Miscoding Potential of Tamoxifen-Derived DNA Adducts: α-(N^2-Deoxyguanosinyl)tamoxifen

Shinya Shibutani* and Lakkaraju Dasaradhi

Department of Pharmacological Sciences, State University of New York at Stony Brook, Stony Brook, New York 11794-8651

Received January 31, 1997; Revised Manuscript Received July 15, 1997[⊗]

ABSTRACT: The treatment of tamoxifen, widely used as adjuvant chemotherapy for breast cancer, increases significantly the risk of developing endometrial cancer. The miscoding properties of tamoxifen-derived DNA adducts, α-(N^2-deoxyguanosinyl)tamoxifens (dG-N^2-tamoxifen), have been explored, using an *in vitro* experimental system to quantify base substitutions and deletions. Site-specifically modified oligodeoxynucleotides containing an epimer of *trans*- and *cis*-forms of dG-N^2-tamoxifens were prepared postsynthetically and used as templates in primer extension reactions catalyzed by mammalian DNA polymerases α, *β*, and *δ*. Pol α catalyzed incorporation of dCMP and dAMP opposite all four stereoisomers of dG-N^2-tamoxifen, accompanied by lesser amounts of dGMP. In contrast, pol *δ* catalyzed preferential incorporation of dCMP, a correct base, opposite the lesions; one of the *trans*-forms of dG-N^2-tamoxifens only promoted incorporation of dTMP. Using pol *β*, preferential incorporation of dCMP, along with small amounts of incorporation of dAMP and dGMP, was detected. One- and two base deletions were also observed with pol α and pol *β*. The miscoding specificities and frequencies of dG-N^2-tamoxifens varied depending on the DNA polymerase used. In addition, with pol α and pol *β*, large amounts of 5-base deletions were preferentially formed at the *cis*-forms of dG-N^2-tamoxifen, but not at the *trans*-forms of dG-N^2-tamoxifen. We conclude that dG-N^2-tamoxifen adducts have high miscoding potentials.

Tamoxifen, an antiestrogen drug, is widely employed for the therapy of breast cancer (Jordan, 1993) and used prophylactically in a study of healthy women with a positive family history of this disease (Nayfield *et al*., 1991; Powles *et al*., 1993). Unfortunately, breast cancer patients treated with tamoxifen have an increased incidence of endometrial cancer (Seoud *et al*., 1993; Fischer *et al*., 1994; van Leeuwen *et al*., 1994). Tamoxifen also induced hepatocellular carcinomas in rodents (Williams *et al*., 1993; Greaves *et al*., 1993; Hard *et al*., 1993) and gave rise to DNA adducts in livers of rodents (Han & Liehr, 1992; White *et al*., 1992). DNA damage, if unrepaired (Carthew *et al*., 1995), may lead to mutations that accumulate in genomic DNA and facilitate development of human cancers. DNA adducts have been detected in the endometrial tissue obtained from breast cancer patients treated with tamoxifen (Hemminki *et al*., 1996). In 1996, tamoxifen has been classified as a possible human carcinogen by the International Agency for Research on Cancer (IARC, 1996).

Several mechanisms have been proposed to explain the formation of DNA adducts induced by tamoxifen. Oxidative species such as 4-hydroxytamoxifen quinone methide may promote the reaction with DNA (Moorthy *et al*., 1996). Alternatively, α-hydroxylation of tamoxifen and its metabolites, tamoxifen *N*-oxide, *N*-desmethyltamoxifen, and 4-hydroxytamoxifen, respectively, are capable of forming DNA adducts (Potter *et al*., 1994; Phillips *et al*., 1994ab; Poon *et al*., 1995). However, α-hydroxylation of tamoxifen has only a low level of reactivity to DNA *in vitro* (Phillips *et al*., 1994a). The formation of tamoxifen−DNA adducts was inhibited by sulfotransferase inhibitors (Randerrath *et al*., 1994). Recently, we found that α-sulfate tamoxifens are highly reactive to DNA, forming four diastereoisomers of α-(N^2-deoxyguanosinyl)tamoxifen (dG-N^2-tamoxifen):[1] the epimeric center of each *trans*- and *cis*-form of dG-N^2-tamoxifen is at the α-carbon (the structures in Figure 1) (Dasaradhi & Shibutani, 1997). Similar results were observed for α-acetoxytamoxifen (Osborne *et al*., 1996; Dasaradhi & Shibutani, 1997). The *trans*-forms of dG-N^2-tamoxifen were found to be major adducts in the liver of rats treated with tamoxifen (Osborne *et al.*, 1996).

In this paper, site-specifically modified oligodeoxynucleotides containing a single stereoisomer of dG-N^2-tamoxifen were prepared postsynthetically and used as templates in primer extension reactions catalyzed by DNA pol α, pol *β*, and pol *δ*. Fully extended products were analyzed by our *in vitro* experimental system (Shibutani, 1993; Shibutani *et al*., 1996) to quantify the miscoding specificities. This is the first evidence that tamoxifen-derived DNA adducts have high miscoding potentials.

MATERIALS AND METHODS

Materials. [*γ*-^{32}P]ATP (specific activity, >6000 Ci/mmol) was obtained from Amersham Corp. Calf thymus DNA pol α (30 000 units/mg of protein) and human pol *β* (100 000

† This research was supported in part by the Lilly W. Ammann Breast Cancer Research Award, the School of Medicine, SUNY at Stony Brook (to S.S.) and Grant CA17395 (to A.P. Grollman) from the National Institutes of Health.

* To whom correspondence should be addressed. Telephone: (516)-444-8018. Fax: (516)-444-3218. E-mail: shinya@pharm.som.sunysb.edu.

⊗ Abstract published in *Advance ACS Abstracts,* October 1, 1997.

[1] Abbreviations: dG, 2′-deoxyguanosine; dNTP, 2′-deoxynucleoside triphosphate; dG-N^2-tamoxifen, α-(N^2-deoxyguanosinyl)tamoxifen; 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.

