Miscoding Properties of Model Estrogen−DNA Adducts in Reactions Catalyzed by Mammalian and *Escherichia coli* DNA Polymerases[†,‡]

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ABSTRACT: The miscoding properties of the model estrogen-derived DNA adducts, N^2-[3-methoxyestra-1,3,5(10)-trien-6-yl]-2′-deoxyguanosine (dG-N^2-3MeE) and N^6-[3-methoxyestra-1,3,5(10)-trien-6-yl]-2′-deoxyadenosine (dA-N^6-3MeE), have been explored, using an *in vitro* experimental system to quantify base substitutions and deletions. Site-specifically modified oligodeoxynucleotides containing a single dG-N^2-3MeE or dA-N^6-3MeE were prepared postsynthetically and used as templates in primer extension reactions catalyzed by *Escherichia coli* and mammalian DNA polymerases. When the 3′ → 5′ exonuclease free (exo$^-$) Klenow fragment of DNA polymerase I was used, dG-N^2-3MeE promoted mostly one- and two-base deletions, along with small amounts of incorporation of dAMP, dGMP, and dCMP opposite the lesion. dA-N^6-3MeE promoted the incorporation of dTMP opposite the lesion as well as two-base deletions, accompanied by the incorporation of dAMP. Using pol α, primer extension reactions were blocked at dG-N^2-3MeE; however, dA-N^6-3MeE promoted preferential incorporation of dTMP opposite the lesion with small amounts of incorporation of dCMP and deletions. Primer extension reactions catalyzed by pol δ were blocked at these lesions. When pol β was used, dG-N^2-3MeE produced small amounts of incorporation of dAMP and deletions. dA-N^6-3MeE promoted preferential incorporation of dTMP, along with incorporation of dCMP and two-base deletions. The miscoding specificities and frequencies varied depending on the DNA polymerase used. These results indicate that estrogen−DNA adducts have miscoding potential.

Estrogens and their metabolites have been implicated in the etiology of breast cancer (Zumoff, 1993) and have been shown to induce several different tumors in rodents [reviewed in IARC Monographs (1979)]. Like other steroid hormones, estrogens are thought to act by regulating gene expression. The mechanism of the carcinogenic effect of estrogens is unknown and may relate to promotion and/or initiation of cancer (Henderson *et al*., 1988; Fishman *et al*., 1995). DNA damage is an initiating event in human cancer and may lead to mutations (Lawley, 1994). DNA adducts have been found in the tissues of rodents treated with natural and synthetic estrogens (Liehr *et al*., 1986; Gladek & Liehr, 1989; Liehr, 1990). However, the chemical structures of these adducts are unknown, and their mutagenic properties have not been investigated.

Endogenous and exogenous estrogens undergo oxidation by cytochrome P450 enzymes, mainly at C-2, C-4, and C-6 (Martucci & Fishman, 1993). Estrogen 2- and 4-hydroxylases metabolize estrogens to catecholestrogens, which, in turn, are oxidized to form semiquinones and quinones by cytochrome P450 (Martucci & Fishman, 1993). The 2,3- and 3,4-quinones of estrogens are thought to be reactive intermediates that directly bind to DNA (Dwivedy *et al*., 1992). When estrone 2,3-quinone (E_1-2,3-Q)[1] was reacted with dG or dA, N^2-(2-hydroxyestron-6-yl)-2′-deoxyguanosine (2-OHE_1-6-N^2-dG) and N^6-(2-hydroxyestron-6-yl)-2′-deoxyadenosine (2-OHE_1-6-N^6-dA) (the structures in Figure 1), respectively, were formed (Stack *et al*., 1996). Reaction of estrone 3,4-quinone (E_1-3,4-Q) with dG produced 7-[4-hydroxyestron-1(α,β)-yl]guanine, with loss of deoxyribose (Stack *et al*., 1996).

The C-6 of estrogens can also be oxidized (Breuer *et al*., 1966) by a mechanism similar to that of bile acids and androgens (Zimniak *et al*., 1991). 6-Hydroxyestrogens were

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[1] Abbreviations: 3MeE-6α-S, pyridinium 3-methoxyestra-1,3,5(10)-trien-6α-yl sulfate; 3MeE-6β-S, pyridinium 3-methoxyestra-1,3,5(10)-trien-6β-yl sulfate; 3MeE-6α-OH, 3-methoxy-6α-hydroxyestra-1,3,5(10)-triene; 3MeE-6β-OH, 3-methoxy-6β-hydroxyestra-1,3,5(10)-triene; dG-N^2-6α-3MeE, N^2-[3-methoxyestra-1,3,5(10)-trien-6α-yl]-2′-deoxyguanosine; dG-N^2-6β-3MeE, N^2-[3-methoxyestra-1,3,5(10)-trien-6β-yl]-2′-deoxyguanosine; dG-N^2-3MeE, a mixture of dG-N^2-6α-3MeE and dG-N^2-6β-3MeE; dA-N^6-6α-3MeE, N^6-[3-methoxyestra-1,3,5(10)-trien-6α-yl]-2′-deoxyadenosine; dA-N^6-6β-3MeE, N^6-[3-methoxyestra-1,3,5(10)-trien-6β-yl]-2′-deoxyadenosine; dA-N^6-3MeE, a mixture of dA-N^6-6α-3MeE and dA-N^6-6β-3MeE; A-N^6-6α-3MeE, N^6-[3-methoxyestra-1,3,5(10)-trien-6α-yl]adenine; A-N^6-6β-3MeE, N^6-[3-methoxyestra-1,3,5(10)-trien-6β-yl]adenine; E_1, estrone; 6-OHE_1, 6-hydroxyestrone; E_1-2,3-Q, estrone 2,3-quinone; E_1-3,4-Q, estrone 3,4-quinone; 2-OHE_1-6-N^2-dG, N^2-(2-hydroxyestron-6-yl)-2′-deoxyguanosine; 2-OHE_1-6-N^6-dA, N^6-(2-hydroxyestron-6-yl)-2′-deoxyadenosine; dG, 2′-deoxyguanosine; dA, 2′-deoxyadenosine; A, adenine; dNTP, 2′-deoxynucleoside triphosphate; exo$^-$, 3′ → 5′ exonuclease free Klenow fragment; pol I, DNA polymerase I; 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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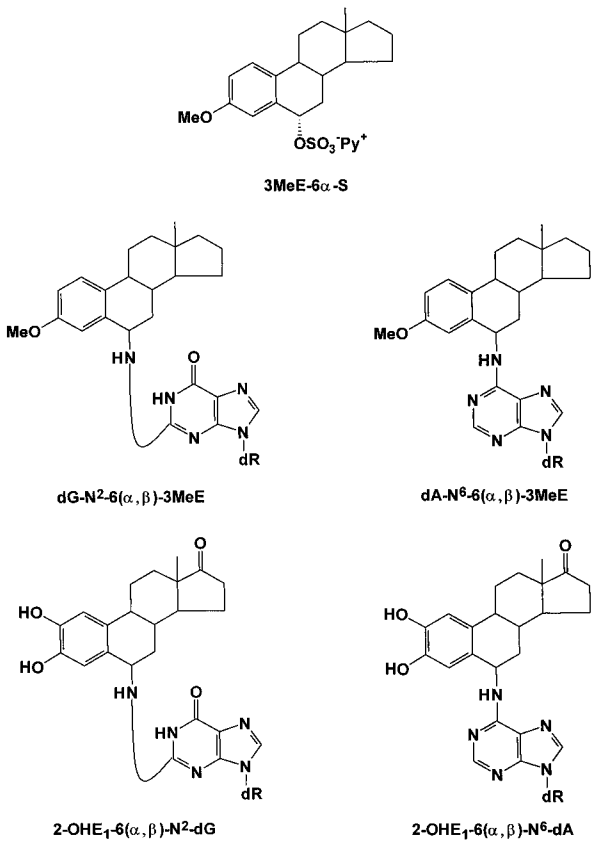

FIGURE 1: Structures of 3MeE-6α-sulfate and related estrogen−DNA adducts.

