of equilenin, and have been detected in women receiving ERT. To explore the miscoding property of 4-OHEN-dC adduct, site-specifically modified oligodeoxynucleotides (Pk-1, Pk-2, Pk-3, and Pk-4) containing a single diastereoisomer of 4-OHEN-dC were prepared by a postsynthetic method. Among them, major 4-OHEN-dC-modified oligodeoxynucleotides (Pk-3 and Pk-4) were used to prepare the templates for primer extension reactions catalyzed by DNA polymerase (pol) α, pol η, and pol κ. Primer extension was retarded one base prior to the lesion and opposite the lesion; stronger blockage was observed with pol α, while with human pol η or pol κ, a fraction of the primers was extended past the lesion. Steady-state kinetic studies showed that both pol κ and pol η inserted dCMP and dAMP opposite the 4-OHEN-dC and extended past the lesion. Never or less-frequently, dGMP, the correct base, was inserted opposite the lesion. The relative bypass frequency past the 4-OHEN-dC lesion with pol η was at least 3 orders of magnitude higher than that for pol κ, as observed for primer extension reactions. The bypass frequency past the dA·4-OHEN-dC adduct in Pk-4 was 2 orders of magnitude more efficient than that past the adduct in Pk-3. Thus, 4-OHEN-dC is a highly miscoding lesion capable of generating C → T transitions and C → G transversions. The miscoding frequency and specificity of 4-OHEN-dC were strikingly influenced by the adduct stereochemistry and DNA polymerase used.

Estrogen replacement therapy (ERT)[1] is widely used for postmenopausal women to decrease menopausal symptoms and to protect against osteoporosis (*1*). More than 40% of U.S. women in this group currently receive ERT. Premarin (Wyeth-Ayerst), composed of approximately 30% equilin, 10% equilenin, and other estrogens, is frequently used for this purpose (reviewed in ref *2*). However, ERT increases the risk of developing breast (*3*, *4*), ovarian (*5*), and endometrial cancers (*4*). The risk of cancers increased depending on duration of ERT (*5*−*7*). The occurrence of endometrial hyperplasia was observed in postmenopausal women receiving ERT (*8*). Treating hamsters for 9 months with equilin or equilenin resulted in 100% tumor incidence and many tumor foci in kidney (*9*).

Equilin and equilenin are metabolized to 4-hydroxyl and 2-hydroxyl forms (*2*). 4-Hydroxyequilenin (4-OHEN) is rapidly autoxidized to an *o*-quinone and reacts readily with DNA, resulting in the formation of unique dC, dA, and dG adducts (*2*, *10*) (Figures 1 and 2). The equine estrogen-derived DNA adducts were detected in breast tumor and adjacent normal tissues of several patients receiving ERT and in paraffin-embedded breast tumor tissues (*11*). 4-Hydroxyequilin (4-OHEQ) is also autoxidized to an *o*-quinone, which isomerizes to 4-OHEN *o*-quinone (Figure 1); therefore, 4-OHEQ produces identical DNA adducts as those observed with 4-OHEN (*12*). Using ^{32}P-postlabeling/polyacrylamide gel electrophoresis (PAGE) analysis (*13*), we have observed that 4-OHEQ is highly reactive to DNA; large amounts of 4-OHEN-dC adduct, accompanied by lesser amounts of 4-OHEN-dA adduct, were detected. During redox cycling between 4-OHEN, *o*-quinone and its semiquinone radicals generate superoxide, hydrogen peroxide, and ultimately reactive hydroxyl radicals (*14*). When 4-OHEN was incubated with DNA or exposed to breast cancer cells, increased formation of 8-oxo-7,8-dihydro-2′-deoxyguanosine

[†] This research was supported by Grant ES09418 (to S.S.) from the National Institute of Environmental Health Sciences and Grant-in-aid (to M.Y.) from Japan Society for the Promotion of Science (JSPS, Japan).

* To whom correspondence should be addressed. Phone: 631-444-8018. Fax: 631-444-3218. E-mail: shinya@pharm.sunysb.edu.

[‡] SUNY at Stony Brook.

[⊥] Kyoto University.

[∥] Osaka University, RIKEN, and CREST, JST.

[1] Abbreviations: ERT, estrogen replacement therapy; 4-OHEQ, 4-hydroxyequilin; 4-OHEN, 4-hydroxyequilenin; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; dNTP, 2′-deoxynucleoside triphosphate; pol η, DNA polymerase η; pol κ, DNA polymerase κ; pol $\kappa\Delta$C, 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; CD, circular dichroism.

10.1021/bi049273n CCC: $27.50
Published on Web 08/06/2004

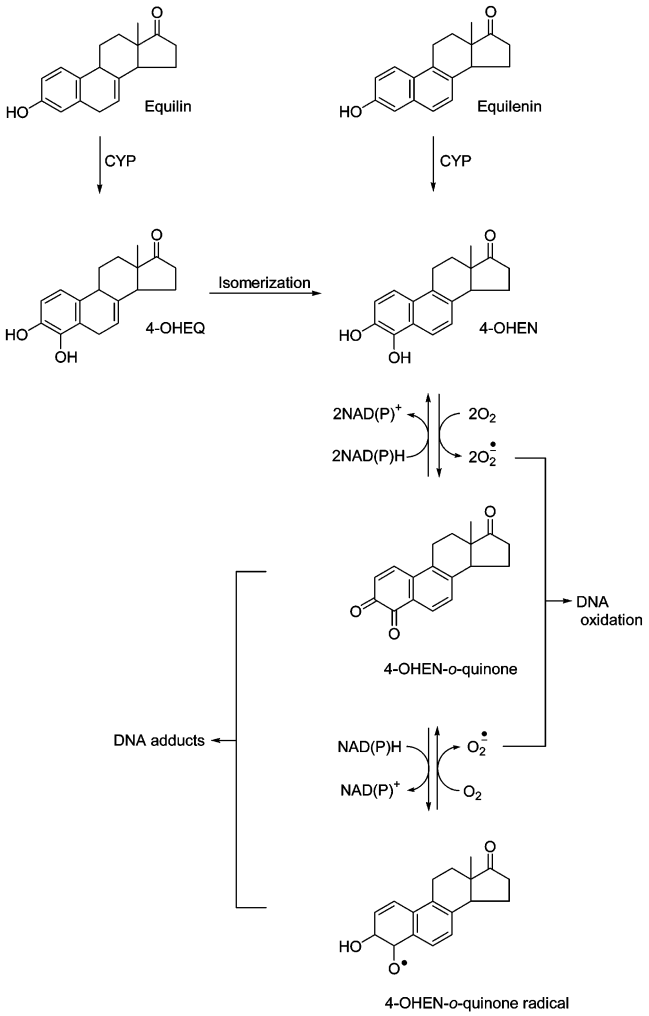

FIGURE 1: DNA damage mediated through 4-OHEN and 4-OHEQ.

(8-oxodG) adduct was observed in DNA (*14*−*17*). Particularly, when 4-OHEN was injected directly into the mammary fat pads of rats, 4-OHEN-dA and 4-OHEN-dG adducts, in addition to increased formation of 8-oxodG, were detected in the mammary tissue using liquid chromatography−tandem mass spectrometry (LC/MS/MS) (*18*).

When a double-stranded pMY189 shuttle vector carrying a bacteria suppressor tRNA gene, *supF*, was exposed to 4-OHEQ and propagated into human fibroblast cells, a high frequency of mutations were observed (*19*). The majority of the 4-OHEQ-induced mutations occurred at the C:G site; C:G → G:C and C:G → A:T mutations were detected with lesser numbers of C:G → T:A transitions. 4-OHEQ was mutagenic in human cells and promotes mutations primarily at C:G pairs in 5′-TC/AG-5′ sequences (*19*).