S0006-2960(97)00243-2 CCC: $14.00

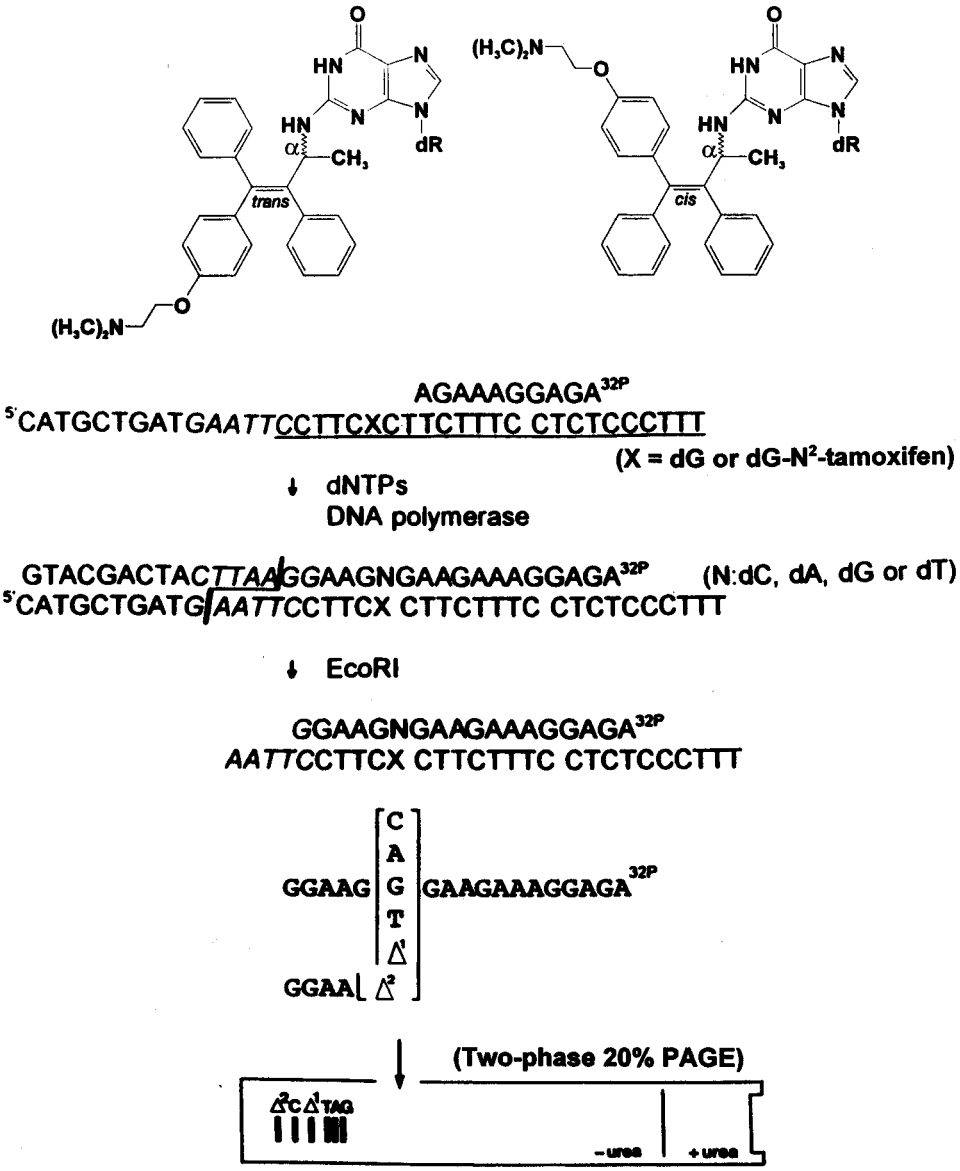

FIGURE 1: Diagram of methods used to determine miscoding specificities. Structures of *trans*- or *cis*-forms of dG-N^2-tamoxifens used in this study are shown. The epimeric center of each *trans*- and *cis*-form of dG-N^2-tamoxifen is at the α-carbon. Unmodified or dG-N^2-tamoxifen-modified 38-mer templates were annealed with ^{32}P-labeled 10-mer primer. Primer extension reactions catalyzed by DNA polymerase were conducted in the presence of four dNTPs. The fully extended products formed during DNA synthesis were recovered from the gel, cleaved with *Eco*RI, and subjected to electrophoresis on two-phase 20% polyacrylamide gels (15 × 72 × 0.04 cm), as described in Materials and Methods. To determine the miscoding specificities, the mobilities of reaction products were compared with those of 18-mer standards containing dC, dA, dG, or dT opposite the lesion and 1 base (Δ^1) or 2 base (Δ^2) deletions.

units/mg of protein) were from Molecular Biology Resources, Inc.; cloned exo$^-$ Klenow fragment of *Escherichia coli* DNA polymerase I (21 200 units/mg of protein) was purchased from United State Biochemical Corp.; T4 polynucleotide kinase was from Stratagene; *Eco*R I restriction endonuclease (100 units/mL) and T4 DNA ligase (400 units/mL) were from New England BioLabs. DNA pol δ and proliferating cell nuclear antigen (PCNA) were provided by Dr. Matsumoto (Matsumoto *et al*., 1994). A waters 990 HPLC instrument, equipped with a photodiode array detector, was used for the separation and purification of the oligodeoxynucleotides.

Synthesis of Oligodeoxynucleotides. Oligodeoxynucleotides used as DNA template, primer, and standard markers were prepared by solid-state synthesis on an automated DNA synthesizer (Takeshita *et al*., 1987). (*Z*)-α-Sulfate tamoxifen was synthesized as reported previously (Dasaradhi & Shibutani, 1997). Standard of dG-N^2-tamoxifens were prepared by reacting dG with (*Z*)-α-sulfate tamoxifen, as described previously (Dasaradhi & Shibutani, 1997). DNA template containing a single dG-N^2-tamoxifen was prepared by reacting 200 μg of unmodified 24-mer containing a single dG (5′CCTTCGCTTCTTTCCTCTCCCTTT) with 4.0 mg of (*Z*)-α-sulfate tamoxifen for 2 h at 37 °C in 500 μL of 50 mM NaH_2PO_4-Na_2HPO_4 buffer, pH 7.0. After the reaction, the sample was extracted twice with 500 μL of butanol. The aqueous fraction was evaporated to dryness and then subjected to HPLC. The dG-N^2-tamoxifen-modified oligomers were isolated on a Water's reverse-phase μBondapak C_{18} (0.78 × 30 cm), using a linear gradient of 0.05 M triethylammonium acetate, pH 7.0, containing 10 → 50% acetonitrile with an elution time of 60 min and a flow rate of 2.0 mL/min (Shibutani *et al*., 1991a). The modified oligomers were further purified by HPLC and by electrophoresis on 20% polyacrylamide denaturing gel (PAGE) (35 × 42 × 0.04 cm) (Shibutani *et al*., 1993a). 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 20% PAGE containing 7 M urea (35 × 42 × 0.04 cm or 15 × 72 × 0.04 cm) to establish homogeneity. The position of the oligomers was established by autoradiography, using Kodak Xomat XAR film.

