Effects of the Anticancer Drug *cis*-Diamminedichloroplatinum(II) on the Activities of Calf Thymus DNA Polymerase ϵ[†]

Lin Huang,[‡] John J. Turchi,[‡,§] Alan F. Wahl,[∥] and Robert A. Bambara*[,‡]

Department of Biochemistry, School of Medicine and Dentistry, University of Rochester, Rochester, New York 14642, and Department of Cellular and Molecular Biology, Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, Connecticut 06492

Received June 2, 1992; Revised Manuscript Received October 13, 1992

ABSTRACT: DNA polymerase (pol) ϵ is essential for DNA replication and is thought to be a component of DNA repair systems in eukaryotic cells. The activities of pol ϵ have been examined using a series of synthetic oligonucleotides designed with *cis*-diamminedichloroplatinum(II) (*cis*-DDP)-modified specific guanine residues. Pol ϵ was incapable of synthesis over *cis*-DDP-modified single guanine or adjacent guanine residues present in the template strand. Both single and double guanines modified by *cis*-DDP present at the 3′-OH end of a primer strand completely inhibited the synthetic activity of pol ϵ and, in addition, sequestered pol ϵ at the platinated 3′-OH termini. The sequestering of pol ϵ on *cis*-DDP modified DNA may interfere with the function of this enzyme in DNA repair in vivo. The intrinsic 3′ to 5′ proofreading exonuclease activity of pol ϵ was also examined. Pol ϵ was capable of degrading a single-strand template with internal *cis*-DDP-modified guanines up to, but not through, the platinated nucleotides. A single platinated guanosine was sufficient to block the 3′ to 5′ exonuclease activity of pol ϵ. These results suggest that *cis*-DDP–DNA adducts inhibit DNA synthesis mediated by DNA polymerase ϵ and that platinated sites can arrest the nuclease of pol ϵ, a function exhibited during DNA repair.

cis-Diamminedichloroplatinum(II) (*cis*-DDP,[1] cisplatin) is one of the most widely used anticancer drugs for treatment of solid tumors (Rosenberg, 1985; Loehrer & Einhorn, 1984). However, in some cases, after initially successful response to the drug, resistance to *cis*-DDP develops in the tumor cells (de Graeff et al., 1988; Scanlon et al., 1989b; Andrews & Howell, 1990). A considerable amount of evidence indicates that although adducts between *cis*-DDP and protein, or *cis*-DDP and RNA, exist, *cis*-DDP exhibits its pharmacological activity by adducting to DNA, with resulting inhibition of DNA metabolism (Harder et al., 1976; Sherman & Lippard, 1987). The *cis*-DDP–DNA adducts formed are predominantly intrastrand cross-links between N7 atoms of adjacent guanines (60–65%), between adenine and guanine bases (20–25%), and between two guanines separated by one nucleotide (3–7%). Interstrand cross-links between guanine bases (~1%) and monoadducts (~2%) are also present (Eastman, 1983; Dijt et al., 1988; Fichtinger-Schepman et al., 1985; Schaller et al., 1987).

It has been postulated that the cytotoxic effects of *cis*-DDP result from inhibition of DNA replication (Harder & Rosenberg, 1970). Pinto and Lippard (1985) have used a DNA replication mapping technique to demonstrate that *cis*-DDP-modified adjacent guanine adducts inhibit the synthetic activity of the large fragment of *E. coli* pol I. Monofunctional lesions did not appear to block DNA synthesis by *E. coli* pol I and eukaryotic DNA pol α from *Drosophila melanogaster* and calf thymus (Hoffmann et al., 1989), suggesting that they may not contribute to the cytotoxic effects of *cis*-DDP.

Recent studies with three eukaryotic DNA polymerases, α, δ, and ϵ, indicated that they are all required for yeast chromosomal DNA replication (Johnson et al., 1985; Boulet et al., 1989; Morrison et al., 1990) and involved in DNA repair (Bambara & Jessee, 1991; Nishida et al., 1988; Dresler et al., 1988; Miller & Chinault, 1982). Salles et al. (1983) reported inhibition of cellular DNA replication in mouse L1210 cells by *cis*-DDP. *cis*-DDP inhibited SV40 replication in vivo (Ciccarelli et al., 1985) and in vitro (Heiger-Bernays et al., 1990). This inhibition could have resulted from *cis*-DDP interference with any or all of the DNA polymerases mentioned above.

One of the mechanisms of acquired *cis*-DDP resistance is believed to be induction of increased DNA repair ability (Masuda et al., 1988, 1990; Sibghat-Ullah et al., 1989; Eastman & Schulte, 1988; Parker et al., 1991). Several groups have demonstrated that DNA repair-deficient cell lines exhibit markedly enhanced sensitivity to *cis*-DDP (Fraval et al., 1978; Meyn et al., 1982; Alazard et al., 1982). Aphidicolin, a specific inhibitor of DNA polymerases α, δ, and ϵ, has shown a dose-dependent capacity to inhibit DNA repair activity and partially restored the sensitivity of *cis*-DDP-resistant cell lines (Masuda et al., 1988; Katz et al., 1990). An elevated pol α mRNA level in resistant cell lines implicates this enzyme in the repair of *cis*-DDP damage (Katz et al., 1990; Scanlon et al., 1989a), even though its polymerase activity was inhibited by *cis*-DDP–DNA adducts in vitro (Hoffmann et al., 1989; Villani et al., 1988). The effects of *cis*-DDP on DNA polymerase δ and ϵ have not been reported. DNA polymerase ϵ also has a repair function, having been isolated from HeLa cell extracts as an enzyme that catalyzed DNA repair synthesis induced by UV irradiation (Nishida et al., 1988; Dresler et al., 1988). In the current paper we have examined the direct effect of *cis*-DDP-modified DNA on the polymerase activity and 3′ to 5′

[†] This research was supported by Grants GM24441 (primarily) and CA 46148, both from the National Institutes of Health.

* Author to whom correspondence should be addressed [phone (716) 275-3269; fax (716) 271-2683].

[‡] University of Rochester.

[§] Postdoctoral Fellow supported by National Institutes of Health Training Grant T32-CA09363.

[∥] Bristol-Myers Squibb.

[1] Abbreviations: pol, DNA polymerase; *cis*-DDP, *cis*-diamminedichloroplatinum(II); SV40, simian virus 40; *E. coli*, *Escherichia coli*; PCNA, proliferating cell nuclear antigen; D:O ratio, molar ratio of drug molecules to oligonucleotides.

0006-2960/93/0432-841$04.00/0

exonuclease activity of pol ϵ using a series of oligomers with known platinated sites. We have demonstrated that pol ϵ was essentially inactive on *cis*-DDP-modified DNA and was sequestered at the platinated 3′-OH terminus of a primer strand.

