Effect of DNA Polymerases and High Mobility Group Protein 1 on the Carrier Ligand Specificity for Translesion Synthesis past Platinum−DNA Adducts†

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ABSTRACT: Translesion synthesis past Pt−DNA adducts can affect both the cytotoxicity and mutagenicity of the platinum adducts. We have shown previously that the extent of replicative bypass in vivo is influenced by the carrier ligand of platinum adducts. The specificity of replicative bypass may be determined by the DNA polymerase complexes that catalyze translesion synthesis past Pt−DNA adducts and/or by DNA damage-recognition proteins that bind to the Pt−DNA adducts and block translesion replication. In the present study, primer extension on DNA templates containing site-specifically placed cisplatin, oxaliplatin, JM216, or chlorodiethylenetriamine−Pt adducts revealed that the eukaryotic DNA polymerases β, ζ, γ, and human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) had a similar specificity for translesion synthesis past Pt−DNA adducts (dien $\gg$ oxaliplatin $\geq$ cisplatin $>$ JM216). Primer extension assays performed in the presence of high mobility group protein 1 (HMG1), which is known to recognize cisplatin-damaged DNA, revealed that inhibition of translesion synthesis by HMG1 also depended on the carrier ligand of the Pt−DNA adduct (cisplatin $>$ oxaliplatin $=$ JM216 $\gg$ dien). These data were consistent with the results of gel-shift experiments showing similar differences in the affinity of HMG1 for DNA modified with the different platinum adducts. Our studies show that both DNA polymerases and damage-recognition proteins can impart specificity to replicative bypass of Pt−DNA adducts. This information may serve as a model for further studies of translesion synthesis.

The clinical efficacy of the anticancer drugs cisplatin and carboplatin is diminished by acquired and intrinsic tumor resistance. Platinum anticancer agents with different carrier ligands have been developed in an attempt to overcome these problems. Platinum complexes with 1,2-diamminocyclohexane[1] carrier ligand (such as oxaliplatin and ormaplatin) and amminecyclohexylamine carrier ligands (such as JM216) are often effective in cells resistant to platinum complexes with *cis*-diammine carrier ligands (such as cisplatin and carboplatin) (see Figure 1 for structures). Oxaliplatin and JM216 are in clinical trial and show promise for the treatment of cisplatin-resistant tumors. Once inside the cell, platinum compounds lose their leaving ligands, bind to cellular DNA, and form several stable Pt−DNA adducts, the majority of which are represented by intrastrand cross-links between adjacent guanosines. Both NMR and crystallographic structural studies have revealed that when a platinum complex such as cisplatin or JM216 binds to the N7 atoms of adjacent guanine residues on duplex DNA, it distorts the double helix

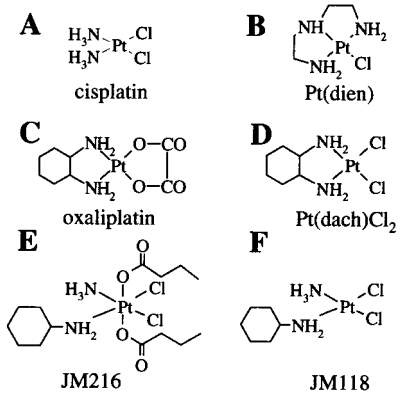

FIGURE 1: Chemical structure of the platinum complexes used in this study. $Pt(dach)Cl_2$ and JM118 are biotransformation products of oxaliplatin and JM216, respectively.

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[1] Abbreviations: cisplatin, *cis*-diamminedichloroplatinum(II); carboplatin, *cis*-diammine(cyclobutanedicarboxylato)platinum(II); oxaliplatin, (*trans*-*R*,*R*)-diaminocyclohexaneoxalatoplatinum(II); $Pt(dach)Cl_2$, (*trans*-*R*,*R*)-diaminocyclohexanedichloroplatinum(II); ormaplatin, *trans*-diaminocyclohexanetetrachloroplatinum(IV); JM216, *cis*-amminediacetatodichloro(cyclohexylamine)platinum(IV); JM118, cis-amminedichloro-(cyclohexylamine)platinum(II); dach, (*trans*-*R*,*R*)-1,2-diaminocyclohexane; dien, chlorodiethylenetriamine; HIV-1 RT, human immunodeficiency virus 1 reverse transcriptase; HMG, high mobility group.

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by causing a bend toward the major groove and a widening and flattening of the minor groove (*1*, *2*). The existing data (*3*, *4*) suggest that subtle conformational differences between cisplatin, oxaliplatin, and JM216 adducts are likely. These differences in the structural distortion caused by Pt−DNA adducts with various carrier ligands are likely to affect their ability to block DNA replication.

Postreplication repair is an important mechanism of DNA damage tolerance (*5*). It is best defined as replication of damaged DNA without the introduction of potentially lethal secondary lesions (e.g., gaps or discontinuities in the nascent DNA), and/or the repair of those secondary lesions following replication (*5*, *6*). A great deal of what has been called postreplication repair actually occurs during replication, rather than following it (*5*). For that reason we use the term "replicative bypass" as synonymous with postreplication repair and the term "translesion synthesis" to define the replication through and beyond the damaged site on the template DNA by individual polymerases. Data obtained in our laboratory provide strong evidence that cells can replicate past Pt adducts and that this replicative bypass consistently discriminates between platinum compounds with *cis*-diammine carrier ligands and dach carrier ligands (*7*−*9*). Our data also suggest that this carrier ligand specificity of replicative bypass contributes to the lack of cross-resistance to oxaliplatin in cisplatin-resistant cell lines (*7*, *8*).

The simplest explanation for the carrier ligand specificity of replicative bypass would be differences in the ability of the polymerase(s) involved in translesion synthesis to catalyze synthesis past platinum adducts with different ligands. Unfortunately, the polymerases involved in replicative bypass of Pt−DNA adducts in human cells in vivo are not known. Cisplatin adducts have been shown to completely block primer elongation by DNA polymerases α, δ, and ϵ (*10*−*12*). Furthermore, addition of the accessory proteins PCNA or replication protein A to the reactions with DNA polymerase δ and ϵ did not restore their capacity to elongate past the platinum adduct (*10*, *12*). However, both in vitro (*10*, *13*) and cell culture (*14*, *15*) experiments suggest that mammalian DNA polymerase β may be able to displace a stalled replication complex and perform error-prone translesion synthesis past Pt−DNA adducts. Similarly, both genetic (*16*, *17*) and in vitro (*18*) evidence has implicated yeast DNA polymerase ζ, which is a complex of the Rev3 and Rev7 gene products (*18*), in error-prone translesion synthesis past bulky DNA adducts. Recent data suggest the existence of a human pol ζ with properties similar to those of the yeast enzyme (*19*, *20*). The effect of platinum adducts on DNA replication by human DNA polymerase γ is not currently known. It has been reported that higher levels of initial binding and decreased removal of cisplatin−DNA adducts result in preferential cisplatin adduct accumulation in mitochondrial DNA as compared to nuclear DNA (*21*, *22*). Therefore, it is reasonable to speculate that, as the only DNA polymerase present in mitochondria, pol γ may be able to perform translesion replication past Pt−DNA adducts. Finally, Hoffmann et al. (*13*) have reported efficient translesion synthesis past cisplatin−GG adducts by HIV-1 RT.

In addition to DNA polymerase complexes that catalyze translesion synthesis past Pt−DNA adducts, the specificity of replicative bypass may be determined by DNA damage-recognition proteins that selectively bind to the Pt−DNA adducts and block the replication. Data from several laboratories have shown that the human mismatch repair complex MutSα can bind to cisplatin−DNA adducts in double-stranded DNA, even if there is no mismatch opposite the Pt−DNA adduct (*23*, *24*). In this sense the MutSα complex is functioning as a "damage recognition" complex rather than a "mismatch recognition" complex. Our laboratory has recently shown that defects in mismatch repair lead to increased replicative bypass of cisplatin adducts but not oxaliplatin adducts (*9*). These data suggest a general model in which platinum damage recognition proteins could impart specificity to replicative bypass, either by removing the newly synthesized DNA (as likely occurs with the mismatch repair system) or by physically blocking translesion synthesis. High mobility group (HMG) domain-containing proteins can recognize DNA structures distorted by platinum complexes and, once bound, induce additional conformational changes in DNA structure (*25*−*27*). Hoffmann et al. (*28*) have shown that HMG1 can block translesion replication past cisplatin−DNA adducts. HMG1 has been shown to bind to both cisplatin and oxaliplatin adducts (*29*), but its relative affinity for those adducts has not been previously reported.

The present study was designed to evaluate whether the carrier ligand specificity of replicative bypass could be determined either by the DNA polymerases that catalyze translesion replication or by the damage-recognition proteins that selectively bind to the Pt−DNA adducts and block translesion synthesis. To test whether DNA polymerases in general were capable of discriminating between Pt−DNA adducts with different carrier ligands, we performed primer extension experiments with enzymes from all four DNA polymerase families (*30*): human DNA polymerase β from the terminal transferase family, yeast DNA polymerase ζ from the pol α family, human DNA polymerase γ from the pol I family, and viral polymerase HIV-1 RT from the reverse transcriptase family. We have shown that eukaryotic DNA polymerases of different fidelity, processivity, structural complexity, and in vivo function vary in their ability to perform translesion synthesis past Pt−DNA adducts and that the carrier ligand of platinum adducts on DNA plays a significant role in determining the blocking potential of the lesion. To test whether platinum damage recognition proteins in general might be capable of imparting specificity to replicative bypass, we determined the ability of HMG1 to block translesion synthesis past Pt−DNA adducts with different carrier ligands. We have shown that the carrier ligands of Pt−DNA adducts affect the ability of HMG1 to bind to the adducts and block translesion synthesis past the adducts.

