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The Efficiency and Fidelity of Translesion Synthesis past Cisplatin and Oxaliplatin GpG Adducts by Human DNA Polymerase β*

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DNA polymerase β (pol β) is the only mammalian DNA polymerase identified to date that can catalyze extensive bypass of platinum-DNA adducts *in vitro*. Previous studies suggest that DNA synthesis by pol β is distributive on primed single-stranded DNA and processive on gapped DNA. The data presented in this paper provide an analysis of translesion synthesis past cisplatin- and oxaliplatin-DNA adducts by pol β functioning in both distributive and processive modes using primer extension and steady-state kinetic experiments. Translesion synthesis past Pt-DNA adducts was greater with gapped DNA templates than with single-stranded DNA templates. In the processive mode pol β did not discriminate between cisplatin and oxaliplatin adducts, while in the distributive mode it displayed about 2-fold increased ability for translesion synthesis past oxaliplatin compared with cisplatin adducts. The differentiation between cisplatin and oxaliplatin adducts resulted from a K_m-mediated increase in the efficiency of dCTP incorporation across from the 3′-G of oxaliplatin-GG adducts. Rates of misincorporation across platinated guanines determined by the steady-state kinetic assay were higher in reactions with primed single-stranded templates than with gapped DNA and a slight increase in the misincorporation of dTTP across from the 3′-G was found for oxaliplatin compared with cisplatin adducts.

Decreased DNA synthesis during replication is responsible in part for the chemotherapeutic effect of drugs which form bulky adducts on the DNA. Mammalian cells contain a post-replication repair pathway that allows cells to overcome replication blocks caused by these adducts. Post-replication repair can be either error-free, which would increase cellular tolerance of bulky adducts, or error-prone, which would increase the mutagenicity of bulky adducts. Previous studies have shown that mammalian cells possess the ability to replicate DNA containing cisplatin (*cis*-diamminedichloroplatinum(II))[1] adducts and that cisplatin-resistant cell lines have a greater ability to replicate past Pt-DNA adducts than their parental cisplatin-sensitive cell lines (1–4). This suggests that some cellular DNA polymerases must be able to replicate Pt-containing DNA relatively well and tolerant cells might have either elevated levels of the polymerases responsible for lesion bypass or mutant DNA polymerases with enhanced translesion replication activity.

DNA polymerase β has been suggested as a candidate for the role of a DNA polymerase that performs error-prone translesion synthesis of cisplatin-DNA adducts (5, 6). Pol β is the only mammalian DNA polymerase identified to date that has been shown to catalyze extensive replicative bypass of Pt-DNA adducts *in vitro* (5–8). In addition, pol β is able to elongate the arrested replication products of polymerases α and δ, thus showing its capacity to successfully complete stalled replication (5). The full-length DNA products of translesion synthesis by pol β contain a high frequency of mutations in the vicinity of the Pt-DNA adducts (6). On the basis of these data, Hoffmann *et al.* (5, 6) have suggested that pol β could be involved in error-prone translesion replication past Pt-DNA adducts *in vivo*. In support of this hypothesis, increased cisplatin resistance (9–12) and an increased rate of both spontaneous and cisplatin-induced mutagenesis (12) have been observed in cell lines with increased pol β expression. This has lead to the suggestion that overexpression of pol β could be associated with cisplatin resistance and a mutator phenotype (12–14). If this hypothesis is correct it could be significant, since some spontaneously occurring tumors appear to overexpress pol β (15), and pol β gene amplification has been detected in tumors from patients that have failed cisplatin therapy (9). Although cell lines expressing pol β antisense RNA show decreased sensitivity to cisplatin (16), no increased sensitivity to cisplatin has been seen in pol β-deleted cells (17). Thus, the available data suggest that pol β overexpression might represent an important mechanism of platinum drug resistance and mutagenicity, but it is not yet clear whether pol β makes a significant contribution to error-prone translesion synthesis at normal levels of expression.

Although to date there is no direct evidence showing that pol β actually carries out error-prone DNA synthesis past bulky DNA adducts *in vivo*, one can envision at least two models for its involvement in translesion synthesis during *in vivo* replication. 1) Pol β might substitute for a replication complex that had stalled at Pt-DNA lesion sites during chromosomal replication. After error-prone translesion synthesis, pol β would dissociate. Then the replication complex could reassociate at the nascent primer terminus downstream from the lesion and complete DNA replication (18). 2) Alternatively, pol β might be involved in the error-prone filling of single-stranded gaps in the daughter strand opposite the lesion. These gaps could be formed as a part of lagging strand synthesis or as a result of activation of alternative origins of replication in damaged chromosomes after the progression of the active replication fork is blocked by a DNA lesion (19).

Both the extent and fidelity of translesion synthesis by pol β

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[1] The abbreviations used are: cisplatin, *cis*-diamminedichloroplatinum(II); oxaliplatin, (*trans-R,R*)-1,2-diaminocyclohexaneoxalatoplatinum(II); Pt(dach)Cl_2, (*trans-R,R*)-1,2-diaminocyclohexanechloroplatinum(II); dach, (*trans-R,R*)-1,2-diaminocyclohexane.

could be affected by the DNA substrate (single-stranded *versus* gapped). It has been shown that DNA synthesis by pol β is distributive on DNA substrates in which primer is annealed to a single-stranded template, while pol β conducts processive synthesis on short gapped DNA templates with a 5′-phosphate at the end of the gap (16, 17, 20–22). With gaps of 2–6 nucleotides pol β exhibits increased catalytic efficiency (23–25) compared with primed single-stranded DNA. However, the overall base substitution fidelity of pol β appears to be very similar with 2–6 nucleotide gaps and single-stranded templates (23, 24).

The platinum complex (*trans-R,R*)1,2-diamminocyclohexaneoxalatoplatinum(II) (oxaliplatin) is currently in clinical trial for the treatment of tumors with intrinsic and acquired resistance to cisplatin. Both cisplatin and oxaliplatin form the same types adducts on the DNA, with the most abundant being Pt(GpG) intrastrand diadducts (26–29). However, the adducts formed by oxaliplatin contain (*trans-R,R*)1,2-diamminocyclohexane (dach) carrier ligands, while the adducts formed by cisplatin contain *cis*-diammine carrier ligands. Previous data from our laboratory suggest that mammalian cells are able to replicate DNA in the presence of platinum-DNA adducts and that the extent of replicative bypass *in vivo* can be influenced by the carrier ligand of the adducts (1, 2, 4). We also have shown recently that pol β can discriminate between cisplatin and oxaliplatin adducts *in vitro*. The present study was designed to evaluate whether the efficiency and carrier ligand specificity of replicative bypass past Pt-DNA adducts by pol β could be determined by the mode of translesion synthesis (distributive synthesis on single-stranded DNA *versus* processive synthesis on gapped DNA) and whether the fidelity of replicative bypass depends on the carrier ligand of the Pt-DNA adducts and/or on the DNA template structure.

