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DNA polymerase β bypasses *in vitro* a single d(GpG)–cisplatin adduct placed on codon 13 of the *HRAS* gene

[calf thymus DNA polymerases α, β, δ, and ε/*cis*-diamminedichloroplatinum(II)/lesion bypass]

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ABSTRACT **We have examined the capacity of calf thymus DNA polymerases α, β, δ, and ε to perform *in vitro* translesion synthesis on a substrate containing a single d(GpG)–cisplatin adduct placed on codon 13 of the human *HRAS* gene. We found that DNA synthesis catalyzed by DNA polymerases α, δ, and ε was blocked at the base preceding the lesion. Addition of proliferating cell nuclear antigen to DNA polymerase δ and replication protein A to DNA polymerase α did not restore their capacity to elongate past the adduct. On the other hand, DNA polymerase β efficiently bypassed the cisplatin adduct. Furthermore, we observed that DNA polymerase β was the only polymerase capable of primer extension of a 3′-OH located opposite the base preceding the lesion. Likewise, DNA polymerase β was able to elongate the arrested replication products of the other three DNA polymerases, thus showing its capacity to successfully compete with polymerases α, δ, and ε in the stalled replication complex. Our data suggest (*i*) a possible mechanism enabling DNA polymerase β to bypass a d(GpG)-cisplatin adduct *in vitro* and (*ii*) a role for this enzyme in processing DNA damage *in vivo*.**

Among the possible mechanisms of mutagenesis is error-prone replication by cellular DNA polymerases past a DNA lesion followed by fixation of the mutation during subsequent rounds of replication. In eukaryotic cells, it is still unclear whether the replicative DNA polymerases α, δ, and ε can carry out translesion synthesis either alone or with the help of accessory proteins. Alternatively, a separate DNA polymerase may be required for this process. For example, genetic evidence in the yeast *Saccharomyces cerevisiae* indicates that a putative DNA polymerase, the product of the *REV3* gene, may be involved in error-prone translesion synthesis but not in normal DNA replication (1). DNA polymerase β is one of the five mammalian polymerases identified to date and is believed to function primarily in the repair of damaged DNA (2). However, DNA polymerase β may also have a role in replicative synthesis, since the enzyme can substitute for DNA polymerase I during DNA replication in *Escherichia coli* (3), and it is required for the conversion of single-stranded M13 DNA to double-stranded DNA in *Xenopus* oocytes and in oocyte nuclear extracts (4).

cis-Diamminedichloroplatinum(II) (cisplatin) is an anticancer agent widely used in the treatment of ovarian, testicular, head, and neck carcinomas (5). It is believed that this compound exerts its cytotoxic properties by forming stable lesions on DNA, primarily intrastrand cross-links at the N-7 positions of adjacent guanine bases [d(GpG)–cisplatin or Pt–d(GpG)] (6). Replicative bypass of cisplatin adducts has been described in bacteria (7, 8) and in eukaryotic cells (9). Recent work in our laboratory has demonstrated that a single-stranded DNA vector bearing a unique intrastrand bifunctional adduct Pt–d(GpG) at codon 13 of the human protooncogene *HRAS* is replicated in simian COS-7 cells and that such translesion synthesis may be mutagenic (10).

To our knowledge, the capacities of the major mammalian replication enzymes to bypass the Pt–d(GpG) lesion have not been compared. To address this question, we have investigated the ability of purified calf thymus DNA polymerases α, β, δ, and ε to catalyze *in vitro* the bypass synthesis of a single Pt–d(GpG) adduct placed on codon 13 of the human *HRAS* protooncogene, the same sequence used for our previous *in vivo* studies (10). Results show that only DNA polymerase β is capable of *in vitro* translesional synthesis and indicate that its ability to initiate DNA replication opposite the cisplatin adduct may be a key step in this process.

MATERIALS AND METHODS

Materials. Calf thymus DNA polymerases α-primase (11), β (12), δ, and ε (13); proliferating cell nuclear antigen (PCNA; ref. 14); and replication protein A (RPA; ref. 15) were purified as described. One unit of DNA polymerase corresponds to 1 nmol of dNTPs incorporated at 37°C in 60 min. All oligonucleotides were synthesized on Cyclone Plus DNA synthesizer from MilliGen/Biosearch and purified on 20% polyacrylamide gel. [γ-^{32}P]ATP was from Du Pont/NEN, and T4 polynucleotide kinase and DNA ligase were from United States Biochemical. Deoxyribonucleotides were purchased from Pharmacia. *Aci* I restriction enzyme was from New England Biolabs.