MATERIALS AND METHODS

Enzymes. Recombinant human pol β, purified as described previously (*31*), 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 with poly(dA)−$(dT)_{20}$ as a template-primer). HIV-1 RT was purchased from from Boehringer Mannheim (specific activity 5 units/μg; one unit is the enzyme activity that incorporates 1 nmol of [^{3}H]TMP into acid-insoluble products in 10 min at 37 °C with poly(A)−$(dT)_{15}$ as substrate). Yeast pol ζ was purified as a Gst−Rev3p−Rev7p complex as described by

Nelson et al. (*18*). The His-tagged recombinant catalytic subunit of human DNA polymerase γ was purified from baculoviral-infected Sf9 cells by chromatography over Ni−NTA followed by MonoQ on a Pharmacia FPLC as described previously (*32*). An exonuclease-deficient mutant of pol γ has been made previously by site-directed mutagenesis and expressed as an untagged mature protein in baculovirus (*32*). To allow rapid large-scale purification of this exonuclease-deficient mutant, a His-tagged construct was prepared by replacing the wild-type *Bam*HI−*Not*I fragment of pHugpQE9 (*32*) with a *Bam*HI−*Not*I fragment of Exo-100/103hugpVL carrying the mutations rendering pol γ exonuclease-deficient. The *Eco*RI−*Not*I fragment of the resulting plasmid, Exo-pQSL4.2, was then subcloned into the baculovirus vector pVL1393 for expression in Sf9 cells. The recombinant baculovirus clone Exo-pQVSL11.4 was then cotransfected into Sf9 cells, and His-tagged exonuclease-deficient recombinant human DNA polymerase γ was purified over a Ni−NTA column followed by FPLC MonoQ as described previously (*32*). The polymerase γ activity eluted at ~0.22 M KCl and active fractions were pooled, dialyzed (1 mL, 0.96 mg/mL), and frozen in small aliquots at −80 °C. Neither intrinsic nor extrinsic exonuclease activity was observed with this purified enzyme. The specific activity of both the exonuclease-proficient and -deficient pol γ was 32 units/ng (one unit is the amount of enzyme required to catalyze the incorporation of 1 pmol of dTMP into TCA-precipitable DNA in 1 h at 37 °C on poly(rA)−oligo(dT) in the standard assay). HMG1 from calf thymus was purified as described by Turchi et al. (*33*).

Construction of Platinum Adduct-Containing Templates for the Primer Extension Assay. Primer−templates were constructed from synthetic oligonucleotides essentially as described by Hoffmann et al. (*34*). The structures of the platinum complexes used in these studies are shown in Figure 1. Cisplatin was obtained from Sigma; Pt(dach)Cl_2 was provided by Dr. S. D. Wyrick (UNC); JM118 was provided by Dr. C. F. J. Barnard (Johnson Matthey Technology Centre); and dien-Pt was provided by Dr. E. L. Mamenta (UNC). All platination reactions were carried out with aquated derivatives of the platinum complexes to facilitate their reaction with the DNA (*35*). Oxaliplatin and JM216 are difficult to convert to aquated complexes in vitro. However, their biotransformation products, Pt(dach)Cl_2 (Figure 1D) and JM118 (Figure 1F), are readily converted to aquated derivatives. Thus, the aquated complexes were obtained by overnight stirring in the dark at room temperature of a solution containing either cisplatin (Figure 1A), Pt(dach)-Cl_2 (Figure 1D), JM118 (Figure 1F), or dien-Pt (Figure 1B) and 1.98 equiv of silver nitrate. The precipitated silver chloride was removed by filtration through 0.2 μM syringe filters. The final concentration of aquated derivatives of the cisplatin, Ptdach(Cl_2), and dien−Pt complexes was 40 mM. Due to the low solubility of JM118 in water (*3*), the final concentration of its aquated complex was 4 mM. Platination of a 12-mer oligonucleotide containing a single GG sequence within a *Stu*I restriction site (TCTA*GG*CCTTCT) was performed for 2 h at 37 °C in the dark. The drug-to-oligonucleotide ratio that allowed maximal conversion of undamaged 12-mer to monoplatinated 12-mer was determined experimentally in incubations with 0.5 nmol of oligonucleotide and various molar ratios of the aquated platinum complexes. For the platinum compounds used in this study a 2:1 ratio of aquated platinum complex to oligonucleotide proved to be optimal. The incubations were then scaled up to 10 nmol of oligonucleotide for template preparations. 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 on Centri-spin-10 columns (Princeton Separations, Inc).

The template sequences used for these experiments are shown in Figure 2. The 90-mer templates that were initially constructed were similar to the template used previously by Hoffmann et al. (*34*), except for the sequence surrounding the platinum adduct. The 44-mer templates were used subsequently in order to facilitate single-base resolution of replication products on polyacrylamide gels. Site-specifically platinated 90-mers were obtained by ligation of a 14-mer (left end), purified singly platinated 12-mer, and 64-mer (right end) with a 65-mer as a scaffold (Figure 2A, I). Construction of the site-specifically platinated 44-mers was performed similarly: platinated 12-mers were ligated with a 14-mer (left end) and an 18-mer (right end) with a 35-mer as a scaffold (Figure 2A, II). Control 44-mer and 90-mer templates were prepared with unplatinated 12-mer by the same procedure. After 16 h of ligation at 16 °C by T4 DNA ligase, templates were purified by MicroSpin G-25 columns (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 test the stability of the platinum adducts during replication studies. Platinum-containing templates were end-labeled with ^{32}P and hybridized with a complementary oligonucleotide of the same length. These double-stranded oligonucleotides were incubated under conditions similar to those used in primer extension assays and then restricted with *Stu*I. The small level of restriction observed in these studies (1−3% for templates containing cisplatin adducts, 2−5% for templates containing oxaliplatin adducts, 2−3% for templates containing JM216 adducts, and 6−9% for templates containing dien−Pt adducts) was independent of the incubation time and therefore probably was due to incomplete blockage of restriction by the platinum adduct. However, we cannot eliminate the possibility that the observed restriction also might be due to a small percentage of unplatinated DNA in our template preparation. Therefore, *Stu*I cleavage was used in all primer extension assays described below, and only *Stu*I-resistant elongation products were quantitated as translesion synthesis.

For primer extension on 90-mer templates, 17-mer primers were used. For primer extension on 44-mer templates, 22-mer primers were used. The primers were 5′-end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, according to the manufacturer's protocol. DNA substrates were prepared by hybridizing labeled primer to the templates (undamaged or platinated) at a 1:1.2 molar ratio in an annealing buffer [50 mM Tris-HCl (pH 8) and 100 mM NaCl] and heating 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). To study the effect of HMG-1 protein on bypass

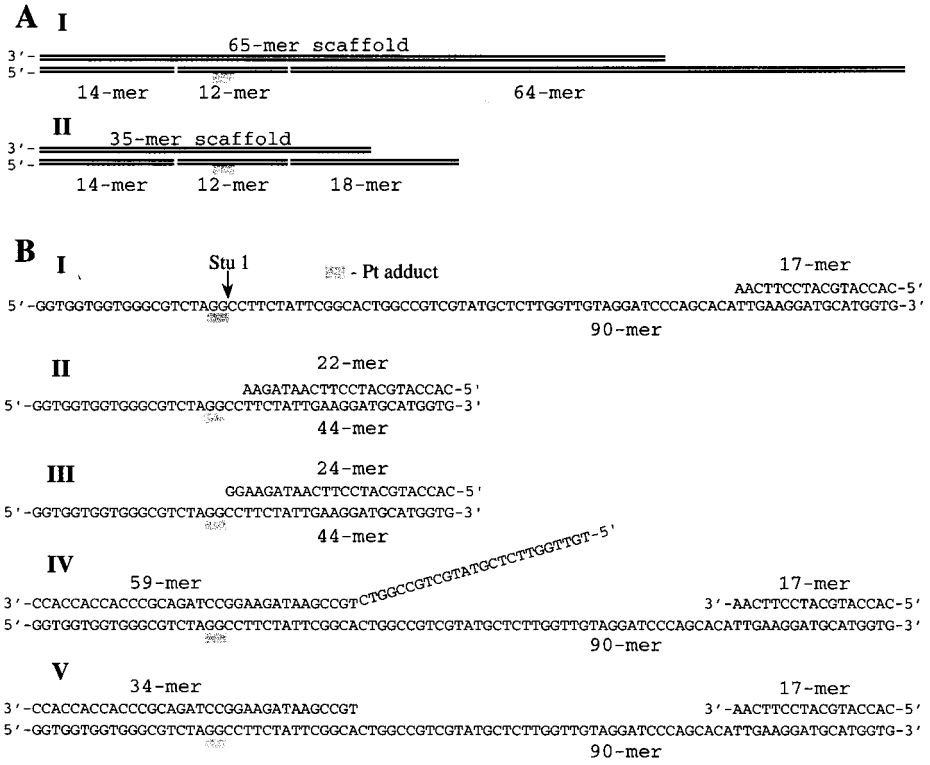

FIGURE 2: Structures and sequences of oligonucleotide substrates with site-specific platinum adduct. (A) Schematic representation of the construction of the site-specifically modified 90-mer and 44-mer templates. (B) The 17/90 (I) and 22/44 (II) primer−templates were used in the primer-extension assay. The 24/44 primer−templates (III) were utilized in the misincorporation assay in the presence of individual nucleotides. The forked-like template (IV) and template with a double-stranded region (V) were used to study the effect of HMG1 on translesion replication. The site-specifically modified oligonucleotides were constructed and hybridized to the primers as described in Materials and Methods.

replication, forked-like DNA (template IV, Figure 2B) analogous to one described by Hoffmann et al. (*28*, *34*) and DNA with a double-stranded binding region (template V, Figure 2B) were constructed by hybridizing the 17/90 primer−template to either partially complementary 59-mer or complementary 34-mer. Platinated 44-mer templates with the same sequence as shown in Figure 2, II were used to create double-stranded DNA for the gel shift assay.