EXPERIMENTAL PROCEDURES

Construction of Platinum Adduct-containing Templates—Primer-templates were constructed from synthetic oligonucleotides as described previously (8). Briefly, cisplatin was obtained from Sigma; Pt(dach)Cl_2 (the biotransformation product of oxaliplatin) was provided by Dr. S. D. Wyrick (University of North Carolina). All platination reactions were carried out with aquated derivatives of the platinum complexes obtained by overnight stirring in the dark at room temperature of a solution containing either cisplatin or Pt(dach)Cl_2 and 1.98 equivalents of silver nitrate. The final concentration of aquated derivatives of the platinum complexes was 40 mM. Platination of a 12-mer oligonucleotide containing a single GG sequence within a *Stu*I restriction site (TCTAGGCCTTCT) was performed for 2 h at 37 °C in the dark with the 2:1 drug to oligonucleotide ratio. The oligonucleotides containing a single platinum adduct were separated from unplatinated impurities by electrophoresis on a 20% polyacrylamide gel. DNA was eluted from gel slices and desalted using CENTRI-SPIN™-10 columns (Princeton Separations, Inc).

The templates used for primer extension and steady-state kinetic experiments are listed in Fig. 1. Site-specifically platinated 44-mer templates were constructed as described previously (8): platinated 12-mers were ligated with a 14-mer (left end) and an 18-mer (right end) using a 35-mer as a scaffold. Control 44-mer templates were prepared using unplatinated 12-mer by the same procedure. After 16-h ligation at 16 °C by T4 DNA ligase, templates were purified by MicroSpin G-25 columns (Amersham Pharmacia Biotech), restricted by *Stu*I to ensure the absence of any unplatinated oligonucleotides, and purified on 12% denaturing polyacrylamide gels. Control experiments were performed to ensure the purity of platinated templates as described previously (8). *Stu*I cleavage was used in all primer extension assays described below, and only *Stu*I-resistant elongation products were quantitated as translesion synthesis to control for any loss of platinum adducts during the incubation. *Stu*I cleaves >95% of undamaged templates elongated past the *Stu*I restriction site, but it is almost completely blocked by the presence of either cisplatin or oxaliplatin adducts (8).

The 22-, 24-, or 25-mer primers were 5′-end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP, according to the manufacturer's protocol. 14- and 16-mer downstream oligonucleotides used to form 6- or 5-base-gapped templates were 5′-end-phosphorylated with unlabeled ATP. DNA substrates were prepared by annealing undamaged or platinated 44-mer templates with ^{32}P-labeled primers alone or in combination with downstream oligonucleotides at a 1.2:1: (2) molar ratio. Hybridization was done by heating the mixture of required oligonucleotides in an annealing buffer (50 mM Tris-HCl (pH 8), 100 mM NaCl) for 10 min at 80 °C followed by slow cooling to room temperature over a period of about 2 h. Annealing efficiencies were >95%, as evidenced by the different mobility of the ^{32}P-labeled primers before and after hybridization to the template on nondenaturing polyacrylamide gels (data not shown).

Primer Extension Assays—Recombinant human pol β was generously provided by Dr. S. Wilson (NIEHS) (specific activity 1.4 units/μg). One unit of polymerase activity is defined as the amount of enzyme required for the incorporation of 1 nmol of dTMP in 1 min at 25 °C using poly(dA)-p(dT)20 as a template-primer). Primer extension assays were performed as described previously (8) using 22/44 and 24/44 primer-templates with or without 16- or 14-mer downstream oligonucleotides (Fig. 1, *A–D*). 200 fmol of primer-template (expressed as primer termini) was incubated with pol β at 37 °C in 10-μl reactions containing 500 μM dNTPs unless otherwise indicated. Reaction times and enzyme concentrations are indicated in the figures. Reactions were terminated by heating at 80 °C for 10 min. Each reaction mixture was split into two aliquots, one of which was incubated with *Stu*I restriction enzyme. Aliquots were mixed with 0.7 volume of formamide loading dye solution containing 500 mM EDTA, 0.1% xylene cyanol, and 0.1% bromphenol blue in 90% formamide. Before loading onto the gel, the reactions were denatured by heating at 100 °C for 5 min and immediately transferred to ice for 5 min. Products were resolved by denaturing polyacrylamide gel electrophoresis (8 M urea, 16% acrylamide, 4 h at 2000 V) and then visualized and quantified using a Molecular Dynamics PhosphorImager and ImageQuant software. The extent of translesion synthesis on damaged templates was calculated as the sum of elongation products past the platinum adducts as a percent of total primer termini (elongated and unelongated).

Steady-state Polymerization Kinetics—Steady-state kinetic parameters K_m and V_{max} for dCTP incorporation were measured in standing-start reactions as described previously (8) using the 44-mer templates hybridized to 24- or 25-mer primers alone or in combination with downstream 16-mer oligonucleotides (Fig. 1, *B, D, E*, and *F*). 150 fmol of DNA substrates were replicated at 37 °C in 10-μl reaction mixtures containing 5 fmol of pol β and variable concentrations of dCTP. Initial time course studies indicated that under standard conditions the reaction velocity is linear (*i.e.* conforms to steady-state kinetics) for up to 12 min for single-stranded templates and up to 6 min for gapped templates (data not shown). Less than 20% of the primers were extended under the steady-state conditions ensuring single hit conditions. As expected in steady state, V_{max} values were proportional to enzyme concentration (data not shown).

The velocity of dCTP incorporation opposite the template 3′-G and 5′-G sites was determined using the equation $v = (I_{3'\text{-G}} + I_{5'\text{-G}})/tI_{-1}$ with templates B and D (Fig. 1) for the 3′-G or $v = I_{5'\text{-G}}/tI_{-1}$ with templates E and F for the 5′-G (where t is the reaction time in min; I_{-1}, $I_{3'\text{-G}}$, and $I_{5'\text{-G}}$ are the integrated gel band intensities at sites −1 (corresponding to unextended primer, 3′-G, and 5′-G, respectively). The relationship between v and dCTP concentration conformed to a Michaelis-Menten equation, as indicated by linearity in a Hanes-Woolf plot of [dCTP]/v *versus* [dCTP]. V_{max} (the maximum value of reaction velocity) and K_m (dCTP concentration at which the reaction velocity is half-maximal) were determined from a Hanes-Woolf plot by linear least squares fit. The efficiency of nucleotide insertion by polymerase (f) was calculated as V_{max}/K_m. To facilitate comparison of values for different Pt adducts, the relative insertion efficiency f_{rel} also is reported: $f_{rel} = f_{Pt}/f_{control}$.