Newly discovered human DNA polymerases including pol η and pol κ are associated with translesion synthesis past DNA adducts (reviewed in refs *20* and *21*). Pol η and pol κ are highly expressed in human testis, ovary and uterus where steroid hormones are produced, including estrogen (*22*−*25*). If equine estrogen-derived DNA adducts are formed in such reproductive organs, the newly found DNA polymerases may miscode the lesion during DNA synthesis, leading to an increased risk of developing breast, ovarian, and endometrial cancers.

To explore the miscoding property of the major 4-OHEN-dC adducts, site-specifically modified oligodeoxynucleotides containing a single diastereoisomer of 4-OHEN-dC were prepared by a postsynthetic method and used as templates for primer extension reactions catalyzed by human pol η or a truncated form of human pol κ (pol $\kappa\Delta$C). Steady-state kinetic studies were also performed to measure the relative bypass frequency past the 4-OHEN-dC lesions.

MATERIALS AND METHODS

General. [γ-^{32}P]ATP (specific activity > 6000 Ci/mmol) and dNTPs were obtained from Amersham Corp.; T4 polynucleotide kinase and *Eco*RI restriction endonuclease were purchased from New England BioLabs. Calf thymus DNA pol α (30 000 units/mg) was purchased from Chimerx.

Synthesis of 4-OHEN-dC-Modified Oligodeoxynucleotides. Oligodeoxynucleotides DNA templates (5′TTTGTCTTTT and 5′TTTGTCTTTTCTTCTTTCCTCTCCC), primers (5′GAAAGAAGAA, 5′GAAAGAAGAAAA, and 5′GAAAGAAGAAAAN, where N is C, A, G, or T), and standard markers were prepared by solid-state synthesis on an automated DNA synthesizer. 4-OHEN was prepared using an established protocol (*10*, *26*). Two hundred micrograms of 5′TTTGTCTTTT was reacted at 37 °C for 16 h with 1 mg of 4-OHEN in 1 mL of 25 mM potassium phosphate buffer, pH 7.4. The reaction mixture was centrifuged, and the supernatant was evaporated to dryness and subjected to HPLC. The 10-mer oligomers containing a single 4-OHEN-dC adduct (5′TTTGTC$^{4-\text{OHEN}}$TTTT) were isolated on a reverse-phase μBondapak C_{18} column (0.39 cm × 30 cm, Waters), using a linear gradient composed of 0.05 M triethylammonium acetate (pH 7.0) containing 10−30% acetonitrile, an elution time of 60 min, and a flow rate of 1.0 mL/min. Five micrograms of 15-mer (5′CTTCTTTC-CTCTCCC) was phosphorylated at the 5′ terminus using 7.5 μL of T4 polynucleotide kinase (10 units/μL) and 3 μL of 10 mM ATP, mixed with 4-OHEN-dC-modified 10-mer (3 μg, 5′TTTGTC$^{4-\text{OHEN}}$TTTT) and a complementary 22-mer oligomer (10 μg, 5′AGAGGAAAGAAGAAAAGACAAA), and then incubated at 4 °C overnight in 100 μL of 50 mM Tris-HCl (pH 7.5) containing 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 2.5 μg of BSA, and 3 μL of T4 DNA ligase (400 units/μL). The 25-mer product was isolated using HPLC as described previously. The unmodified and modified 25-mers were purified using HPLC and gel electrophoresis (*27*) and used as templates for primer extension reactions and kinetic studies. HPLC was performed using 515 HPLC pump, 996 photodiode array detector, and pump control module. Concentrations of oligodeoxynucleotides were calculated from their UV absorption spectra in solution, measured with a Hewlett-Packard 8452A diode array spectrophotometer.

Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) Analysis. Samples were run on a Voyager-DE STR MALDI-TOF mass spectrometer system (Applied Biosystems, Framingham, MA) operated in the linear mode. Samples were dissolved in a 50% solution of acetonitrile (ANC)/water containing hydroxypicolinic acid (5 mg/mL) and ammonium citrate at an 8:1 ratio and dried on sample plate. A nitrogen laser operating at 337 nm and a 3 ns pulse rate was employed. The accelerating voltage was set to 20 kV, and a delay of 450 ns was used to accelerate ions into the flight tube of the mass spectrometer. The mass scale (*m*/*z* 1000−10 000) was calibrated in the

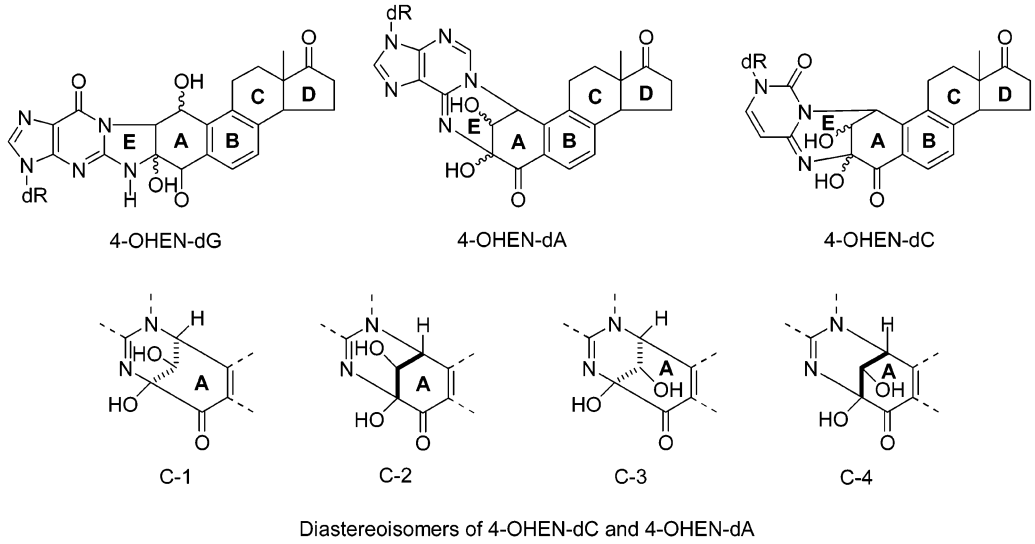

FIGURE 2: Structures of diastereoisomers of 4-OHEN-DNA adducts.

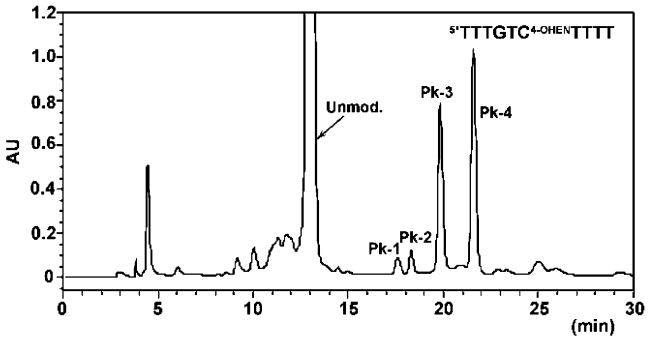

FIGURE 3: HPLC separation of oligodeoxynucleotides containing a single 4-OHEN-dC adduct. Two hundred micrograms of 5′TTTGTCTTTT was reacted at 37 °C for 16 h with 4-OHEN (1 mg in 50 μL of acetone) in 1 mL of 25 mM potassium phosphate buffer, pH 7.4. After centrifugation, the supernatant was evaporated to dryness and subjected to HPLC. The 10-mer oligomers containing an isoform of 4-OHEN-dC adduct (5′TTTGTC$^{4-\text{OHEN}}$TTTT) were isolated on a reverse-phase μBondapak C_{18} column (0.39 cm × 30 cm, Waters) using a linear gradient composed of 0.05 M triethylammonium acetate (pH 7.0) containing 10−30% acetonitrile, an elution time of 60 min, and a flow rate of 1.0 mL/min.

positive ion mode with a mixture of standard oligodeoxynucleotides, and approximately 100 laser shots were used to produce each spectrum.