Primer Extension Assays. Primer extension assays were performed with 17/90 or 22/44 primer−templates (Figure 2B, I and II). Primer−template (200 fmol, expressed as primer termini) was incubated with different DNA polymerases at 37 °C in 10-μL reactions containing 500 μM dNTPs (unless otherwise indicated). Reaction times and enzyme concentrations, indicated in the figures, were adjusted for each substrate to optimize translesion synthesis. Primer extension by pol β was carried out as described by Singhal and Wilson (*36*). The reaction with HIV-1 RT was carried out as described by Hoffmann et al. (*13*). The primer extension reaction with pol ζ was performed as described by Nelson et al. (*18*). Replication by pol γ was performed in the presence of 25 mM HEPES (pH 7.6), 7 mM $MgCl_2$, 0.1 mM EDTA, and 50 μg of acetylated BSA/mL. 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. Cleavage by *Stu*I enzyme revealed that >95% of the product synthesized on platinated templates was resistant to the restriction digestion, while product synthesized on control templates was restricted completely. Aliquots were mixed with 0.7 volume of formamide loading dye solution containing 500 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue in 90% formamide. Before being loaded onto the gel, the reactions were denatured by heating at 100 °C for 5 min and immediately transferred into ice for 5 min. Products were resolved by denaturing polyacrylamide gel electrophoresis (8 M urea and 16% acrylamide, 2 h at 400 V for 90-mer and 4 h at 2000 V for 44-mers) and then visualized and quantified by using a Molecular Dynamics PhosphorImager and ImageQuant software.

The extent of translesion synthesis on damaged templates was calculated as follows:

$$\%\ \text{translesion synthesis} = \frac{\text{bypass product following } \textit{Stu}\text{I restriction}}{\text{total primer termini}} \times 100 \quad (1)$$

where bypass product equals chain elongation past the stop sites caused by the platinum adduct. Total primer termini are defined as the sum of elongated and unelongated primers.

The efficiency of translesion synthesis relative to synthesis on undamaged DNA templates was calculated as follows:

$$\text{relative translesion synthesis (\%)} = \frac{\text{translesion synthesis on damaged DNA}}{\text{synthesis on undamaged DNA}} \times 100 \quad (2)$$

where translesion synthesis on damaged DNA is calculated by eq 1 and synthesis on undamaged DNA equals chain elongation past the sites corresponding to the stop sites on damaged templates, also calculated as a percent of total primer termini. The calculation with eq 2 could only be made for those enzyme concentrations and incubation times for

which replication was incomplete (i.e., not all of the primers had been fully elongated) on both the control and damaged templates. Thus, for most enzymes efficiency of translesion synthesis relative to synthesis on undamaged DNA templates was determined for 15 min incubations at very low enzyme concentrations.

Misincorporation Assays. For the misincorporation assays, 24-mer primers were hybridized with control and damaged 44-mer templates. These 24/44 primer−templates (Figure 2B, III) were incubated with 1 mM of each dNTP individually for 15 min with 1.4 ng of HIV-1 RT. Reactions were terminated and products were analyzed as described above.

Primer Extension in the Presence of HMG-1 Protein. For the assays to determine the effect of HMG1 on translesion synthesis, 300 fM of the forked-like primer−templates (IV, Figure 2B) or primer−templates with a double-stranded binding region (V, Figure 2B) were incubated with different amounts of HMG-1 protein for 20 min on ice, followed by 20 min at room temperature in the polymerase reaction buffer (total volume 7 μL). Primer extension reactions were started by the addition of 3 μL of the reaction buffer containing 300 μM dNTP mixture and 150 ng of HIV-1 RT. After a 2-h incubation at 37 °C, reactions were stopped. The samples were resolved on 12% denaturing gels and analyzed as described above.

Electrophoretic Mobility Shift Assay. Gel-shift assays were performed with control and platinated double-stranded 44-mer templates (template II in Figure 2B, hybridized to the 44-mer complementary strand). Binding reactions were carried out with the indicated amount of HMG1 in a final reaction mixture of 20 μL containing 20 mM HEPES (pH 7.0), 1 mM DTT, 0.001% Nonidet P-40, 50 μg/mL BSA, 50 mM NaCl, 2 mM $MgCl_2$, 25 ng calf thymus competitor DNA, and 50 fmol of ^{32}P-labeled double-stranded DNA for 30 min at room temperature to achieve equilibrium. Following incubation, gel loading dye was added to the reactions and the reaction products were separated by 6% native polyacrylamide gel electrophoresis at 125 V for 1.5 h and then visualized and quantified by using a Molecular Dynamics PhosphorImager and ImageQuant software. Results are the average of two individual experiments.

RESULTS

Translesion synthesis, i.e. replication past DNA lesions that block chain elongation by the normal replicases, is likely to be influenced by the type of DNA lesion. To examine polymerase activity on platinated DNA, substrates of different structure, templates containing cisplatin, oxaliplatin, JM216, and monofunctional dien−Pt adducts were constructed. To determine the molecular details of DNA replication at the adducted site, in vitro reactions were conducted with eukaryotic DNA polymerases β, ζ, γ, or HIV-1 RT in the presence of a mixture of all four dNTPs. Enzyme concentrations were varied in order to assess both the maximal possible amount of translesion synthesis by each DNA polymerase and the efficiency of translesion synthesis relative to the synthesis on the undamaged template.

Effect of Pt−GG Adducts on Primer Extension Catalyzed by Human Polymerase β. The initial experiments with pol β involved analysis of the extension of a 17-mer primer on a 90-mer template (Figure 2B, I), which is similar to one of the substrates used by Hoffmann et al. (*34*). The in vitro experiments on this templates demonstrated a high efficiency for bypass of cisplatin adducts by pol β, as described previously (*10*). One band corresponding to the product blocked at the sites of the adduct was observed, in agreement with the data reported by Hoffmann et al. (*10*). Since shorter templates allow a clearer examination of replication products by sequencing gel electrophoresis at single-nucleotide resolution, 22/44 primer−templates were also prepared (Figure 2B, II). Comparison of in vitro replication experiments using the 90-mer and 44-mer templates revealed that the efficiency of translesion synthesis by pol β was not affected by the position of the primer relative to the Pt−GG adduct (data not shown). Furthermore, with 44-mer templates it became clear that DNA synthesis was inhibited at several different positions relative to the platination sites (see below). Therefore, 22/44 primer−templates were used for most of the subsequent experiments.

The elongation of 22-mer primers on 44-mer templates for 15 min by 0.43−430 ng of pol β is shown in Figure 3A. When untreated primer−template was used at low enzyme concentration, the enzyme failed to reach the end of the template strand and there were intense bands corresponding to nearly every nucleotide in the sequence, which is consistent with the distributive nature of pol β (Figure 3A, lanes 1−3). With increasing enzyme concentrations, bands corresponding to the intermediate pause sites became relatively weak and a major band corresponding to the full-length product was observed (seen in Figure 3A, lanes 4−6). At least 14 ng of pol β was required to extend most of the primers to full-size product in 15 min. Furthermore, at high enzyme concentration a non-template-directed +1 addition at the 3′ terminus was detected on both undamaged (Figure 3A, lanes 5 and 6) and damaged templates (Figure 3A, lane 10). The product of this non-template-directed nucleotidyl transfer activity accumulated with time (Figure 3B, lanes 11−14).

Primer extension on damaged templates produced multiple shorter elongation products in addition to the full-length product (Figure 3A, lanes 7−10, and Figure 3B, lanes 11−22). Relative distribution of the products of replication depended on the structure of platinum adduct, enzyme concentration, and reaction time. Figure 3A (lanes 7−10) shows primer extension on templates containing a cisplatin−GG adduct with 14−430 ng of pol β for 15 min, and Figure 3C summarizes translesion synthesis past all three Pt−DNA diadducts at those enzyme concentrations. At low and intermediate enzyme concentrations, in vitro replication resulted in a strong stop site one base 3′ to the lesion (Figure 3A, lanes 7−9, band −1). Stop sites across from the 3′ and 5′Gs of the Pt−GG adduct were also observed. The stop site across from the 3′G(Pt) became more prominent with increasing enzyme concentration, while the stop site across from the 5′G(Pt) remained weak at all concentrations tested. At the highest enzyme concentration, additional strong pause sites appeared one and two bases 5′ to the lesion (Figure 3A, lane 10, bands +1 and +2).