Misincorporation Assays—To measure nucleotide misinsertion opposite Pt-GG adducts, DNA substrates shown in Fig. 1, *B, D, E*, and *F* were used. 150 fmol of these DNA substrates were incubated with 5 fmol of pol β and 5 mM of each dNTP individually for 15 and 30 min for control and damaged templates, respectively. Reactions were terminated, and products were analyzed as described above. The kinetics of dTTP misincorporation opposite platinated and undamaged GG sites using templates shown in Fig. 1, *C*, *E*, and *F*, were determined in reactions similar to ones described in the previous section. To detect extension products resulting from nucleotide misincorporation, it was necessary to increase incubation times from 1–2 to 15–30 min. The dTTP misinsertion efficiency (f_{mis}) was determined as the ratio of dTTP to dCTP insertion efficiencies.

FIG. 1. **Primed single-stranded and gapped DNA substrate configurations used in primer-extension and kinetic studies.** The site-specifically modified DNA templates were constructed using combination of synthetic oligonucleotides as described under "Experimental Procedures."

RESULTS

In a previous study (8) we established the carrier-ligand specificity of pol β catalyzed translesion synthesis using primer-templates where the primer was placed either 2 or 53 bases before the GG sites containing the platinum adduct. In those studies, oxaliplatin-GG adducts were bypassed to a greater extent than cisplatin-GG adducts, and the placement of the primer relative to the Pt-GG adduct had no effect on the carrier-ligand specificity of translesion synthesis. To study the effect of template structure on the extent and specificity of translesion synthesis by pol β in more detail, we have performed the primer extension assay using undamaged or site-specifically platinated templates of different structure (Fig. 1, *A–D*). All these DNA substrates contained the same 44-mer template. In addition to a 22-mer primer used previously in primer extension assays (Fig. 1*A*), we have utilized a 24-mer primer (Fig. 1*B*) to examine the capacity of pol β to initiate DNA synthesis from the base preceding the lesion. The same experiments were also carried out using 6-base gapped DNA templates with the lesion either in the center of the gap (Fig. 1*C*) or at the beginning of the gap (Fig. 1*D*).

Fig. 2, *A–H*, shows the time course for elongation of 22-mer or 24-mer primers on 44-mer single-stranded and gapped DNA templates, either undamaged or containing a single cisplatin adduct. The positions of primers, full-length reaction product (44-mer), and template sequence are indicated. The enzyme to DNA substrate ratio was approximately 1:4 in these experiments, which corresponds to the minimum amount of pol β necessary to ensure measurable translesion synthesis on all templates. As expected for such reaction conditions, distinct bands corresponding to practically every nucleotide in the sequence were clearly visible when undamaged single-stranded templates were used (Fig. 2, *A* and *E*). Such a pattern is consistent with the distributive nature of pol β. Singhal and Wilson (30) have shown previously that pol β fills gaps of up to 6 nucleotides by a processive mechanism that requires a phosphate group at the 5′-end of the gap. In agreement with these findings we observed no strong pause sites within the 6-nucleotide gaps on undamaged DNA substrates (Fig. 2, *C* and *G*). At the pol β concentrations used in these experiments, some strand displacement was observed with gapped DNA templates. The extent of strand displacement depended on the DNA substrate utilized. For example, with the 22/44/16 DNA template (Fig. 1*C*) there was a major accumulation of products varying in length from 28 nucleotides (corresponding to the end of the gap) to 32 nucleotides (corresponding to strand displacement of four nucleotides). This was accompanied by significant time-dependent strand displacement to the end of the template (Fig. 2*C*). However, with the 24/44/14 DNA substrate (Fig. 1*D*) the major stop sites varied in length from 30 nucleotides (end of the gap) to 32 nucleotides (strand displacement of two nucleotides) (Fig. 2*G*). This was accompanied by limited strand displacement of the remaining duplex DNA, which did not increase with time. The difference in strand displacement between the 22/44/16 and 24/44/14 DNA substrates is most likely due to the stability of the duplex region immediately downstream of the gap. For the 24/44/14 DNA substrate, the first 5 base pairs of the duplex region downstream of the gap are all G·Cs, whereas the 22/44/16 DNA substrate contains an A·T base pair located 2 bases downstream from the gap.

Primer extension on all damaged templates was substantially reduced (Fig. 2, *B, D, F,* and *H*). When single-stranded templates were used, the decrease in the amount of full-length product was mainly due to a strong inhibition of dNTP incorporation opposite the template 3′-G(Pt) (24-mer band), Fig. 2, *B* and *F*) and 3′ to the lesion (22- and 23-mer bands), Fig. 2*B*). Weaker inhibition of dNTP incorporation opposite the 5′-G(Pt) (25-mer band), Fig. 2, *B* and *F*) and of chain extension from 5′-G(Pt) (26-mer band), Fig. 2, *B* and *F*) was also observed. Primer extension on damaged gapped DNA templates produced multiple stop sites, although the relative distribution of the products of replication was somewhat different from that seen with single-stranded templates. Stop sites 3′ from the lesion weakened. This was most apparent for the DNA substrates with the Pt adduct in the center of the gap (compare the 23- and 24-mer bands in gel 2D (gapped DNA) with gel 2B (single-stranded DNA)). In addition, the stop site across from the 3′-G(Pt) became more prominent and additional pause sites 1 and 2 nucleotides beyond the adduct appeared. This was most apparent for the DNA substrates with the Pt adduct at the beginning of the gap (compare the 27- and 28-mer bands in gel 2H (gapped DNA) and gel 2F (single-stranded DNA)). Some strand displacement was seen with both gapped DNA templates with platinum adducts. However, in contrast to the pattern of strand displacement for undamaged templates, less strand displacement was seen for the 22/44/16 DNA template (Fig. 2*D*) than for 24/44/14 DNA template (Fig. 2*H*).