Construction and Purification of the 60-mer and the 60-mer–Pt Oligonucleotide Substrates. Four oligonucleotides (8-mer, 14-mer, 38-mer, and the complementary 52-mer) were annealed by mixing an excess of the 14-mer, 52-mer, and 5′-phosphorylated 38-mer with either the 5′-phosphorylated 8-mer or the 5′-phosphorylated 8-mer–Pt adduct oligonucleotides, which had been purified and characterized as described (16). After ligation the resulting 60-mer or 60-mer–Pt substrates were purified on a 20% polyacrylamide/7 M urea/30% formamide denaturing gel. These templates contain the sequence of the protooncogene *HRAS* from codon 7 to codon 14 (nucleotide 2 to nucleotide 25). For DNA synthesis assays, these templates were hybridized to a 5′-^{32}P-labeled 17-mer synthetic primer situated at nucleotides 44–60 (Fig. 1*A*).

***In vitro* DNA Replication Assays.** In assays for calf thymus DNA polymerases α, δ, and ε, 15-μl reaction mixtures contained 20 mM Hepes (pH 7.8); 3 mM $MgCl_2$; 1 mM dithiothreitol; and 500 μM each of dATP, dCTP, dGTP, and dTTP. For calf thymus DNA polymerase β, the reactions (15 μl) contained 25 mM Hepes (pH 8.5); 125 mM NaCl; 5 mM

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Abbreviations: PCNA, proliferating cell nuclear antigen; RPA, replication protein A.

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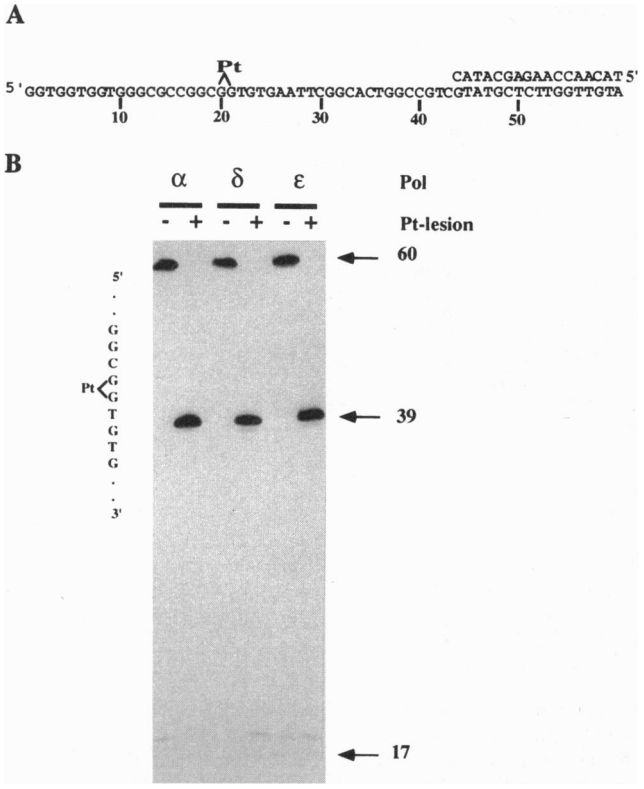

FIG. 1. *In vitro* replication of the 60-mer and the 60-mer–Pt substrates by calf thymus DNA polymerases α, δ, and ε. (*A*) The 60-mer–Pt substrate annealed to a 17-nucleotide-long primer. "Pt" indicates the intrastrand bifunctional adduct. (*B*) One-quarter picomole of 5′-^{32}P-labeled and primed 60-mer or 60-mer–Pt templates were replicated at 37°C for 2 hr by 5 units of DNA polymerase α, 0.1 unit of DNA polymerase δ, or 0.1 unit of DNA polymerase ε. The positions of the 17-mer (primer), 39-mer (product of synthesis to the dT base preceding the Pt lesion), and 60-mer (full-size product) are indicated by arrows. To determine the exact location of the arrest sites, the intact 60-mer substrate was sequenced by the dideoxy method (17) and migrated on the gel next to the reaction products. The printed sequence on the left side corresponds to the template containing the Pt adduct (Pt). Pol indicates DNA polymerases.