formed from estrone or estradiol by rodent hepatic and renal microsomes (Haaf *et al*., 1992; Suchar *et al*., 1995). We have proposed (Takagi *et al*., 1991) that the hydroxyl group at C-6 is sulfonated, as shown for the carcinogens, estragole (Phillip *et al*., 1981), and 7,12-dimethylbenz[*a*]anthracene (Watabe *et al*., 1982), and that the highly reactive benzyl esters may form covalent adducts with DNA. Pyridinium 3-methoxyestra-1,3,5(10)-trien-6α-yl sulfate (3MeE-6α-S, Figure 1) or 3MeE-6β-S was synthesized as a model-activated form of estrogen (Takagi *et al*., 1991). In fact, 3MeE-6α-S or 3MeE-6β-S was reactive, forming several modified nucleosides such as N^2-[3-methoxyestra-1,3,5(10)-trien-6-yl]-2′-deoxyguanosine (dG-N^2-3MeE) and N^6-[3-methoxyestra-1,3,5(10)-trien-6-yl]-2′-deoxyadenosine (dA-N^6-3MeE) (Figure 1) (Hirai et al., 1994; Itoh *et al*., 1996). The binding positions of 3MeE-6α-S or 3MeE-6β-S to dG and dA were similar to that induced by E_1-2,3-Q. Thus, dG-N^2-3MeE and dA-N^6-3MeE may also be used as model DNA adducts for exploring the mutagenic specificities of estrogens.

In this paper, we have prepared site-specifically modified oligodeoxynucleotides containing a single dG-N^2-3MeE or dA-N^6-3MeE. Using these modified oligomer templates, miscoding properties of model estrogen−DNA adducts were investigated, in an *in vitro* experimental system that can quantify all base substitutions and deletions (Shibutani, 1993). This is the first evidence that estrogen−DNA adducts have miscoding potential.

EXPERIMENTAL PROCEDURES

Materials and Methods. Organic chemicals used for the synthesis of oligodeoxynucleotides were supplied by Aldrich Chemical Co. [γ-^{32}P]ATP (specific activity, >6000 Ci/mmol) was obtained from Amersham Corp. Cloned exo$^-$ Klenow fragment of *Escherichia coli* DNA polymerase I [21 200 units/(mg of protein)] was purchased from United State Biochemical Corp. Calf thymus DNA pol α [30 000 units/(mg of protein)] and human pol β [100 000 units/(mg of protein)] were from Molecular Biology Resources, Inc. T4 polynucleotide kinase was from Stratagene. *Eco*RI restriction endonuclease (100 units/μL) and T4 DNA ligase (400 units/μL) were from New England BioLabs. HPLC grade acetonitrile, triethylamine, and distilled water were purchased from Fisher Chemical. A Waters 990 HPLC instrument, equipped with a photodiode array detector, was used for the separation and purification of the oligodeoxynucleotides.

Table 1 Sequence of oligodeoxynucleotides[a]

Number	Sequence 5'	3'
1	CCTTCGCTTCTTTCCTCTCCCTTT	
2	CCTTCACTTCTTTCCTCTCCCTTT	
3	CCTTC**X**CTTCTTTCCTCTCCCTTT	
4	CATGCTGATGAATTCCTTC**X**CTTCTTTCCTCTCCCTTT	
5	CATGCTGATGAATT	
6	GAAGCGAAGGAATTCATC	
7	AGAGGAAAGA	
8	AGAGGAAAGAAG	
9	AGAGGAAAGAAG**N**	
10	AGAGGAAAGAAG**N**GAAGG	
11	AGAGGAAAGAAGGAAGG	
12	AGAGGAAAGAAGAAGG	

[a]Sequence of templates, primers, and standard markers. X = dG-N^2-3MeE, dA-N^6-3MeE, dG, or dA; N = dC, dA, dG, or dT.

Synthesis of Model Estrogen-Modified Nucleosides. Pyridinium 3-methoxyestra-1,3,5(10)-trien-6α-yl sulfate (3MeE-6α-S) and pyridinium 3-methoxyestra-1,3,5(10)-trien-6β-yl sulfate (3MeE-6β-S) were synthesized according to the previous paper (Takagi *et al*., 1991). 2′-Deoxyguanosine (dG, 1.4 g) was reacted with 3MeE-6α-S (0.7 g) in a reaction solution of 270 mL of 50 mM NaH_2PO_4/Na_2HPO_4 buffer at pH 7.0 and 30 mL of tetrahydrofuran. The reaction mixture was stirred at 37 °C for 20 min and extracted four times with 200 mL of ethyl acetate (EtOAc). The combined EtOAc fractions were back-extracted with distilled water, dried over anhydrous Na_2SO_4, and evaporated to dryness. N^2-[3-Methoxyestra-1,3,5(10)-trien-6α-yl]-2′-deoxyguanosine (dG-N^2-6α-3MeE, 1 mg) and N^2-[3-methoxyestra-1,3,5(10)-trien-6β-yl]-2′-deoxyguanosine (dG-N^2-6β-3MeE, 9 mg) were isolated by HPLC, using an ODS-80T_M column (0.75 × 30 cm, Toso, Tokyo). The column was eluted with MeOH/H_2O (3:1, v:v) at a flow rate of 2.0 mL/min. When 3MeE-6β-S was used as a reactive form, a similar product was obtained. 2′-Deoxyadenosine (dA, 1.77 g) was reacted with 3MeE-6α-S (1.0 g) using the same reaction conditions described for dG. N^6-[3-Methoxyestra-1,3,5(10)-trien-6β-yl]-2′- deoxyadenosine (dA-N^6-6β-3MeE, 6.0 mg) was isolated as a major product by HPLC (Hirai et al., 1994). N^6-[3-Methoxyestra-1,3,5(10)-trien-6α-yl]adenine (A-N^6-6α-3MeE) and N^6-[3-methoxyestra-1,3,5(10)-trien-6β-yl]adenine (A-N^6-6β-3MeE) were synthesized according to the previous paper (Itoh *et al*., 1996).

Synthesis of Oligodeoxynucleotides. DNA template, primer, and standard markers, listed in Table 1, were prepared by solid-state synthesis on an automated DNA synthesizer (Takeshita *et al*., 1987). DNA template containing a single

dG-N^2-3MeE or dA-N^6-3MeE was prepared by reacting 1.0 mg of unmodified 24-mer containing a single dG (sequence 1 in Table 1) or dA (sequence 2) with 4.0 mg of 3MeE-6α-S for 30 min at 37 °C in 500 μL of 50 mM NaH_2PO_4/Na_2HPO_4 buffer at pH 7.0. After the reaction, the samples were centrifuged and the supernatants were subjected to HPLC. The dG-N^2-3MeE- or dA-N^6-3MeE-modified and unmodified oligomers were isolated on a Waters reverse-phase μBondapak C_{18} column (0.39 × 30 cm), using a linear gradient of 0.05 M triethylammonium acetate (pH 7.0) containing 10 to 50% acetonitrile with an elution time of 60 min and a flow rate of 1.0 mL/min as described elsewhere (Shibutani *et al*., 1991a). DNA templates and primers were further purified by electrophoresis on a 20% polyacrylamide gel in the presence of 7 M urea (35 × 42 × 0.04 cm) (Shibutani *et al*., 1993). The oligomers recovered from PAGE were again subjected to HPLC to remove urea. Oligomers were labeled at the 5′ terminus by treating with T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP (Maniatis *et al*., 1982) and subjected to electrophoresis to establish homogeneity. The position of the oligomers was established by autoradiography, using Kodak Xomat XAR film.

Enzymatic Digestion. A dG-N^2-3MeE- or dA-N^6-3MeE-modified 24-mer oligodeoxynucleotide (3.0 μg, sequence 3) was digested with nuclease P1 (2 units) and alkaline phosphatase (3 units) as described previously (Shibutani *et al*., 1993). Methanol extract obtained from the digested sample was evaporated to dryness and analyzed by HPLC, using a Supelcosil LC-18S column (0.46 × 25 cm, Supelco, Inc.). The column was eluted at 40 °C with 50 mM $(NH_4)H_2PO_4$ (pH 2.5) containing 0 to 20% methanol over 20 min and subsequently 20 to 75% over 5 min at a flow rate of 1.0 mL/min. The eluate of dA-N^6-3MeE was collected, evaporated to dryness, and hydrolyzed for 30 min at 60 °C with 50 μL of 0.1 N HCl and 2.5 μL of DMSO to determine the ratio of 6α- and 6β-isomeric form of dA-N^6-3MeE. The sample was subjected to HPLC, eluting at 40 °C with 50 mM $(NH_4)H_2PO_4$ (pH.2.5)/MeOH (25:75) at a flow rate of 1.0 mL/min.