To determine the maximal extent of translesion synthesis past cisplatin, oxaliplatin, and JM216 adducts by pol β, the time course for translesion synthesis was determined with 500 ng of enzyme (Figure 3B, lanes 11−22). For the cisplatin and oxaliplatin adducts, the stop sites observed at early

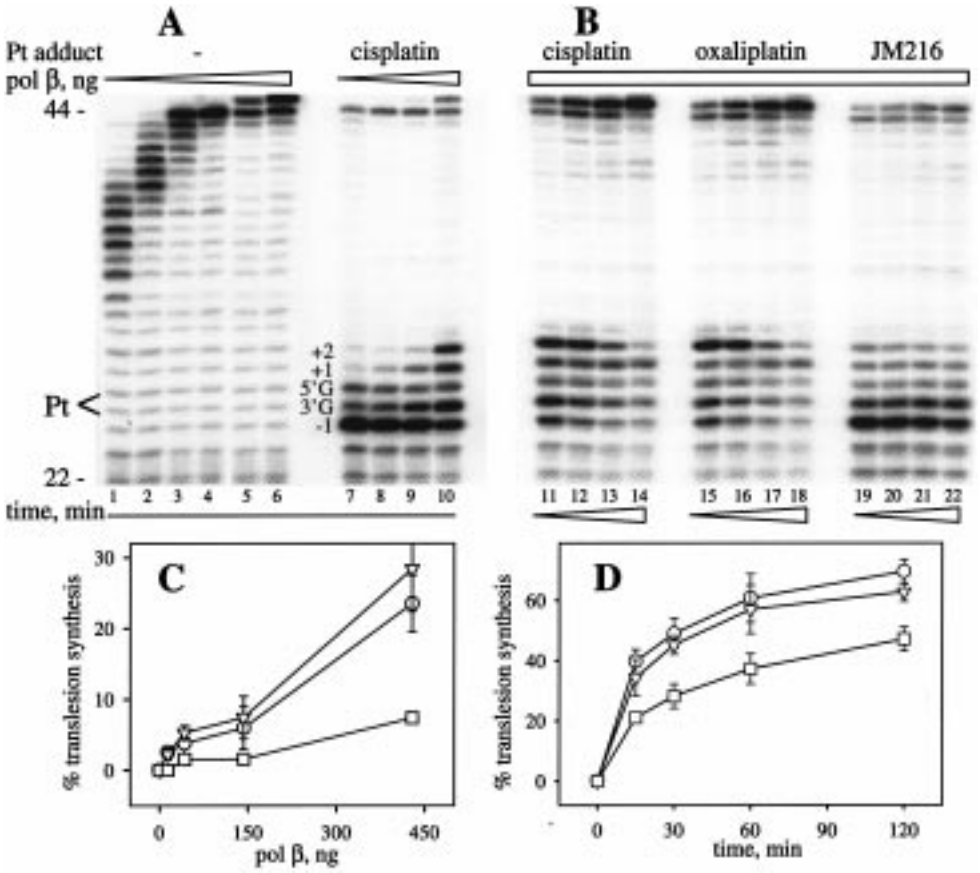

FIGURE 3: Primer extension activity of pol β. (A) Representative gel showing primer extension by different amounts of pol β using the 22/44 primer−templates (Figure 2B, II) with and without cisplatin adduct. Enzyme amounts were 0.43, 2.5, 14, 44, 137, and 430 ng for control templates (lanes 1−6) and 14, 44, 137, and 430 ng for templates with cisplatin adduct (lanes 7−10) in 10 μL reaction mixtures. Reaction time was 15 min. Gels for templates with oxaliplatin and JM216 adducts are not shown. (B) Time-course experiments conducted for 15, 30, 60, and 120 min using all three platinated templates and 500 ng of pol β (lanes 11−22). The graphs show the enzyme concentration dependence (C) or time course (D) of translesion synthesis past oxaliplatin (▽), cisplatin (○), and JM216 (□) adducts as a percent of total primer termini calculated according to eq 1. Data are means (± standard error) from six different experiments with three independent template preparations.

incubation times were reduced by increasing the length of the reaction (Figure 3B, lanes 11−18). However, a very strong block of DNA synthesis by the JM216 adduct at the base preceding the lesion and at the first platination site remained for all reaction times tested (Figure 3B, lanes 19−22). An enzyme concentration- (Figure 3C) and time-dependent (Figure 3D) increase in the translesion synthesis for all damaged templates was evident. Translesion synthesis appeared to be similar for the templates with cisplatin and oxaliplatin adducts at this enzyme concentration; however, the JM216 adduct was a much more effective block to translesion synthesis.

The extent of translesion synthesis past cisplatin adducts was comparable to that reported previously by Hoffmann et al. (*10*, *13*) under similar conditions. However, because 500 ng of pol β represents an approximately 60-fold excess of enzyme over primer−template, it is likely that these conditions are not physiological. Thus, we also tested the specificity of bypass of Pt−DNA adducts using 2 ng of pol β, which represents an approximately 4-fold excess of primer−template over enzyme. These data are shown in Figure 4. At this enzyme concentration, an additional stop site two bases 3′ to the Pt−GG adduct was observed. The intensity of this stop site was not significantly different for the templates with cisplatin and oxaliplatin adducts than for the undamaged template (Figure 4A, compare lanes 1−4 with lanes 5−12). However, for the JM216 adduct, this appeared to represent an additional strong stop site at low pol β concentrations (Figure 4A, lanes 13−16). At this enzyme concentration, total translesion synthesis was much lower and cisplatin adducts were about 2.5-fold more effective than oxaliplatin adducts at blocking translesion synthesis (Figure 4B). To determine translesion synthesis as a percentage of chain elongation on undamaged template, primer extension experiments were performed for 15 min with 2−5 ng of pol β (data not shown). Under these conditions, primer extension on both undamaged and damaged template was incomplete and, therefore, relative translesion synthesis was measurable. Translesion synthesis relative to synthesis on an undamaged template (calculated by eq 2) ranged from 2% to 12%, depending on the carrier ligand of the platinum complex (Table 1).

Effect of Pt−GG Adducts on Primer Extension Catalyzed by Human Immunodeficiency Virus Type 1 Reverse Transcriptase. Primer extension assays of control templates and templates with the cisplatin adduct using 5−150 ng of HIV-1 RT for 15 min are shown in Figure 5A (lanes 1−8). Replication on undamaged templates was more processive with HIV-1 RT than with pol β. The amount of extended primer at 15 min ranged from 15% at 5 ng of HIV-1 RT (Figure 5A, lanes 1 and 2) to 100% at 50 or more ng of enzyme (Figure 5A, lanes 3 and 4). In addition, HIV-1 RT,

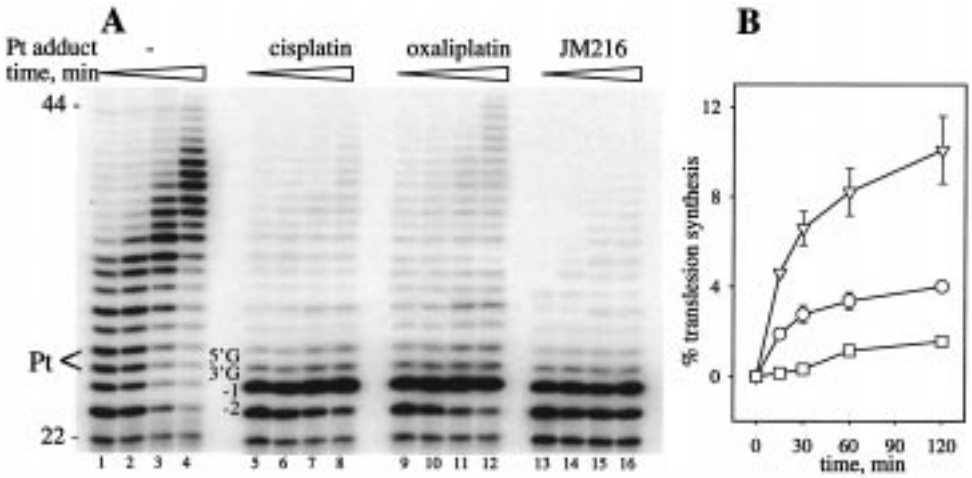

FIGURE 4: Primer extension activity of pol β using the 22/44 primer−templates (Figure 2B, II) and low enzyme concentrations. (A) Time-course experiments were conducted for 15, 30, 60, and 120 min using undamaged (lanes 1−4) or platinated (lanes 5−16) templates and 1.9 ng of pol β (B). The data for graphical representation were obtained as described in Figure 3 from three different experiments using two independent template preparations.

Table 1: Translesion Synthesis past Pt−DNA Adducts Relative to Chain Elongation on Control Templates[a]

enzyme	enzyme-to-template ratios[b]	platinum adduct		
		oxaliplatin	cisplatin	JM216
pol β	0.2:1−0.7:1	11−12	5−6	2−3
HIV-1 RT	0.2:1−0.5:1	11−14	9−14	3−5
pol γ	0.6:1−1:1	11−15	7−9	3−5
pol ζ	0.7:1−1:1	7−9	4−5	1−3

[a] Pol β, (2−7 ng), 5−12.5 ng of HIV-1 RT, 17−28 ng of pol γ, and 32−45 ng of pol ζ was used to determine translesion synthesis on damaged templates relative to chain elongation on control templates calculated by eq 2. Reactions with pol β, HIV-1 RT, and pol γ were performed for 15 min; reactions with pol ζ were performed for 2 h. Synthesis is expressed as a percentage. [b] Enzyme-to-template ratios were estimated by assuming that the enzymes were fully active. Thus, these values represent maximum enzyme-to-template ratios.

like pol β, catalyzed a non-template-driven one-base addition to the 3′ terminus of blunt-ended template-primers (Figure 5, lanes 2 and 3). Similar blunt-end addition by HIV-1 RT has been previously reported (*37*).

Three major stop sites were observed after replication on the damaged templates (shown in Figure 5A, lanes 5−8, for templates containing the cisplatin adduct). Synthesis at low enzyme concentrations produced stop sites one base 3′ to the adduct and opposite the 3′G(Pt) (Figure 5A, lanes 5 and 6). At higher enzyme concentration and longer reaction times, an increasing number of products terminated opposite the 5′G(Pt). The time course for translesion synthesis past all three Pt−DNA diadducts at 150 ng of HIV-1 RT is shown in Figure 5B, lanes 9−20. The stop sites were very similar for the cisplatin and oxaliplatin adducts. However, with the JM216 adduct, no stop site was observed opposite the 5′G-(Pt). The extent of translesion synthesis past cisplatin adducts was comparable to that reported previously by Hoffmann et al. (*10*, *13*) with similar amounts of enzyme. As with pol β at high enzyme concentration (Figure 3), the JM216 adducts were the most effective at blocking translesion synthesis, while the cisplatin and oxaliplatin adducts did not differ significantly in their ability to block replication (Figure 5C,D). However, unlike pol β the specificity of HIV-1 RT for translesion synthesis past cisplatin and oxaliplatin adducts did not change at lower enzyme concentrations (data not shown). For HIV-1 RT translesion synthesis relative to synthesis on an undamaged template could be estimated for 15-min incubations containing 4−13 ng of enzyme (Table 1). The relative translesion synthesis was comparable to that seen with pol β.