Enzymatic Digestion. Three micrograms (approximately 9090 pmol of deoxynucleosides) of dG-N^2-tamoxifen-modified 24-mer was digested with nuclease P1 (2 units) and alkaline phosphatase (3 units) as described previously (Shibutani *et al*., 1993a). The sample was extracted with methanol. Methanol extracts were evaporated to dryness, and the product was dissolved in distilled water containing 10% of ethanol and analyzed by HPLC, using μBondapak C_{18} (0.39 × 30 cm). The column was eluted with a linear gradient of 0.05 M triethylammonium acetate, pH 7.0, containing 0 → 4% acetonitrile over 20 min, and subsequently 4 → 50% over 30 min at a flow rate of 1.0 mL/min. The detection limit was 5 ng (15 pmol) of deoxynucleoside (approximately 0.16% of 3 μg of oligomer digested) using a photodiode array detector.

Primer Extension Reactions. Two micrograms of dG-N^2-tamoxifen-modified 24-mer (5′CCTTCXCTTCTTTCCTCTCCCTTT, X = dG-N^2-tamoxifen) was phosphorylated at the 5′ terminus using 5 μL of T4 kinase (10 units/μL) and 2 μL of 10 mM ATP (Maniatis *et al*., 1982) and then ligated to a 14-mer (2.4 μg, 5′CATGCTGATGAATT) at 8 °C overnight using 2 μL of T4 DNA ligase (400 units/μL), 2 μL of 10 mM ATP, and a 18-mer template (3.0 μg, 5′GAAGCGAAGGAATTCATC). The resultant 38-mer (5′CATGCTGATGAATTCCTTCXCTTCTTTCCTCTCCCTTT, X = dG-N^2-tamoxifen) 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 10-mer (0.5 pmol, 5′AGAGGAAAGA), primer extension reactions catalyzed by DNA polymerases were conducted at 25 °C in 10 μL of a buffer containing all four dNTPs (100 μM each) (Shibutani *et al*., 1991b). The buffer for pol α or pol β consisted of 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 2 mM dithiothreitol, and BSA (0.5 μg/μL). The buffer for pol δ (0.014 units) 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. The reaction samples were subjected to 20% PAGE containing 7 M urea (35 × 42 × 0.04 cm).

Quantitation of Miscoding Specificity. As reported previously (Shibutani *et al*., 1996), the fully extended products obtained during DNA synthesis were recovered from the gel, annealed with an unmodified 38-mer containing dG at the X position (5′CATGCTGATGAATTCCTTCXCTTCTTTCCTCTCCCTTT), 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 1. 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). Bands were identified by autoradiography and the radioactivities were measured by β-phosphoimager (Molecular Dynamic Inc.).

dNTP Incorporation Opposite Lesions and the Chain Extension. Unmodified or modified 38-mer templates were primed with a ^{32}P-labeled 12-mer (5′AGAGGAAAGAAG) for the determination of dNTP insertion opposite the lesion or with a ^{32}P-labeled 13-mer (5′AGAGGAAAGAAGN, N = dC, dA, dG, or dT) for the determination of the chain extension. The amounts of dNMP incorporation opposite the lesions were measured at 25 °C in the presence of a single dNTP and DNA polymerase. The amounts of chain extension from dN·dG-N^2-tamoxifen pairs were measured at 25 °C in the presence of all four dNTPs. The percentage of dNMP incorporation or chain extension was determined by gel electrophoresis (Shibutani *et al*., 1993a).

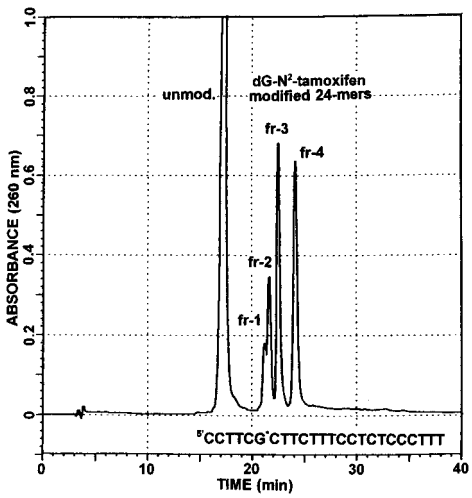

FIGURE 2: HPLC separation of 24-mer oligodeoxynucleotides containing a single dG-N^2-tamoxifen. Two hundred micrograms of a 24-mer containing a single dG (5′CCTTCGCTTCTTTCCTCTCCCTTT) was reacted with 4.0 mg of (*Z*)-α-sulfate tamoxifen, as described in Materials and Methods. The dG-N^2-tamoxifen-modified 24-mers were isolated on a μBondapak C_{18}, using a linear gradient of 0.05 M triethylammonium acetate, pH 7.0, containing 10 → 50% acetonitrile with an elution time of 60 min and a flow rate of 2.0 mL/min.

RESULTS

Preparation of Site-Specifically Modified Oligodeoxynucleotides Containing a Single Stereoisomer of dG-N^2-Tamoxifens. When a 24-mer oligodeoxynucleotide containing a single dG was reacted with (*Z*)-α-sulfate tamoxifen, four tamoxifen-modified 24-mers were isolated from the corresponding oligodeoxynucleotide (t_R = 17.3) by HPLC (Figure 2). Fr-3 (t_R = 22.5 min) and fr-4 (t_R = 23.9) of the modified 24-mer were completely separated from the other fractions. However, fr-1(t_R = 21.1) was not separated completely from fr-2 (t_R = 21.5). To minimize the cross-contamination, fr-1 was collected before the highest point of the peak and fr-2 was collected after the highest point of the peak. The yield of fr-1, fr-2, fr-3, and fr-4 were 1.9, 5.8, 12.6, and 12.1%, respectively. These modified oligodeoxynucleotides were further purified by HPLC twice and by gel electrophoresis.

The modified 24-mers were analyzed by 20% polyacrylamide gels (15 × 72 × 0.04 cm) after labeling with ^{32}P at the 5′ terminus (Figure 3). All modified 24-mers migrated much slower than the unmodified oligomer. The migration of fr-2 was much faster than that of fr-1 on the gel although fr-2 was not separated completely from fr-1 using HPLC. Cross-contamination between fr-1 and fr-2 was not detected. The migration of fr-3 was slightly slower than that of fr-4 on the gel, but fr-3 was completely separated from fr-4 using HPLC. The detection limit on the gel was 0.03% of the labeled oligomers. Thus, all modified 24-mers were purified to homogeneity using a combination of HPLC and gel electrophoresis.