The extent of translesion synthesis past the cisplatin-GG and oxaliplatin-GG adducts was calculated as described under

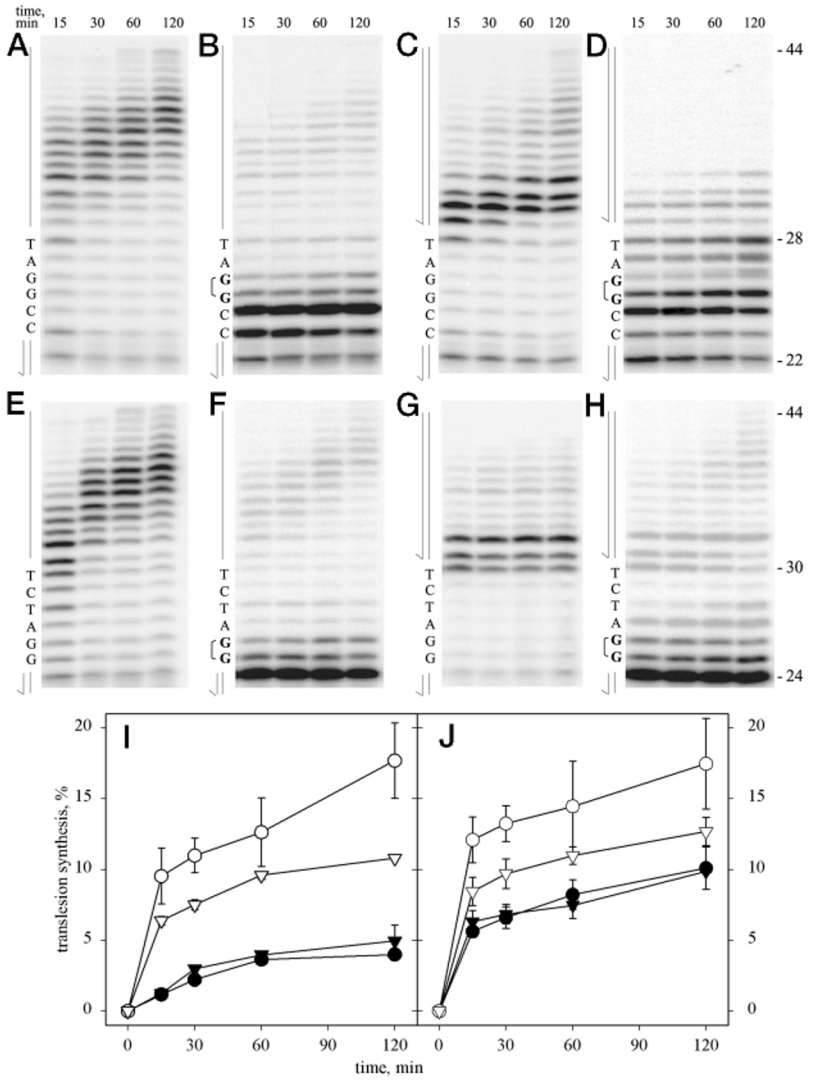

FIG. 2. **Primer extension activity of pol β using DNA substrates of different structure.** Time course experiments were conducted using 150 fmol of undamaged templates (*A, C, E,* and *G*) or templates containing a single cisplatin-GG adduct (*B, D, F,* and *H*) and 40 fmol of pol β. Reactions with 5′-^{32}P-end-labeled primer situated 2 bases before (*A–D*) or immediately before (*E–H*) the Pt-GG adduct were performed using primed single-stranded (*A, B, E,* and *F*) or gapped (*C, D, G,* and *H*) DNA substrates. Template sequences are shown to the *left* of the gels. Platinated guanines are indicated. The length of the primer and the position corresponding to the end of the gap are shown on the *right*. The extent of translesion synthesis past cisplatin (*I*) and oxaliplatin (*J*) (gels not shown) adducts was calculated as a percent of total primer termini as described under "Experimental Procedures." The primer extension reactions were performed using 22/44 (●)- and 24/44 (▼) primed single-stranded templates and 22/44/16 (○) and 24/44/14 (▽) gapped DNA templates (Fig. 1). Data are the means (±S.E.) from four different experiments using two independent template preparations.

"Experimental Procedures" and is summarized in Fig. 2, *I* and *J* (the stop sites were identical for cisplatin- and oxaliplatin-GG adducts, so the gels for the experiment with oxaliplatin adduct are not shown). Levels of translesion synthesis past platinum adducts (sum of products longer than 26 nucleotides (Fig. 2, *B, D, F*, and *H*) as a percent of total primer termini) appeared to be similar for both the 22/44 and 24/44 single-stranded DNA templates (Fig. 2, *I* and *J*, *closed symbols*). The extent of translesion synthesis past Pt-DNA adducts was increased 1.5–3-fold with gapped DNA templates (Fig. 2, *I* and *J*, *open symbols*) relative to single-stranded primed DNA templates. Furthermore, a higher extent of translesion replication was observed for DNA templates with the Pt adduct in the center of the gap (Fig. 2, *I* and *J*, *open circles*) compared with DNA templates with the Pt adduct at the beginning of the gap (Fig. 2, *I* and *J*, *open triangles*).

In agreement with our previous data on pol β-catalyzed primer extension at low enzyme concentrations (8), cisplatin adducts were about 2.5-fold more effective than oxaliplatin adducts at blocking translesion synthesis on both 22/44 and 24/44 single-stranded primed templates (compare Fig. 2, *I* and *J*, *closed symbols*). However, with both gapped DNA substrates, there was relatively little difference in the extent of translesion synthesis between templates with cisplatin-GG and oxaliplatin-GG adducts (Fig. 2, *I* and *J*, *open symbols*).

We have also shown previously that when the enzyme is present in excess to primed single-stranded DNA templates, pol β does not appear to discriminate between cisplatin- and oxaliplatin-DNA adducts. Thus, we examined the effect of polymerase concentration on replicative bypass using the DNA substrates described above (Fig. 3). At high enzyme concentrations (2.5–25-fold excess over primer-template), pol β did not differentiate between cisplatin and oxaliplatin adducts for any DNA substrate tested (data not shown); therefore data are presented for cisplatin-damaged templates only. Fig. 3 shows the enzyme concentration-dependence and time course for translesion synthesis past cisplatin-GG adducts using all four DNA constructs. The extra band seen at 45 nucleotides is characteristic of primer extension reactions with high concentrations of pol β and is most likely due to the intrinsic nucleotidyl transferase activity of pol β (8). With both 22/44 and 24/44 primed single-stranded templates at pol β to template ratios of 25:1, strong stop sites 1 and 2 nucleotides past the Pt-GG adducts were observed in addition to the stop sites 3′ to the Pt-GG adduct and across the lesion (Fig. 3, *A* and *C*). For both gapped templates, the stop site immediately preceding the Pt-GG adduct was decreased in intensity and the stop site immediately following the Pt-GG adduct was increased in intensity compared with the single-stranded templates (compare Fig. 3, *B* and *D,* with 3, *A* and *C*). Finally, the stop sites 1 and 2 nucleotides past the Pt-GG adducts were considerably weaker and strand displacement was significantly greater for the template with the Pt-GG adduct at the beginning of the gap compared with the template with the Pt-GG adduct in the