MATERIALS AND METHODS

Materials. Synthetic oligonucleotides and cisplatin were kindly supplied by Bristol-Myers Squibb (Wallingford, CT). Deoxyribonucleotides were purchased from Pharmacia (Piscataway, NJ). [γ-^{32}P]ATP, [α-^{32}P]dGTP, and [^{3}H]dTTP were from New England Nuclear (Boston, MA). T4 polynucleotide kinase and Sequenase (version 2.0) were from United States Biochemical (Cleveland, OH). Poly(dA)$_{3000}$ and oligo(dT)$_{16}$ were from Midland Scientific (Midland, TX). Phosphodiesterase from snake venom or calf spleen was purchased from Boehringer Mannheim (Indianapolis, IN). All of the other reagents were obtained from Sigma Chemical (St. Louis, MO).

DNA Substrates. Poly(dA)·oligo(dT)$_{16}$ was prepared as described by Turchi et al. (1992). The structures of specific DNA substrates used in this study are shown in Figure 1. The substrates were purified by 18% polyacrylamide/7 M urea sequencing gel electrophoresis (Sambrook et al., 1989) before use.

Platination of DNA Substrates. Purified oligonucleotides (shown in Figure 1) were incubated at 37 °C for 16–18 h in the presence of *cis*-DDP in 3 mM NaCl and 1 mM Na_2HPO_4 (pH 7.5) in the dark. To achieve full platination, the molar ratio of drug molecules to oligonucleotides (D:O) used in the initial incubation was 10:1 (5 nmol:0.5 nmol). Unbound platinum was then removed by Sephadex G-50 spin column chromatography (Penefsky, 1977). The platinated DNA was separated from unplatinated DNA using 18% polyacrylamide/7 M urea sequencing gel electrophoresis. This results in an efficient separation because platination slows the migration of DNA (Marrot & Leng, 1989). The platinated DNA band was excised from the gel, and the DNA was eluted and purified further by NENsorb 20 cartridge chromatography (New England Nuclear). The DNA was quantified by UV spectroscopy, and the amount of *cis*-DDP bound to the oligomer was determined by atomic absorption spectroscopy using a Perkin-Elmer 5100 PC equipped with graphite tube furnace. The molar ratios of drug to oligonucleotide (D:O ratios) are shown in the legend to Figure 1. The values generally fall somewhat below one, most likely because a small amount of nonoligonucleotide contamination, if it absorbs at 260 nm, would cause a systematic error that would lower the D:O ratio.

DNA Polymerases. Pol ϵ was purified from calf thymus to a specific activity of 550 units/mg as previously described (Siegal et al., 1992). The pol ϵ used in this study was the 145-kDa form, which has similar structural and enzymological characteristics to calf thymus pol ϵ isolated in other laboratories (Focher et al., 1989; Kesti & Syvaoja, 1991). One unit of activity is incorporation of 1 nmol of nucleotides in a 60-min reaction at 37 °C.

DNA Synthesis Assay. Self-primed fold-back oligomers [substrates a–f in Figure 1) were 5′ end labeled using [γ-^{32}P]-ATP (3000 Ci/mmol) and T4 polynucleotide kinase. The assay for DNA synthesis was carried out in synthesis assay buffer: 50 mM Tris-HCl (pH 7.5), 8 mM $MgCl_2$, 5% glycerol, 5 mM β-mercaptoethanol, and 250 μg/mL BSA. One picomole of labeled DNA oligomer molecules was used for each reaction. Reactions were initiated by adding 1 mM of each dNTP and 0.2 unit of pol ϵ to a total volume of 25 μL. They were terminated by addition of 20 μL of gel loading buffer [90% formamide, 10 mM EDTA (pH 8.0), 0.1% xylene cyanol, 0.1% bromophenol blue]. Reaction products were analyzed by 18% polyacrylamide/7 M urea sequencing gel electrophoresis, unless stated otherwise, and visualized by autoradiography.

Polymerase Sequestering Assay. Platinated fold-back oligomeric DNA was tested for ability to bind and sequester polymerase molecules from further catalysis. This DNA was used at a nucleotide concentration of 3.6 μM per reaction. After a 5-min preincubation at 37 °C with 0.15 unit of pol ϵ in DNA synthesis assay buffer as given under DNA Synthesis Assay, 40 μM poly(dA)·oligo(dT)$_{16}$ (in nucleotide) and 25 μM [^{3}H]dTTP (4.0 Ci/mmol) were added. The reactions (25 μL) were incubated for an additional 15 min at 37 °C and then were terminated by addition of EDTA to a final concentration of 50 mM. Each sample was passed through a Sephadex G-50 spin column to remove unincorporated [^{3}H]-dTTP and quantified by liquid scintillation counting.

Measurement of the Rate of the Pol ϵ Transfer between the Templates. Pol ϵ (0.2 unit) was allowed to prebind to 1 pmol of unlabeled platinated DNA for 5 min at 37 °C in DNA synthesis assay buffer. Then an equal amount of 5′ end labeled DNA substrate e and 1 mM dTTP were added. The amount of pol ϵ that transferred from the unlabeled to the labeled DNA templates was determined by measurement of polymerization on the labeled substrate. Polymerization was quantitated by measurement of the amount of extension of the labeled oligomer over a time course. The samples were analyzed on a 12% polyacrylamide/7 M urea sequencing gel and visualized by autoradiography. The full-length products extended by pol ϵ were quantified by scanning laser densitometry.

Exonuclease Assay. The 3′ to 5′ exonuclease activity of pol ϵ was assessed using single-stranded oligonucleotides (g–j), since these are better exonuclease substrates than oligomers that fold back to form double-stranded primer-template structures (Sabatino et al., 1990). The substrates were labeled at the 5′ end using [γ-^{32}P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase, so that degradation products of the reaction would retain the radiolabel. The assay conditions were the same as for DNA synthesis, except that dNTPs were not included. These same assay conditions were also applied for reactions involving phosphodiesterase from snake venom (30 microunits) or from calf spleen (12 milliunits), except that the DNA substrates (a–d) were labeled at the 3′ end by Sequenase (version 2.0) and [α-^{32}P]dGTP (3000 Ci/mmol) for the reactions with phosphodiesterase from calf spleen. The degradation products were separated on an 18% polyacrylamide/7 M urea sequencing gel, and the distribution of products was visualized by autoradiography.