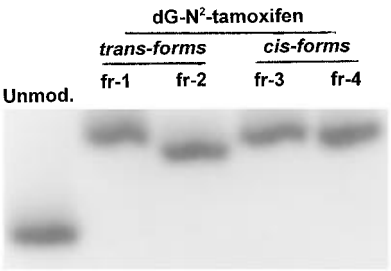

FIGURE 3: Polyacrylamide gel electrophoresis of 24-mer containing a single dG-N^2-tamoxifen. Oligodeoxynucleotides were labeled with ^{32}P, as described in Materials and Methods, then subjected to electrophoresis on 20% polyacrylamide (15 × 72 × 0.04 cm).

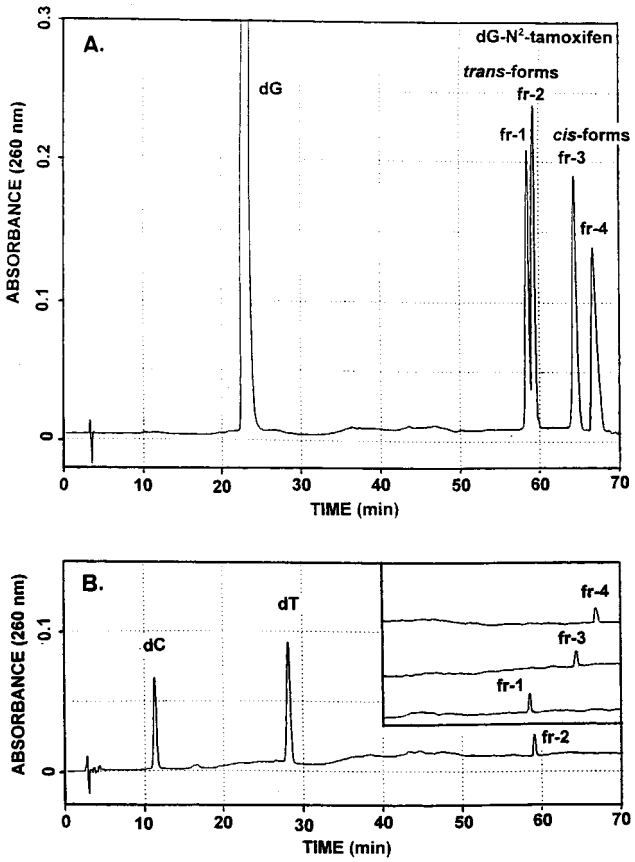

FIGURE 4: Enzymatic digestion of dG-N^2-tamoxifen-modified 24-mer. (A) dG was reacted with (*Z*)-α-sulfate tamoxifen, as described previously (Dasaradhi & Shibutani, 1997). The reaction mixture containing four stereoisomeric dG-N^2-tamoxifens was passed through a μBondapak C_{18} column. The column was eluted with 0.05 M triethylammonium acetate, pH 7.0, containing 0 → 4% acetonitrile over 20 min and subsequently 4 → 50% acetonitrile over 30 min at a flow rate of 1.0 mL/min, as described in Materials and Methods. (B) Three micrograms of fr-2 of the tamoxifen-modified 24-mer was digested with nuclease P1 and alkaline phosphatase. Methanol extract of the digested sample was evaporated to dryness and analyzed by HPLC. A part of HPLC profile of fr-1, fr-3, or fr-4 is shown as an inset of this figure.

When the modified 24-mers were digested enzymatically, the retention time of tamoxifen adduct obtained was compared with that of standards of stereoisomers of dG-N^2-tamoxifens (Dasaradhi & Shibutani, 1997), as shown in Figure 4A. When fr-2 of the modified 24-mer was digested, the retention time (t_R = 59.3) of the adduct isolated was consistent with that of an epimer of the *trans*-forms of dG-N^2-tamoxifen (Figure 4B). We also confirmed that fr-1 of the modified 24-mer contains only an epimer of the *trans*-forms of dG-N^2-tamoxifen (t_R = 58.4) and that fr-3 and fr-4 contain only an epimer of the *cis*-forms of dG-N^2-tamoxifen (t_R = 64.5 or 67.2) (Figure 4B). The retention times of dC, dG, dT, and dA were 11.7, 23.0, 28.2, and 36.6, respectively. The molar ratio of dC (10.6−11.3):dT (12.1−12.4) obtained from the digested samples was consistent with the theoretical value (dC:dT = 11:12) of nucleoside composition of the 24-mers. Although the detection limit was 5 ng of deoxynucleoside, no contaminations were detected. These dG-N^2-tamoxifen-modified oligomers were ligated to a 14-mer for the preparation of 38-mer templates.

Table 1: Miscoding Specificities of dG-N^2-Tamoxifen Adducts[a]

	C (%)	A (%)	G (%)	T (%)	Δ^1 (%)	Δ^2 (%)
			pol α			
dG	78.0					
dG-N^2-tamoxifen						
fr-1	14.6	4.9	0.79		1.0	1.4
fr-2	1.5	1.6	0.24		0.30	0.60
fr-3	1.0	1.5	0.29		1.3	1.6
fr-4	2.3	2.3	0.70		0.53	3.3
			pol β			
dG	74.5					
dG-N^2-tamoxifen						
fr-1	4.0	0.33	0.15	0.32	1.2	0.72
fr-2	0.91	0.15	0.04		0.13	0.29
fr-3	1.2	0.13	0.14		0.59	0.29
fr-4	1.5	0.15	0.10		0.33	1.1
			pol δ			
dG	13.8					
dG-N^2-tamoxifen						
fr-1	6.5			0.30		
fr-2	1.4					
fr-3	0.70					
fr-4	0.40					

[a] Data were obtained from Figure 6. C, A, G, T, Δ^1, and Δ^2 represent the amount of the fully extended product containing dC, dA, dG, dT, and 1 and 2 base deletions opposite the lesion produced from the starting primer, respectively.

Miscoding properties of dG-N^2-tamoxifen DNA adducts. Primer extension reactions catalyzed by mammalian DNA polymerases were conducted in the presence of all four dNTPs on a 38-mer template containing a single stereoisomer of dG-N^2-tamoxifens. Using unmodified templates, primer extension reactions catalyzed by pol α, a mammalian replicative enzyme (Kornberg & Baker, 1992), occurred rapidly to form the fully extended products (Figure 5A). However, using dG-N^2-tamoxifen-modified templates, the primer extensions were blocked opposite the lesions. Some of the primers passed the lesions to form the fully extended products. The amounts of fully extended products of fr-1, fr-2, fr-3, and fr-4 were 23.0, 4.2, 4.2, and 9.1%, respectively (Figure 5A).