Finally, the product terminating opposite the 3′G(Pt) consisted of two bands for all of the Pt−DNA adducts tested. Because the 3′-terminal nucleotide has a significant influence on oligonucleotide mobility, the presence of doublets suggests that HIV-1 RT catalyzed a significant degree of misincorporation opposite the 3′G of the Pt−DNA adduct. Therefore, we repeated extension experiments in the presence of each nucleotide individually to obtain qualitative information about misincorporation opposite the 3′G. Primer extension assays were performed with a 24-mer primer that ended just before the template GG site (Figure 2B, III). Qualitatively, primer extension studies with individual dNTPs revealed a similar tendency of HIV-1 RT to misincorporate nucleotides across all three types of platinum diadducts; therefore, results are shown only for templates damaged by cisplatin. The correct dCTP was the base preferentially incorporated opposite the GG sequence, and misincorporation of dCTP across from A and T bases 5′ to the GG was also detected (Figure 5E, lane 1). A significant degree of misincorporation of dTTP was observed across from 3′G, resulting in a 25-mer band (Figure 5E, lane 3). Since after misincorporation of two bases the next position in the template contains an A, incorporation of dTTP is favored at that position. Therefore, additional misincorporation of dTTP followed by correct incorporation resulted in the appearance of a weak 27-mer (Figure 5E, lane 3). Smaller amounts of dATP and dGTP misincorporation opposite the GG sequence were also observed (Figure 5E, lanes 2 and 4). These data support the hypothesis that the doublet observed opposite the 3′G during translesion synthesis on platinated templates by HIV-1 RT (Figure 5A,B) was due to misincorporation.

Effect of Pt−GG Adducts on Primer Extension Catalyzed by Human Polymerase γ. When exonuclease-proficient pol γ was used in primer extension experiments, significant digestion of the primer was observed for all templates except templates containing JM216 adducts (data not shown). Thus,

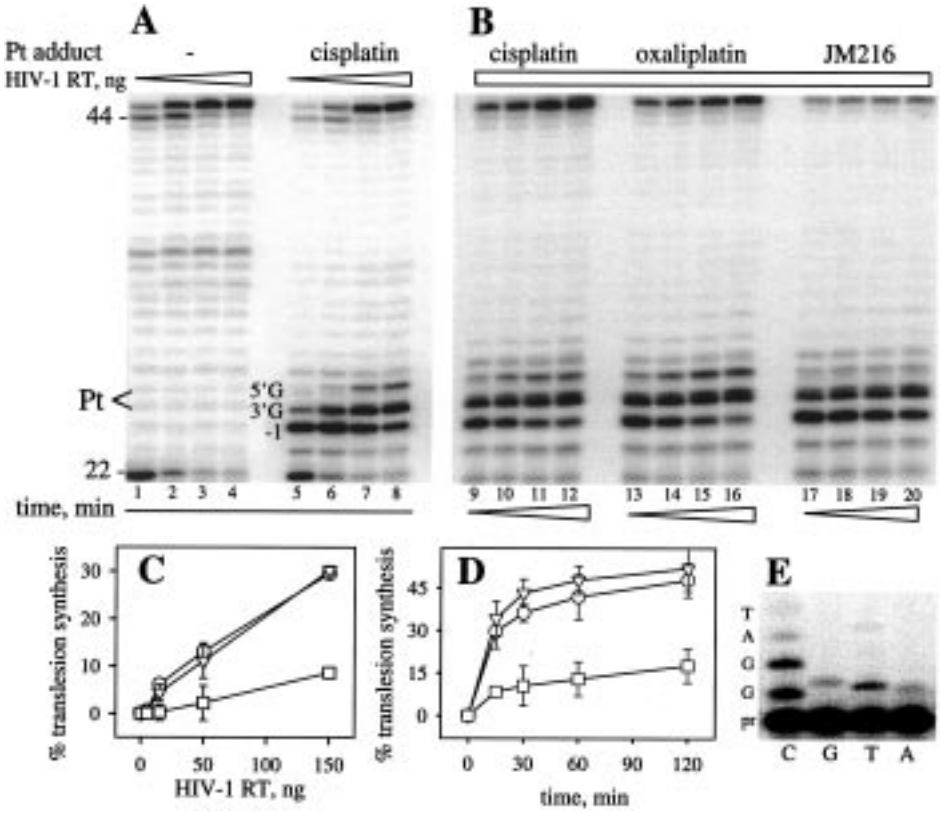

FIGURE 5: Primer extension activity of HIV-1 RT. (A) Representative gel showing in vitro primer extension by HIV-1 RT performed for 15 min with 5, 15, 50, and 150 ng of enzyme for both the control templates (lanes 1−4) and templates with cisplatin adduct (lanes 5−8). 22/44 primer−templates (Figure 2B, II) were used in these experiments. Gels for templates with oxaliplatin and JM216 adducts are not shown. (B) Time-course experiments with all three platinated 22/44 primer−templates incubated for 15, 30, 60, and 120 min with 150 ng of enzyme (lanes 9−20). (C,D) The data for graphical representation were obtained as described in Figure 3 from six different experiments with three independent template preparations. (E) Specificity of nucleotide incorporation by 1.4 ng of HIV-1 RT across GG sites on 24/44 primer−templates (Figure 2B, III) with a cisplatin adduct. Nucleotide misincorporation studies were performed for 15 min with individual dNTPs present at 1 mM concentration. (C, incubation with 1 mM dCTP; G, incubation with 1 mM dGTP; T, incubation with 1 mM dTTP; and A, incubation with 1 mM dATP.) Template sequence is indicated on the left (pr = primer).

to simplify interpretation of the data, exonuclease-deficient pol γ was used for most of these studies. Elongation of 22-mer primers on control templates for 15 min with 18−150 ng of pol γ is shown in Figure 6A (lanes 1−4). The processivity of pol γ appeared to be similar to that of HIV-1 RT in that no strong intermediate pause sites were detected. However, exonuclease-deficient pol γ differed from the other enzymes in that the major elongation product was 45 nucleotides long, even at low enzyme concentrations when more than half of the primers were not extended. This was true for both control (Figure 6A, lanes 1−4) and platinated (Figure 6A, lanes 5−16, and Figure 6B, lanes 17−20) templates. The addition of an extra base to the end of the template was not seen with exonuclease-proficient pol γ (data not shown).

Figure 6A (lanes 5−16) shows primer extension for 15 min on templates containing all three Pt−DNA diadducts, using 18−150 ng of pol γ. At all enzyme levels a major termination site was observed one base 3′ to the lesion (Figure 6A, lanes 5−16). As the enzyme concentration was increased, a second major stop site was observed across from the 3′G(Pt), along with a minor stop site across from the 5′G(Pt) and an increase in full-length DNA (Figure 6A, lanes 7, 8, 11, 12, 15, and 16). With 150 ng of pol γ essentially all of the primer was elongated to the position of the adduct (Figure 6A, lanes 8, 12, and 16). With higher amounts of enzyme, little further translesion synthesis was observed (data not shown).

The time course for translesion synthesis past the oxaliplatin adduct with 150 ng of pol γ is shown in Figure 6B and graphically for all three Pt−DNA diadducts in Figure 6D. Although some elongation was observed from the −1 position to the stop site opposite the 3′ G(Pt) during the first 15 min of incubation (Figure 6B, lanes 17−20), the extent of translesion synthesis past the stop sites reached near-maximal levels between 5 and 10 min (Figure 6B, lanes 18−19) and increased little during the next 2 h of incubation (Figures 6B, lanes 20−23, and 6D). As with low levels of pol β, the order of translesion synthesis was oxaliplatin > cisplatin > JM216 (Figure 6C,D). Although exonuclease-proficient pol γ catalyzed extensive primer degradation, the relative efficiency of translesion synthesis past cisplatin, oxaliplatin, and JM216 adducts was the same as for the exonuclease-deficient pol γ (data not shown). Therefore, the 3′ to 5′ exonuclease activity normally associated with pol γ does not appear to affect the specificity of translesion synthesis past Pt−DNA adducts.

With the exonuclease-deficient pol γ it was possible to compare synthesis on damaged and undamaged templates with 15-min incubations containing 18−28 ng of enzyme (Table 1). When calculated as a percentage of synthesis on an undamaged template, the initial ability of pol γ to catalyze