RESULTS

Pol ϵ Was Incapable of Synthesis over cis-DDP-Modified dG or dGG Residues in a Template Strand. Hoffmann et al. (1989) have shown that monofunctional *cis*-DDP adducts do not appear to affect pol α mediated DNA synthesis in vitro. To determine the direct effect of template mono- or bifunctional *cis*-DDP-modified adducts on DNA synthesis catalyzed by pol ϵ, a group of self-priming fold-back oligomers with either single- or double-dG residues in the template strands were employed (substrates a–d in Figure 1). Figure 2 shows that pol ϵ could not synthesize past either single- or double-

DNA SUBSTRATES

(a) T^{TC}ATTTAAATTATA 3'
C_{TT}TAAATTTAATATCTCTT**G**TCTTC 5'

(b) T^{TC}ATTTAAATTATA 3'
C_{TT}TAAATTTAATATCTCTT**GG**TCTTC 5'

(c) T^{TC}ATTTAAATTATA 3'
C_{TT}TAAATTTAATATCTCTTTCTTC**G**CCCTC 5'

(d) T^{TC}ATTTAAATTATA 3'
C_{TT}TAAATTTAATATCTCTTTCTTC**GG**CCCTC 5'

(e) T^{TT}TTTATATATAT**GG** 3'
T_{TT}AAATATATATACCAAAAAAAAA 5'

(f) T^{TT}TTTATATATAT**G** 3'
T_{TT}AAATATATATACCAAAAAAAAA 5'

(g) 5'TTTTTTTTTTTTTTTTT**G** 3'

(h) 5'TTTTTTTTTTTTTTTTT**GG** 3'

(i) 5'TTTTTTTTTTT**G**TTTTTT 3'

(j) 5'TTTTTTTTTTT**GG**TTTTT 3'

FIGURE 1: DNA substrates used in this study. The dG residues that are subject to platination are shown in bold. For those substrates that are self-complementary (a–f), the expected double-stranded primer-template configurations are shown. The molar ratios (D:O) of bound *cis*-DDP to oligonucleotides a–j are 0.87, 0.81, 1.26, 0.77, 0.92, 0.54, 0.51, 0.68, 0.89, and 0.76, respectively.

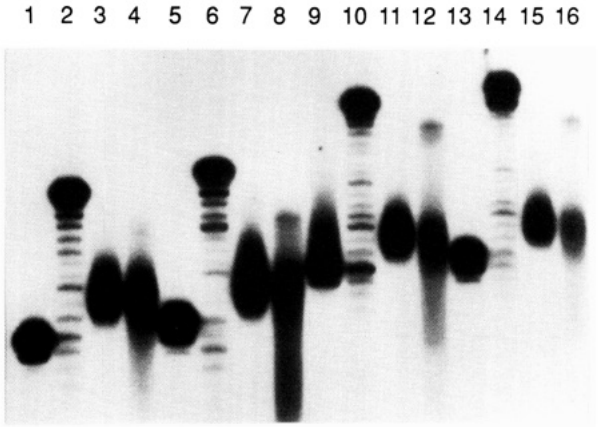

FIGURE 2: DNA polymerase ϵ was incapable of synthesis over *cis*-DDP-modified single dG or double dG in the template strand. Extension reactions were performed as described under Materials and Methods. Shown are the electrophoretic analyses of extension reactions containing DNA substrates a (lanes 1 and 2), b (lanes 5 and 6), c (lanes 9 and 10), and d (lanes 13 and 14) and the platinated substrates a (lanes 3 and 4), b (lanes 7 and 8), c (lanes 11 and 12), and d (lanes 15 and 16). Reaction specifications are as follows: lanes 1, 3, 5, 7, 9, 11, 13, and 15, no enzyme added; lanes 2, 6, 10, and 14, 0.2 unit of pol ϵ added to nonplatinated DNA; lanes 4, 8, 12, and 16, 0.2 unit of pol ϵ added to platinated DNA.

platinated dG residues in a template strand. The extension of nonplatinated DNA (lanes 2, 6, 10, and 14) can be clearly observed on this high-percentage sequencing gel. The extension of platinated DNA (lanes 4, 8, 12, and 16) was completely stopped at the platinated dG (lanes 4 and 12) or dGG (lanes 8 and 16). In addition, the total extension observed on the platinated DNA was less efficient than synthesis on an equivalent, but nonplatinated, template. This product profile suggests that initiation of synthesis is also significantly inhibited by the template platinated dG.

Phosphodiesterases from either snake venom or calf spleen were employed to further define the structure of *cis*-DDP–DNA adducts on DNA substrates a–d. The enzyme digestion products were analyzed using 18% polyacrylamide/7 M urea sequencing gel electrophoresis (Sambrook et al., 1989). Figure 3A–D shows the digestion of substrates a–d in the 3′ to 5′ direction using snake venom phosphodiesterase. Judging from the degradation of nonplatinated DNA, the nuclease digested platinated substrate a up to 31–33 and substrate b up to 32–34 nucleotides from the 3′ end, and substrate c up to 36–38 and substrate d up to 37–39 nucleotides from the 3′ end. Consequently, digestion terminated two to four or one to three nucleotides away from the platinated dG or dGG residues, respectively. Figure 3E shows digestion of substrates a–d in the 5′ to 3′ direction using phosphodiesterase from calf spleen. Under the conditions employed, the nuclease digests nonplatinated DNA from the 5′ end up to the double-strand region (lanes 2, 6, 10, and 14); the platinated DNA molecules are only lightly digested since there are just five nucleotides from the 5′ end to the platinated sites (lanes 4, 8, 12, and 16). These phosphodiesterase digestion experiments demonstrate that the substrates were quantitatively platinated. The observation that digestion can approach close to the dG residues in either direction indicated that the level of intrastrand cross-links between dA and dG residues was undetectably low. Similarly, the sizes of the limit digestion products demonstrate that the level of interstrand cross-links between dG or dA with dG was undetectably low. This suggests that the *cis*-DDP on the single dG residues can form a stable monofunctional adduct on the DNA substrates utilized in this study.