$MgCl_2$; and 500 μM each of dATP, dCTP, dGTP, and dTTP. At the end of the reaction, 5 μl of stopping buffer (90% formamide/0.1% xylene cyanol/0.1% bromophenol blue/0.1 mM EDTA) was added. Samples were boiled for 5 min and loaded to a 15% polyacrylamide/7 M urea/30% formamide gel. The amounts of templates and enzymes as well as the incubation times are indicated in the figure legends.

RESULTS

Calf Thymus DNA Polymerases α, δ, and ε Cannot Bypass the d(GpG)–Cisplatin Adduct Either Separately or in Combination with Accessory Proteins. To study translesion synthesis by DNA polymerases α, δ, and ε, we investigated the extension of a 5′-phosphorylated 17-mer oligonucleotide primer on untreated or cisplatin-damaged 60-mer templates. After these substrates were replicated by the enzymes in the presence of high concentrations of nucleotide triphosphates to maximize the opportunity of bypass events (18), the newly synthesized DNA products were separated by polyacrylamide gel electrophoresis and visualized by autoradiography (Fig. 1*B*). When untreated substrate was used, all of the available primers were extended to full-size (60-mer) products, as expected for efficient replication by the three DNA polymerases. In the case of the cisplatin-modified 60-mer substrate, all of the reaction products migrated as a 39-mer DNA, indicating complete arrest of replication opposite to the thymidine base (dT) immediately preceding the unique Pt–d(GpG) adduct. Additional experiments performed either for longer incubation times or with 10 units of DNA polymerase α, 6 units of DNA polymerase δ, and 1.3 units of DNA polymerase ε did not show detectable translesion synthesis (data not shown; also see Fig. 4).

Many accessory proteins participate in eukaryotic DNA replication. One of the components of this machinery is RPA, also known as human single-stranded DNA-binding protein or replication factor A (19). This factor has been shown to reduce pausing by DNA polymerase α at specific sites in single-stranded templates (20, 21). We hypothesized that the presence of RPA might stimulate DNA polymerase α to replicate across the Pt–d(GpG) bifunctional adduct. Bypass of the cisplatin adduct was not observed when DNA synthesis was carried out with 5 units of DNA polymerase α in the presence of 0.25 pmol of platinated templates and 50, 100, or 200 ng of RPA protein—concentrations known to alleviate pausing of DNA polymerase α on M13 single-stranded DNA (21) (data not shown). Another accessory factor that may modulate bypass replication at a lesion is PCNA, which enhances the processivity of DNA polymerase δ (22) and has been shown to promote DNA synthesis past *cis-syn*- and *trans-syn*-cyclobutane-thymine dimers by calf thymus DNA polymerase δ *in vitro* (23). Polymerase δ did not bypass the Pt–d(GpG) adduct in the presence of comparable amounts of PCNA to those reported (23) (data not shown).

Taken together, these results show that purified calf thymus DNA polymerases α, δ, and ε stop one base prior to the Pt–d(GpG) adduct during *in vitro* replication of the 60-mer substrate.

Calf Thymus DNA Polymerase β Can Bypass the d(GpG)–Cisplatin Adduct. DNA polymerase β may be necessary in the final steps of eukaryotic genomic DNA replication for gap-filling. We therefore decided to study the progression of DNA synthesis by this DNA polymerase on the single-stranded 60-mer and 60-mer–Pt substrates. At least 5 units of DNA polymerase β were required to extend all primers to full size on the unplatinated substrate (data not shown) so that 5 units or more were used in our experiments. Fig. 2*A* shows the replication of 0.12 pmol of treated oligonucleotide by increasing amounts of enzyme. In contrast to what was observed for DNA polymerases α, δ, and ε, DNA synthesis by DNA polymerase β proceeded past the Pt–d(GpG) damage. At the lowest concentration of enzyme used, two main classes of products were observed, one arrested by the lesion and the other corresponding to full-length chains (bypass products). The 3′ termini of the interrupted products were localized opposite the platinated guanines, in contrast to the position of the arrest sites detected for DNA polymerases α, δ, and ε, which stopped prior to the adduct (compare lanes α and β of Fig. 2). By increasing the amount of DNA polymerase β, efficient translesion synthesis was achieved with a concomitant disappearance of the stop sites (Fig. 2*A*). Similar results were obtained by performing a kinetic study with 0.12 pmol of template and 15 units of enzyme (Fig. 2*B*). Here, one can see that the arrest sites observed at early incubation times were greatly reduced by increasing the length of the reaction. Replication of the 60-mer substrates creates an *Aci* I restriction site, 5′-GCGG-3′, which includes the GG sequence bearing the adduct. Following replication of intact and platinated substrates by DNA polymerase β, the reaction products were subjected to digestion by *Aci* I. Gel analysis of the digestion products revealed that the full-length products of replication of the platinated 60-mer (but not of unplatinated control template) were resistant to the action of the restriction enzyme (data not shown). Given the known capacity of the Pt–d(GpG) adduct to inhibit cleavage by restriction enzymes (24), this result strongly suggests that the cisplatin lesion is still present