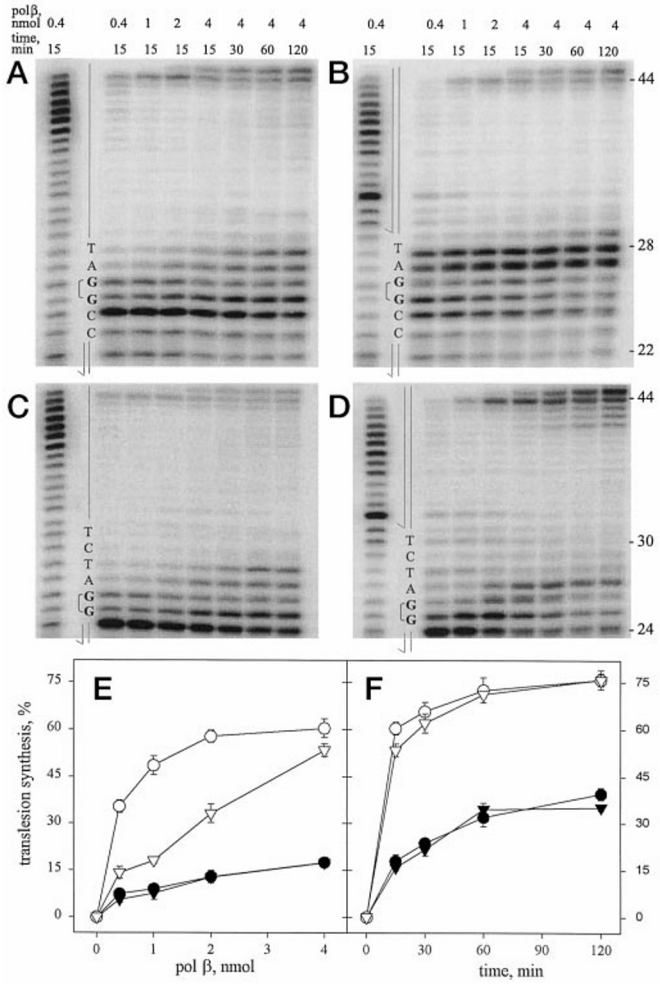

FIG. 3. **Primer extension activity of pol β at high enzyme concentrations.** Enzyme concentration dependence and time course experiments were carried out using 150 fmol of DNA templates with 5′-^{32}P-end-labeled primer situated 2 bases before (*A* and *B*) and immediately before (*C* and *D*) the cisplatin-GG adduct on single-stranded (*A* and *C*) or gapped (*B* and *D*) DNA substrates. Primer extension by 0.4 nmol of pol β on control templates (first lane in each gel) were performed for 15 min. Template sequences are shown to the *left* of the gels. Platinated guanines are indicated. The length of the primer and the position corresponding to the end of the gap are shown on the *right*. The graphs show the enzyme concentration dependence at 15 min (*E*) or the time course with 4 nmol of pol β (*F*) for translesion synthesis past cisplatin adducts as a percent of total primer termini. These data were obtained using 22/44 (●)- and 24/44 (▼) primed single-stranded templates and 22/44/16 (○)- and 24/44/14 (▽) gapped DNA templates (Fig. 1). Data are means (±S.E.) from four different experiments using two independent template preparations.

center of the gap. For example, with the DNA substrate containing the Pt adduct at the beginning of the gap, no stop site was observed at the end of the gap and strand displacement was essentially complete (Fig. 3*D*). In contrast, with the DNA substrate containing the Pt adduct in the center of the gap, a strong stop site at the end of the gap and significantly less strand displacement were observed (Fig. 3*B*).

The extent of translesion synthesis past cisplatin adducts with high levels of pol β appeared to be identical for 22/44 and 24/44 primed single-stranded DNA substrates (Fig. 3, *A* and *C*; Fig. 3, *E* and *F*, *closed symbols*). The effect of adding the 14- or 16-mer 5′-phosphorylated downstream oligonucleotides to create 6-base pair gaps (Fig. 3) was very similar to the effects observed at lower pol β concentrations (Fig. 2). The extent of translesion synthesis was increased with both gapped templates compared with single-stranded templates (Fig. 3, *E* and *F*, *open symbols versus closed symbols*). The most significant effect of pol β concentration was on the relative extent of translesion synthesis with the two gapped templates. At enzyme to template ratios of up to 12:1, gapped DNA with the lesion in the center of the gap was a better substrate for translesion synthesis than gapped DNA with the lesion at the beginning of the gap (Fig. 3*E*). However, at enzyme to template ratios of greater than 12:1, the total amount of translesion synthesis past Pt-GG sites was equal for these two DNA substrates at all reaction times tested (Fig. 3*F*).

Steady-state Kinetics of dCTP Insertion Opposite GG Sites on Damaged and Undamaged Templates—As shown above, oxaliplatin-GG adducts were bypassed more readily than cisplatin-GG adducts when the primer extension assay was performed with single-stranded DNA substrates under conditions of low enzyme concentration (Fig. 2, *I* and J, *closed symbols*) and/or limiting dNTP concentrations (data not shown). However, during processive gap-filling synthesis, replication past cisplatin and oxaliplatin adducts did not differ significantly (Fig. 2, *I* and *J*, *open symbols*). Goodman and colleagues (31) have shown that the steady-state kinetic assay is more sensitive than the primer extension assay for determining whether a polymerase is able to discriminate between adducts and can provide information about the mechanism of discrimination between adducts. This assay is performed under limiting enzyme concentrations and is more representative of true intracellular conditions than the primer extension assay. To characterize the extent of lesion bypass more quantitatively, kinetic experiments were performed on undamaged templates and templates with cisplatin and oxaliplatin adducts. In the previous primer-extension experiments, the strongest inhibitory effect of platinum adducts on DNA replication by pol β at low enzyme concentrations was reduced incorporation across from the 3′- and 5′-platinated guanines. Therefore, steady-state kinetic assays of dCTP incorporation opposite these major stop sites were performed. Our study was done using standing start reaction conditions where nucleotide insertion is measured at the first template site adjacent to the original primer 3′ terminus. Although the absolute values of kinetic parameters, V_{max} and K_m, derived from the standing start assay have limited mechanistic significance, the ratios of V_{max}/K_m for damaged *versus* undamaged templates are reliable measurements of the effect of different lesions on polymerase activity (32). All experiments were carried out in the presence of 30-fold excess of template over enzyme. Prior to kinetic studies, experiments were performed to determine the enzyme concentrations, dNTP concentrations, and times for which product accumulation was linear as a function of time.