Pol ϵ Was Completely Inactive on cis-DDP-Modified DNA with Single or Double dG at the 3′-OH End of a Primer Strand. The capacity of pol ϵ to synthesize from a platinated primer was examined. The fold-back, self-priming substrates c and f, with double or single dGs, respectively, at the 3′-OH end (Figure 1), were employed. Figure 4A shows DNA synthesis by pol ϵ on substrate e. Full-length products were synthesized from substrate e with increasing incubation time from 0 to 20 min (lanes 1–5). However, synthesis on platinated substrate e, examined under the same conditions, was completely inhibited by the *cis*-DDP-modified double dG at the 3′-OH end (lanes 6–10). Lanes 11–15 show the same experiment using a mixture of platinated and nonplatinated substrates e. Nonplatinated DNA was extended as in lanes 1–5, indicated by a reduction of the starting 41mer (lowest band). Platinated DNA was resistant to extension by pol ϵ, indicated by the invariable appearance of the middle band, which represents the platinated starting substrate. A comparison of lanes 11–15 with lanes 1–5 shows that there is a reduction in the extension products band intensity. This suggests that, in the mixing experiment, some of the pol ϵ was sequestered by the platinated DNA substrate. Therefore, less pol ϵ was available for extension of the nonplatinated DNA substrate. This phenomenon will be considered further in the next section. Results of the mixing experiment also rule out the possibility that the inhibition of pol ϵ results from contamination by free *cis*-DDP or other factors in the reaction.

The same set of experiments described above was carried out on substrate f, which has a single dG at the 3′-OH end. Figure 4B shows the outcome of extensions of platinated and nonplatinated substrate f by pol ϵ. The results throughout were essentially identical to those obtained with substrate e. Therefore, a monofunctional *cis*-DDP–dG lesion at the 3′-OH end of a primer is sufficient to block the synthetic activity of pol ϵ.

Pol ϵ Was Sequestered at the cis-DDP-Modified 3′-OH Terminus of a Fold-Back Oligomer. The next experiments

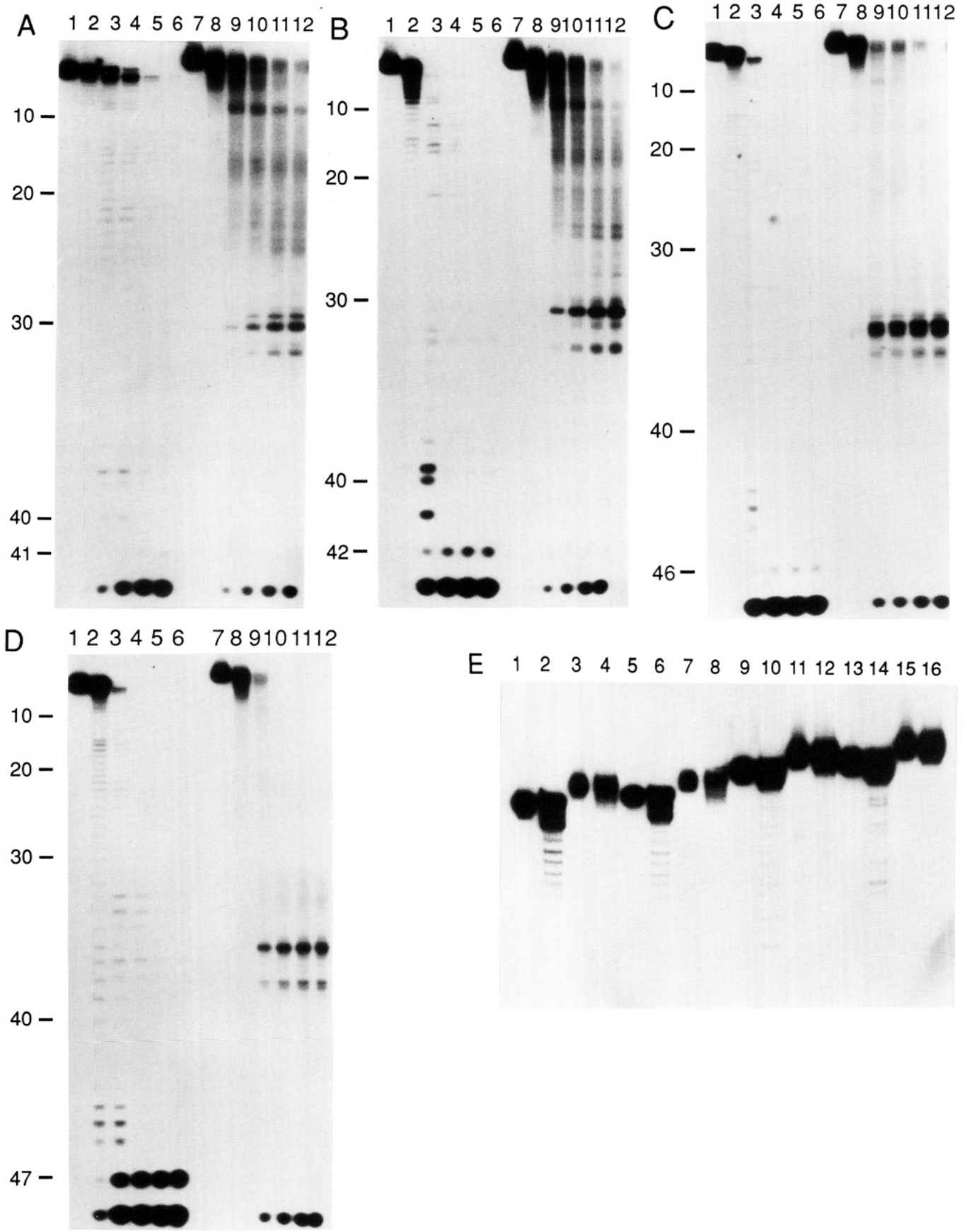

FIGURE 3: Digestion of DNA substrates a–d by directional phosphodiesterases. Panels A–D show results from substrates a–d, respectively, after digestion by snake venom phosphodiesterase. In each of these panels, lanes 1–6 contain nonplatinated DNA and lanes 7–12 contain platinated DNA. Nuclease (30 microunits) was added, and digestion was allowed to proceed for the following times as described under Materials and Methods prior to fractionation by electrophoresis: 0 min, lanes 1 and 7 (no enzyme control); 1 min, lanes 2 and 8; 5 min, lanes 3 and 9; 10 min, lanes 4 and 10; 20 min, lanes 5 and 11; 30 min, lanes 6 and 12. The numbers on the left ordinate of panels A–D indicate the number of nucleotides removed from the 3′-OH end of substrates a–d, respectively. Panel E shows the results of digestion of substrates a–d by calf spleen phosphodiesterase. Lanes 1, 5, 9, and 13 contain substrates a, b, c, and d, respectively, which had not been exposed to enzyme. Lanes 3, 7, 11, and 15 are no-enzyme controls for platinated substrates a–d. Nuclease (12 milliunits) was added to nonplatinated DNA (lanes 2, 6, 10, and 14) or platinated DNA (lanes 4, 8, 12, and 16) and incubated for 30 min at 37 °C prior to electrophoresis. The highest mobility bands in panels A–D are free phosphate generated by a phosphatase contamination in the nuclease.