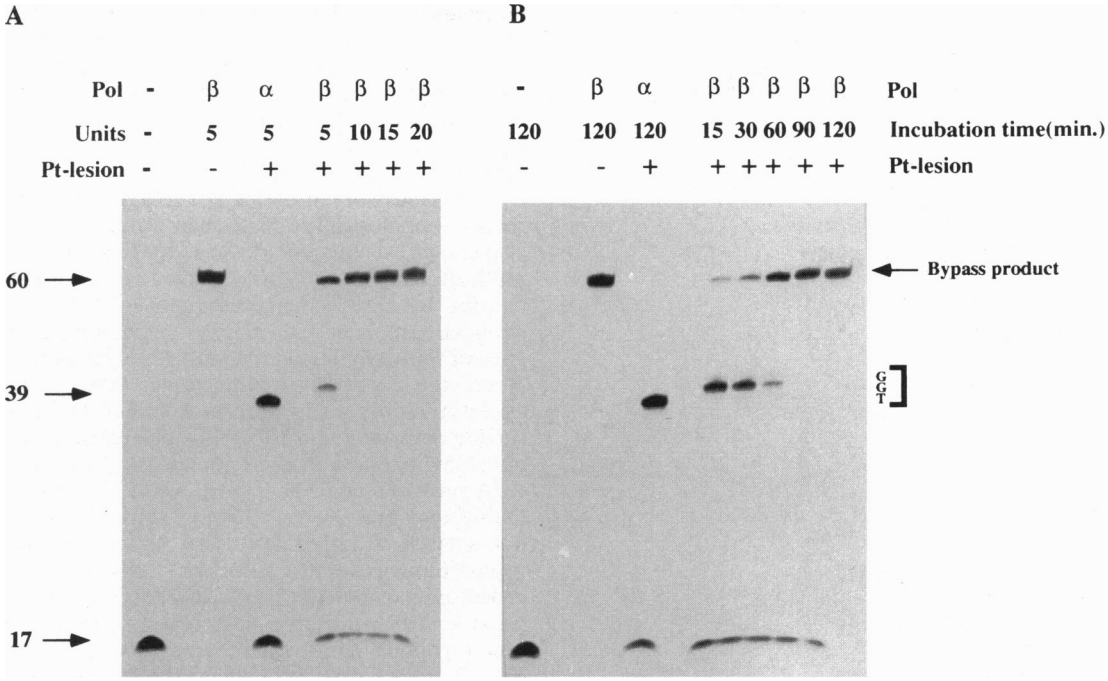

FIG. 2. Replication across the Pt–d(GpG) lesion by calf thymus DNA polymerase β. (*A*) Effect of increasing amounts of DNA polymerase β on primer extension of the 60-mer–Pt substrate. Cisplatin-damaged substrate (0.12 pmol) was replicated for 2 hr at 37°C by different amounts of DNA polymerase β as indicated above each lane. In lane α, 0.12 pmol of cisplatin-damaged substrate was replicated for 2 hr at 37°C by 5 units of DNA polymerase α. Pol indicates DNA polymerases. (*B*) Kinetics of primer extension on the 60-mer–Pt substrate by DNA polymerase β. DNA synthesis was carried out with 15 units of DNA polymerase β with 0.12 pmol of cisplatin-damaged substrate at 37°C for the times indicated above the lanes. Arrows in *A* and *B* indicate positions of the primer and reaction products as described in Fig. 1. The bracket on the right side of *B* indicates the position in the template of the two guanines involved in the cisplatin adduct and of the dT preceding them. In lane α, 0.12 pmol of cisplatin-damaged substrate was replicated for the indicated time by 5 units of DNA polymerase α. Pol indicates DNA polymerases.

in the product synthesized by DNA polymerase β on the platinated substrate.