The kinetic parameters for dCTP insertion opposite the template 3′- and 5′-Gs using control and damaged primed single-stranded and gapped DNA substrates (Fig. 1, *B, D, E*, and *F*) are presented in Table I. As anticipated, the catalytic efficiency (V_{max}/K_m) for dCTP incorporation opposite the 3′- and 5′-Gs was enhanced substantially with 5-nucleotide and 6-nucleotide gapped DNA compared with primed single-stranded DNA substrates, mainly due to increases in V_{max}. The observed 4-fold increase in catalytic efficiency on gapped DNA relative to single-stranded DNA is comparable with that reported previously for 6-nucleotide gaps (24). Chagovetz *et al*. (24) reported that the increase in catalytic efficiency with 6-nucleotide gapped DNA was predominantly K_m-mediated. The apparent discrepancy between this report and our data may relate to differences in the assays used to measure kinetic parameters. Chagovetz *et al.* (24) used the running start kinetic assay. Since the kinetic parameters in our experiments were determined by the standing start assay, the V_{max}-governed discrimination might not

TABLE I

Steady-state kinetic analysis of dCTP insertion opposite Pt-GG adducts

Standing start kinetic assays were performed using 5 fmol of pol β and 150 fmol of primer-templates. Data were obtained using 22/44 and 24/44 primed single-stranded templates and 22/44/16 and 24/44/14 gapped DNA templates (Fig. 1). Incubation time was 1 min for control gapped DNA substrates, 2 min for platinated gapped DNA substrates, 5 min for control primed single-stranded DNA substrates, and 10 min for platinated primed single-stranded DNA substrates. dCTP concentrations ranged from 1 to 8 μM for the control and from 32 to 1024 μM for the platinated templates. Kinetic parameters (K_m and V_{max}) and insertion efficiency (V_{max}/K_m) for dCTP incorporation by pol β were determined using Hanes-Woolf plots (see "Experimental Procedures"). The relative insertion efficiency was determined as $f_{rel} = f_{Pt}/f_{control}$. Data are means (±S.E.) from five to six different experiments using two independent template preparations. nt, nucleotide.

Target (template)	Pt adduct	V_{max}	K_m	V_{max}/K_m	f_{rel}
		min^{-1}	μM	$M^{-1}\ min^{-1}$	
3′-G (primed single-stranded DNA)	Control	0.022 ± 0.002	2.6 ± 0.3	8300 ± 470	
	Cisplatin	0.020 ± 0.002	234 ± 22	90 ± 12	0.011
	Oxaliplatin	0.019 ± 0.001	110 ± 14	181 ± 25	0.022
5′-G (primed single-stranded DNA)	Control	0.021 ± 0.002	3.2 ± 0.7	6460 ± 174	
	Cisplatin	0.014 ± 0.001	95 ± 8	141 ± 22	0.027
	Oxaliplatin	0.016 ± 0.001	96 ± 6	165 ± 24	0.032
3′-G (6-nt gap)	Control	0.125 ± 0.039	3.9 ± 1.7	32,000 ± 3000	
	Cisplatin	0.083 ± 0.004	36.8 ± 2.3	2200 ± 200	0.069
	Oxaliplatin	0.096 ± 0.003	39.6 ± 3.2	2400 ± 270	0.075
5′-G (5-nt gap)	Control	0.090 ± 0.014	3.3 ± 0.7	27,000 ± 6000	
	Cisplatin	0.092 ± 0.015	77 ± 6	1300 ± 290	0.048
	Oxaliplatin	0.100 ± 0.011	80 ± 7	1450 ± 250	0.054

necessarily represent the differences in the rate of polymerization from one site to the next, but could also be due to the differences in the rate of enzyme-DNA dissociation or association (32).

Pt-GG adducts had little effect on the V_{max} for dCTP insertion opposite either the 3′- or 5′-Gs (Table I). On the other hand, Pt-GG adducts increased the K_m values for dCTP insertion substantially (Table I). The increase in the K_m values was greater with single-stranded DNA templates (30–90-fold) than with gapped DNA substrates (10–25-fold). Thus, the use of gapped DNA led to a considerable increase (2–3.5-fold) in the efficiency of nucleotide insertion across both 3′-G(Pt) and 5′-G(Pt) relative to single-stranded DNA. With a single-strand DNA template, the K_m for insertion of dCTP opposite the 3′-G of an oxaliplatin-GG adduct was about one-half that for insertion of dCTP opposite the 3′-G of a cisplatin-DNA adduct, while the V_{max} values for both reactions were very similar (Table I). This resulted in overall 2-fold increase in insertion efficiency across from the 3′-G of an oxaliplatin adduct. However, no significant differences were evident between templates with cisplatin and oxaliplatin adducts in the relative insertion efficiency of dCTP opposite the 5′-G(Pt) on single-stranded DNA or opposite either the 3′-G(Pt) and 5′-(G)Pt on gapped DNA (Table I).

Nucleotide Misincorporation Opposite GGs on Undamaged and Platinated Templates—pol β is known to lack associated proofreading activity and to exhibit high infidelity during DNA replication *in vitro* (33, 34). It is reasonable to expect that the presence of platinum adducts on DNA might influence polymerase fidelity and that the conformation of the adduct might be important in determining the miscoding potential of the lesion. Therefore, a kinetic fidelity assay to measure single nucleotide incorporation opposite the template 3′-G and 5′-G was performed on damaged and control single-stranded and gapped DNA substrates. Initially, extension studies by pol β were performed in the presence of 5 mM of either dTTP, dGTP, and dATP to obtain qualitative information about misincorporation across from the platinum adducts (data not shown). Primer extension assays were performed for 15 min on control templates and for 30 min on damaged templates with a 30-fold excess of DNA substrate over enzyme using primers which ended just prior the template 3′-G and 5′-G sites. Both single-stranded and gapped templates were utilized. Some misincorporation of T was detected for both control and damaged templates, except for the incorporation across from the 5′-G on single-stranded templates. However, none of the other nucleotides was inserted opposite the 3′-G and 5′-G sites by pol β under the conditions of these assays with any of the DNA templates used.