Enzymatic Digestion. A 25-mer oligodeoxynucleotide containing a single 4-OHEN-dC (0.75−1.5 μg) was digested to deoxynucleoside 3′-monophosphates at 37 °C for 3 h in 100 μL of 17 mM sodium succinate (pH 7.0) containing 8 mM $CaCl_2$ using micrococcal nuclease (3 units) and spleen phoshodiesterase (0.1 units). To remove 3′-monophosphate from deoxynucleoside 3′-monophosphates, the reaction mixture was further incubated at 37 °C for 2 h with alkaline phosphatase (3 units) in 250 μL of 8 mM Tris-HCl (pH 8.5) containing 0.8 mM zinc sulfate. One milliter of ethanol was added to the reaction mixture, and the mixture was centrifuged to precipitate digestion enzymes at 12 000*g* for 20 min. The supernatant was evaporated to dryness and analyzed by HPLC using a μBondapak C_{18} column (0.78 cm × 30 cm, Waters). Elution was carried out using a linear gradient of distilled water containing 10% MeOH over 2 min, 10−35% MeOH over 3 min, 35−43% MeOH over 25 min, and 43−90% MeOH over 5 min at a flow rate of 2.0 mL/min. Diastereoisomers (fr-1, fr-2, fr-3, and fr-4) of 4-OHEN-dC were prepared as described previously (*10*) and used as standards.

Stability of 4-OHEN-dC-Modified Oligodeoxynucleotide. Each isoform of 4-OHEN-dC-modified 10-mer (10 pmol, 5′TTTGTC$^{4-\text{OHEN}}$TTTT) was labeled with ^{32}P (*28*) and incubated at 37 °C for 24 h in 100 μL of 50 mM Tris-HCl (pH 7.4). During the incubation, a fraction (1 pmol) was collected at 1, 3, 5, 8, and 24 h from the reaction mixture and stored at −70 °C until analysis. The samples were subjected to 20% polyacrylamide gel electrophoresis (PAGE) (35 cm × 42 cm × 0.04 cm). The radioactivity of extended products was measured by β-phosphorimager (Molecular Dynamics).

Primer Extension Reactions. Pol η and a truncated form of pol κ (pol $\kappa\Delta$C) were prepared as described previously (*29*, *30*). Pol $\kappa\Delta$C, a catalytically active fragment composed of 560 amino acids, used in this study lacks motifs VIIa and VIIb that denote zinc clusters from intact DINB1 protein (870 amino acids) (*30*). Although pol $\kappa\Delta$C has a lower processivity than full-length pol κ, the miscoding rates on undamaged DNA by pol $\kappa\Delta$C and pol κ were similar (*31*). A 10-mer (5′GAAAGAAGAA) was labeled at the 5′ terminus with T4 polynucleotide kinase and [γ-^{32}P]ATP (*28*). Using 4-OHEN-dC-modified and unmodified 25-mer oligodeoxynucleotide (150 fmol) primed with a ^{32}P-labeled 10-mer (5′GAAAGAAGAA; 100 fmol), we conducted primer extension reactions catalyzed by pol α, pol η, and pol $\kappa\Delta$C at 25 °C for 30 min in a buffer (10 μL) containing four dNTPs (100 μM each) (Figure 2). The reaction buffer for pol α consisted of 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM dithiothreitol (DTT), and BSA (0.5 μg/μL). 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 $\kappa\Delta$C, 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 cm × 42 cm × 0.04 cm). The radioactivity of extended products was measured by β-phosphorimager (Molecular Dynamics).

Steady-State Kinetic Studies. Kinetic parameters associated with nucleotide insertion opposite 4-OHEN-dC lesion and

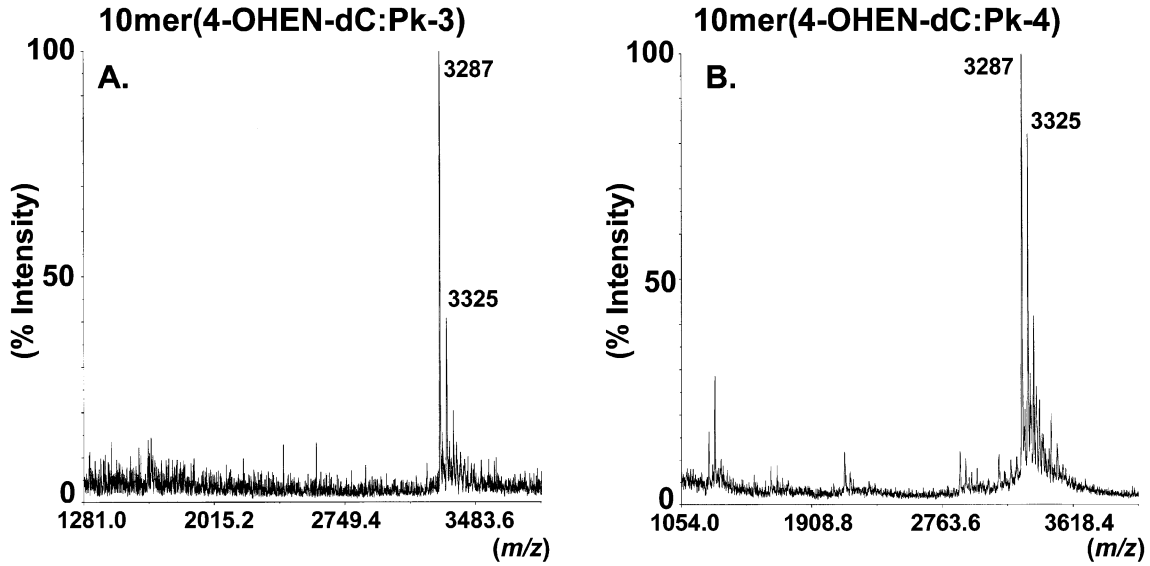

FIGURE 4: MALDI-TOF mass spectra of 4-OHEN-dC-modified oligodeoxynucleotides. The molecular weight of Pk-3 (A) and Pk-4 (B) of 4-OHEN-dC-modified 10-mer oligomers (5′TTTGTC^{4-OHEN}TTTT) was measured using MALDI-TOF mass spectrometry.

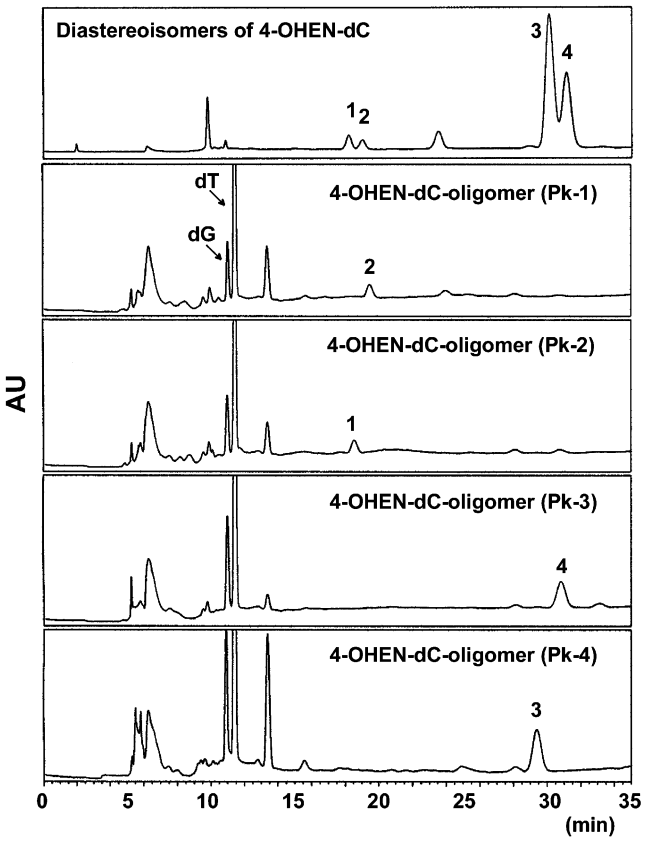

FIGURE 5: Determination of 4-OHEN-dC isoform incorporated into 4-OHEN-modified oligomers. The peaks 1, 2, 3, and 4 represent standards of diastereoisomers of 4-OHEN-dC. 4-OHEN-dC-modified oligodeoxynucleotides (5′TTTGTC^{4-OHEN}TTTT, 0.75 μg for Pk-1 and Pk-2, 1.5 μg for Pk-3 and Pk-4) were digested at 37 °C for 3 h with spleen phosphodiesterase (0.05 units) and alkaline phosphates (30 milliunits) in a buffer, as described in Materials and Methods. The resulting deoxynucleosides were analyzed using HPLC, using a μBondapak C_{18} column (0.78 cm × 30 cm, Waters). Elution was carried out using a linear gradient of distilled water containing 10% MeOH over 2 min, 10−35% MeOH over 3 min, 35−43% MeOH over 25 min, and 43−90% MeOH over 5 min at a flow rate of 2.0 mL/min.