To quantify the miscoding specificities, the fully extended products were recovered from the gel, cleaved with *Eco*R I, and then subjected to two-phase gel electrophoresis. Standards representing products containing dC, dA, dG, or dT opposite the lesion or 1- and 2-base deletions were completely resolved on the gel, based on their different migration (Figure 6, panel A, lane 2; panel B, lanes 7 and 12; panel C, lane 13). DNA synthesis on unmodified templates led to the expected incorporation of dCMP opposite dG (Figure 6A, lane 1). As shown in Figure 6A and Table 1, the epimers of *trans*-forms (fr-1 and fr-2) and the epimers of *cis*-forms (fr-3 and fr-4) of dG-N^2-tamoxifen adducts promoted the incorporation of dCMP (1.0−14%) and dAMP (1.5−4.9%) opposite the lesions, accompanied by small amounts of incorporation of dGMP. One and two base deletions were also detected.

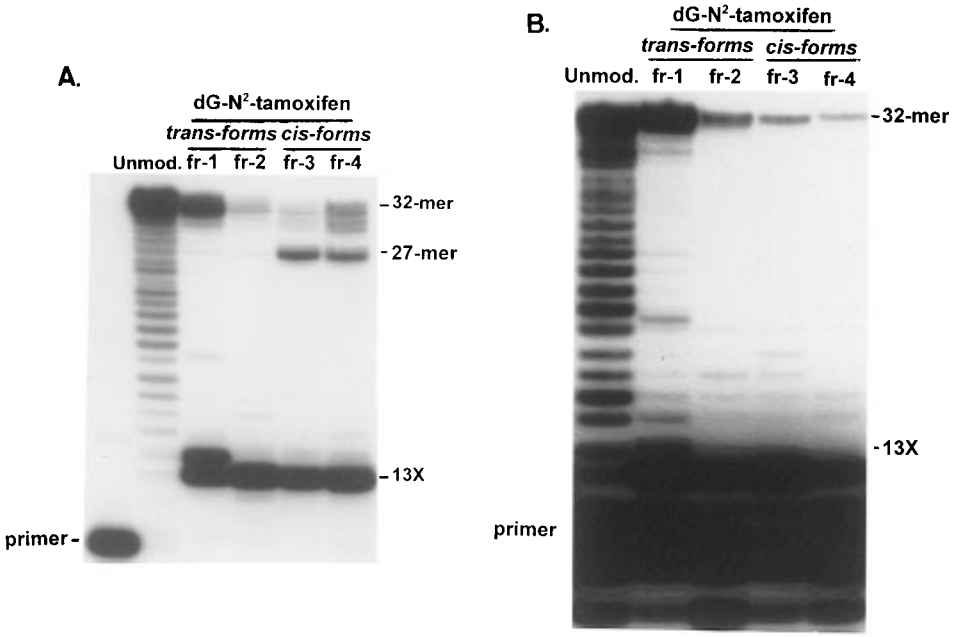

FIGURE 5: Primer extension reactions catalyzed by pol α or pol δ. Using a dG-*N*²-tamoxifen-modified 38-mer template (5′CATGCTGATGAATTCCTTCXCTTCTTTCCTCTCCCTTT, X = dG or dG-*N*²-tamoxifen) primed with a ³²P-labeled 10-mer (5′AGGAAAGA), primer extension reactions catalyzed by pol α (A) or pol δ (B) were conducted for 1 h at 25 °C in 10 μL of a buffer containing four dNTPs (100 μM each), (A) using 0.3 unit of pol α for the unmodified template and 2.4 units for dG-*N*²-tamoxifen-modified templates, and (B) using 0.014 unit of pol δ for unmodified and dG-*N*²-tamoxifen-modified templates, as described in Materials and Methods. The reaction samples were subjected to 20% PAGE containing 7 M urea (35 × 42 × 0.04 cm).

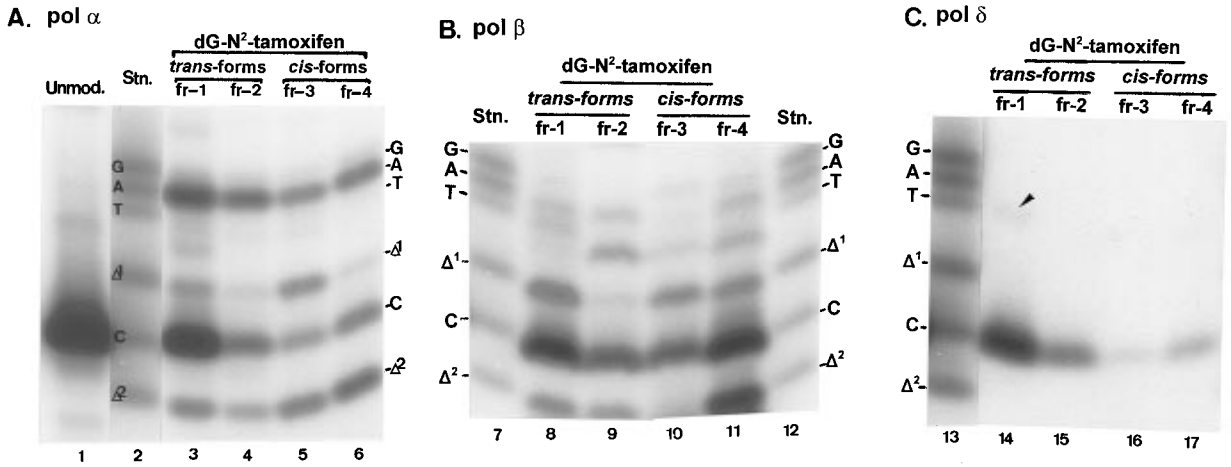

FIGURE 6: Quantitation of miscoding specificities induced by dG-*N*²-tamoxifen. Primer extension reactions catalyzed by pol α (A) and pol δ (C) were conducted, as described in the legend of Figure 5. Primer extension reactions catalyzed by pol β (B) were carried out for 1 h at 25 °C, using 2.5 units for dG-*N*²-tamoxifen-modified templates. The reaction samples were subjected to 20% PAGE containing 7 M urea (35 × 42 × 0.04 cm). The fully extended products obtained were recovered from the gel, cleaved with *Eco*RI, and subjected to electrophoresis on two-phase 20% polyacrylamide gels (15 × 72 × 0.04 cm), as described in the legend of Figure 1.