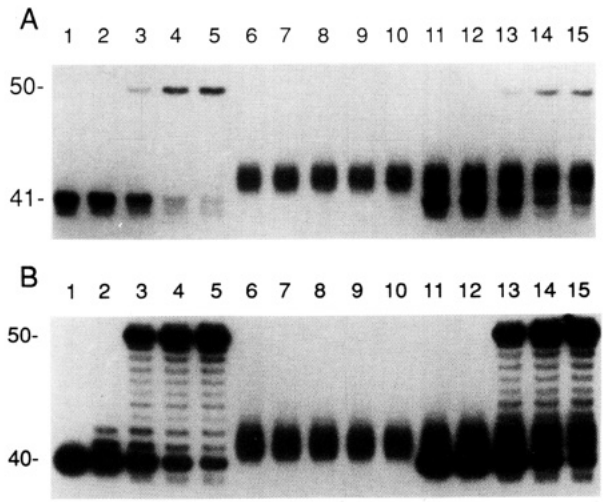

FIGURE 4: Pol ϵ was completely inactive for synthesis on *cis*-DDP-modified single or double dGs at the 3′-OH end of a primer strand, on an unmodified template. (A) Nonplatinated (lanes 1–5), platinated (lanes 6–10), and mixed platinated and unplatinated (lanes 11–15) DNA substrates e were used in the reactions. The extension reaction by pol ϵ was performed in 25 μL. Aliquots (5 μL) were removed at 0 (lanes 1, 6, and 11), 1 (lanes 2, 7, and 12), 5 (lanes 3, 8, and 13), 10 (lanes 4, 9, and 14, and 20 min (lanes 5, 10, and 15) and added to 5 μL of the formamide reaction stop mixture described under Materials and Methods, heated at 90 °C for 5 min, and fractionated by electrophoresis. (B) The experiments were done under the same conditions, and in the same order, as in panel A, except using substrate f (Figure 1). The numbers on the left ordinate indicate the lengths of the DNA substrates before and after the extension assays.

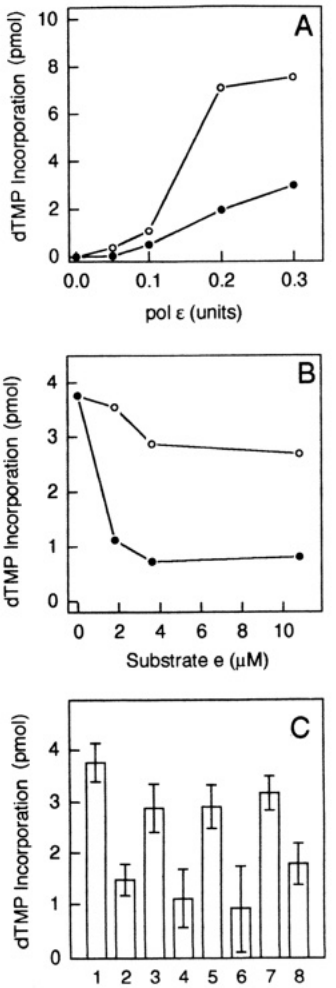

FIGURE 5: DNA polymerase ϵ was sequestered at the 3′-OH ends of fold-back primer-templates. The experiments were performed as described under Materials and Methods. In both panels A and B, open circles represent substrate e and solid circles represent platinated substrate e used as the DNA being tested for ability to sequester pol ϵ. [^{3}H]dTMP (pmol) incorporation vs units of pol ϵ and concentration of sequestering DNA are plotted in panels A and B, respectively. In panel A, 1.8 μM sequestering DNA e was used. In panel B, 0.15 unit of pol ϵ was used. (Panel C) Bars 1 and 2 had no sequestering DNA added. DNA synthesis was assayed on 40 μM poly(dA)·oligo(dT)$_{16}$ alone (bar 1) or 3.6 μM substrate e alone (bar 2). No synthesis had taken place on the platinated substrate e alone. Bars 3–8 represent pol ϵ preincubated with sequestering DNA (3.6 μM) of the following types: 3, substrate e; 4, platinated substrate e; 5, substrate e plus 10 μM free *cis*-DDP; 6, substrate f; 7, substrate f plus 1 mM dGTP; 8, platinated substrate f. Then, for bars 3–8, poly(dA)·oligo(dT)$_{16}$ (40 μM) was added for continued synthesis in the standard sequestering assay.

were designed to determine whether platinated 3′-OH termini on a primer strand bind pol ϵ and sequester it from synthesizing on additional unmodified primer-templates. The ability of pol ϵ to dissociate from platinated DNA and to subsequently utilize poly(dA)·oligo(dT)$_{16}$ as a substrate to incorporate [^{3}H]-dTMP was measured. In Figure 5A, 1.8 μM DNA substrate e was preincubated with increasing concentrations of pol ϵ and then initiated with [^{3}H]dTTP and a 22-fold excess concentration of poly(dA)·oligo(dT)$_{16}$ to measure subsequent DNA synthesis. At all levels of polymerase added, incorporation of [^{3}H]dTMP was higher with nonplatinated vs platinated substrate e (Figure 5A). The decrease in [^{3}H]-dTMP incorporation must have resulted from a sequestering or trapping of pol ϵ at the platinated 3′-OH end of the fold-back oligomer. This phenomenon was further examined by titrating the concentration of platinated substrate e in a similar reaction. Results in Figure 5B demonstrate that higher concentrations of platinated DNA result in a decreased level of synthesis by pol ϵ. This observation provides further evidence that the platinated 3′-OH end sequestered pol ϵ.

To further clarify the mechanism by which pol ϵ was sequestered at platinated 3′-OH ends, several control experiments were carried out. Figure 5C shows that adding free *cis*-DDP into the reaction mixture has no effect on pol ϵ synthetic activity (bar 5). This observation is consistent with the report of Bernges and Holler (1988) using *E. coli* DNA polymerase I and that of Harder et al. (1976) using DNA polymerase α. Platinated substrate f, having a monofunctional adduct, also sequestered pol ϵ at its 3′-OH end (bar 8). Interestingly, nonplatinated substrate f had the maximal inhibitory effect of the substrates tested (Figure 5C, bar 6) in the sequestering assay. In this case, the reaction mixture contained [^{3}H]dTTP as the only deoxynucleoside triphosphate. Extension of substrate f requires dGTP. Once dGTP was supplied, synthesis in the assay with substrate f reached a similar level as that with substrate e (Figure 5C, bar 7). We interpret these results to mean that the lack of the appropriate dNTPs for synthesis on a fold-back substrate causes the polymerase to become trapped or sequestered at the 3′ end of the substrate. The result implies that the platinated terminus sequesters pol ϵ, not because of any special characteristic of the adduct but because the adduct allows the polymerase to bind, but not extend, the 3′-OH terminus. Although platinated substrate e and nonplatinated substrate f both sequester pol ϵ, the two events are not necessarily occurring via the same mechanism.