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 (0.2−10 ng) or pol $\kappa\Delta$C (0.05−10 ng) were incubated at 25 °C for 2 min in 10 μL of Tris-HCl buffer (pH 8.0) using a 25-mer template (150 fmol; 5′TTTGTXTTTTCTTCTTTCCTCTCCC, where X is dC or 4-OHEN-dC) primed with a ^{32}P-labeled 12-mer (100 fmol; 5′GAAAGAAGAAAA). Reaction mixtures containing a 25-mer template (150 fmol) primed with a ^{32}P-labeled 13-mer (100 fmol; 5′GAAAGAAGAAAAN, where N is C, A, G, or T) with varying amounts of dATP (0−500 μM) and either pol η (0.2−10 ng) or pol $\kappa\Delta$C (0.05−10 ng) were used to measure chain extension. The reaction samples were subjected to 20% denaturing PAGE (35 cm × 42 cm × 0.04 cm). The Michaelis constants (K_m) and maximum rates of reaction (V_{max}) were obtained from Hanes−Woolf plots. The V_{max} is corrected for the different enzyme concentrations. Frequencies of dNTP insertion (F_{ins}) and chain extension (F_{ext}) were determined relative to the dG·dC base pair according to the equation $F = (V_{max}/K_m)_{[wrong\ pair]}/(V_{max}/K_m)_{[correct\ pair=dG\cdot dC]}$ (*32*, *33*).

RESULTS

Preparation of Oligodeoxynucleotides Containing a Single 4-OHEN-dC Adduct. When calf thymus DNA was reacted with 4-OHEQ (*13*, *19*) or 4-OHEN (data not shown) and analyzed by ^{32}P-postlabeling/PAGE, 4-OHEN-dC adduct was detected as a major adduct, along with lesser amounts of 4-OHEN-dA adduct. Only when large amounts of 4-OHEN was used, a faint of 4-OHEN-dG adduct was detected. We have attempted to prepare oligodeoxynucleotides containing a single 4-OHEN-dC adduct. Since 4-OHEN can react preferentially with dC and dA residues in DNA, the sequence context to use for preparing the site-specific oligomer is limited. Therefore, a 10-mer oligodeoxynucleotide (5′TTTGTCTTTT) was reacted with 4-OHEN under neutral pH conditions. Two minor (t_R = 17.6 min for Pk-1 and t_R = 18.3 min for Pk-2) and two major (t_R = 19.8 min for Pk-3 and t_R = 21.6 min for Pk-4) products representing 10-mers containing a single 4-OHEN-dC were isolated by HPLC (Figure 3).

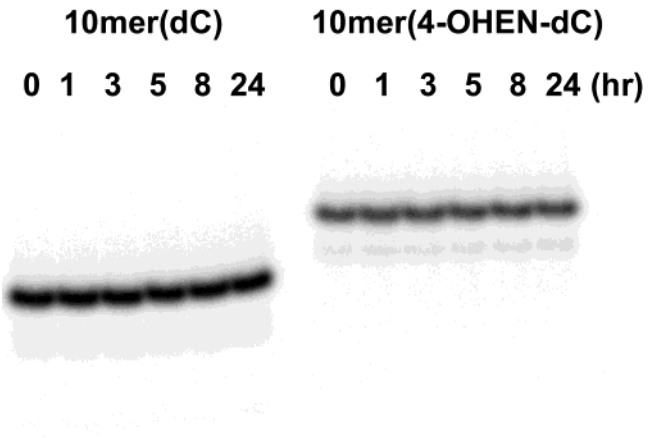

FIGURE 6: Stability of 4-OHEN-dC-modified oligodeoxynucleotide. Each isoform of 4-OHEN-dC-modified 10-mer (10 pmol, 5′TTTGTC^{4-OHEN}TTTT) was labeled with ^{32}P and incubated at 37 °C for 24 h in 100 μL of 50 mM Tris-HCl, pH 7.4. A fraction (1 pmol) was collected at 1, 3, 5, 8, and 24 h from the reaction mixture and stored at −70 °C. The samples were subjected to 20% polyacrylamide gel electrophoresis (PAGE) (35 cm × 42 cm × 0.04 cm). The radioactivity of the oligomers was measured by β-phosphorimager (Molecular Dynamics).

Mass Spectrometry of Oligodeoxynucleotides Containing a Single 4-OHEN-dC Adduct. The molecular weight of products isolated by HPLC was measured using a Voyager-DE STR MALDI-TOF mass spectrometer system. The parent ions of all four 4-ONEN-dC-modified 10-mers exhibited a *m*/*z* 3287, identifying the molecular mass as 3286 Da (for example, Pk-3 and Pk-4 in Figure 4). Since potassium forms an adduct with the deprotonated phosphate, peaks (*m*/*z* 3325) representing $[M + K]^+$ were also detected. Thus, the reaction products are identified as 10-mers containing a single diastereoisomer of 4-OHEN-dC.

Presence of Diastereoisomers of 4-OHEN-dC Incorporated into the 4-OHEN-Modified Oligomers. Enzymatic digestion analysis was used to determine which diastereoisomer is incorporated into the modified oligomers. Each product of 4-OHEN-dC-modified oligodeoxynucleotides (5′TTTGTC^{4-OHEN}TTTT, 0.75 μg for Pk-1 and Pk-2, 1.5 μg for Pk-3 and Pk-4) was digested with spleen phosphodiesterase and alkaline phosphates in a buffer. The resulting deoxynucleosides were analyzed using HPLC (Figure 5). The minor modified oligomers (Pk-1 and Pk-2) contain fr-2 and fr-1 of monomeric 4-OHEN-dC diastereoisomers, respectively, and major modified oligomers (Pk-3 and Pk-4) contain fr-4 and fr-3 diastereoisomers, respectively. The ratio of dG/dT/4-OHEN-dC was 1:8:1, which is consistent with constituents of the oligomer.

Stability of 4-OHEN-dC-Modified Oligodeoxynucleotides. A 10-mer oligodeoxynucleotide (5′TTTGTXTTTT, where X is dC or 4-OHEN-dC) was labeled with ^{32}P at the 5′-terminus. The migration of 4-OHEN-dC-modified oligomers on the gel was slower than that of the corresponding unmodified oligomers (Figure 6). These oligomers were incubated at 37 °C for 24 h in a Tris-HCl buffer (pH 7.5). If the 4-OHEN-dC adduct is depurinated or degraded, the shorter fragments could be observed on the gel. During 24 h incubation, no significant degradation was detected. This indicates that 4-OHEN-dC-modified oligomers are stable under the experimental conditions used for mutagenesis studies.