Primer Extension Reactions. Using a ^{32}P-labeled 10-mer (0.5 pmol, sequence 7 in Table 1) primed with a 24-mer template (0.75 pmol, sequence 2), primer extension reactions, catalyzed by DNA polymerases, were carried out at 25 °C in 10 μL of a buffer containing all four dNTPs (100 μM each) (Shibutani *et al*., 1991b). The reaction buffer for exo$^-$ Klenow fragment consisted of 50 mM Tris-HCl (pH 8.0), 8 mM $MgCl_2$, and 5 mM 2-mercaptoethanol. The 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). DNA pol δ and proliferating cell nuclear antigen (PCNA) were provided by Dr. Matsumoto (Matsumoto *et al*., 1994). The buffer for pol δ (0.014 unit) 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 formamide dye and heating to 95 °C for 3 min. Samples were subjected to electrophoresis on a 20% polyacrylamide gel containing 7 M urea (35 × 42 × 0.04 cm). Bands were identified by autoradiography and excised from the gel, and the radioactivities were measured with a Wallac liquid scintillation counter.

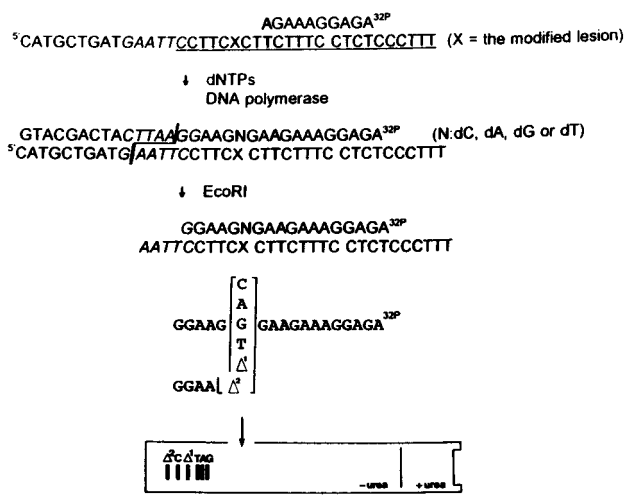

FIGURE 2: Diagram of the primer extension methods and analysis of reaction products.

Quantitation of Miscoding Specificity. Three micrograms of dG-N^2-3MeE- or dA-N^6-3MeE-modified 24-mer (sequence 3) was phosphorylated at the 5′ terminus using 7 μL of T4 kinase and 3 μL of 10 mM ATP (Maniatis *et al*., 1982) and then ligated to a 14-mer (3.6 μg, sequence 5) at 8 °C overnight using 3 μL of T4 DNA ligase, 2 μL of 10 mM ATP, and a 18-mer template (4.5 μg, sequence 6). The resultant 38-mer (sequence 4) was isolated by HPLC as described above (Shibutani *et al*., 1991a). Using a 38-mer template (0.75 pmol) primed with a ^{32}P-labeled 12-mer (0.5 pmol, sequence 8), primer extension reactions catalyzed by DNA polymerases were conducted at 30 °C for 1 h in the presence of four dNTPs. The reaction samples were subjected to 20% PAGE with 7 M urea (35 × 42 × 0.04 cm). The fully extended products were recovered from the gel, annealed with an unmodified 38-mer (sequence 4, X = C), and cleaved with *Eco*RI restriction enzyme (100 units) at 30 °C for 1 h and subsequently at 15 °C for 1 h as shown in Figure 2. To quantify all base substitutions and deletions, the 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 (Shibutani, 1993). The ^{32}P-labeled products (GGAAGNGAAGAAAGGAGA^{32P}) can be separated from an unlabeled 28-mer (5′AATTCCTTCXCTTCTTTCCTCTCCCTTT, X = C) in the upper phase and then completely resolved on the lower phase. The detection limit was 0.03% of the starting primer (Shibutani, 1993).

dNTP Incorporation Opposite Lesions and the Chain Extension. Unmodified or modified templates (sequences 1−3) were primed with a ^{32}P-labeled 12-mer (sequence 8) for the determination of dNTP insertion opposite the lesion or with a ^{32}P-labeled 13-mer (sequence 9) for the determination of the chain extension. The amounts of dNMP incorporation opposite the lesions were measured at 30 °C in the presence of a single dNTP. The amounts of chain extension were measured at 30 °C in the presence of a single dGTP. The percentage of dNMP incorporation or extension was determined by gel electrophoresis as described previously (Shibutani *et al*., 1993).

RESULTS

Quantitative Method for Analyzing Miscoding Properties in Vitro. We established an *in vitro* experimental system to

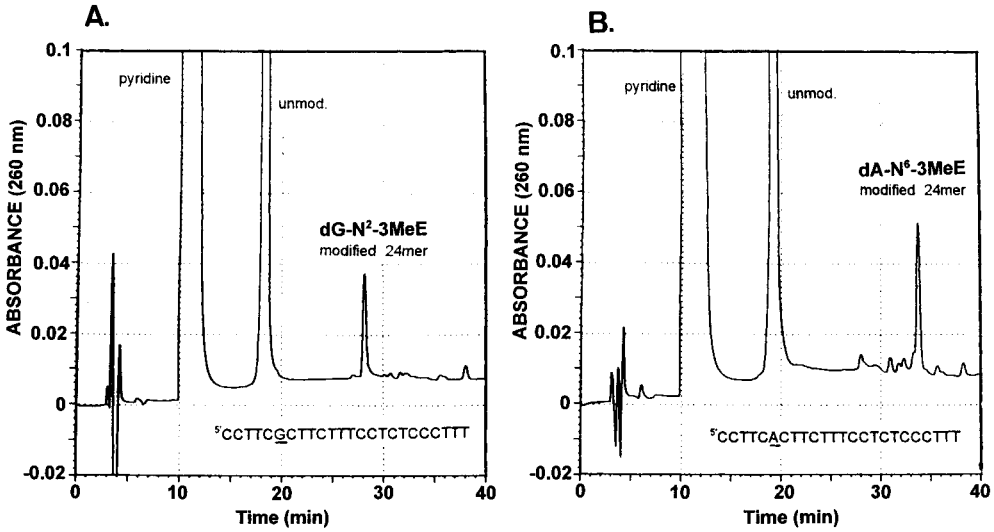

FIGURE 3: HPLC separation of 24-mer oligodeoxynucleotide containing a single dG-N^2-3MeE or dA-N^6-3MeE. One milligram of 24-mer containing a single dG or dA was reacted for 30 min at 37 °C with 4.0 mg of 3MeE-6α-S in 500 μL of 50 mM phosphate buffer at pH 7.0. After the centrifugation, one-tenth of the reaction sample containing dG-modified 24-mer (A) or one-fifth of the reaction sample containing dA-modified 24-mer (B) was subjected to HPLC as described in Experimental Procedures.

quantify base substitutions and deletions (Shibutani, 1993). Template oligomers used for this assay contain three purine bases that can be modified by 3MeE-6α-S, an activated form of the model estrogen (Itoh *et al.*, 1996). To avoid multiple modification of templates, new sequences of 24-mer template oligomers containing a single purine base (sequences 1 and 2 in Table 1), underlined in Figure 2, were designed. Standards representing products containing dC, dA, dG, or dT opposite the lesion or one- and two-base deletions were completely resolved on the two-phase gel, depending on their different migrations (Figure 2). In some cases, DNA polymerases generated blunt-end additions (Clark *et al.*, 1987) during DNA synthesis, interfering with the quantitative determination of miscoding properties (Shibutani *et al.*, 1996). Therefore, using a 38-mer template containing an *Eco*RI site, fully extended products produced during DNA synthesis were cleaved with *Eco*RI and were subjected to two-phase gel electrophoresis to quantify miscoding properties (Figure 2). Since *Eco*RI cut the fully extended products efficiently (>98%), this new experimental system allows us to quantify all base substitutions and deletions induced by model estrogen-derived adducts.