These data show that purified calf thymus DNA polymerase β can efficiently bypass the Pt–d(GpG) adduct during *in vitro* replication of the 60-mer substrate.

Calf Thymus DNA Polymerase β Can Initiate Replication Opposite the d(GpG)–Cisplatin Adduct. One key step in the Pt–d(GpG) bypass mechanism might be the ability of DNA polymerase β to initiate DNA synthesis from the base preceding the lesion, where elongation by DNA polymerases α, δ, and ε is interrupted. To evaluate this hypothesis, we constructed a 60-mer–Pt substrate hybridized to a 39-nucleotide-long complementary primer, whose 3′ dA terminus is positioned opposite the dT base prior to the lesion (see Fig. 3*A*). This substrate, as well as the untreated control template, was then extended by DNA polymerases α, δ, ε, and β, and the products were resolved by gel electrophoresis (Fig. 3*B*). Efficient primer extension was catalyzed by all enzymes on undamaged substrate, generating a full-length 60-mer product. However, DNA synthesis initiation from the position opposite the cisplatin adduct occurred only when DNA polymerase β was used (Fig. 3*B*, lane β). In experiments performed under conditions similar to those described for Fig. 3 but without dNTPs, we observed degradation by the 3′ → 5′ exonuclease activity of the DNA polymerases δ and ε of the 39-mer primer when hybridized to the platinated substrate (data not shown). These results indicate that in the replication experiments, the failure of DNA polymerases δ and ε to extend the primer hybridized to the platinated substrate is not due to their inability to bind it.

Taken together, these results suggest a possible mechanism enabling *in vitro* bypass of the Pt–d(GpG) adduct by calf thymus DNA polymerase β (see *Discussion*).

Calf Thymus DNA Polymerase β Can Extend Interrupted DNA Chains Synthesized by DNA Polymerases α, δ, and ε. The data presented so far lead to the question of whether DNA polymerase β could replicate through the Pt–d(GpG) adduct by competing with the DNA polymerases for the primer–template junction. Therefore, we decided to test whether DNA polymerase β was able to extend the interrupted products of synthesis by DNA polymerases α, δ, and ε. Primer extension experiments on cisplatin-damaged 60-mer substrate were first carried out with DNA polymerases α, δ, and ε for 2 hr at 37°C as described in Fig. 1. Only DNA polymerase β performed partial DNA translesion synthesis at this pH, which was optimal for DNA polymerases α, δ, and ε (Fig. 4, lane β). Addition of DNA polymerase β extended the interrupted

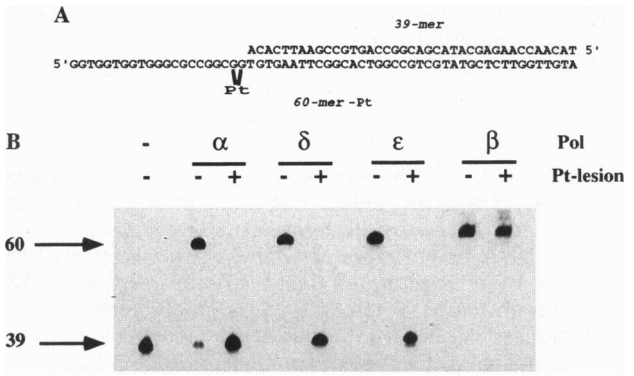

FIG. 3. Primer extension from the 3′-OH of dA at the platinated site by calf thymus DNA polymerases α, β, δ, and ε. (*A*) 60-mer–Pt substrate annealed to a 39-nucleotide-long primer. $\overset{\vee}{\text{Pt}}$ indicates the intrastrand bifunctional adduct. (*B*) Substrate (0.12 pmol) was replicated for 2 hr at 37°C by 5 units of DNA polymerase α, 0.5 unit of DNA polymerase δ, 1.3 units of DNA polymerase ε, or 15 units of DNA polymerase β. Arrows indicate the positions of the 39-mer primer and of full-length reaction products. Pol, DNA polymerases.