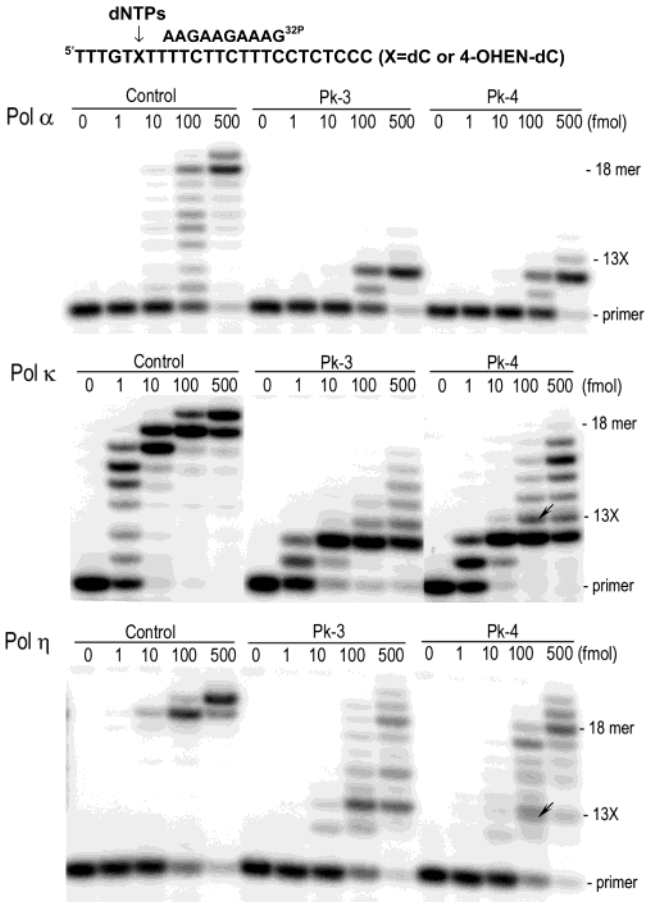

FIGURE 7: Primer extension reactions catalyzed by DNA polymerases on 4-OHEN-dC-modified DNA templates. Unmodified, 4-OHEN-dC (Pk-3 or Pk-4)-modified 25-mer templates were annealed to a ^{32}P-labeled 10-mer primer (^{32P}-GAAAGAAGAA). Primer extension reactions catalyzed by variable amounts (0−500 fmol) of pol α, pol $\kappa\Delta$C, or pol η were conducted at 25 °C for 30 min in the presence of four dNTPs using variable amounts of enzymes. One-third of the reaction mixture was subjected to PAGE (35 cm × 42 cm × 0.04 cm). The radioactivity of extended products was measured by β-phosphorimager. 13X marks the location opposite the 4-OHEN-dC.

Primer Extension Reactions past 4-OHEN-dC Adduct by DNA Polymerases. The 4-OHEN-dC-modified 10-mers were ligated to a 15-mer (5′CTTCTTTCCTCTCCC) for preparation of 25-mer DNA templates that can be used for primer extension reactions catalyzed by mammalian DNA polymerases. Since 4-OHEN-dC adduct was primarily detected in calf thymus DNA exposed to 4-OHEN (*13*, *19*) and Pk-3 and Pk-4 of 4-OHEN-dC-modified oligomers were major products (Figure 3), primer extension reactions catalyzed by DNA pol α, pol η, and pol $\kappa\Delta$C were conducted in the presence of four dNTPs on the templates prepared from Pk-3 and Pk-4. Primer extension occurred rapidly on unmodified templates to form fully extended products (Figure 7). However, with the 4-OHEN-dC-modified templates (Pk-3 and Pk-4), primer extension was retarded one base before the lesion and opposite the lesion; stronger blocking was observed with pol α (100 and 500 fmol). When the same molar amounts of pol η or pol $\kappa\Delta$C were used, a fraction of the primers was extended past the lesion. The primer extension past the 4-OHEN-dC lesion in Pk-4 oligomer was much faster than that for Pk-3 oligomer. When the amount of pol α and pol η was increased, blunt-end addition to the

Table 1: Kinetic Parameters for Nucleotide Insertion and Chain Extension Reactions Catalyzed by DNA pol κ and pol η

Insertion dNTP ↓ **AAAAGAAGAAAG^{32P}**
5'TTTGTXTTTTCTTCTTTCCTCTCCC

Extension dATP ↓ **NAAAAGAAGAAAG^{32P}**
5'TTTGTXTTTTCTTCTTTCCTCTCCC

	insertion			extension			
N:X[a]	K_m (μM)[b]	V_{max} (% min^{-1})[b]	F_{ins}[c]	K_m (μM)[b]	V_{max} (% min^{-1})[b]	F_{ext}[c]	$F_{ins}F_{ext}$[c]
			Pol κ				
G:C	1.18 ± 0.26	370 ± 1	**1.0**	1.58 ± 0.12	217 ± 6.7	**1.0**	**1.0**
			X = 4-OHEN-dC (Pk-3)				
C:X	126 ± 7	0.26 ± 0.12	**6.48×10^{-6}**	6.30 ± 0.79	1.29 ± 0.01	**1.49×10^{-3}**	**9.64×10^{-9}**
A:X	108 ± 41	0.32 ± 0.10	**5.72×10^{-6}**	14.9 ± 0.11	0.10 ± 0.01	**4.83×10^{-5}**	**2.76×10^{-10}**
G:X	*d*	*d*	***d***	50.6 ± 1.47	0.14 ± 0.01	**1.94×10^{-5}**	***d***
T:X	*d*	*d*	***d***	30.5 ± 4.79	0.14 ± 0.01	**3.34×10^{-5}**	***d***
			X = 4-OHEN-dC (Pk-4)				
C:X	165 ± 5	0.41 ± 0.01	**7.77×10^{-6}**	4.47 ± 1.12	1.01 ± 0.01	**1.69×10^{-3}**	**1.31×10^{-8}**
A:X	114 ± 28	0.26 ± 0.04	**7.11×10^{-6}**	23.3 ± 0.28	0.81 ± 0.05	**2.52×10^{-4}**	**1.79×10^{-9}**
G:X	*d*	*d*	***d***	340 ± 112	0.43 ± 0.05	**9.59×10^{-6}**	***d***
T:X	*d*	*d*	***d***	9.66 ± 0.75	0.07 ± 0.01	**5.61×10^{-5}**	***d***
			Pol η				
G:C	0.10 ± 0.04	23.5 ± 4.1	**1.0**	0.80 ± 0.12	48.7 ± 2.2	**1.0**	**1.0**
			X = 4-OHEN-dC (Pk-3)				
C:X	17.9 ± 6.8	0.83 ± 0.02	**2.13×10^{-4}**	35.6 ± 2.94	0.35 ± 0.10	**1.60×10^{-4}**	**3.40×10^{-8}**
A:X	2.72 ± 1.6	1.43 ± 0.24	**2.56×10^{-3}**	25.0 ± 4.23	0.35 ± 0.03	**2.30×10^{-4}**	**5.90×10^{-7}**
G:X	4.81 ± 0.84	0.63 ± 0.12	**5.50×10^{-4}**	34.6 ± 8.79	0.13 ± 0.02	**6.23×10^{-5}**	**3.43×10^{-8}**
T:X	14.0 ± 0.69	1.69 ± 0.06	**5.10×10^{-4}**	*d*	*d*	***d***	***d***
			X = 4-OHEN-dC (Pk-4)				
C:X	69.8 ± 16.4	4.37 ± 0.06	**2.72×10^{-4}**	3.00 ± 1.22	0.68 ± 0.01	**4.00×10^{-3}**	**1.09×10^{-6}**
A:X	8.72 ± 5.88	4.36 ± 0.03	**2.73×10^{-3}**	1.58 ± 0.54	1.69 ± 0.18	**1.83×10^{-2}**	**5.00×10^{-5}**
G:X	19.7 ± 5.21	3.10 ± 0.15	**6.91×10^{-4}**	43.7 ± 5.20	0.82 ± 0.04	**3.05×10^{-4}**	**2.11×10^{-7}**
T:X	60.4 ± 6.48	3.86 ± 0.26	**2.72×10^{-4}**	19.4 ± 0.48	0.61 ± 0.09	**5.10×10^{-4}**	**1.39×10^{-7}**

[a] X = dC or 4-OHEN-dC lesion. [b] Data expressed as mean ± S. D. obtained from three independent experiments. [c] Frequencies of nucleotide insertion (F_{ins}) and chain extension (F_{ext}) were estimated by the equation: $F = (V_{max}/K_m)_{[wrong\ pair]}/(V_{max}/K_m)_{[correct\ pair=dG:dC]}$. [d] Not determined.

fully extended reaction product was observed (*34*, *35*). As indicated by an arrow, at least two bands were observed opposite the 4-OHEN-dC lesion (position 13), indicating that incorrect nucleotides were incorporated opposite the lesion.