Preparation of Site-Specifically Modified Oligodeoxynucleotides Containing a Single Model Estrogen Adduct. When a 24-mer oligodeoxynucleotide containing a single dG (sequence 1 in Table 1) or dA (sequence 2) was reacted with 3MeE-6α-S, dG-N^2-3MeE- (t_R = 28.2 min) and dA-N^6-3MeE-modified (t_R = 33.8 min) 24-mers were isolated, respectively, from the corresponding oligomers by HPLC (Figure 3). The yields of dG-N^2-3MeE- and dA-N^6-3MeE-modified 24-mers were 2.7 and 1.7%, respectively.

When a mixture of four deoxynucleosides (dN), dG-N^2-6α-3MeE, dG-N^2-6β-3MeE, and dA-N^6-6β-3MeE was subjected to HPLC, all standard compounds were separated completely (Figure 4A). After the dG-N^2-3MeE-modified 24-mer was digested enzymatically, dG-N^2-6α-3MeE (t_R = 41.8 min) and dG-N^2-6β-3MeE (t_R = 42.9 min) were detected with a 1:5.5 ratio (Figure 4B). The molar ratio of dC and dT (11.3:12.4) obtained was consistent with the theoretical value (dC:dT = 11:12) of the nucleoside composition of the 24-mer. When the dA-N^6-3MeE-modified 24-mer was digested, a peak representing dA-N^6-3MeE (t_R = 62.0 min) was detected (Figure 4C). The fraction of dA-N^6-3MeE was collected, hydrolyzed using the acidic condition, and subjected to HPLC using a different elution condition. As shown in Figure 5A, a standard of N^6-(3MeE-6β-yl)adenine (A-N^6-6β-3MeE) can be separated from N^6-(3MeE-6α-yl)adenine (A-N^6-6α-3MeE). A-N^6-6β-3MeE was only detected in the recovered sample (Figure 5B). Thus, dA-N^6-3MeE-modified oligodeoxynucleotide contained only dA-N^6-6β-3MeE. These modified 24-mer oligomers, underlined in Figure 2, were further purified by gel electrophoresis and ligated to a 14-mer to prepare 38-mer templates used for the *in vitro* mutagenesis studies.

Miscoding Properties of 3MeE-6α-S-Derived DNA Adducts. Primer extension reactions catalyzed by DNA polymerases were conducted in the presence of all four dNTPs on a 38-mer template containing a single dG-N^2-3MeE or dA-N^6-3MeE lesion. Using unmodified templates, primer extension reactions catalyzed by pol α occurred rapidly, forming the fully extended products (Figure 6A). However, using dG-N^2-3MeE- and dA-N^6-3MeE-modified templates, the primer extensions were blocked one base before the lesions and opposite the lesions. Some of the primers passed dG-N^2-3MeE (0.1%) and dA-N^6-3MeE (1.5%), respectively, to form the fully extended products (Figure 6A).

Using large amounts of pol α and longer reaction times, fully extended products formed during DNA synthesis were recovered from the gel, cleaved with *Eco*RI enzyme, and subjected to the two-phase gel electrophoresis to quantify the miscoding specificities. Standards representing products containing dC, dA, dG, or dT opposite the lesion or one- and two-base deletions were completely resolved on the gel (Figure 7, lanes 2 and 4 and Figure 9, lanes 2 and 5). DNA synthesis on unmodified templates led to the expected incorporation of dCMP opposite dG (Figure 9, lane 1) and dTMP opposite dA (Figure 7, lane 1). Excess amounts of reaction mixture obtained from synthesis on the unmodified templates were subjected to the two-phase gel (Figure 7, lane 1, and Figure 9, lane 1); therefore, overloading caused

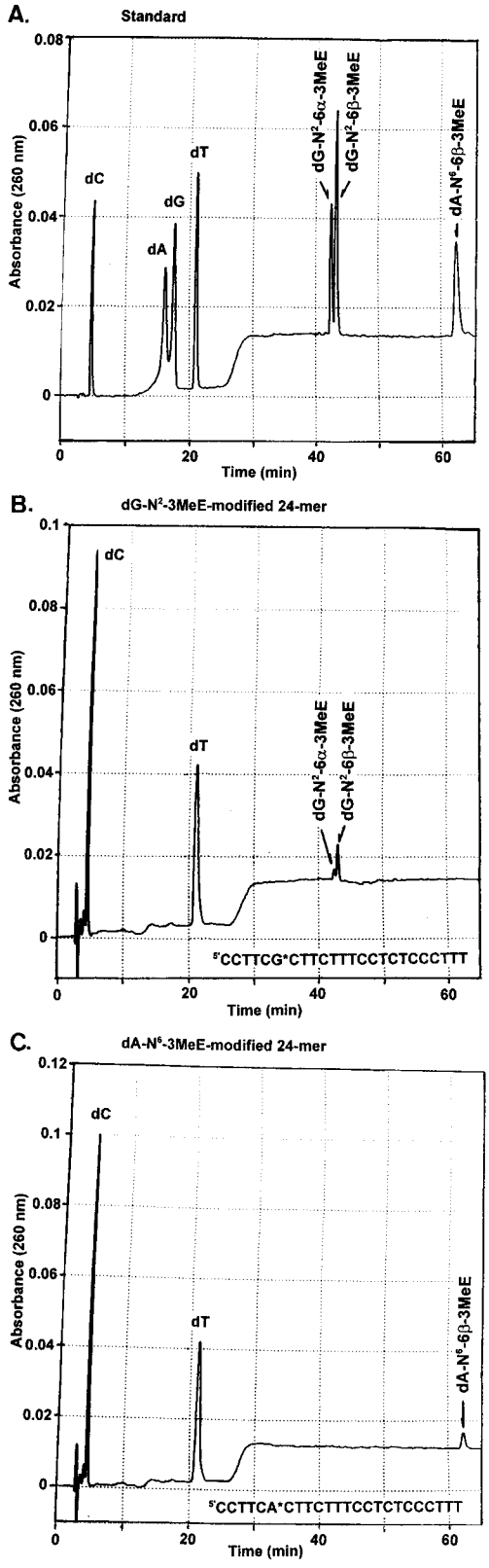

FIGURE 4: Enzymatic digestion of dG-N^2-3MeE- or dA-N^6-3MeE-modified 24-mer. (A) A standard mixture of dNs, dG-N^2-6α-3MeE, dG-N^2-6β-3MeE, and dA-N^6-6β-3MeE was passed through a Supelcosil LC-18S column. The column was eluted at 40 °C with 50 mM $(NH_4)H_2PO_4$ (pH 2.5) containing 0−20% methanol over 20 min and subsequently 20−75% over 5 min at a flow rate 1.0 mL/min, as described in Experimental Procedures. Three micrograms of dG-N^2-3MeE- (B) or dA-N^6-3MeE-modified (C) 24-mer was digested with nuclease P1 and alkaline phosphatase. Methanol extract of the digested sample was evaporated to dryness and analyzed by HPLC as described in Experimental Procedures.