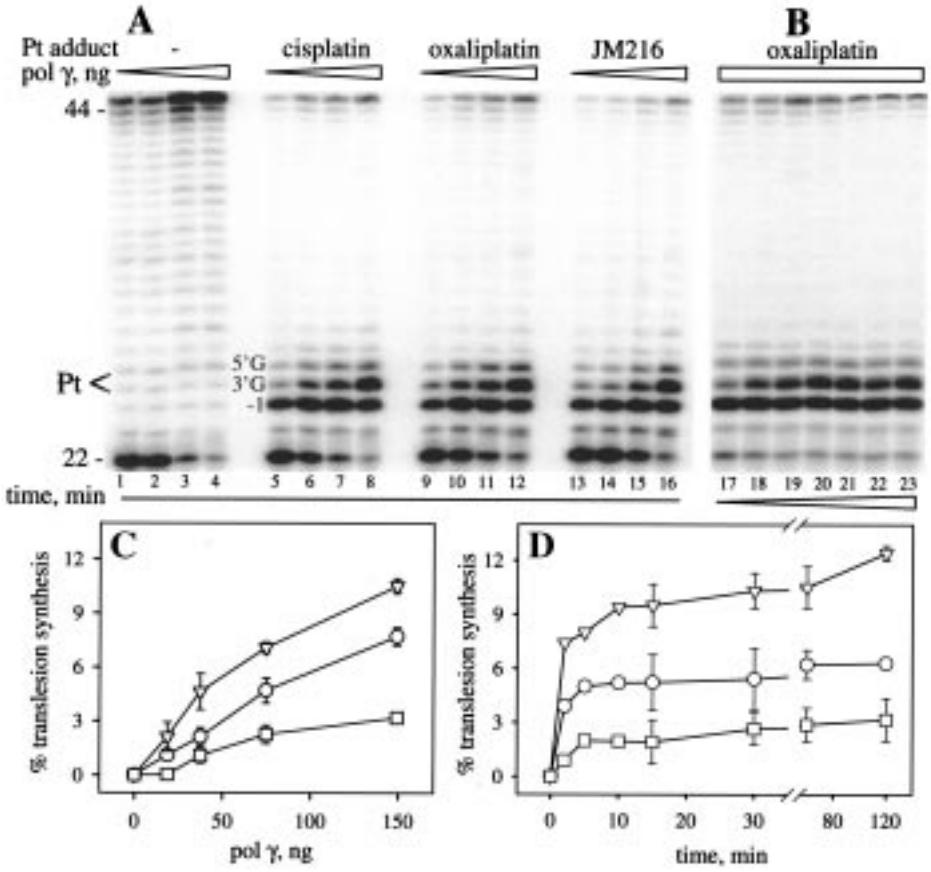

FIGURE 6: Primer extension activity of pol γ. (A) Primer extension was performed for 15 min in the presence of 18−150 ng of pol γ for both the control (lanes 1−4) and all three platinated (lanes 5−16) 22/44 primer−templates (Figure 2B, II). **B**. Representative gel showing time-course experiments with 30 ng of pol γ performed for 2, 5, 10, 15, 30, 60, and 120 min on 22/44 primer−templates (Figure 2B, II) with oxaliplatin adduct (lanes 17−23). Gels for templates with cisplatin and JM216 adducts are not shown. (C, D) Data for graphical representation were obtained as described in Figure 3 from four different experiments with two independent template preparations.

translesion synthesis past Pt−DNA diadducts was very similar to that of pol β and HIV-1 RT. However, the maximum extent of translesion synthesis that could be achieved was much less than for pol β and HIV-1 RT, and could not be increased by increasing either enzyme concentration or time of incubation. Thus, several experiments were performed to determine what was limiting for translesion synthesis by pol γ. The same extent of translesion synthesis was seen with the 17/90, 24/44, and 22/44 primer−templates (data not shown). Thus, the extent of translesion synthesis was not influenced by the placement of the original primer relative to the Pt−DNA adduct. To determine whether the lack of a time-dependent increase beyond 10 min could be due to the instability of pol γ in the incubation mixture, the 22/44 primer−template containing a oxaliplatin adduct was incubated at 37 °C for 15 min with 150 ng of enzyme. At that point all of the primer had been extended to the Pt adduct and total translesion synthesis was 13%. The addition of 150 ng of fresh enzyme to this incubation mixture did not produce any additional translesion synthesis over the next 15 min. These data show that translesion synthesis was not limited by the amount of active enzyme in the incubation mixture. However, when the incubation mixture was heated at 80 °C for 10 min and then slowly cooled to 37 °C before the addition of 150 ng of fresh enzyme, an additional 10% translesion synthesis was produced over the next 15 min. These data suggest that once the primers reach the position of the platinum adduct, most of the pol γ is complexed to the end of the DNA chain in an inactive form. In addition, these data show that if pol γ were to dissociate from the growing DNA chain in the vicinity of the adduct, it clearly has the ability to rebind and perform additional elongation. Finally, it is possible that an inactive enzyme DNA complex could be formed by multiple copies of pol γ bound to the DNA in the presence of the Pt−DNA adduct. Thus, to determine whether these effects were an artifact of the high (150 ng) levels of enzyme used, the primer extension assay was repeated with 18 ng of pol γ (which represents an enzyme to primer−template ratio of approximately 0.6) and the 22/44 primer−template containing a oxaliplatin adduct. Under these conditions, the reaction was again essentially complete by 15 min even though only 60% of the primer had been elongated to the position of the adduct. Thus, all the available data suggest that when it initially encounters a Pt−DNA adduct, pol γ has a finite probability of catalyzing translesion synthesis past Pt−DNA adducts. The initial ability of pol γ to bypass the adduct is determined by the carrier ligand of the adduct (Figure 6) and is about the same as for pol β and HIV-1 RT. However, unlike pol β and HIV-1 RT, those molecules of pol β that do not bypass the adduct appear to form an inactive complex with the template and the growing DNA chain at the position of the Pt−DNA adduct.

Effect of Pt−GG Adducts on Primer Extension Catalyzed by Yeast Polymerase ζ. The pattern of products synthesized by 15−30 ng of pol ζ in a 2-h incubation with undamaged and platinated templates is shown in Figure 7. Replication

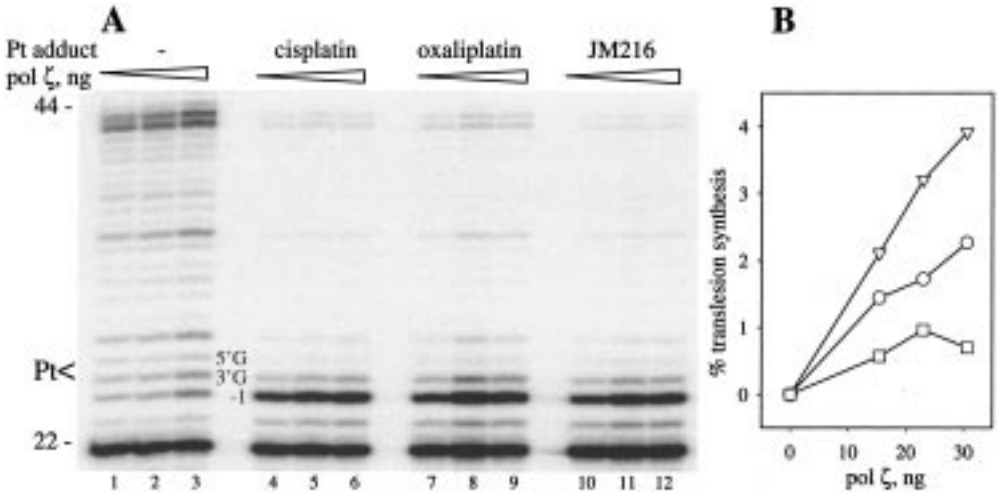

FIGURE 7: Primer extension activity of pol ζ as a function of enzyme concentration. (A) In vitro replication by pol ζ was performed for 2 h with 15.5, 23, and 30.5 ng of enzyme for control and platinated 22/44 primer−templates (Figure 2B, II). (B) Data for graphical representation were obtained as described in Figure 3.

with pol ζ on an undamaged template was moderately distributive, as a number of intermediate stop sites were observed (Figure 7A, lanes 1−3). Overall, replication by pol ζ was not as efficient as for the other polymerases tested. Pol ζ was active over only a relatively narrow range of enzyme concentrations, and a low level of primer utilization was seen even on undamaged templates (Figure 7A, lanes 1−3). At higher enzyme concentrations an inhibition of polymerase activity was observed even on undamaged templates. This could indicate the formation of enzyme−DNA complexes that are not competent for dNTP insertion at high enzyme concentrations. Furthermore, unlike the other DNA polymerases tested, pol ζ tended to form products that terminated 1 or 2 bases before the end of the template, although some full-length product was observed at the highest enzyme concentration (Figure 7A, lane 3). Nelson et al. (*18*) have reported this phenomenon previously for other templates.

With the damaged substrates (Figure 7A, lanes 4−12), the same amount of primer was extended as with undamaged templates. Because the efficiency of primer extension by pol ζ was low, it was difficult to quantitate the extent of translesion synthesis on damaged templates at low enzyme concentrations and short incubation times. Figures 7A (lanes 4−12) and 7B show the extent of translesion synthesis past Pt−DNA diadducts during a 2-h incubation with 15−30 ng of pol ζ. All three Pt diadducts showed a strong stop site one base prior to the Pt-GG adduct, with a weaker stop site opposite the 3′ G(Pt). No detectable stop site was observed opposite the 5′ G(Pt). These observations indicate that the 3′G(Pt) was a very strong block to replication by pol ζ. However, if nucleotides were incorporated opposite the adduct, further chain elongation proceeded normally. The ability of pol ζ to catalyze translesion synthesis past Pt−DNA diadducts varied in the order oxaliplatin > cisplatin > JM216 (Figure 7B), which is similar to the pattern observed with pol γ and low concentrations of pol β. When translesion synthesis was calculated relative to synthesis on an undamaged template (eq 2), pol ζ catalyzed translesion synthesis past Pt−DNA diadducts with only slightly less efficiency than the other polymerases studied (Table 1). The relative extent of translesion synthesis past Pt−DNA adducts by pol ζ was also similar to the extent of translesion synthesis past *cis*,*syn*-cyclobutane thymine dimers when calculated in a similar manner (*18*). However, because the amount of primer extension was low for pol ζ even on undamaged templates, the maximum extent of translesion synthesis (calculated by eq 1) was much less for pol ζ than for the other polymerases tested. The reasons for the relatively low activity of pol ζ and the decrease in enzyme activity observed at high enzyme concentrations are not known. However, it is possible that accessory factors exist that might increase the activity and/or processivity of pol ζ.