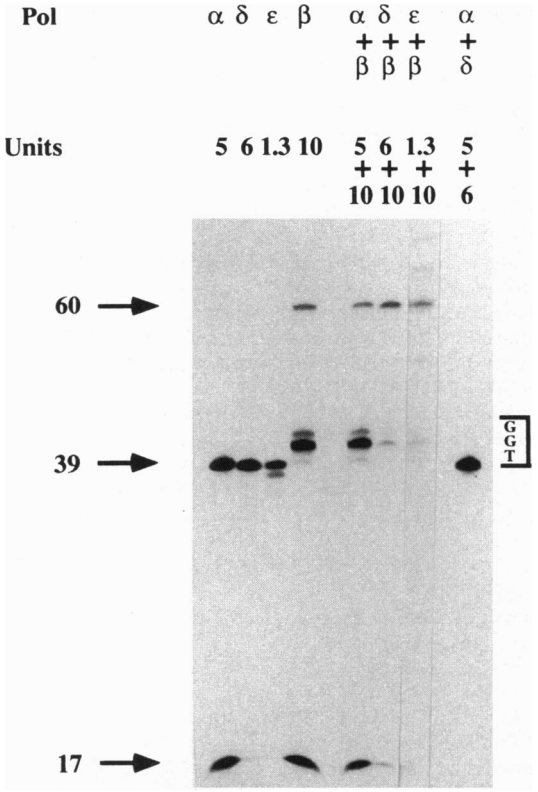

FIG. 4. Interrupted chains synthesized by DNA polymerases α, δ, and ε can be extended by DNA polymerase β. 5′-^{32}P-labeled-primed 60-mer-Pt substrates (0.25 pmol) were extended by different amounts of DNA polymerases α, δ, ε, and β as indicated at the top of the lanes. Reactions were incubated for 2 hr at 37°C under assay conditions described in *Materials and Methods*, except that DNA polymerase β buffer was Hepes (pH 7.8) (lanes α, δ, ε, and β). After 2 hr of incubation, 10 units of DNA polymerase β together with 100 mM NaCl (final concentration) were added, and the reaction continued for an additional 2 hr (lanes α+β; δ+β; ε+β). In lane α+δ, 6 units of DNA polymerase δ were added to a reaction already incubated 2 hr with DNA polymerase α; the incubation was then prolonged for an additional 2 hr. Arrows and bracket are as described in Fig. 2.

chains synthesized by DNA polymerases α, δ, and ε to an extent comparable to DNA polymerase β alone (Fig. 4, compare lane β to lane α+β, δ+β, and ε+β). In control reactions, addition of fresh DNA polymerases α, δ, or ε did not result in translesion synthesis (see, for example, lane α+δ of Fig. 4).

These results indicate that *in vitro* DNA polymerase β can replace DNA polymerases α, δ, and ε in the stalled replication complex and extend 3′ termini of interrupted products through the d(GpG)–cisplatin adduct.

DISCUSSION

Replication of damaged DNA represents a major mechanism for generating mutations. Bypass synthesis of unrepaired lesions at mutagenic hot-spot codons of transforming genes may be an important factor in both the initiation and development of human cancer. Slow repair has been seen in the p53 gene at positions frequently mutated in skin cancer (25), and the replication machinery may pass through the damaged codons at mutation hot spots with a high frequency of misincorporation. We have recently reported that a vector containing a single Pt–d(GpG) adduct placed on codon 13 of the human *HRAS* protooncogene was efficiently replicated in monkey cells and that translesion synthesis could contribute to *HRAS*-activating mutations induced by the drug (10). In the present study, we investigated whether purified eukaryotic DNA polymerases could perform *in vitro* translesion synthesis on the same platinated sequence used *in vivo*. For this purpose, we have constructed a 60-mer oligonucleotide containing part of the human *HRAS* sequence and a well-defined Pt–d(GpG) intrastrand bifunctional adduct at the d(GGT) sequence of codon 13 (see Fig. 1*A*).

When we performed primer extension experiments on this damaged template with purified calf thymus DNA polymerases α, δ, and ε, we failed to observe any bypass (see Fig. 1*B*). For all of these enzymes, a strong stop was seen at the position one base prior to the adduct site, and we detected no incorporation opposite the damage. The fact that we observed the same arrest position by the three enzymes suggests that the size of the products obtained with DNA polymerases δ and ε is not a consequence of degradation by their proofreading activities, since such activity is not present in the form of the DNA polymerase α used here. Inhibition of DNA polymerase ε elongation by a unique Pt–d(GpG) lesion placed on short synthetic oligomers has been described by Huang *et al.* (26). Our data show that DNA polymerase ε inhibition by the biadduct can also be obtained on codon 13 of the human *HRAS* protooncogene, a hot spot for mutagenesis *in vivo*.