Comparison of the capacities of pol α, pol δ, and pol ϵ to utilize platinated DNA was also studied. As pointed out above, DNA polymerases α, δ, and ϵ have all been implicated in both DNA replication and repair. However, the three polymerases exhibited different behaviors in the polymerase sequestering assay. Pol α, like pol ϵ, displayed reduced [^{3}H]dTMP incorporation when platinated substrate e was used as the

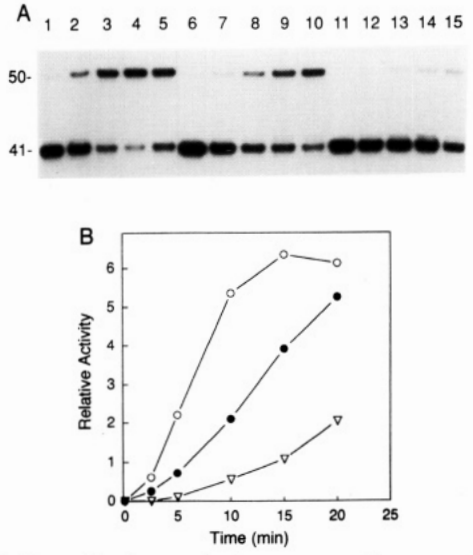

FIGURE 6: Rate of pol ϵ transfer from one template to another. The experiments were performed as described under Materials and Methods. (A) Lanes 1–5, no sequestering DNA was added; lanes 6–10, unlabeled platinated substrate e was in the preincubation; lanes 11–15, unlabeled substrate f was in the preincubation. The extension of labeled substrate e was done for 2.5 (lanes 1, 6, and 11), 5 (lanes 2, 7, and 12), 10 (lanes 3, 8, and 13), 15 (lanes 4, 9, and 14), and 20 min (lanes 5, 10, and 15). (B) The full-length products in (A) were quantitated by densitometry and plotted vs extension time of substrate e by pol ϵ. Results are presented as open circles (lanes 1–5), solid circles (lanes 6–10), and open triangles (lanes 11–15).

sequestering DNA. In contrast, pol δ/PCNA exhibited similar [^{3}H]dTMP incorporation in the presence of nonplatinated or platinated DNA substrate e (data not shown).

Measurement of the Relative Rates of the Pol ϵ Transfer between Templates. Results of the experiment described above reveal a possible complicating factor associated with use of substrate e in the sequestering assay. Since the template portion of substrate e is a dA homopolymer, [^{3}H]dTMP can be incorporated into this DNA. No synthesis can occur on platinated substrate e because of the inhibitory effect of the adduct. Consequently, the difference in [^{3}H]dTMP incorporation observed in the assay when platinated and nonplatinated substrate e are compared may not directly reflect synthesis resulting from pol ϵ transfer to poly(dA)·oligo(dT)$_{16}$.

To more accurately quantitate the relative transfer rates from substrates e and f, pol ϵ was first allowed to prebind to unlabeled substrate e or f, and then an equivalent amount of 5′ end labeled substrate e and unlabeled dTTP was added. The rate of pol ϵ transfer from the unlabeled DNA to labeled DNA was assessed by measurement of full-length extension of the primer on the labeled DNA. The amount of full-length product synthesis over a time course was determined by electrophoresis (Figure 6A). Quantitation of the full-length product bands by densitometry is shown in Figure 6B. Comparison of polymerase-transfer rates shows that transfer was significantly slower when platinated substrate e was used as the sequestering DNA (Figure 6A, lanes 6–10). The transfer rate from substrate f was even slower (lanes 11–15) in this reaction lacking dGTP. These results are consistent with those presented in Figure 5C.

cis-DDP Inhibits Pol ϵ 3′ to 5′ Exonuclease Activity. Inhibition of pol ϵ synthetic activity by platination at the 3′-OH end of primers suggested that the intrinsic 3′ to 5′ exonuclease activity of pol ϵ, which also acts at primer termini, may be affected. This hypothesis was examined by using substrates g–j (see Figure 1), which are designed to be completely single stranded under our assay conditions.

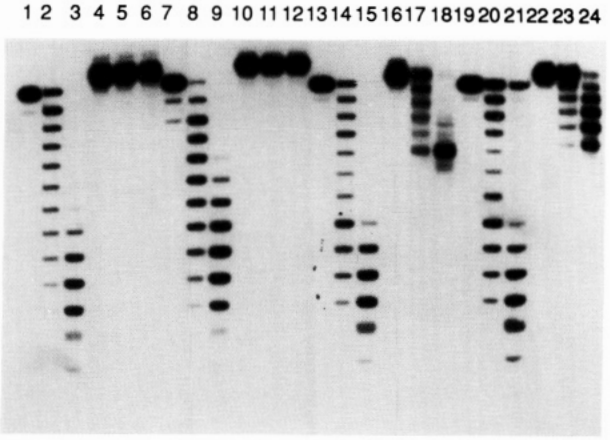

FIGURE 7: Intrinsic 3′ to 5′ exonuclease activity of pol ϵ was inhibited by platinated adducts. The experiments were performed as described under Materials and Methods. Reactions contained substrate g (lanes 1–3) or platinated substrate g (lanes 4–6); substrate h (lanes 7–9) or platinated substrate h (lanes 10–12); substrate i (lanes 13–15) or platinated substrate i (lanes 16–18); substrate j (lanes 19–21) or platinated substrate j (lanes 22–24). Reaction times were 15 s (lanes 1, 4, 7, 10, 13, 16, 19, and 22), 5 min (lanes 2, 5, 8, 11, 14, 17, 20, and 23), and 20 min (lanes 3, 6, 9, 12, 15, 18, 21, and 24).

Substrate g has a single dG at the 3′ end, while substrate h has a double dG at the 3′ end. Figure 7, lanes 1–12, shows the results of exposure of substrates g and h to pol ϵ. Lanes 1–3 and 7–9 show that pol ϵ 3′ to 5′ exonucleolytic degradation of the nonplatinated substrates g and h occurred over the experimental time course. After platination, however, no degradation occurred over the same time course (lanes 4–6 for substrate g and lanes 10–12 for substrate h). This observation is consistent with inhibition of proofreading activity reported by Bernges and Holler (1988) using *E. coli* pol I and that reported by Rampino and Johnston (1991) using bacteriophage T4 polymerase.