Effect of HMG-1 Protein Binding on Replication past Pt−DNA Adducts. Hoffmann et al. (*28*) demonstrated that HMG1 can inhibit translesion replication past cisplatin−DNA adducts. Thus, we performed in vitro primer extension assays in the presence of HMG1 to determine whether binding of damage-recognition proteins would influence the ability of purified polymerases to catalyzed translesion synthesis past Pt−DNA adducts. Since HMG domain proteins recognize platinum adducts only in double-stranded DNA sequences (*38*) we constructed templates IV and V for these experiments (Figure 2B). Template IV is similar to the one used by Hoffmann et al (*34*). The noncomplementary region of that template provided a "fork-like" structure that was required to obtain efficient translesion synthesis past cisplatin−DNA adducts by cell extracts. However, since it was not known whether the noncomplementary region would be required for efficient translesion synthesis on double-stranded DNA by purified enzymes, the experiments were also performed with template V, which is analogous to template IV except that it does not contain the 25-bp noncomplementary region. Purified pol β could not be tested in these experiments using templates with partially duplex DNA molecules because it produced large amount of an aberrant DNA product, analogous to one observed by Hoffmann et al. (*34*). However, HIV-1 RT utilized these templates as efficiently and with the same specificity as it utilized single-stranded templates. Thus, the ability of HMG1 protein to block translesion synthesis past Pt−DNA adducts was determined with HIV-1 RT.

DNA preincubation with HMG1 protein caused an inhibition of translesion synthesis by HIV-1 RT, as indicated by

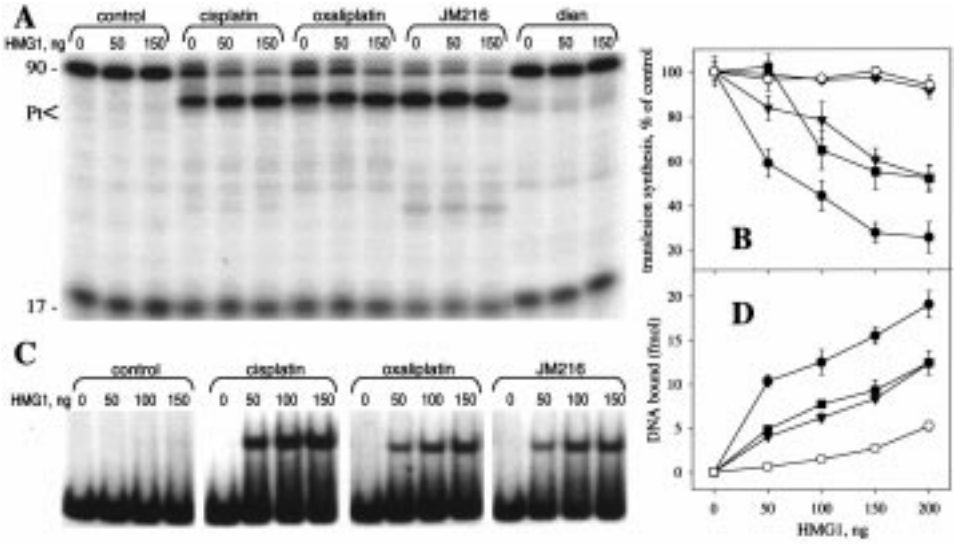

FIGURE 8: Binding of HMG1 protein to platinated DNA and its effect on translesion synthesis past platinum−DNA adducts by HIV-1 RT. (A) Representative gel showing inhibition of HIV-1 RT-catalyzed translesion synthesis by 50 and 150 ng of HMG1. The primer extension assays were performed on the forked-like 90-mer template (Figure 2B, IV). (B) Quantitative analysis of the inhibition of translesion synthesis past platinum−DNA adducts by 50, 100, 150, and 200 ng of HMG1. The translesion synthesis was calculated according to eq 1. In the absence of HMG1, the extent of translesion synthesis was 50.7% ± 2.2% for cisplatin (●), 49.4% ± 2.0% for oxaliplatin (▼), 17.6% ± 1.2% for amminecyclohexylamine-Pt (■), and 100% ± 3.1% for dien-Pt (◆) adducts. The extent of translesion synthesis in the absence of HMG1 was assigned a value of 100% to compare the effect of HMG1 binding on translesion synthesis on the templates with different platinum adducts and on the inhibition of chain elongation on control template (○). No differences between amount of translesion synthesis in the absence of HMG1 and in the inhibition of translesion synthesis by HMG1 were detected when the forked-like template IV or the template with double-stranded binding site V (Figure 2B) was used. Therefore, data obtained on these templates were averaged together. Data are means (± standard error) from eight different experiments with a total of four independent template preparations (two of each kind of template). (C) Representative gel showing differential binding of HMG1 (50, 100, and 150 ng) to 50 fmol of platinated DNA. Gel shift assays were performed with ^{32}P-labeled double-stranded 44-mers without and with site-specifically placed platinum adducts as discussed in Materials and Methods. (D) Results of the gel shift assays using 50, 100, 150, and 200 ng of HMG1 protein. The symbols are the same as in panel B.

decreased amount of full-length products and accumulation of termination products at the lesion sites (a representative gel is shown in Figure 8A). The effect of HMG1 on translesion synthesis past cisplatin, oxaliplatin, JM216, and dien adducts was identical for substrates IV and V, so the data obtained with both substrates were averaged for the graph shown in Figure 8B. As can be seen from Figure 8B, DNA synthesis on undamaged template and on templates with the dien-Pt adduct was not affected by up to 200 ng of HMG-1. However, 50 ng of HMG1 inhibited translesion replication on templates with the cisplatin adduct by about 40% (Figure 8B). About 150 ng of HMG1 was required to give the same level of inhibition of translesion synthesis for templates with oxaliplatin and JM216 adducts. Therefore, translesion replication was inhibited to a greater extent when the template was modified by cisplatin compared to the other platinum bifunctional complexes. Since the efficiency of translesion synthesis past platinum adducts by HIV-1 RT in the absence of HMG1 was dach = cisplatin > JM216, HMG1 had clearly altered the specificity of translesion synthesis by HIV-1 RT. The ability of HMG1 to bind to platinum adducts was assessed by a gel-shift assay with double-stranded 44-mer template (the sequence of the damaged strand is shown in Figure 2B, II). These data revealed that, in agreement with translesion synthesis results, HMG1 bound to cisplatin adducts with about 2-fold greater affinity than to oxaliplatin and JM216 adducts, whereas only a minimal shift of undamaged template was observed under the identical conditions (Figure 8C,D). These studies suggest that platinum-damage recognition proteins such as HMG1 can discriminate between cisplatin, oxaliplatin, and JM216 adducts and can impart specificity to the replicative bypass of these adducts.

DISCUSSION

Comparison of Primer Extension on Control and Platinated Templates by Different DNA Polymerases. Most of the DNA polymerases that have been studied previously catalyze very limited translesion synthesis past Pt−DNA adducts (*35*, *39*−*41*). In contrast, pol β and HIV-1 RT have been reported to catalyze extensive translesion synthesis past Pt−DNA adducts (*10*, *13*). The mechanistic basis for these differences has not been explored previously. Thus, we compared the efficiency of translesion synthesis for all four enzymes included in this study at short reaction times and low enzyme concentrations. Under these conditions the ability to catalyze translesion synthesis past Pt−DNA adducts, as a percent of synthesis on undamaged templates, was similar for all four enzymes (Table 1). However, in agreement with previous observations, the eukaryotic DNA polymerases in this study varied considerably in the maximum amount of translesion synthesis they were able to catalyze in vitro. For pol β and HIV-1 RT, the extent of translesion synthesis could be increased significantly by increasing both the amount of enzyme and the time of incubation. On the other hand, maximum translesion synthesis by pol γ and pol ζ was much less. The activity of the exonuclease-deficient pol γ was high enough that the mechanism of the limited translesion synthesis by the purified enzyme could be examined in detail. The available data suggest that, on its initial approach to the adduct, pol γ had a finite probability of catalyzing translesion synthesis past

the Pt−DNA adduct. However, those molecules of pol γ that were initially unsuccessful at bypassing the adduct appeared to be sequestered in an inactive form at the 3′ end of the growing DNA chain in the vicinity of the Pt−DNA adduct. These observations are consistent with the model proposed by Suo et al. (*41*) for polymerase pausing at sites of secondary structure or bulky adducts. In that model, a small percentage of polymerase molecules bind productively to the damaged DNA and rapidly incorporate the next nucleotide to form an elongated product. The rest of the polymerase molecules are bound nonproductively to the DNA. Those molecules remain bound to the DNA and are slowly converted to the productive state; or they slowly dissociate from the DNA, leaving a 3′ end available for rebinding. Our data would suggest that pol β, HIV-1 RT, pol ζ, and pol γ are similar with respect to the percent of polymerase molecules that are capable of binding productively to DNA containing Pt−DNA diadducts. The biggest difference between these enzymes would appear to be in their ability to convert nonproductively bound enzyme to an active form at the site of Pt−DNA adducts. This hypothesis will need to be tested by pre-steady-state kinetic analysis of translesion synthesis past Pt−DNA adducts by these enzymes.

Effect of Carrier Ligand on the Extent of Translesion Synthesis. The efficiency of translesion synthesis was also strongly influenced by the structure of platinum complex. We did not observe a substantial block of DNA synthesis by the monofunctional dien−Pt adduct for any polymerases employed in this study (data not shown). The Pt−GG diadducts retarded primer extension by all four polymerases, but the extent of translesion synthesis depended on the carrier ligand of the adduct (Figures 3−7). JM216 adducts were more effective than both cisplatin− and oxaliplatin−DNA adducts at blocking translesion synthesis by all four eukaryotic DNA polymerases used in this study. Hartwig and Lippard (*40*) have shown that two conformational isomers of the JM216−GG adducts form in a 2:1 ratio, the most abundant with the cyclohexylamine ligand oriented in the 3′ direction and the less abundant with cyclohexylamine ligand oriented in the 5′ direction (*40*). We have not separated the 5′ and 3′ isomers of the JM216 adducts for this study, so we do not know which of the isomers is responsible for the strong block to translesion synthesis that we have observed in these experiments. We plan to analyze differences in translesion synthesis of isomers of JM216 adducts in ongoing studies.