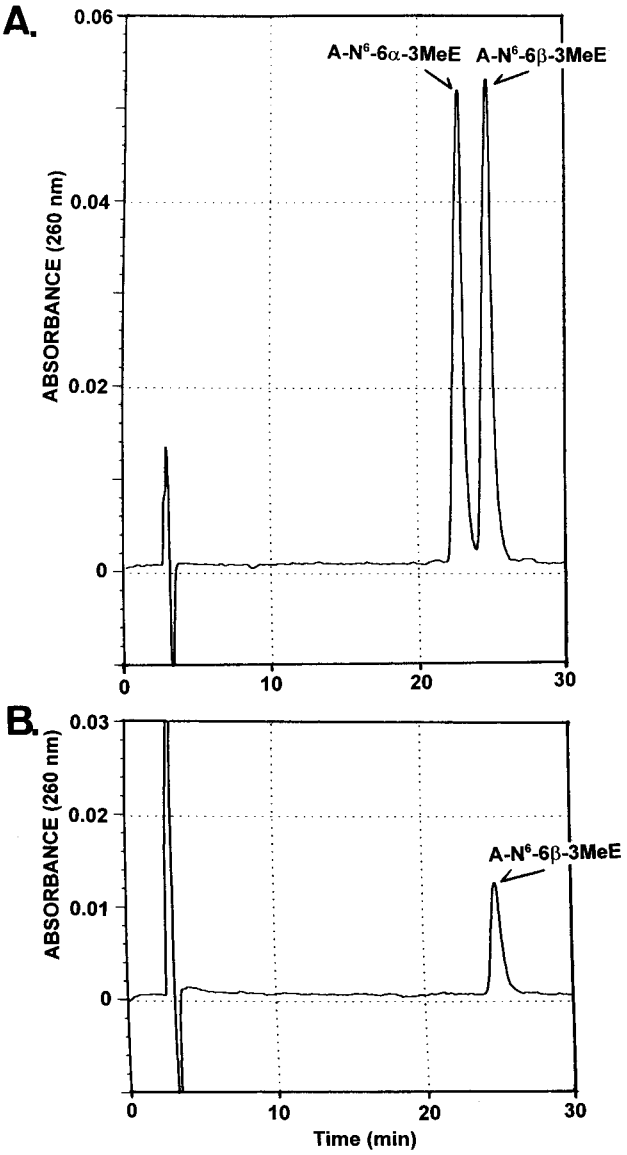

FIGURE 5: HPLC separation of N^6-(3MeE-6β-yl)adenine and N^6-(3MeE-6α-yl)adenine. The peak appearing at 62 min in Figure 4C was recovered from several separate samples, evaporated to dryness, and hydrolyzed for 30 min at 60 °C with a mixture of 50 μL of 0.1 N HCl and 2.5 μL of DMSO. The sample was subjected to HPLC, eluting at 40 °C with 50 mM $(NH_4)H_2PO_4$ (pH 2.5)/MeOH (25:75) at a flow rate of 1.0 mL/min (B) and compared with standard markers of A-N^6-6β-3MeE and A-N^6-6α-3MeE (A).

broadening of bands below and above the major bands. In addition, since amounts of pol α or pol β 2 or 3 times higher were used for the primer extension reactions on unmodified templates, very small amounts of misincorporation of dCMP was seen opposite dA (Figure 7, lane 1). With pol α, a mammalian replicative enzyme, dA-N^6-3MeE promoted preferential incorporation of dTMP (8.7%) opposite the lesion, accompanied by small amounts of one- (0.25%) and two-base (1.0%) deletions (Figure 7, lane 3). With longer exposure, a trace of incorporation of dCMP (0.04%) was also observed (data not shown). However, amounts of fully extended products that passed dG-N^2-3MeE were insufficient to quantify the miscoding property.

Using the reaction condition containing a single dNTP, the frequencies of dNMP insertion opposite dG-N^2-3MeE or dA-N^6-3MeE were determined, using a ^{32}P-labeled 12-mer primed template. The frequencies of chain extension

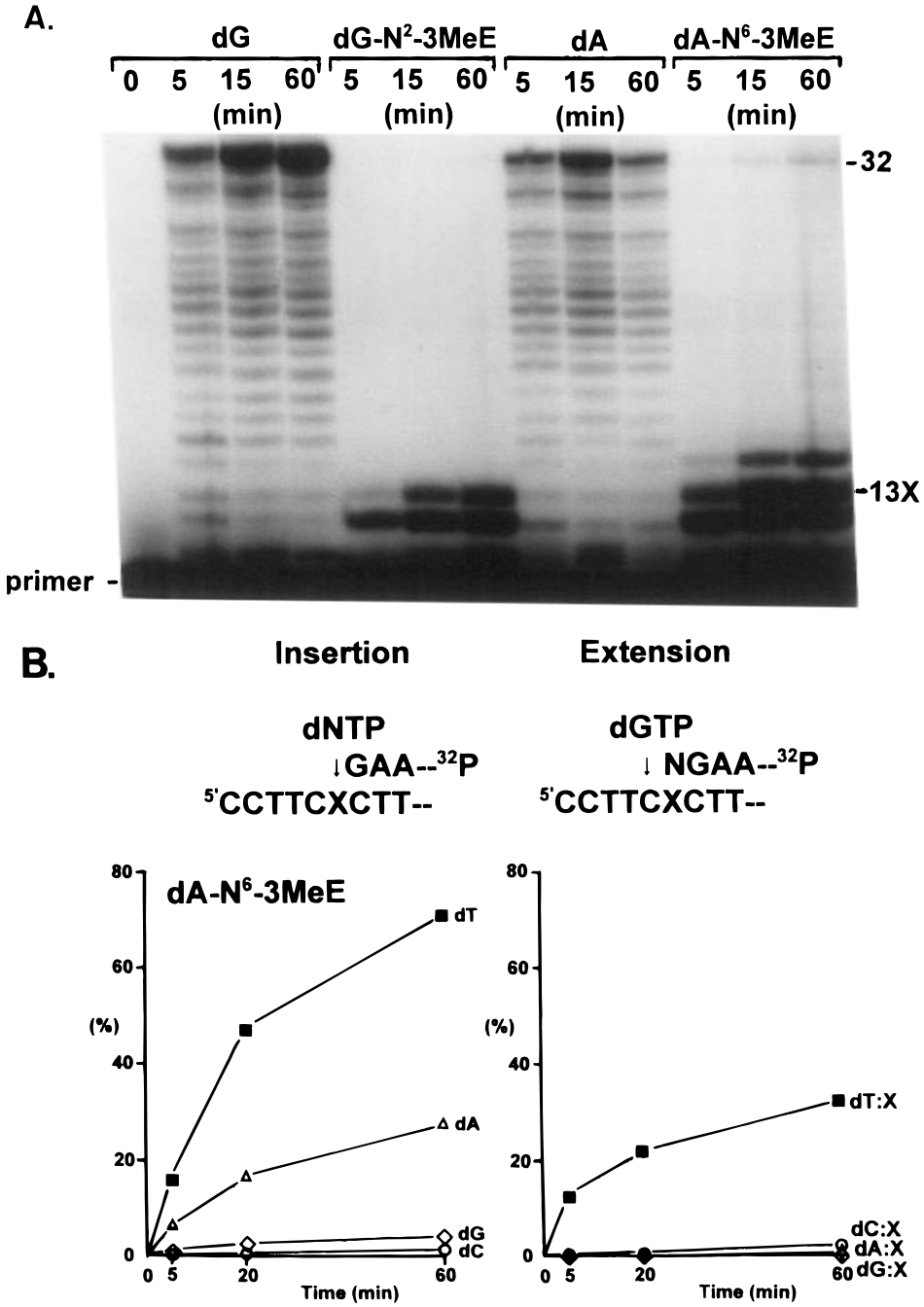

FIGURE 6: Primer extension reactions catalyzed by pol α. (A) Using unmodified or dG-N^2-3MeE- or dA-N^6-3MeE-modified 38-mer templates (sequence 4 in Table 1) primed with a 5′-^{32}P-labeled 10-mer (sequence 7), primer extension reactions were conducted at 25 °C, using 0.2 unit of pol α for unmodified templates and 2.4 units for dG-N^2-3MeE- and dA-N^6-3MeE-modified templates as described in Experimental Procedures. One-third of the reaction mixture was subjected to denaturing 20% polyacrylamide gel electrophoresis (35 × 42 × 0.04 cm). 13X shows the location opposite the dG-N^2-3MeE or dA-N^6-3MeE lesion. (B) Using a dA-N^6-3MeE-modified 24-mer template (sequence 3 in Table 1) primed with a 5′-^{32}P-labeled 12-mer (sequence 8) or 5′-^{32}P-labeled 13-mer (sequence 9), nucleotide insertion opposite the lesion or the chain extension was measured at 30 °C, using 1.2 units of pol α as described in Experimental Procedures. One-third of the reaction mixture was subjected to denaturing 20% polyacrylamide gel electrophoresis (35 × 42 × 0.04 cm).

were measured, using a ^{32}P-labeled 13-mer primed template. When dG-N^2-3MeE-modified template was used, the incorporation of dCMP (4.5%), a correct base, only was detected; however, no significant chain extension reactions were observed (data not shown). In contrast, with dA-N^6-3MeE-modified template, dTMP, a correct base, was inserted preferentially opposite the lesion, accompanied by the incorporation of dAMP (Figure 6B). The order of dNTP incorporation opposite dA-N^6-3MeE was dTMP > dAMP ≫ dGMP and dCMP. In the chain extension reaction, the dT·dA-N^6-3MeE pair extended much further than the other pairs (Figure 6B). Thus, the fully extended products containing dT opposite dA-N^6-3MeE were formed preferentially (Figure 7, lane 3).