When pol β was used, the primer extensions were blocked 1 base before the lesions and opposite the lesions (Figure 7A). Some of the primers [6.6% (fr-1); 1.5% (fr-2); 2.4% (fr-3); 3.2% (fr-4)] passed the lesions in 60 min, respectively, to form the fully extended products. The amounts of fully extended products increased with an increase of incubation times. The fully extended products were analyzed to explore the miscoding specificities by using the two-phase gel electrophoresis. Each of dG-*N*²-tamoxifen adducts promoted preferential incorporation of dCMP (0.9−4.0%) and 1 (0.13−1.2%) and 2 base (0.29−1.1%) deletions (Figure 6B and Table 1). Small amounts of misincorporation of dAMP, dGMP, or dTMP were also detected, but the frequencies varied depending on the isomer of the dG-*N*²-tamoxifen used (Figure 6B).

Using pol δ, another replicative enzyme (Kornberg & Baker, 1992), primer extension reactions were blocked 1 base before dG-*N*²-tamoxifen lesions (Figure 5B). Some of the primers passed the lesions to form the fully extended products. Preferential incorporation of dCMP, a correct base, was observed opposite the lesions (Figure 6C and Table 1). Fr-1 of the *trans*-form of dG-*N*²-tamoxifen only promoted small amounts of misincorporation of dTMP (see arrow, 0.3%). No deletions were detected. The miscoding specificities obtained by pol δ were much different from that of pol α or pol β.

Formation of Deletions. With pol β, large amounts of 27-mer products were produced during DNA synthesis on fr-3 and fr-4 of dG-*N*²-tamoxifen-modified templates (Figure 7A). Since Maxam−Gilbert sequence analysis (Maxam & Gilbert, 1980) showed that the 27-mer product lacks 5 bases opposite the dG-*N*²-tamoxifen lesions (data not shown), the 27-mer corresponds to the 5 base deletion (Δ^5). The amounts of Δ^5 formed by fr-3 and fr-4 at 60 min were 74 and 37%,

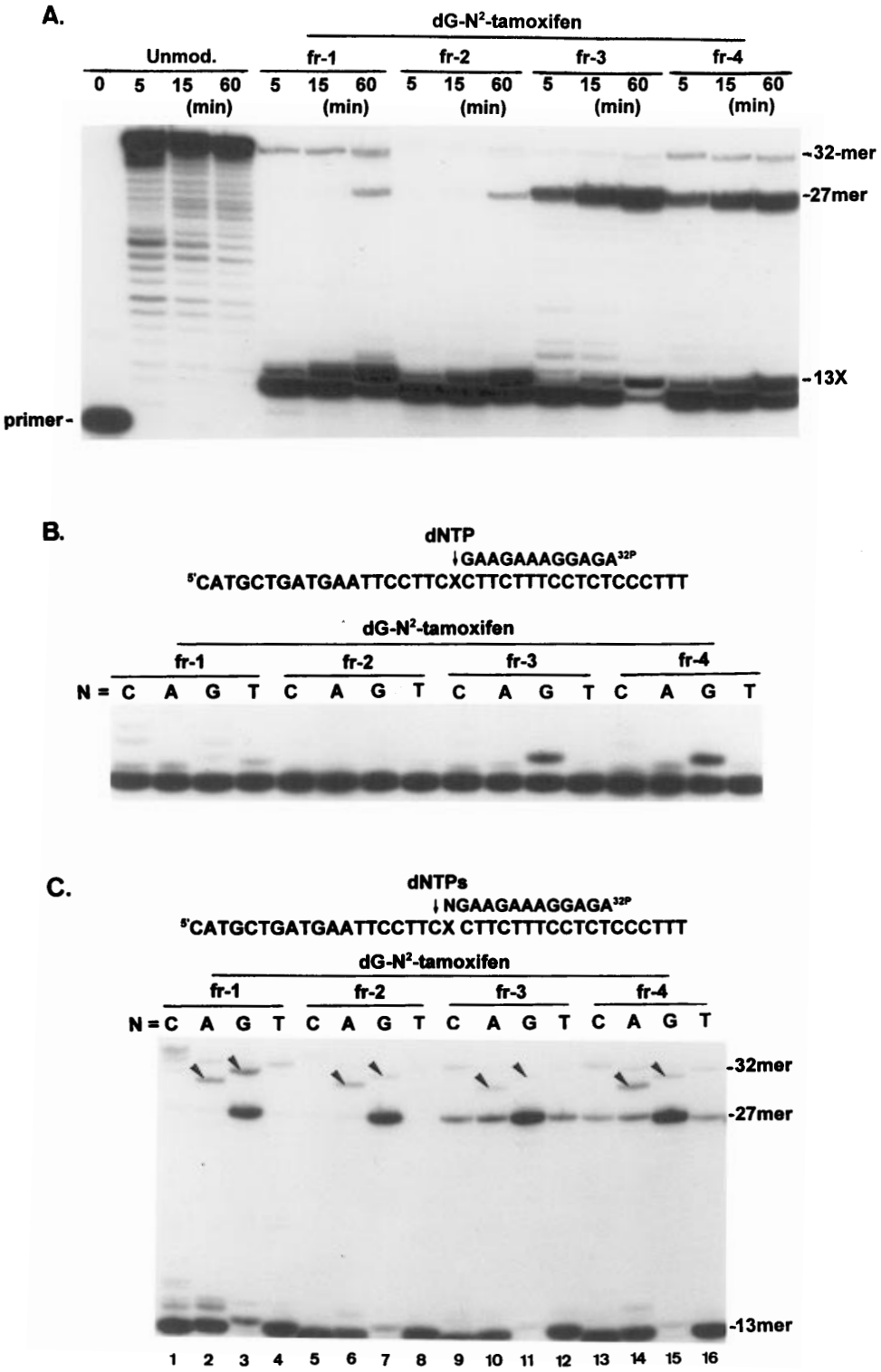

FIGURE 7: Primer extension on a 38-mer template catalyzed by pol β. (A) Using a dG-N^2-tamoxifen-modified 38-mer template primed with a ^{32}P-labeled 10-mer, primer extension reactions were conducted at 25 °C under the reaction condition containing four dNTPs, using 0.2 unit of pol β for unmodified templates and 2.0 units for dG-N^2-tamoxifen-modified templates. (B) Using a dG-N^2-tamoxifen-modified 38-mer template primed with a ^{32}P-labeled 12-mer (5′AGAGGAAAGAAG), nucleotide insertion opposite the lesion was measured for 1 h at 25 °C under the reaction condition containing a single dNTP and 0.5 unit of pol β. (C) Using a dG-N^2-tamoxifen-modified 38-mer template primed with a ^{32}P-labeled 13-mer (5′AGAGGAAAGAAGN, N = dC, dA, dG, or dT), chain extension from the 3′-primer terminus was conducted for 1 h at 25 °C under the reaction condition containing four dNTPs and 1.5 units of pol β.

respectively, while those formed by fr-1 and fr-2 were 3.8 and 2.7% (Figure 7A). Using pol α, fr-3 and fr-4 also promoted 8.6 and 5.9% of Δ^5, respectively: however, both fr-1 and fr-2 promoted only 0.4% of Δ^5 (Figure 5A). No 5 base deletion was observed with pol δ.