We further investigated whether accessory factors involved at the eukaryotic DNA replication fork could promote bypass synthesis. RPA, known to reduce DNA polymerase α pausing (21), did not appear to alleviate DNA polymerase α arrest at the site of the lesion. PCNA, which functions to increase the processivity of DNA polymerase δ and has been shown to promote its bypass synthesis of UV lesions (23), did not help DNA polymerase δ in replicating the Pt–d(GpG) adduct.

In contrast to polymerases α, δ, and ε, DNA polymerase β is capable of catalyzing the incorporation of nucleotides across the damaged guanines and of elongating efficiently past the position of the Pt–d(GpG) adduct to reach full-length 60-mer products (Fig. 2). This finding may implicate DNA polymerase β in the previously observed survival of single-stranded simian virus 40-based shuttle vectors bearing the same damaged *HRAS* sequence (10).

To our knowledge, there has been no previous report that a purified eukaryotic DNA polymerase can efficiently synthesize past a Pt–d(GpG) adduct, one normally considered to be a strong block to DNA replication. DNA polymerase β has been reported to copy past apurinic sites (27) and to synthesize *in vitro* through a *cis-syn* thymine dimer placed on codon 61 of *HRAS* gene (28); however, in contrast to what we observed with the Pt–d(GpG) adduct, the translesion replication of a *cis-syn* dimer does not appear to be specific to DNA polymerase β, since it is also bypassed by DNA polymerase α (28) and DNA polymerase δ in the presence of PCNA (23). It is possible that translesion synthesis observed here may be influenced by the type of lesion and the sequence context. Hence, it remains to be determined whether the bypass observed at codon 13 of the *HRAS* protooncogene, hot spot for mutagenesis, could be obtained with the same adduct placed on a different codon or for another blocking lesion.

What properties of these enzymes might be responsible for their different capacities to bypass the d(GpG)–cisplatin lesion? One feature that distinguishes DNA polymerase β is its small size compared to DNA polymerases α, δ, and ε (29). Recently the crystal structure of polymerase β has been solved (30, 31). It showed structural similarity with two other small polymerases with which it has no sequence homology, the Klenow fragment of *E. coli* polymerase I [PolI(Kf)] and human immunodeficiency virus (HIV) reverse transcriptase (RT) (32). We have observed that PolI(Kf) and RT can also replicate past the Pt–d(GpG) lesion on the templates used in this study (data to be published elsewhere). Others have observed the capacity of PolI(Kf) to bypass *in vitro* the Pt–d(GpG) adduct in a different sequence context (33, 34). Apparently these enzymes not only have structural homology,

but their capacity to bypass certain DNA damage appears to be a common functional property that may distinguish proteins with the Pol β/PolI(Kf)/RT structure from larger and more complex DNA polymerases.

All of the DNA polymerases investigated probably dissociate from the platinated template. In agreement with this hypothesis, Huang *et al.* demonstrated that the Pt–d(GpG) adduct, when placed on the template strand, does not sequester DNA polymerase ε (26). Given its distributive mode of DNA synthesis (29), DNA polymerase β likely dissociates and reassociates frequently with the substrate. The particular ability of polymerase β to catalyze *in vitro* extension from the position prior to the platinated guanines appears to reflect its capacity to reinitiate DNA replication at the site opposite the adduct, while DNA polymerases α, δ, and ε appear to be unable to reinitiate DNA synthesis at this position (see Fig. 3). Furthermore, experiments shown in Fig. 4 indicate that DNA polymerase β was able to compete with DNA polymerases α, δ, and ε at the stalled replication complex. We propose that the simple subunit composition of polymerase β (30, 31) may favor reassociation of the enzyme to a primer–template junction with damaged bases. Taken together, our results suggest a possible role of DNA polymerase β in the bypass of d(GpG)–cisplatin DNA lesions *in vivo*. In this respect, it is interesting to note that induction of DNA polymerase β mRNA by DNA-damaging agents has been described in CHO cells (35).

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