Kinetic Studies on 4-OHEN-dC-Modified Templates. Steady-state kinetic studies were performed to explore the miscoding properties of 4-OHEN-dC adducts. The frequencies of dNTP insertion (F_{ins}) and chain extension (F_{ext}) by pol $\kappa\Delta$C and pol η were measured opposite the 4-OHEN-dC within the linear range of the reaction (Table 1). Typical Hanes−Woolf plots obtained with pol η for dGTP insertion opposite dC or 4-OHEN-dC (Pk-3 and Pk-4) and dATP insertion opposite the 4-OHEN-dC are shown in Figure 8. When pol $\kappa\Delta$C was used for Pk-3, the F_{ins} value for dCMP (6.48×10^{-6}) was not significantly different from that for dAMP (5.72×10^{-6}) (Table 1). However, F_{ext} for the dC·4-OHEN-dC pair was 31-fold higher than that for the dA·4-OHEN-dC pair. Therefore, the relative bypass frequency past the dC·4-OHEN-dC pair was more efficient than that for the dA·4-OHEN-dC pair. F_{ins} values for dGMP, the correct base, and dTMP were unable to be determined. Similar results were observed with Pk-4. Pol κ may promote C → G transversions, along with C → T transitions, at the 4-OHEN-dC lesion.

With pol η, the F_{ins} for dAMP opposite the 4-OHEN-dC lesion in Pk-3 was 4.7 times higher than that for dGMP, the correct base, and other nucleotides. The F_{ext} for the dA·4-OHEN-dC pair was also 3.7 times higher than that for the dG·4-OHEN-dC pair. Therefore, relative bypass frequency past the dA·4-OHEN-dC pair was 17 times more efficient than that for the dG·4-OHEN-dC pair. A similar phenomenon was observed with Pk-4. The relative bypass frequency past the dA·4-OHEN-dC pair in Pk-4 (5.00×10^{-5}) was 240 times higher than that for the dG·4-OHEN-dC pair (2.11×10^{-7}) and 2 orders of magnitude higher than that for the dA·4-OHEN-dC pair in Pk-3 (5.90×10^{-7}). Pol η may promote primarily C → T transitions, along with C → G transversions at the 4-OHEN-dC lesion.

DISCUSSION

Site-specific mutagenesis techniques were used to explore the miscoding potential of diastereoisomers of major 4-OHEN-dC DNA adducts. 4-OHEN-dC in the oligomers (Pk-3 and Pk-4) showed miscoding potential during translesion synthesis catalyzed by human pol κ and pol η, both of which are highly expressed in reproductive organs (*22*−*25*). On the basis of steady-state kinetic studies, pol κ incorporated dCMP and dAMP opposite the 4-OHEN-dC, indicating that this enzyme is associated with generating C → G transversions and C → T transitions. Pol η incorporated dAMP, followed by dCMP, opposite the lesion, suggesting that the enzyme promotes primarily C → T transitions, along with C → G transversions. Surprisingly, insertion of dGMP, the correct base, opposite the 4-OHEN-dC lesion was highly inefficient. The relative bypass frequency past 4-OHEN-dC with pol η was more efficient than that with pol $\kappa\Delta$C, as observed for primer extension reactions with similar molar concentrations. Therefore, 4-OHEN-dC may be a highly miscoding lesion; the frequency and specificity varies depending on DNA polymerase used.

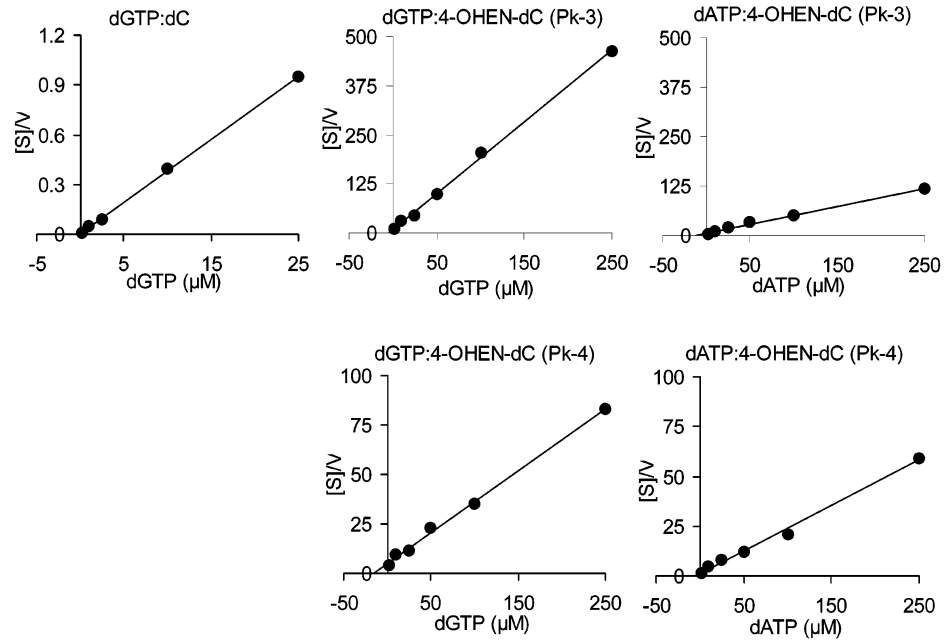

FIGURE 8: Hanes−Woolf plots of deoxynucleotide insertion opposite the 4-OHEN-dC lesion. With use of 25-mer templates (150 fmol; 5′CCTTCXCTTCTTTCCTCTCCCTTT, where X is dC or 4-OHEN-dC) primed with a ^{32}P-labeled 12-mer (100 fmol; 5′GAAAGAAGAAAA), kinetic parameters associated with dGTP or dATP insertion opposite 4-OHEN-dC lesion were determined. The reaction mixtures containing dGTP or dATP (0−500 μM) and pol η (0.2 ng for dGTP insertion opposite dC, 10 ng for dGTP insertion opposite 4-OHEN-dC, and 5 ng for dATP insertion opposite 4-OHEN-dC) were incubated at 25 °C for 2 min in 10 μL of Tris-HCl buffer (pH 8.0). The reaction samples were subjected to 20% denaturing PAGE (35 cm × 42 cm × 0.04 cm). The K_m and V_{max} were obtained from Hanes−Woolf plots, as described in Materials and Methods.

When a pMY189 shuttle vector carrying the *supF* gene was exposed to 4-OHEQ and transfected into human fibroblast cells, 4-OHEQ-induced mutations occurred mainly at C:G pairs. C:G → G:C and C:G → A:T transversions were detected primarily, along with lesser numbers of C:G → T:A transitions (*19*). Therefore, these mutations could be promoted by C, G, or both damaged by 4-OHEQ. 4-OHEN-dC was a major equine estrogen-DNA adduct, followed by lesser amounts of 4-OHEN-dA adduct (*13*, *19*), and almost no 4-OHEN-dG was detected in the DNA reacted with either 4-OHEQ or 4-OHEN (*19*). Therefore, C:G → G:C and C:G → T:A mutations observed at C:G pairs in the *supF* gene may result from miscoding occurring at 4-OHEN-dC adducts during DNA synthesis catalyzed by pol η, pol κ, or both.