Under the condition where the reaction contained a single dNTP, pol β inserted large amounts of dGMP opposite the *cis*-forms of dG-N^2-tamoxifen (fr-3 and fr-4), along with small amounts of incorporation of dAMP. In contrast, the amount of incorporation of dGMP opposite the *trans*-forms of dG-N^2-tamoxifen (fr-1 and fr-2) was low (Figure 7B). Primer extension reactions catalyzed by pol β were conducted using a 13-mer primer containing dC, dA, dG, or dT at the 3′ terminus with this base positioned opposite dG-N^2-tamoxifen. The primer extension reactions from all dG·dG-N^2-tamoxifen pairs occurred rapidly, forming 27-mer prod-

ucts containing 5 base deletions (Figure 7C; lanes 3, 7, 11, and 15). Small amounts of 31-mer products containing a 1 base deletion were also detected (see arrows). When the primers extended from dA·dG-N^2-tamoxifen pairs, small amounts of 30-mer products containing 2 base deletions were observed (see arrows, lanes 2, 6, 10, and 14).

DISCUSSION

Since a reactive allylic carbocation of the *trans*- and *cis*-forms of tamoxifen can be converted to that of the *cis*- and *trans*-forms respectively, through an intermediate (Dasaradhi & Shibutani, 1997), four modified oligodeoxynucleotides containing epimers of each *trans*- and *cis*-forms of dG-N^2-tamoxifen were formed when (*Z*)-α-sulfate tamoxifen was reacted. All dG-N^2-tamoxifen-modified oligomers were purified to homogeneity using a combination of HPLC and gel electrophoresis and used for *in vitro* mutagenesis studies.

DNA pol α and δ have been known to be responsible for chromosomal replication and pol β is associated with repair in mammalian cell (Kornberg & Baker, 1992). The miscoding specificities of each *trans*- and *cis*-form of dG-N^2-tamoxifen adduct, observed during translesional synthesis by mammalian DNA polymerases, are explored. All base substitutions and deletions targeted to the site of the lesion were detected by our established *in vitro* experimental system (Shibutani, 1993; Shibutani *et al.*, 1996). DNA pol α showed the preferential misincorporation of dAMP opposite all isomers of dG-N^2-tamoxifen and 1 and 2 base deletions, accompanied by small amounts of misincorporation of dGMP. Pol β also promoted deletions and small amounts of misincorportaion of dAMP and dGMP. In contrast, with pol δ, preferential incorporation of dCMP, a correct base, was observed opposite the lesions: fr-1 of dG-N^2-tamoxifen only promoted small amounts of misincorporation of dTMP. No deletions were detected with pol δ. The 3′ → 5′ exonuclease function of pol δ (Wang, 1991) may efficiently remove incorrect dNTPs inserted opposite the lesions to prevent the formation of deletions. In addition, when the exonuclease-free Klenow fragment of *E. coli* DNA pol I (exo$^-$) was used, 1 and 2 base deletions were preferentially observed at the dG-N^2-tamoxifen lesions, along with small amounts of misincorporation of dAMP and dGMP (S. Shibutani, unpublished data). Thus, the miscoding specificities and frequencies varied depending on the DNA polymerase used.

With pol α, pol β, or pol I exo$^-$, 1 and 2 base deletions were frequently detected opposite dG-N^2-tamoxifens. A following mechanism was proposed for frameshift (deletion) mutations (Kunkel, 1990; Shibutani & Grollman, 1993a). When a nucleotide is inserted opposite the lesion, if the extension is temporarily delayed or blocked, and if a suitable neighboring base capable of pairing with the newly inserted base was 5′ to the lesion, deletions could be produced preferentially. Primer extension reactions catalyzed by DNA polymerases were blocked at the dG-N^2-tamoxifens lesions. With the reaction condition containing a single dNTP, pol β (Figure 7B), pol α and exo$^-$ (data not shown) were shown to insert dAMP and dGMP opposite dG-N^2-tamoxifen adducts. In addition, when dG or dA was positioned opposite the lesion, 1 or 2 base deletions were produced (see arrows, Figure 7C). Thus, when dGMP is inserted opposite the lesion, the newly inserted dGMP could be paired with dC 5′ to the lesion to form 1 base deletions (Figure 8A). The newly inserted dAMP could be paired with dT 2 bases 5′ to the lesion to form 2 base deletions (Figure 8A).

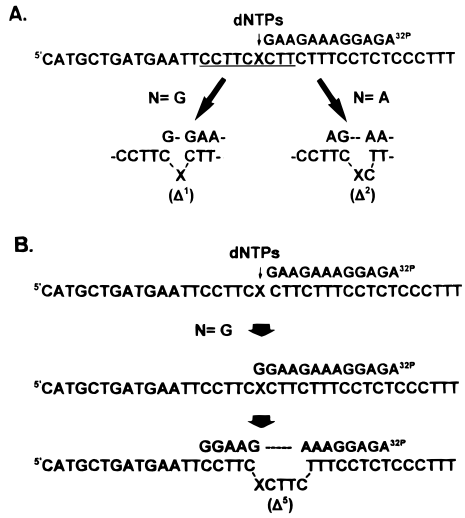

FIGURE 8: Proposed mechanism of 1, 2, and 5 base deletions. X represents dG-N^2-tamoxifen lesion.

With pol β, the *cis*-forms of dG-N^2-tamoxifen (fr-3 and fr-4) particulaly promoted large amounts of 5 base deletions, while the *trans*-forms of dG-N^2-tamoxifen (fr-1 and fr-2) promoted small amounts of 5 base deletions (Figure 7A). All primer extensions from the dG·dG-N^2-tamoxifen pairs occurred rapidly to form 5 base deletions (Figure 7C). However, dGMP was preferentially inserted only opposite the *cis*-forms of dG-N^2-tamoxifen (fr-3 and fr-4) (Figure 7B). Thus, the newly inserted dGMP opposite the lesions and its 5′ flanking bases, dGAAG, of the primer could be paired with dCCTTC 5′ to the lesion to form 5 base deletions (Figure 8B). A similar phenomenon was observed with pol α. This result indicates that our proposed mechanism of 1 and 2 base deletions (Shibutani & Grollman, 1993a) can be used to predict the long-patch deletions.

dG-N^2-modified DNA adducts induced by 2-acetylaminofluorene (Shibutani & Grollman, 1993b), 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (Shibutani *et al.*, 1993b), and model estrogen (Shibutani *et al.*, 1997) are highly blocking lesions. However, dG-N^2-tamoxifen allowed high amounts of primer extension past the lesions, and has strong miscoding potentials. G → T and G → C transversions and deletions are predicted in mammalian cells. Thus, dG-N^2-tamoxifen lesions result in mutations and pose a potential risk to women treated with tamoxifen. This result raises concerns about the use of tamoxifen as a chemopreventive agent for healthy women as well as its use in treatment of breast cancer.