Substrates i and j were designed to study additional aspects of pol ϵ 3′ to 5′ exonuclease activity on platinated DNA. Substrate i contains an internal single dG 7 nucleotides upstream of the 3′ end of the oligomer. Substrate j has a double dG six nucleotides upstream of the 3′ end of the oligomer. Figure 7, lanes 13–24, shows that pol ϵ was capable of degrading these substrates from the 3′ end but stopped just before the platinated dG residues. For substrates i or j, this resulted in removal of six (lanes 16–18) or five (lanes 21–24) nucleotides, respectively. This experiment demonstrates that a single platinated dG residue is sufficient to block the 3′ to 5′ exonuclease activity of pol ϵ.

We also examined the ability of platinated substrates g–j to sequester pol ϵ. We did not observe any significant difference in behavior between platinated and nonplatinated substrates g–j in the polymerase sequestering assay, demonstrating that platinated single-strand DNA does not sequester pol ϵ (data not shown).

DISCUSSION

We have used synthetic oligomers having *cis*-DDP adducts at specific sites to examine the effects of platination of DNA on the activities of calf pol ϵ. Pol ϵ has recently been demonstrated to be essential for DNA replication and involved in DNA repair in eukaryotic cells [reviewed in Bambara and Jessee (1991)]. Consequently, its behavior when encountering *cis*-DDP adducts is relevant to the metabolic effects of *cis*-DDP as an antitumor agent.

We have demonstrated that pol ϵ is unable to traverse *cis*-DDP-modified single dG or bifunctionally modified dG dimers

in the template strand. The polymerase is completely inactive for synthesis on substrates in which the 3′-OH nucleotides of the primer strand are *cis*-DDP-modified single- or double-dG residues. Furthermore, pol ϵ is sequestered to 3′-OH modified primer-templates, resulting in inhibition of its ability to move to other primer-templates in the reaction mixture that are able to sustain synthesis. In addition, the 3′ to 5′ exonuclease of pol ϵ is unable to degrade past single- or double-platinated residues in single-stranded DNA. This latter observation suggests that the nuclease is unable to participate in removal of *cis*-DDP–DNA adducts in vivo.

Previous studies of the effects of platinated sites on DNA synthesis in vitro were reported by Pinto and Lippard (1985) and Villani et al. (1988). As substrate, they used partially platinated single-strand bacteriophage M13 with a unique synthetic primer. They demonstrated that bifunctional platinated sites (on dGG or dAG) inhibited the synthetic activity of *E. coli* DNA polymerase I and eukaryotic DNA polymerase α. Our results demonstrate that *cis*-DDP adducts have the capacity to inhibit all three eukaryotic nuclear DNA polymerases. Furthermore, compared to previous studies, we have used specifically modified oligonucleotides as substrates. These are advantageous in that the effects of platination in a precise location can be examined. Their size also allows convenient examination of products of synthesis or degradation by electrophoresis, at single-nucleotide resolution.

DNA polymerase ϵ is incapable of synthesis over a platinated single dG in the template strand of substrates a and c, as well as substrates that had bifunctional lesions (b and d) (Figure 2). This is surprising since a recent paper from Comess et al. (1992) showed that several prokaryotic polymerases can traverse major bifunctional cisplatin adducts formed in vitro. However, they also found that, among the polymerases they tested, bacteriophage T4 DNA polymerase, which has a very active 3′ to 5′ exonuclease, was the most strongly inhibited by bifunctional *cis*-DDP adducts. Possibly, the strong intrinsic 3′ to 5′ exonuclease activity of pol ϵ contributes to the failure of bypass synthesis as observed in our system. We also note that the efficiency with which pol ϵ utilized platinated DNA substrates was low. The decreased efficiency derives not from blockage at the platinated site but because most of the DNA is not utilized for synthesis at all. This conclusion is supported by results showing that platinated substrates a–d do not sequester pol ϵ (L. Huang, J. Turchi, and R. Bambara, unpublished observation). These observations suggest that *cis*-DDP–DNA adducts are inhibiting initial binding of the polymerase to the primer-template. A possible mechanism for this blocking of initial binding is alteration of DNA conformation.

We observed that monofunctional platination of dG at the 3′-OH end of substrate f strongly blocks DNA synthesis (Figure 4B). However, it may have been argued that the major platinum adducts were not monofunctional in the experiments using substrate f. The possibility existed that, under the thorough platination conditions utilized, *cis*-DDP could form intrastrand cross-links between adenosine and guanosine separated by a third nucleotide, i.e., d(-ANG-), in substrate f, according to the scenario of Eastman (1983). However, the studies of *cis*-DDP–DNA adducts by Reedijk and colleagues, both in vitro (Fichtinger-Schepman et al., 1985) and in vivo (Dijt et al., 1988), showed that they did not find such products. The five types of platinated adducts that they found were *cis*-DDP bound to either two adjacent guanosines, adjacent adenosine and guanosine, two guanosines separated by one or more nucleotides, two guanosines in opposite strands, or monofunctional guanosine. Also, our results from 3′ to 5′ exonuclease assays (Figure 7) demonstrate that monofunctional platinated adducts at 3′-dG residues are capable of inhibiting pol ϵ activity. The substrates used in those experiments were verifiably monofunctional since they had no adenosine residues and had appropriate electrophoretic mobility as monomeric oligonucleotides.

Our results demonstrate that pol ϵ can be sequestered at the platinated 3′-OH end of a fold-back oligomer. Pol ϵ synthesizes DNA by a highly processive mechanism (Bambara & Jessee, 1991). One might think that both primer- and template-based blockage of synthesis would result in the polymerase remaining inactively bound to the substrate DNA. However, platination of the template strand may induce dissociation of the polymerase, allowing it to move to other primer-templates that can sustain synthesis. A platinated 3′-OH residue also sequesters DNA polymerase α but not δ. DNA polymerase α, with its tightly associated primase and low processivity, is believed to be involved in DNA replication initiation and subsequent lagging strand synthesis (Tsurimoto et al., 1990). DNA polymerase δ, with high processivity in the presence of PCNA, an auxiliary protein, is thought to elongate the primed leading strand (Tsurimoto et al., 1990). An interesting possibility is that platinum adducts interfere differentially with leading and lagging strand synthesis.

The 3′ to 5′ exonuclease activity of pol ϵ was capable of degrading up to, but not through, platinated dG or dGG residues. This activity could, in vivo, generate a DNA primer-template with a platinated 3′-OH end, similar to the substrates that sequester pol ϵ. The result would be a decrease in DNA synthesis during chromosomal replication or repair. That could account, in part, for the chemotheraputic effect of *cis*-DDP.