A steady-state kinetic analysis of dTTP incorporation was then performed to characterize the misincorporation efficiency more quanitatively (Table II). Since no detectable dTTP misincorporation across from the 5′-G on single-stranded templates was found in the primer extension studies, kinetic experiments were not performed with the 25/44 DNA substrate. Therefore, the frequency of dTTP misincorporation on single-stranded *versus* gapped DNA could only be compared for the 3′-G. The efficiency (V_{max}/K_m) of dTTP incorporation opposite the undamaged 3′-G residue for 6-nucleotide gapped DNA substrate was about 10 times higher compared with the single-stranded DNA, mainly due to a decrease in apparent K_m values. This enhanced dTTP incorporation efficiency resulted in about 2.4-fold enhanced misinsertion frequency (f_{mis}) compared with single-stranded DNA. The presence of a platinum lesion on the DNA template caused a significant (15–25-fold) increase in the misinsertion frequency opposite the 3′-G site for single-stranded substrates, but not for 6-nucleotide gapped DNA (Table II). In addition, no significant differences in the frequency of dTTP misincorporation opposite the 5′-G(Pt) compared with an undamaged 5′-G for 5-nucleotide gapped DNA was detected. The low frequency of T-G mispair formation with all DNA templates was determined by both a decrease in V_{max} and an increase in K_m for dTTP incorporation (Table II) compared with dCTP incorporation (Table I) opposite a template G.

For both single-stranded and gapped DNA substrates, the efficiency of dTTP incorporation (V_{max}/K_m) and frequency of misinsertion (f_{mis}) opposite the 3′-G (Pt) were both approximately 1.7-fold higher for oxaliplatin adducts than for cisplatin adducts (Table II). The greater efficiency of dTTP misincorporation across from the 3′-G(Pt) of oxaliplatin adducts compared with cisplatin adducts on single-stranded templates was determined by both lower K_m and higher V_{max} values, while for gapped DNA substrates this difference was primarily due to an increase in V_{max}. Misincorporation opposite the 5′-G of a cisplatin adduct on the gapped substrate was too low for reliable determination of kinetic parameters, and dTTP misincorporation opposite the 5′-G(Pt) on single-stranded templates was too low to be detected for both cisplatin and oxaliplatin adducts.

TABLE II

Steady-state kinetic analysis of dTTP insertion opposite Pt-GG adducts

Standing start kinetic assays were performed using 5 fmol of pol β and 150 fmol of primer-templates. Data were obtained using 22/44 and 24/44 primed single-stranded templates and 22/44/16 and 24/44/14 gapped DNA templates (Fig. 1). Incubation time was 15 min for control gapped and single-stranded DNA templates and 30 min for platinated gapped and single-stranded DNA templates. dTTP concentrations ranged from 20 to 1000 μM for control and from 1000 to 10,000 μM for platinated templates. Kinetic parameters (K_m and V_{max}) and insertion efficiency ($f = V_{max}/K_m$) for nucleotide insertion by pol β were determined using Hanes-Woolf plots (see "Experimental Procedures"). The misinsertion efficiency (f_{mis}) was determined as the ratio of incorrect (dTTP) to correct (dCTP) insertion efficiencies. Data are means (±S.E.) from five to six different experiments using two independent template preparations. nt, nucleotide.

Target (template)	Pt adduct	V_{max}	K_m	V_{max}/K_m	f_{rel}
		min^{-1}	μM	$M^{-1}\ min^{-1}$	
3′-G (primed single-stranded DNA)	Control	0.0027 ± 0.0005	640 ± 150	4.52 ± 0.39	5.4×10^{-4}
	Cisplatin	0.00058 ± 0.00009	820 ± 95	0.76 ± 0.14	84.4×10^{-4}
	Oxaliplatin	0.00104 ± 0.00004	440 ± 57	2.52 ± 0.34	139.2×10^{-4}
5′-G (primed single-stranded DNA)	Control	ND[a]	ND	ND	ND
	Cisplatin	ND	ND	ND	ND
	Oxaliplatin	ND	ND	ND	ND
3′-G (6-nt gap)	Control	0.0020 ± 0.00013	50.3 ± 9.1	41.6 ± 4.1	13×10^{-4}
	Cisplatin	0.0026 ± 0.0003	1700 ± 200	1.5 ± 0.1	6.8×10^{-4}
	Oxaliplatin	0.0054 ± 0.0013	2040 ± 500	2.9 ± 0.5	12.1×10^{-4}
5′-G (5-nt gap)	Control	0.0015 ± 0.00008	260 ± 80	7.85 ± 1.83	2.9×10^{-4}
	Cisplatin	ND	ND	ND	ND
	Oxaliplatin	0.0011 ± 0.00006	880 ± 80	1.30 ± 0.14	9×10^{-4}

[a] ND, not determined. No detectable dTTP misincorporation was observed opposite the 5′-G on primed single-strand DNA. Bands produced as a result of dTTP misincorporation across from the 5′-G of the cisplatin adduct on the gapped DNA template were detectable but were too weak for reliable measurement of kinetic parameters.

DISCUSSION

It has been recently postulated that pol β overexpression facilitates translesion synthesis of cisplatin adducts, leading to increased drug tolerance and induced mutagenesis (12, 13). It is not clear whether pol β at normal levels catalyzes sufficient amounts of translesion synthesis to influence cisplatin cytotoxicity, since pol β-deleted cells do not exhibit increased sensitivity to this drug treatment (17). However, mutation frequency in response to cisplatin treatment was not measured for pol β-deleted cells. Therefore, the available data do not rule out the possibility that pol β may play a role in error-prone translesion synthesis even when expressed at normal levels.

The experiments presented here confirm the previously reported data (5, 6, 8) showing that pol β can catalyze extensive bypass of platinum-DNA adducts in a single-stranded region of DNA. These data may be relevant to pol β's postulated participation in leading strand and most lagging strand translesion synthesis at the replication fork. In the present study we also found that translesion synthesis by pol β is even greater during short gap-filling synthesis (Figs. 2 and 3), which might occur during some lagging strand translesion synthesis (19). In agreement with the primer-extension data, steady-state kinetic analysis also showed that the catalytic efficiency (V_{max}/K_m) of translesion synthesis by pol β was greater with gapped DNA than with single-stranded DNA templates. Thus, when primed single-stranded DNA was used, the efficiency of dCTP incorporation across from both the 3′- and 5′-Gs of Pt-GG adducts was decreased by a factor of 40–100 compared with incorporation across from undamaged guanines, while for gapped DNA only a 13–20-fold decrease was observed. The decrease in dCTP insertion opposite platinated guanines resulted from a substantial increase in the K_m values for dCTP with all damaged DNA substrates (Table I). At the same time, the Pt adducts did not appear to affect the rate of phosphodiester bond formation, since the apparent V_{max} values were similar for all templates.

For the cisplatin-GG adduct, the efficiency (V_{max}/K_m) of dCTP incorporation across from the 5′-G(Pt) was two times higher than that across from the 3′-G(Pt) for primed single-stranded substrates. These data contradict the prediction made by Bradley *et al.* (35) that polymerases should have more difficulty traversing the 5′-nucleotide than the 3′-nucleotide. The crystal structure of the cisplatin-GG adduct shows a greater distortion of the 3′-G·C base pair than the 5′-G·C base pair (36), while the NMR solution structure shows a greater distortion in the vicinity of the 5′-G·C base pair (37). Our data suggest that, when bound to the active site of pol β, the cisplatin-GG adduct may adapt a conformation which is closer to that seen in crystals than to that seen in solution.