ACKNOWLEDGMENT

We thank Dr. Yoshihiro Matsumoto for providing DNA polymerase δ. We are indebted to Dr. Arthur P. Grollman for his encouragement and interest in this program.

REFERENCES

Carthew, P., Martin, E. A., White, I. N. H., DeMatteis, F., Edwards, R. E., Dorman, B. M., Heydon, R. T., & Smith, L. L. (1995) *Cancer Res. 55*, 544−547.

Dasaradhi, L., & Shibutani, S. (1997) *Chem. Res. Toxicol. 10*, 189−196.

Fischer, B., Costantino, J. P., Redmond, C. K., Fisher, E. R., Wickerham, D. L., & Cronin, W. M. (1994) *J. Natl. Cancer Inst. 86*, 527−537.
Greaves, P., Goonetilleke, R., Nunn, G., Topham, J., & Orton, T. (1993) *Cancer Res. 53*, 3919−3924.
Han, X., & Liehr, J. G. (1992) *Cancer Res. 52*, 1360−1363.
Hard, G. C., Iatropoulos, M. J., Jordan, K., Radi, L., Kaltenberg, O. P., Imondi, A. R., & Williams, G. M. (1993) *Cancer Res. 53*, 4534−4541.
Hemminki, K., Rajaniemi, H., Lindahl, B., & Moberger, B. (1996) *Cancer Res. 56*, 4374−4377.
IARC Monographs (1996) Vol. 66. Lyon, IARC.
Jordan, V. C. (1993) *Br. J. Pharmacol. 110*, 507−517.
Kornberg, A., & Baker, T. (1992) *DNA replication*, 2nd ed., pp197−226, W. H. Freeman & Company, New York.
Kunkel, T. A. (1990) *Biochemistry 29*, 8003−8011.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Plainview, NY.
Matsumoto, Y., Kim, K., & Bogenhagen, D. F. (1994) *Mol. Cell. Biol. 14*, 6187−6197.
Maxam, A. M., & Gilbert. W. (1980) *Methods Enzymol. 65*, 499−560.
Moorthy, B., Sriram, P., Pathak, D. N., Bodell, W. J., & Randerath, K. (1996) *Cancer Res. 56*, 53−57.
Nayfield, S. G., Karp, J. E., Ford, L. G., Dorr, F. A., & Kramer, B. S. (1991) *J. Natl. Cancer Inst. 83*, 1450−1459.
Osborne, M. R., Hewer, A., Hardcastle, I. R., Carmichael, P. L., & Phillips, D. H. (1996) *Cancer Res. 56*, 66−71.
Phillips, D. H., Carmichael, P. L., Hewer, A., Cole, K. J., & Poon, G. K. (1994a) *Cancer Res. 54*, 5518−5522.
Phillips, D. H., Potter, G. A., Horton, M. N., Hewer, A., Crofton-Sleigh, C., Jarman, M., & Venitt, S. (1994b) *Carcinogenesis 15*, 1487−1492.
Poon, G. K., Walter, B., Lfnning, P. E., Horton, M. N., & McCague, R. (1995) *Drug Metab. Dispos. 23*, 377−382.
Potter, G. A., McCague, R., & Jarman, M. (1994) *Carcinogenesis 15*, 439−442.
Powles, T. J., Hardy, J. R., Ashley, S. E., Farrington, G. H., Cosgrove, D., Davey, J. R., Dowsett, M., McKinna, J. A., Wash, A. G., Sennet, H. D., Tillyer, C. R., & Treleaven, J. G. (1993) *Br. J. Cancer 60*, 126−131.
Randerrath, K., Moorthy, B., Mabon, N., & Sriram, P. (1994) *Carcinogenesis 15*, 2087−2094.
Seoud, M. A.-F., Johnson, J., & Weed, J. C. (1993) *Obstet. Gynecol. 82*, 165−169.
Shibutani, S. (1993) *Chem. Res. Toxicol. 6*, 625−629.
Shibutani, S., & Grollman, A. P. (1993a) *J. Biol. Chem. 268*, 11703−11710.
Shibutani, S., & Grollman, A. P. (1993b) *Chem. Res. Toxicol. 6*, 819−824.
Shibutani, S., Gentles, R., Johnson, F., & Grollman, A. P. (1991a) *Carcinogenesis 12*, 813−818.
Shibutani, S., Takeshita, M., & Grollman, A. P. (1991b) *Nature 349*, 431−434.
Shibutani, S., Bodepudi, V., Johnson, F., & Grollman, A. P. (1993a) *Biochemistry 32*, 4615−4621.
Shibutani, S., Margulis, L. A., Geacintov, N. E., & Grollman, A. P. (1993b) *Biochemistry 32*, 7531−7541.
Shibutani, S., Suzuki, N., Matsumoto. Y., & Grollman, A. P. (1996) *Biochemistry 35*, 14992−14998.
Shibutani, S., Itoh, S., & Yoshizawa, I. (1997) *Biochemistry 36*, 1755−1765.
Takeshita, M., Chang, C.-N., Johnson, F., Will, S., & Grollman, A. P. (1987) *J. Biol. Chem. 262*, 10171−10179.
van Leeuwen, F. E., Benraadt, J., Coebergh, J. W. W., Kiemeney, L. A. L. M., Diepenhorst, F. W., van den Belt-Dusebout, A. W., & van Tinteren, H. (1994) *Lancet 343*, 448−452.
Wang, T. S.-F. (1991) *Annu. Rev. Biochem. 60*, 513−552.
White, I. N. H., de Matteis, F., Davies, A., Smith, L. L., Crofton-Sleigh, C., Venitt, S., Hewer, A., & Phillips, D. H. (1992) *Carcinogenesis 13*, 2197−2203.
Williams, G. M., Iatropoulos, M. J., Djordjevic, M. V., & Kaltenberg, O. P. (1993) *Carcinogenesis 14*, 315−317.

BI970243C