Oxidative DNA damage is also induced by 4-OHEN or 4-OHEQ. 4-OHEQ is autoxidized to an *o*-quinone, which isomerizes to 4-OHEN *o*-quinone (*12*). Reactive oxygen species generated during redox cycling between 4-OHEN *o*-quinone and its semiquinone radicals react with cellular DNA, resulting in an increased formation of 8-oxodG in vitro and in vivo (*14*−*18*, *36*). Since 8-oxodG generates G → T transversions by several DNA polymerases including pol η and pol κ (*37*−*39*), oxidative lesions may also be involved in the mutagenic events. In fact, 8-oxodG has been known to be mutagenic lesion primarily generating G → T transversions in mammalian cells (*40*, *41*). Generation of 8-oxodG at C:G pairs may contribute to the mutations observed with 4-OHEN.

When pol η was used, the bypass frequency past 4-OHEN-dC in the Pk-4 oligomer was 2 orders of magnitude higher than that for the Pk-3 oligomer, as observed for the primer extension reactions. Enzymatic digestion analysis of each isoform of 4-OHEN-dC-modified oligomers determined that Pk-3 and Pk-4 contain the fr-4 and fr-3 diastereoisomer of 4-OHEN-dC, respectively. Geacintov and his colleagues have recently indicated, using high-resolution 2D NMR spectroscopy, that two 4-OHEN-dC isomers (C-1 or C-3 shown in Figure 2) with positive circular dichroism absorbance at 260 nm ($CD_{260\ nm}$) were oriented toward the 5′-terminus in duplex DNA; the other two isomers (C-2 or C-4 in Figure 2) with negative $CD_{260\ nm}$ were oriented toward the 3′-terminus in duplex DNA (*42*, *43*). Since fr-3 and fr-4 have negative $CD_{260\ nm}$ and positive $CD_{260\ nm}$, respectively (data not shown), fr-3 in Pk-4-oligomer orients in the 3′-direction and fr-4 in Pk-3-oligomer orients in the 5′-direction in duplex DNA. Such adduct stereochemistry may affect the bypass efficiency observed with pol η.

In conclusion, pol κ and pol η can efficiently bypass 4-OHEN-dC, the major equine estrogen-derived adduct, by incorporating dAMP or dCMP opposite the lesion. Since pol η and pol κ are highly expressed in the reproductive organs, such as ovaries and uterus (*22*−*25*), the newly discovered human DNA polymerases are more likely to be associated with mutagenic events generated by equine estrogen DNA damage.

ACKNOWLEDGMENT

We thank Ms. M. Cecilia Torres for synthesizing unmodified oligodeoxynucleotides, Mr. Robert Rieger for MALDI-TOF mass spectrometry analysis, and Dr. Bongsup Cho, University of Rhode Island, for measurement of CD spectra of 4-OHEN-dC isomers.

REFERENCES

1. Grodstein, F., Stampfer, M. J., Colditz, G. A., Willett, W. C., Manson, J. E., Joffe, M., Rosner, B., Fuchs, C., Hankinson, S. E., Hunter, D. J., Hennekens, C. H., and Speizer, F. E. (1997)