With the primer extension assay, primed single-stranded DNA templates, and physiological conditions (low enzyme concentrations (Fig. 2, *B* and *F*) and/or low dNTP concentrations (data not shown)), pol β catalyzes 2.5-fold more translesion synthesis past oxaliplatin adducts than past cisplatin adducts (Ref. 8; Fig. 2). These findings were confirmed by steady-state kinetic experiments that showed a 2-fold higher efficiency of dCTP incorporation opposite the 3′-G of oxaliplatin-GG adducts than opposite the 3′-G of cisplatin-GG adducts. This was due almost entirely to a 2-fold decrease in the K_m for dCTP incorporation opposite the 3′-G for the oxaliplatin adducts compared with cisplatin adducts. In contrast, the efficiency (V_{max}/K_m) of nucleotide insertion across from the 5′-G(Pt) was similar for both cisplatin- and oxaliplatin-GG adducts. Therefore, the increased translesion synthesis past oxaliplatin adducts seen in the primer extension experiments is determined primarily by enhanced nucleotide incorporation across from the 3′-G(Pt). The structure of the oxaliplatin-GG adduct has not been studied in detail, but our kinetic data with pol β suggest that the oxaliplatin- and cisplatin-GG adducts will show the greatest conformational differences in the vicinity of the 3′-G(Pt). Our previous data show that oxaliplatin-GG adducts are bypassed more readily than cisplatin-GG adducts by human pol γ and yeast pol ζ, as well as by human pol β (8). Those data suggest that the differences in bypass of oxaliplatin-GG and cisplatin-GG adducts are most likely due to the conformational differences of the adducts themselves rather than to unique interactions of the adducts with the active site of an individual DNA polymerase.

In primer extension experiments using an excess of enzyme over single-stranded DNA, pol β did not appear to differentiate between cisplatin and oxaliplatin adducts (Ref. 8 and Fig. 3). Similar levels of translesion synthesis past cisplatin and oxaliplatin adducts over a wide range of enzyme concentrations were also found with the primer extension assay during pro-

cessive gap-filling DNA synthesis (Fig. 3). The primer extension data with gapped DNA templates were supported by steady-state kinetic experiments which showed that pol β did not discriminate between cisplatin and oxaliplatin adducts in the efficiency of dCTP incorporation across from either the 3′- or the 5′-G(Pt) of gapped DNA templates (Table I). The fact that both increased enzyme concentration and the switch to a more processive mode of action diminish the differences between oxaliplatin and cisplatin adducts suggests that the ability of pol β to discriminate between cisplatin and oxaliplatin adducts is strongly influenced by the rate of polymerase dissociation from the damaged template.

For the gapped DNA substrates, the distance of the lesion from both the 3′-primer terminus and the 5′ terminus of the downstream oligonucleotide seemed to have a considerable effect on the extent and pattern of translesion synthesis. Our data suggest that association between pol β and gapped DNA may be more difficult when the primer 3′-end is immediately adjacent to the lesion than when the primer is separated from the lesion by two nucleotides. Once the polymerase successfully associates with either of the gapped templates, replicative bypass proceeds processively due to binding of the 8-kDa domain of pol β to the 5′-phosphate moiety at the end of the gap (30). This results in an increased level of replicative bypass of Pt-DNA adducts (Figs. 2 and 3) and an increased efficiency of nucleotide incorporation (V_{max}/K_m) opposite platinated guanines (Table I) on gapped DNA compared with single-stranded DNA. The position of the lesion relative to the end of the gap also influenced the extent of strand displacement. When the Pt adduct was located at the beginning of the gap, strand displacement proceeded with the same effectiveness as with undamaged DNA. However, when the Pt adduct was located in the center of the gap, completion of gap filling and subsequent strand displacement was inhibited (Fig. 3*B*).

Cisplatin is known to be a mutagen in mammalian cells (38). However, the relative mutagenicity of cisplatin and oxaliplatin in mammalian cells has not been reported. Cisplatin-induced mutations in human cells have been detected at both the 5′- and 3′-Gs of Pt-GG adducts, and both G·C → T·A transversions and G·C → A·T transitions have been observed at high frequency (39, 40). Hoffmann *et al.* (6) have demonstrated that replication past cisplatin-GG adducts by calf thymus DNA polymerase β *in vitro* is mutagenic. The present study compared the fidelity of pol β during translesion synthesis past cisplatin and oxaliplatin adducts on DNA substrates of different structure. Under our experimental conditions, dTTP was the only dNTP that was misinserted opposite Gs to a significant degree with either undamaged DNA or DNA containing Pt-GG adducts (data not shown). This observation appears to conflict with previous reports that pol β catalyzes G·T and G·A mispairs with similar efficiency (24). However, previous studies have also shown that the fidelity of pol β is highly dependent on sequence context (23). Thus, the differences between the current and previous studies may be related to differences in sequence context and/or the assays used. With undamaged DNA the misincorporation frequency for dTTP opposite Gs was 2.4-fold greater for gapped DNA than for single-stranded DNA (Table II). A similar increase in misincorporation frequency for G-T mispairing during 6 nucleotide gap filling synthesis was reported by Chagovetz *et al.* (24). Moreover, the overall frequencies of dTTP misinsertion opposite guanine on undamaged single-stranded DNA and 6-nucleotide gapped DNA are in agreement with previous reports (24, 41). In contrast to misincorporation opposite undamaged 3′-G, the fidelity of dNTP insertion opposite 3′-G(Pt) was significantly (about 12-fold) greater with gapped DNA than with single-stranded DNA with both cisplatin- and oxaliplatin-GG adducts (Table II).

The dTTP misincorporation frequency opposite the 3′-G(Pt) with both single-stranded and gapped DNA was 1.7-fold greater for oxaliplatin adducts than for cisplatin adducts (Table II), which reinforces the suggestion that the conformation of cisplatin- and oxaliplatin-GG adducts differs significantly in the vicinity of the 3′-G(Pt). However, it is important to note that dTTP misincorporation does not appear to represent the major mutagenic effect of platinum adducts on pol β. Hoffmann *et al.* (6) have recently shown that the mutational hot spots generated by pol β *in vitro* are situated 5′ to the lesion and that the frequency of frameshift mutations in that region is much greater than the frequency of base substitution mutations. Therefore, these data do not necessarily predict the relative mutagenicity of cisplatin and oxaliplatin adducts in pol β overexpressing cells.

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