Primer Extension Reactions Catalyzed by Pol β and Exo⁻ Klenow Fragment. When pol β, functioning as a repair enzyme, was used in a similar study, the primer extensions were blocked one base before the lesions and opposite the lesions (Figure 8); amounts of primer blocked one base before dG-N^2-3MeE were higher than that of dA-N^6-3MeE,

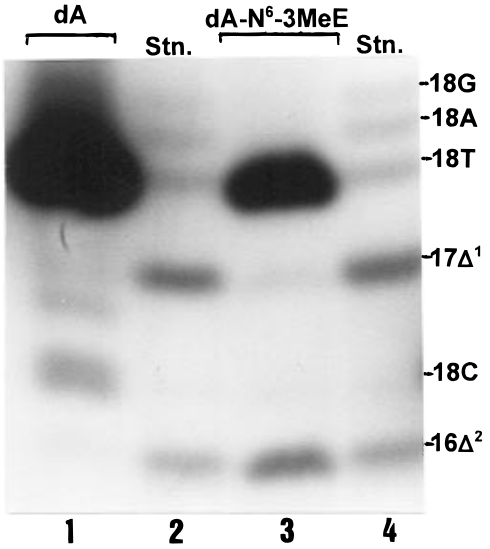

FIGURE 7: Quantitation of miscoding specificities induced by dA-N^6-3MeE. Using experimental conditions similar to those shown in Figure 6A, primer extension reactions were conducted for 3 h at 25 °C, using 0.6 unit of pol α for the unmodified template (lane 1) and 4.8 units for dA-N^6-3MeE-modified template (lane 3). The reaction sample was subjected to denaturing 20% polyacrylamide gel electrophoresis (35 × 42 × 0.04 cm). The fully extended products recovered from the gel were annealed with 38-mer (sequence 4) and cleaved by *Eco*RI as described in Experimental Procedures. The reaction samples were subjected to a two-phase 20% polyacrylamide gel electrophoresis (15 × 72 × 0.04 cm). Mobilities of reaction products were compared with those of 18-mer standards (sequences 10−12) containing dC, dA, dG, or dT opposite the lesion and one-base (Δ^1) or two-base (Δ^2) deletions (lanes 2 and 4, respectively).

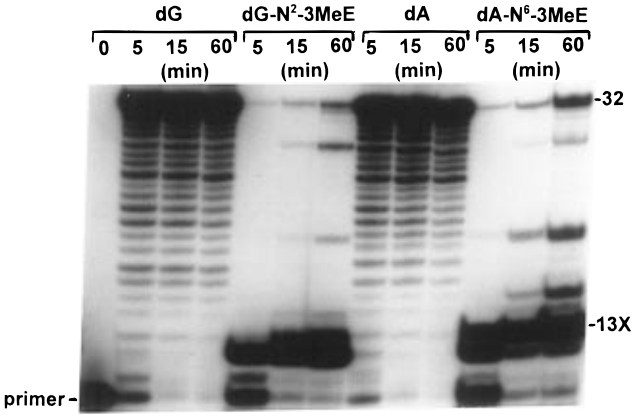

FIGURE 8: Primer extension reactions catalyzed by pol β. Using experimental conditions similar to those shown in Figure 6, primer extension reactions were conducted at 25 °C, using 0.2 unit of pol β for unmodified templates and 2.0 units for dG-N^2-3MeE- and dA-N^6-3MeE-modified templates as described in Experimental Procedures.

while amounts of primer blocked opposite dG-N^2-3MeE were lower than that of dA-N^6-3MeE. A total of 2.4 and 7.2% of primers passed dG-N^2-3MeE and dA-N^6-3MeE, respectively, to form the fully extended products. dG-N^2-3MeE appears to be a stronger blocking lesion than dA-N^6-3MeE.

Through analysis of the fully extended products to explore the miscoding specificities, dG-N^2-3MeE promoted small amounts of incorporation of dAMP (1.2%, see arrow) and one-base deletion (2.1%) (Figure 9, lane 4). dA-N^6-3MeE generated preferential incorporation of dTMP (16%) opposite the lesion, along with small amounts of incorporation of

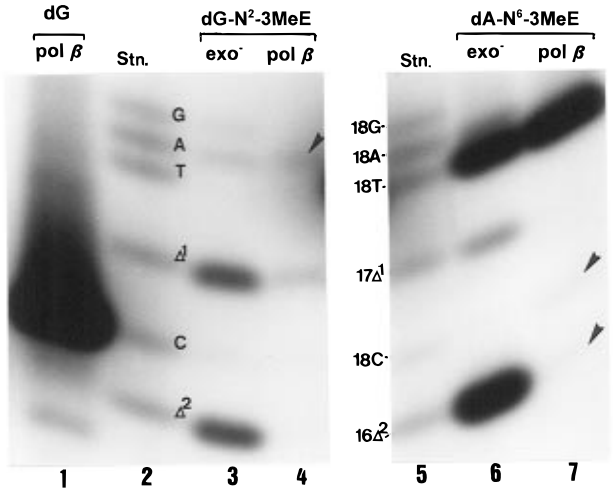

FIGURE 9: Quantitation of miscoding specificities induced by dG-N^2-3MeE and dA-N^6-3MeE. Primer extension reactions were carried out at 25 °C, using 0.5 unit of pol β for unmodified template (lane 1), 4.0 units of pol β for dG-N^2-3MeE- (lane 4) and dA-N^6-3MeE-modified (lane 7) templates, and 5.0 units of exo$^-$ Klenow fragment for dG-N^2-3MeE- (lane 3) and dA-N^6-3MeE-modified (lane 6) templates as described in the legend of Figure 7. The fully extended products recovered from PAGE were cleaved by *Eco*RI and subjected to two-phase gel electrophoresis to compare with standard markers (lanes 2 and 5) as described in Experimental Procedures.

dCMP (0.28%, see arrow) and two-base deletions (0.32%, see arrow) (lane 7).

Using the reaction condition containing a single dNTP, the insertion frequencies of dCMP opposite dG-N^2-3MeE were much higher than that of the other dNMP (Figure 10A); however, the chain extension of the dC·dG-N^2-3MeE pair was strongly blocked (Figure 10A). Thus, the fully extended product containing dCMP opposite the lesion may not be observed (Figure 9, lane 4). On the other hand, the amount of dNMP insertion opposite dA-N^6-3MeE followed the order dTMP ≫ dCMP > dGMP and dAMP (Figure 10A). The frequencies of chain extension of dT·dA-N^6-3MeE and dC·dA-N^6-3MeE were slightly higher than that of other pairs (Figure 10A). Thus, the fully extended products containing dTMP promoted preferentially (Figure 9, lane 7).

Fully extended products formed by the 3′ → 5′ exonuclease free (exo$^-$) Klenow fragment of DNA pol I, a bacteria "repair" polymerase, were analyzed. dG-N^2-3MeE promoted mostly one- (1.3%) and two-base (2.1%) deletions with small amounts of incorporation of dAMP (0.15%), dGMP (0.07%), and dCMP (0.05%) opposite the lesion (Figure 9, lane 3). dA-N^6-3MeE promoted incorporation of dTMP (3.8%) opposite the lesion and two-base deletions (5.7%), along with incorporation of dAMP (0.56%) and one-base deletions (0.55%) (Figure 9, lane 6).