Our data also indicated an approximately 2-fold preference of pol ζ pol γ, and low concentrations of pol β for bypass of oxaliplatin-DNA adducts compared to cisplatin−DNA adducts. However, at high enzyme concentrations pol β did not appear to differentiate between cisplatin and oxaliplatin adducts. These data suggest that ability of pol β to discriminate between cisplatin and oxaliplatin adducts may be determined primarily by the rate of polymerase dissociation from the damaged template. In contrast, translesion synthesis past JM216 adducts was less than for the other two adducts at all enzyme concentrations tested, suggesting that the ability to discriminate between JM216 adducts and the other adducts may be determined primarily by the rate of nucleotide insertion across the damaged sites. These hypotheses will need to be evaluated in future kinetic studies.

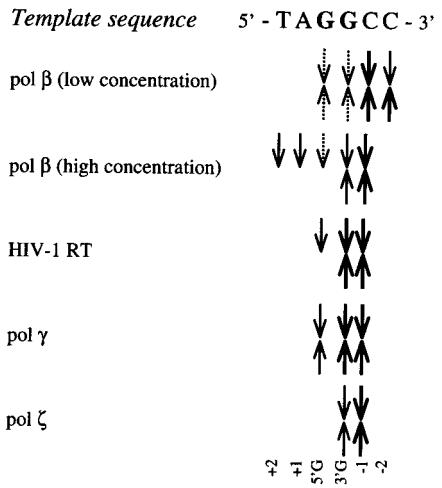

FIGURE 9: Schematic diagram of stop sites by DNA polymerases on 22/44 primer−templates (Figure 2B, II) with site-specifically placed platinum adducts. Stop sites for templates with cisplatin and oxaliplatin adducts are shown by down arrows; stop sites for templates with JM216 adducts are shown by up arrows. The relative intensities of stop sites are represented as weak (broken arrow), medium (continuous arrow), or strong (bold arrow).

Thus, our data demonstrate that eukaryotic DNA polymerases from three different DNA polymerase families, pol β, pol ζ, and pol γ, all share a similar specificity for translesion synthesis past oxaliplatin, cisplatin, and JM216 adducts under physiological conditions. These data suggest that the carrier ligand specificity for translesion synthesis past Pt−DNA adducts may be determined in part by common features of DNA polymerase active sites. However, this specificity is clearly not universal since HIV-1 RT does not discriminate between cisplatin and oxaliplatin adducts. Therefore, if other DNA polymerases are implicated in the bypass of Pt−DNA adducts in vivo, their specificity for translesion synthesis in vitro will need to be examined. For example, it will be interesting to determine the ability and specificity of the recently discovered yeast polymerase η (*42*) to bypass Pt−DNA adducts. This polymerase presumably represents a new class of DNA polymerases and has the ability to efficiently bypass thymine−thymine dimers in vitro (*42*).

Effect of Carrier Ligand on the Sites of Replication Termination. The stop sites observed during primer extension on platinated templates are summarized in Figure 9. The major stop sites (−1, 3′G, and 5′G) have been reported previously for other DNA polymerases with templates containing site-specific cisplatin adducts (*35*, *39*−*41*). In the present study we have also found that at high enzyme concentrations pol β has two additional stop sites at positions +1 and +2 downstream from the lesion. While strong stop sites at these positions have not been previously reported, Suo et al. (*41*) have shown that for T7 DNA polymerase the binding of both DNA substrate and next nucleotide are significantly decreased at the +1 and +2 positions relative to the cisplatin adduct.

The stop sites for the cisplatin and oxaliplatin adducts were generally very similar. In contrast, the stop sites for the JM216 adducts were noticeably different for both HIV-1 RT and pol β. For HIV-1 RT no stop site at 5′G(Pt) was detected. For pol β at low enzyme concentrations, less primer molecules were extended and the band at the −2 position

was stronger than for the other adducts. At high pol β concentrations the stop site at the −1 position was stronger than for the other adducts and no stop sites at the +1 and +2 positions were evident. These data suggest that the JM216 adduct exerts a stronger effect on the proximal side of the adduct than either the cisplatin or oxaliplatin adducts. This is consistent with the recent NMR/molecular modeling analysis of the most abundant isomer of the JM216−GG adduct (*3*). In that structure, the cyclohexylamine ligand lies in the major groove and extends to the base pair 3′ to the adduct.

Role of DNA Polymerases in Determining the Specificity of Replicative Bypass in Vivo. Our findings suggest a model in which the specificity and fidelity of replicative bypass in vivo may be determined in part by one or more polymerases involved in translesion synthesis. Previous studies have shown that the oxaliplatin (*43*) and JM216 (*44*) adducts are likely to be less mutagenic than the cisplatin adducts. We have determined in the present study that two of the polymerases (pol β and pol ζ) that were thought to be involved in error-prone translesion synthesis in vivo are able to discriminate between cisplatin, oxaliplatin, and JM216 adducts. The present investigation was primarily focused on relative extent of translesion synthesis. However, studies are currently in progress to determine the fidelity of translesion synthesis past platinum adducts with different carrier ligands by these polymerases. Error-free replicative bypass has been proposed to occur by a template switching mechanism, which would not involve direct translesion synthesis past the adduct. In this case, the specificity of replicative bypass may be determined by damage-recognition proteins which bind to the Pt−DNA adducts and interfere with error-free bypass (see below), rather than by the interaction of DNA polymerases with Pt−DNA adducts.

Effect of Platinum Damage Recognition Proteins on the Carrier Ligand Specificity of Translesion Synthesis past Pt−DNA Adducts. Since Hoffmann et al. (*28*) have reported that HMG1 inhibits translesion synthesis past cisplatin adducts in vitro, we determined the carrier ligand specificity for this effect. We found that HMG1 protein binds with higher affinity to cisplatin than to oxaliplatin adducts or JM216 adducts (Figure 8C,D). Structural analysis suggests that HMG domain proteins bind to the minor groove of DNA that is bent in the direction of the major groove, as is the case with DNA containing cisplatin adduct (*25*). Once the HMG domain proteins bind to DNA containing a Pt−DNA adduct, they bend it further to create an "induced fit" (*25*, *45*). Previous structural studies (*3*, *4*) have suggested that DNA containing the JM216 and oxaliplatin adducts may have a slightly wider minor groove than DNA containing the cisplatin adducts. Therefore, the binding of the HMG1 to different Pt−DNA adducts may be affected by the width of the minor groove. Alternatively, the differences in the affinity of HMG1 for platinated DNA could be due to the increased bulk or rigidity of dach and amminecyclohexylamine carrier ligands compared to *cis*-diammine ligand, which interferes with the additional bending of the DNA that is seen when HMG1 binds to the cisplatin adduct.

We found that HMG1 binding to the damaged DNA specifically inhibited translesion replication by HIV-1 RT (Figure 8A,B). These findings suggest that binding of platinum damage-recognition proteins may cause a direct block for translesion replication. The ability of HMG1 to block translesion synthesis past cisplatin adducts was about 2.5-fold greater than its ability to block translesion synthesis past oxaliplatin or JM216 adducts, while DNA synthesis on undamaged template and template with the dien−Pt adduct was not affected by up to 200 ng of HMG-1. These data suggest that damage recognition proteins such as HMG1 can influence the specificity of translesion synthesis.

HMG1 itself is very abundant and is not present at different levels in cisplatin-sensitive and -resistant cell lines (*29*). Therefore, HMG1 probably cannot be implicated in imparting specificity to the increased replicative bypass seen in cisplatin-resistant cells. However, to date over 20 damage-recognition proteins have been described that bind with high affinity to cisplatin adducts, and some of these proteins are expressed in a tissue-specific manner (*46*) or are expressed at different levels in cisplatin-sensitive and -resistant cell lines (*47*, *48*). Furthermore, since many of these platinum damage-recognition proteins appear to recognize common structural features of the Pt−DNA adducts, it is likely that other damage-recognition proteins will also discriminate between Pt−DNA adducts with different carrier ligands. For example, one of the HMG domain proteins, ribosomal RNA transcription factor hUBF, has been recently shown to exhibit higher affinity for cisplatin-modified DNA compared to the DNA containing oxaliplatin or ethylenediamine−Pt adducts (*49*). In contrast, TATA binding factor, which is not an HMG domain protein, appears to bind to cisplatin and oxaliplatin adducts with equal affinity (*50*). Thus, it will be important to assess the selectivity of the other damage-recognition proteins for binding to Pt−DNA adducts.

Conclusion. Our data suggest a model in which the carrier ligand specificity for replicative bypass of Pt−DNA adducts could be determined by the interaction of the adducts with the DNA polymerases that catalyze translesion synthesis and/or the damage-recognition proteins that block translesion synthesis past those adducts. We demonstrate that at low enzyme concentration pol β, pol ζ, and pol γ all show the same specificity for translesion synthesis past Pt−DNA adducts with different carrier ligands, i.e., oxaliplatin > cisplatin > JM216. Because pol β and pol ζ have both been implicated in error-prone translesion synthesis, these data are consistent with the hypothesis that the specificity of error-prone replicative bypass and therefore mutagenicity of Pt−DNA adducts may be determined in part by the DNA polymerases involved in error-prone translesion synthesis. Our data also suggest a model in which binding of damage recognition proteins to Pt−DNA adducts can also impart specificity to replicative bypass and/or other processes that may influence the cytotoxicity of Pt−DNA adducts. Studies are currently underway to define the role of DNA polymerases and damage-recognition proteins in determining the specificity of the error-free and error-prone pathways of replicative bypass in human cells.

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