Miller and Chinault (1982) suggested that different DNA polymerases are involved in repairing different types of DNA damage. The inability of the polymerases and associated nucleases used in this study to utilize platinated substrates suggests that other DNA-modifying enzymes may be responsible for removing platinum adducts. A multienzyme complex involving recognition, incision, and displacement or degradation of platinated sections of polymer must be required to effectively repair platinated DNA. In *E. coli*, the multienzyme UvrABC complex can effectively remove intrastrand platinum adducts followed by gap filling synthesis by pol I (Beck et al., 1985; Visse et al., 1992). Experiments are in progress to determine whether other mammalian enzymes involved in DNA replication and repair can metabolize platinated DNA.

REFERENCES

Alazard, R., Germanier, M., & Johnson, N. P. (1982) *Mutat. Res. 93*, 327.
Andrews, P. A., & Howell, S. B. (1990) *Cancer Cells 2*, 35.
Bambara, R. A., & Jessee, C. B. (1991) *Biochim. Biophys. Acta 1088*, 11.
Beck, D. J., Popoff, S., Sancar, A., & Rupp, W. D. (1985) *Nucleic Acids Res. 13*, 7395.
Bernges, F., & Holler, E. (1988) *Biochemistry 27*, 6398.
Boulet, A., Simon, M., Faye, G., Bauer, G. A., & Burgers, P. M. (1989) *EMBO J. 8*, 1849.
Ciccarelli, R. B., Solomon, M. J., Varshavsky, A., & Lippard, S. J. (1985) *Biochemistry 24*, 533.
Comess, K. M., Burstyn, J. N., Essigmann, J. M., & Lippard, S. J. (1992) *Biochemistry 31*, 3975.

de Graeff, A., Slebos, R. J. C., & Rodenhuis, S. (1988) *Cancer Chemother. Pharamcol. 22*, 325.
Dijt, F. J., Fichtinger-Schepman, A. M. J., Berends, F., & Reedijk, J. (1988) *Cancer Res. 48*, 6058.
Dresler, S. L., Gowans, B. J., Robinson-Hill, R. M., & Hunting, D. J. (1988) *Biochemistry 27*, 6379.
Eastman, A. (1983) *Biochemistry 22*, 3927.
Eastman, A., & Schulte, N. (1988) *Biochemistry 27*, 4730.
Fichtinger-Schepman, A. M. J., van der Veer, J. L., den Hartog, J. H. J., Lohman, P. H. M., & Reedijk, J. (1985) *Biochemistry 24*, 707.
Focher, F., Gassmann, M., Hafkemeyer, P., Ferrari, E., Spadari, S., & Hubscher, U. (1989) *Nucleic Acids Res. 17*, 1805.
Fraval, H. N. A., Rawlings, C. J., Roberts, J. J. (1978) *Mutat. Res. 51*, 21.
Harder, H. C., & Rosenberg, B. (1970) *Int. J. Cancer 6*, 207.
Harder, H. C., Smith, R. G., & Leroy, A. F. (1976) *Cancer Res. 36*, 3821.
Heiger-Bernays, W. J., Essigmann, J. M., & Lippard, S. J. (1990) *Biochemistry 29*, 8461.
Hoffmann, J.-S., Johnson, N. P., Villani, G. (1989) *J. Biol. Chem. 264*, 15130.
Johnson, L. M., Snyder, M., Chang, L. M., Davis, R. W., & Campbell, J. L. (1985) *Cell 43*, 369.
Katz, E. J., Andrews, P. A., & Howell, S. B. (1990) *Cancer Commun. 2*, 159.
Kesti, T., & Syvaoja, J. E. (1991) *J. Biol. Chem. 266*, 6336.
Loehrer, P. J., & Einhorn, L. H. (1984) *Ann. Intern. Med. 100*, 704.
Marrot, L., & Leng, M. (1989) *Biochemistry 28*, 1454.
Masuda, H., Ozols, R. F., Lai, G.-M., Fojo, A., Rothenberg, M., & Hamilton, T. C. (1988) *Cancer Res. 48*, 5713.
Masuda, H., Tanaka, T., Matsuda, H., & Kusaba, I. (1990) *Cancer Res. 50*, 1863.
Meyn, R. E., Jenkins, S. F., & Thompson, L. H. (1982) *Cancer Res. 42*, 3106.
Miller, M. R., & Chinault, D. N. (1982) *J. Biol. Chem. 257*, 10204.
Morrison, A., Arai, H., Clark, A. B., Hamatake, R. K., & Sugino, A. (1990) *Cell 62*, 1143.
Nishida, C., Reinhard, P., & Linn, S. (1988) *J. Biol. Chem. 263*, 501.
Parker, R. J., Eastman, A., Bostick-Bruton, F., & Reed, E. (1991) *J. Clin. Invest. 87*, 772.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891.
Pinto, A. L., & Lippard, S. J. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 4616.
Rampino, N. J., & Johnston, P. G. (1991) *Biochem. Biophys. Res. Commun. 179*, 1344.
Rosenberg, B. (1985) *Cancer 55*, 2303.
Sabatino, R. D., Myers, T. W., & Bambara, R. A. (1990) *Cancer Res. 50*, 5340.
Salles, B., Butour, J. L., Lesca, C., & Macquet, J. P. (1983) *Biochem. Biophys. Res. Commun. 112*, 555.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) in *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, NY.
Scanlon, K. J., Kashani-Sabet, M., & Sowers, L. C. (1989a) *Cancer Commun. 1*, 269.
Scanlon, K. J., Kashani-Sabet, M., Miyachi, H., Scowers, L. C., & Rossi, J. (1989b) *Anticancer Res. 9*, 1301.
Schaller, W., Reisner, H., & Holler, E. (1987) *Biochemistry 26*, 943.
Sherman, S. E., & Lippard, S. J. (1987) *Chem. Rev. 87*, 1153.
Sibghat-Ullah, Husain, I., Carlton, W., & Sancar, A. (1989) *Nucleic Acids Res. 17*, 471.
Siegal, G., Turchi, J. J., Jessee, C. B., Mallaber, L. M., Bambara, R. A., & Myers, T. W. (1992) *J. Biol. Chem. 267*, 3991.
Tsurimoto, T., Melendy, T., & Stillman, B. (1990) *Nature 346*, 534.
Turchi, J. J., Siegal, G., & Bambara, R. A. (1992) *Biochemistry 31*, 9008.
Villani, G., Hubscher, U., & Butour, J.-L. (1988) *Nucleic Acids Res. 16*, 4407.
Visse, R., de Ruijter, M., Moolenaar, G. F., & van de Putte, P. (1992) *J. Biol. Chem. 267*, 6736.