The amounts of dNMP insertion opposite dG-N^2-3MeE followed the order dGMP > dAMP ≫ dCMP and dTMP (Figure 10B). The chain extension reactions of all dN·dG-N^2-3MeE pairs were poor. Although the amount of dTMP insertion opposite dA-N^6-3MeE was much lower than that of dAMP and dGMP, dT·dA-N^6-3MeE was extended rapidly, compared to any other pairs (Figure 10B). Thus, the fully extended product containing dTMP was predominantly observed (Figure 9, lane 6). The exo$^-$ Klenow fragment incorporated preferentially dGMP opposite dG-N^2-3MeE, while pol β incorporated dCMP preferentially (Figure 10). In addition, the exo$^-$ Klenow fragment incorporated

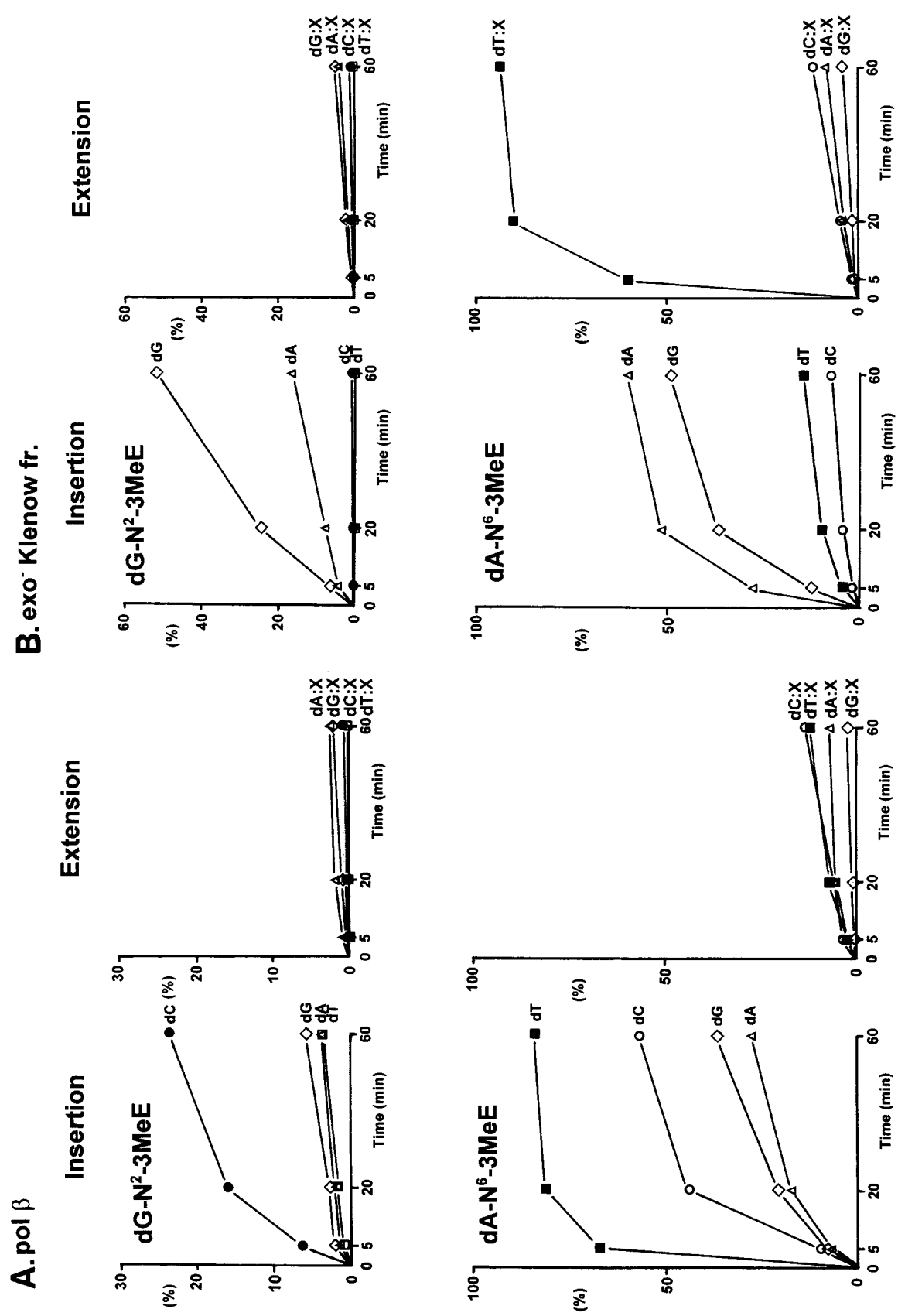

FIGURE 10: Time course of dNTP insertion opposite dG-N^2-3MeE or dA-N^6-3MeE and chain extension. Nucleotide insertion opposite dG-N^2-3MeE or dA-N^6-3MeE and the chain extension were measured at 30 °C, using 1.0 unit of pol β (A) or 1.0 unit of exo$^-$ Klenow fragment (B) as described in the legend of Figure 6B.

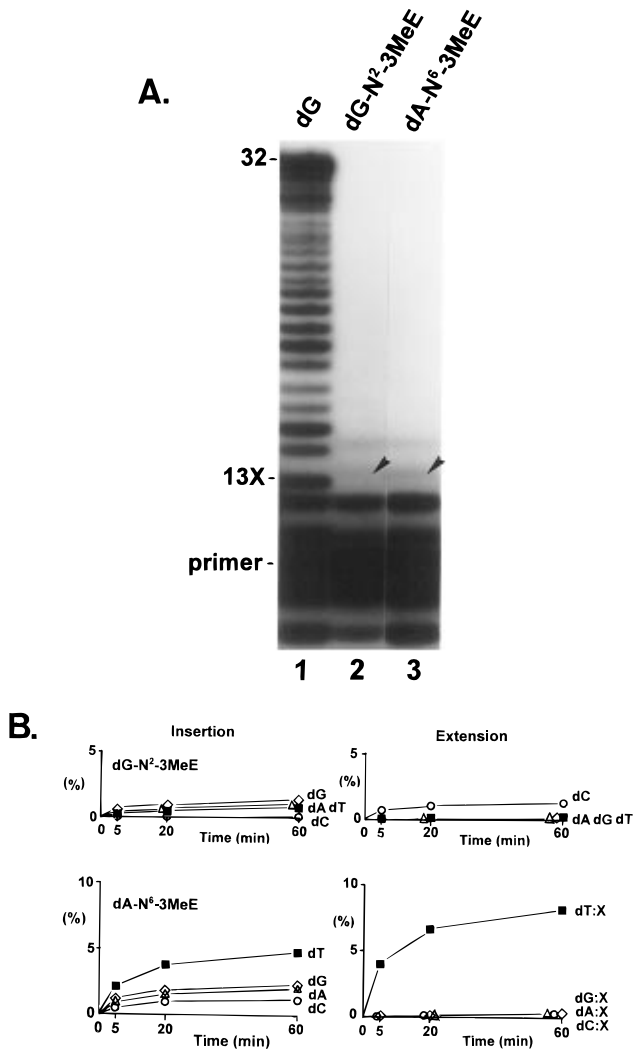

FIGURE 11: Primer extension reactions catalyzed by pol δ. (A) Primer extension reactions catalyzed by 0.014 unit of pol δ were carried out at 25 °C for 1 h in the reaction mixture containing 6 ng of PCNA as described in Experimental Procedures. (B) Nucleotide insertion opposite dG-N^2-3MeE or dA-N^6-3MeE and the chain extension were measured at 30 °C, using 0.007 unit of pol δ and 6 ng of PCNA as described in Experimental Procedures.

predominantly dAMP and dGMP opposite dA-N^6-3MeE, while pol α and pol β incorporated dTMP preferentially (Figures 6B and 10). Thus, the specificities of nucleotide inserted opposite these lesions varied depending on the DNA polymerase used. Similar results were observed in the chain extension reactions.