Postmenopausal hormone therapy and mortality, *N. Engl. J. Med. 336*, 1769−1775.
2. Bolton, J. L., Pisha, E., Zhang, F., and Qiu, S. (1998) Role of quinoids in estrogen carcinogenesis. *Chem. Res. Toxicol. 11*, 1113−1127.
3. Grodstein, F., Stampfer, M. J., Colditz, G. A., Willett, W. C., Manson, J. E., Joffe, M., Rosner, B., Fuchs, C., Hankinson, S. E., Hunter, D. J., Hennekens, C. H., and Speizer, F. E. (1997) Postmenopausal hormone therapy and mortality, *N. Engl. J. Med. 336*, 1769−1775.
4. Colditz, G. A., Hankinson, S. E., Hunter, D. J., Willett, W. C., Manson, J. E., Stampfer, M. J., Hennekens, C. H., Rosner, B., and Speizer, F. E. (1995) The use of estrogens and progestins and the risk of breast cancer in postmenopausal women, *N. Engl. J. Med. 332*, 1589−1593.
5. Lacey, J. V., Jr., Mink, P. J., Lubin, J. H., Sherman, M. E., Troisi, R., Hartge, P., Schatzkin, A., and Schairer, C. (2002) Menopausal hormone replacement therapy and risk of ovarian cancer, *JAMA, J. Am. Med. Assoc. 288*, 334−341.
6. Grady, D., Gebretsadik, T., Kerlikowske, K., Emster, V., and Petitti, D. (1995) Hormone replacement therapy and endometrial cancer risk: a meta-analysis, *Obstet. Gynecol. 85*, 304−313.
7. Steinberg, K. K., Smith, S. J., Thacker, S. B., and Stroup, D. F. (1994) Breast cancer risk and duration of estrogen use: The role of study design in meta-analysis, *Epidemiology 5*, 415−421.
8. Judd, H. L., Mebane-Sims, I., Legault, C., Wasilauskas, C., Johnson, S., Merino, M., Barrett-Connor, B., and Trabal, J. (1996) Effects of hormone replacement therapy on endometrial histology in postmenopausal women, *JAMA, J. Am. Med. Assoc. 275*, 370−375.
9. Li, J. J., Li, S. A., Oberley, T. D., and Parsons, J. A. (1995) Carcinogenic activities of various steroidal and nonsteroidal estrogens in the hamster kidney: relation to hormonal activity and cell proliferation, *Cancer Res. 55*, 4347−4351.
10. Shen, L., Qiu, S., Chen, Y., Zhang, F., van Breemen, R. B., Nikolic, D., and Bolton, J. L. (1998) Alkylation of 2′-deoxynucleosides and DNA by the Premarin metabolite 4-hydroxyequilenin semiquinone radical, *Chem. Res. Toxicol. 11*, 94−101.
11. Embrechts, J., Lemiere, F., Van Dongen, W., Esmans, E. L., Buytaert, P., Van Marck, E., Kockx, M., and Makar, A. (2003) Detection of estrogen DNA-adducts in human breast tumor tissue and healthy tissue by combined nano LC-nano ES tandem mass spectrometry, *J. Am. Soc. Mass Spectrom. 14*, 482−491.
12. Zhang, F., Chen, Y., Pisha, E., Shen, L., Xiong, Y., van Breemen, R. B., and Bolton, J. L. (1999) The major metabolite of equilin, 4-hydroxyequilin, autoxidizes to an o-quinone which isomerizes to the potent cytotoxin 4-hydroxyequilenin-o-quinone, *Chem. Res. Toxicol. 12*, 204−213.
13. Terashima, I., Suzuki, N., and Shibutani, S. (2002) ^{32}P-Postlabeling/polyacrylamide gel electrophoresis assay: application to the detection of DNA adducts, *Chem. Res. Toxicol. 15*, 305−311.
14. Chen, Y., Shen, L., Zhang, F., Lau, S. S., van Breemen, R. B., Nikolic, D., and Bolton, J. L. (1998) The equine estrogen metabolite 4-hydroxyequilenin causes DNA single-strand breaks and oxidation of DNA bases in vitro, *Chem. Res. Toxicol. 11*, 1105−1111.
15. Han, X., and Liehr, J. G. (1995) Microsome-mediated 8-hydroxylation of guanine bases of DNA by steroid estrogens: correlation of DNA damage by free radicals with metabolic activation to quinones, *Carcinogenesis 16*, 2571−2574.
16. Chen, Y., Liu, X., Pisha, E., Constantinou, A. I., Hua, Y., Shen, L., van Breemen, R. B., Elguindi, E. C., Blond, S. Y., Zhang, F., and Bolton, J. L. (2000) A metabolite of equine estrogens, 4-hydroxyequilenin, induces DNA damage and apoptosis in breast cancer cell lines, *Chem. Res. Toxicol. 13*, 342−350.
17. Zhang, F., Yao, D., Hua, Y., van Breemen, R. B., and Bolton, J. L. (2001) Synthesis and reactivity of the catechol metabolites from the equine estrogen, 8,9-dehydroestrone, *Chem. Res. Toxicol. 14*, 754−763.
18. Zhang, F., Swanson, S. M., van Breemen, R. B., Liu, X., Yang, Y., Gu, C., and Bolton, J. L. (2001) Equine estrogen metabolite 4-hydroxyequilenin induces DNA damage in the rat mammary tissues: formation of single-strand breaks, apurinic sites, stable adducts, and oxidized bases, *Chem. Res. Toxicol. 14*, 1654−1659.
19. Yasui, M., Matsui, S., Santosh Laxmi, Y. R., Suzuki, N., Kim, S. Y., Shibutani, S., and Matsuda, T. (2003) Mutagenic events induced by 4-hydroxyequilin in *supF* shuttle vector plasmid propagated in human cells, *Carcinogenesis 24*, 911−917.
20. Goodman, M. F., and Tippen, B. (2000) The expanding polymerase universe, *Nat. Rev. Cell Biol. 1*, 101−109.
21. Kunkel, T. A., Pavlov, Y. I., and Bebenek, K. (2003) Functions of human DNA polymerases *η*, *κ* and *ι* suggested by their properties, including fidelity with undamaged DNA templates, *DNA Repair 3*, 135−149.
22. Gerlach, V. L., Aravind, L., Gotway, G., Schultz, R. A., Koonin, E. V., and Friedberg, E. C. (1999) Human and mouse homologues of *Escherichia coli* DinB (DNA polymerase IV), members of the UmuC/DinB superfamily, *Proc. Natl. Acad. Sci. U.S.A. 96*, 11922−11927.
23. Ogi, T., Kato, T., Jr., Kato, T., and Ohmori, H. (1999) Mutation enhancement by DINB1, a mammalian homologue of the *Escherichia coli* mutagenesis protein dinB, *Genes Cells 4*, 607−618.
24. Velasco-Miguel, S., Richardson, J. A., Gerlach, V. L., Lai, W. C., Gao, T., Russell, L. D., Hladik, C. L., White, C. L., and Friedberg, E. C. (2003) Constitutive and regulated expression of the mouse Dinb (Pol *κ*) gene encoding DNA polymerase *κ*, *DNA Repair 2*, 91−106.
25. Kawamura, K., Bahar, R., Seimiya, M., Chiyo, M., Wada, A., Okada, S., Hatano, M., Tokuhisa, T., Kimura, H., Watanabe, S., Honda, I., Sakiyama, S., Tagawa, M., and O-Wang, J. (2004) DNA polymerase *θ* is preferentially expressed in lymphoid tissues and upregulated in human cancers, *Int. J. Cancer 109*, 9−16.
26. Chang, M., Zhang, F., Shen, L., Pauss, N., Alam, I., van Breemen, R. B., Blond, S. Y., and Bolton, J. L. (1998) Inhibition of glutathione S-transferase activity by the quinoid metabolites of equine estrogens, *Chem. Res. Toxicol. 11*, 758−765.
27. Shibutani, S., Ithoh, S., and Yoshizawa, I. (1997) Miscoding properties of model estrogen-DNA adducts in reactions catalyzed by mammalian and *Escherichia coli* DNA polymerases, *Biochemistry 36*, 1755−1765.
28. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
29. Masutani, C., Araki, M., Yamada, A., Kusumoto, R., Nogimori, T., Maekawa, T., Iwai, S., and Hanaoka, F. (1999) Xeroderma pigmentosum variant (XP−V) correcting protein from HeLa cells has a thymine dimer bypass DNA polymerase activity, *EMBO J. 18*, 3491−3501.
30. Ohashi, E., Ogi, T., Kusumoto, R., Iwai, S., Masutani, C., Hanaoka, F., and Ohmori, H. (2000) Error-prone bypass of certain DNA lesions by the human DNA polymerase *κ*, *Genes Dev. 14*, 1589−1594.
31. Ohashi, E., Bebenek, K., Matsuda, T., Feaver, W. J., Gerlach, V. L., Friedberg, E. C., Ohmori, H., and Kunkel, T. A. (2000) Fidelity and processivity of DNA synthesis by DNA polymerase *κ*, the product of the human *DINB1* gene, *J. Biol. Chem. 275*, 39678−39684.
32. Mendelman, L. V., Boosalis, M. S., Petruska, J., and Goodman, M. F. (1989) Nearest neighbor influences on DNA polymerase insertion fidelity, *J. Biol. Chem. 264*, 14415−14423.
33. Mendelman, L. V., Petruska, J., and Goodman, M. F. (1990) Base mispair extension kinetics, *J. Biol. Chem. 265*, 2338−2346.
34. Clark, J. M., Joyce, C. M., and Beardsley, G. P. (1987) Novel blunt-end addition reactions catalyzed by DNA polymerase I of *Escherichia coli*, *J. Mol. Biol. 198*, 123−127.
35. Terashima, I., Suzuki, N., Dasaradhi, L., Tan, C.-K., Downey K. M., and Shibutani, S. (1998) 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, *Biochemistry 37*, 13807−13815.
36. Liu, X., Yao, J., Pisha, E., Yang, Y., Hua, Y., van Breemen, R. B., and Bolton, J. L. (2002) Oxidative DNA damage induced by equine estrogen metabolites: role of estrogen receptor a, *Chem. Res. Toxicol. 15*, 512−519.
37. Shibutani, S., Takeshita, M., and Grollman, A. P. (1991) Insertion of specific bases during DNA synthesis past the oxidation-damaged base 8-oxodG, *Nature 349*, 431−434.
38. Haracska, L., Prakash, L., and Prakash, S. (2002) Role of human DNA polymerase *κ* as an extender in translesion synthesis, *Proc. Natl. Acad. Sci. U.S.A. 99*, 16000−16005.
39. Zhang, Y., Yuan, F., Wu, X., Rechkoblit, O., Taylor, J. S., Geacintov, N. E., and Wang, Z. (2000) Error-prone lesion bypass by human DNA polymerase *η*, *Nucleic Acids Res. 28*, 4717−4724.
40. Moriya, M. (1993) Single strand shuttle phagemid for mutagenesis studies in mammalian cells: 8-oxoguanine in DNA induces

targeted G:C → T:A transversion in simian kidney cells, *Proc. Natl. Acad. Sci. U.S.A. 90*, 1122−1126.
41. Tan, X., Grollman, A. P., and Shibutani, S. (1999) Comparison of the mutagenic properties of 8-oxo-7,8-dihydro-2′-deoxyadenosine and 8-oxo-7,8-dihydro-2′-deoxyguanosine DNA lesions in mammalian cells, *Carcinogenesis 20*, 2287−2292.
42. Ding, S., Shapiro, R., Geacintov, N. E., and Broyde, S. (2003) Conformation of stereoisomeric base adducts to 4-hydroxyequilenin, *Chem. Res. Toxicol. 16*, 695−707.
43. Geacintov, N. E., Kolbanovskiy, A., Kuzmin, V., Chen, D., Shastry, A., Chang, M., and Bolton, J. L. (2004) Synthesis and characterization of site-specifically modified oligodeoxyribonucleotides containing single lesions derived from the equine estrogen metabolite (4-OHEN), *Proc. Am. Assoc. Cancer Res. 45*, 741−742.

BI049273N