Primer Extension Reactions Catalyzed by Pol δ. Using pol δ, and another mammalian replicative enzyme, primer extension occurred rapidly on an unmodified template, forming fully extended products (14%); however, primer extension reactions were blocked one base before dG-N^2-3MeE or dA-N^6-3MeE (Figure 11A). Small amounts of incorporation of nucleotide(s) were observed opposite dG-N^2-3MeE (0.28%, see arrow) or dA-N^6-3MeE (0.15%, see arrow). Under the condition where the reaction mixture contained a single dNTP, small amounts of incorporation of dAMP, dGMP, and dTMP opposite dG-N^2-3MeE were detected (Figure 11B). No incorporation of dCMP opposite dG-N^2-3MeE was observed. However, the chain extension of the dC·dG-N^2-3MeE pair was much higher than that of other pairs (Figure 11B). In contrast, the amounts of dNMP insertion opposite dA-N^6-3MeE followed the order dTMP > dGMP and dAMP > dCMP. The relative frequencies of chain extension of dT·dA-N^6-3MeE were higher than that of the other pairs (Figure 11B). Some of the primers were extended past the lesions, but no fully extended products were detected (Figure 11A). When large amounts of pol δ were used, most of the primers was degraded by the $3' \rightarrow 5'$ exonuclease activity.

DISCUSSION

Model Estrogen-Derived DNA Adducts. Since hydroxyl groups at C-3, C-6, and/or C-17 of 6-hydroxyestradiol (6-OHE_2) or 6-hydroxyestrone (6-OHE_1) cannot be selectively sulfonated, 3MeE-6α-S was designed as a model reactive intermediate (Takagi *et al*., 1991; Itoh *et al*., 1996). In fact, 3MeE-6α-S reacted with purine bases embedded in oligodeoxynucleotides and formed dG-N^2-3MeE and dA-N^6-3MeE lesions (Figure 3). Thus, the sulfate, located in the benzylic ester site of natural estrogens, could be highly reactive to DNA. When 6-OHE_2 was incubated with adenosine and 3′-phosphoadenosine 5′-phosphosulfate (PAPS) using a cytosol fraction of rat liver, N^6-(estradiol-6β-yl)adenosine was identified as a product (Itoh *et al*., 1993). These observations indicate that C-6 sulfonation of estrogens may be involved in the formation of estrogen−DNA adducts in cells, in addition to the formation of DNA adducts induced by estrogen quinones (Dwivedy *et al*., 1992; Stack *et al*., 1996).

When 24-mers containing a single dG or dA were reacted with 3MeE-6α-S and subjected to HPLC, several minor peaks were observed around the peak of dG-N^2-3MeE- or dA-N^6-3MeE-modified oligodeoxynucleotide (Figure 3). Recently, one of our groups found that 3MeE-6α-S reacts with dC at the low rate and identified that the product was N^4-[3-methoxyestra-1,3,5(10)-trien-6β-yl]-2′-deoxycytidine (dC-N^4-3MeE).[2] Since the 24-mer oligodeoxynucleotides used in this study contained 11 dC bases, the minor peaks may result from one and/or multiple dC-modified oligodeoxynucleotides. The retention time of dC-N^4-3MeE was shorter than that of dG-N^2-3MeE on the HPLC system.[2] However, when dG-N^2-3MeE- or dA-N^6-3MeE-modified oligodeoxynucleotides were digested enzymatically (panels B and C of Figure 4), no other peaks were detected. In addition, the migration of multiple dC-modified oligodeoxynucleotides was slower than that of the single dG- or dA-modified oligodeoxynucleotide on the 20% polyacrylamide denaturing gel (data not shown). Thus, our purification procedures using HPLC and gel electrophoresis should prevent the contamination in the sample of dG- or dA-modified oligodeoxynucleotides.

The dA-modified oligodeoxynucleotide only contained dA-N^6-6β-3MeE (Figures 4C and 5), while the dG-modified oligodeoxynucleotide was constituted with the two isomers, dG-N^2-6α-3MeE and dG-N^2-6β-3MeE, with the ratio of 1:5.5 (Figure 4B). Thus, it is unknown which isomer of dG-N^2-3MeE gave rise to the miscoding.

Miscoding Properties of Estrogen−DNA Adducts. During redox cycling, when estrogen quinones are reduced to catecholestrogen, free radicals produce oxidative damage, including 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in DNA (Han & Liehr, 1994). 8-OxodG lesion has been

[2] S. Itoh and I. Yoshizawa, unpublished data.

Table 2: Summary of Miscoding Properties of DNA Polymerases

	miscoding specificity	
polymerase	dG-N^2-3-MeE	dA-N^6-3MeE
pol I	Δ^1, Δ^2 > A, G, c[a]	t[a], Δ^2 > A, Δ^1
pol α	block	t > Δ^2 > Δ^1, C
pol β	Δ^1 > A	t ≫ C, Δ^2
pol δ	block	block

[a] Lowercase represents a correct base inserted opposite the lesion.

reported to be mutagenic, generating G → T transversions (Wood *et al*., 1990; Shibutani *et al*., 1991b; Moriya, 1993). However, G → A (Mazars *et al*., 1992; Sasa *et al*., 1993) and A → G transitions (Blaszyk *et al*., 1994; Nigro *et al*., 1994) were detected mainly in human breast cancer, along with some G → T transversions (Mazars *et al*., 1992; Coles *et al*., 1992; Biggs *et al*., 1993) and deletions (Sato *et al*., 1992; Hamelin *et al*., 1994). These mutational spectra differ from that induced by 8-oxodG.

The miscoding specificities of dG-N^2-3MeE and dA-N^6-3MeE, observed during DNA synthesis in reactions catalyzed by mammalian and *E. coli* DNA polymerases, are summarized in Table 2. The *in vitro* experimental system used for these studies detects all base substitutions and deletions targeted to the site of the lesion. Although primer extension reactions catalyzed by pol α and δ were blocked at the dG-N^2-3MeE lesion, the misincorporation of dAMP opposite the lesion and deletions were observed with pol β and the exo$^-$ Klenow fragment. The exo$^-$ Klenow fragment also misincorporated small amounts of dGMP opposite the lesion. Thus, G → T and G → C transversions and deletions were predicted to be formed opposite dG-N^2-3MeE *in vivo*. On the other hand, pol α, pol β, and exo$^-$ Klenow fragment promoted preferential incorporation of dTMP, the correct base, opposite dA-N^6-3MeE, accompanied by deletions. In addition, pol α and pol β showed small amounts of misincorporation of dCMP and the exo$^-$ Klenow fragment showed misincorporation of dAMP. A → G transition, A → T transversion, and deletions were predicted to be produced opposite dA-N^6-3MeE in cells. Although the miscoding specificities and frequencies varied depending on the DNA polymerases used, some mutational spectra were similar to that observed in human breast cancer (Mazars *et al*., 1992; Coles *et al*., 1992; Sato *et al*., 1992; Biggs *et al*., 1993; Blaszyk *et al*., 1994; Nigro *et al*., 1994). Thus, DNA adducts generated by endogenous estrogens may be involved in the development of breast cancer.

Mechanism of Deletions. We have proposed a general mechanism for the generation of frameshift deletions, using several mutagenic DNA adducts (Shibutani & Grollman, 1993). When primer extension is blocked at the lesion site, the newly inserted nucleotide opposite the lesion pairs with bases 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 (Shibutani & Grollman, 1993).

Frameshift deletions were frequently produced by model estrogen−DNA adducts during DNA synthesis, using several DNA polymerases (Table 2). Using the reaction conditions containing a single dNTP, dAMP and/or dGMP were observed to be incorporated opposite dG-N^2-3MeE or dA-N^6-3MeE (Figures 6B and 10). In addition, the chain

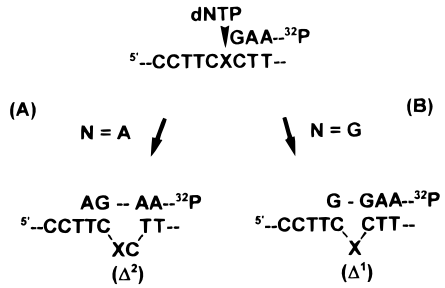

FIGURE 12: Proposed mechanism of one- and two-base deletions. X represents the dG-N^2-3MeE and/or dA-N^6-3MeE lesion.

extension of dA or dG that paired with dG-N^2-3MeE or dA-N^6-3MeE was quite poor (Figures 6B and 10). When dAMP is inserted opposite the lesions, the newly inserted base could be paired with dT two bases 5′ to the lesion to form a two-base deletion (Δ^2) (Figure 12A). When dGMP is inserted opposite the lesions, the newly inserted base could be paired with dC 5′ to the lesion to form a one-base deletion (Δ^1) (Figure 12B).

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