

Mammalian Translesion DNA Synthesis across an Acrolein-derived Deoxyguanosine Adduct

PARTICIPATION OF DNA POLYMERASE η IN ERROR-PRONE SYNTHESIS IN HUMAN CELLS*

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α -OH-PdG, an acrolein-derived deoxyguanosine adduct, inhibits DNA synthesis and miscodes significantly in human cells. To probe the cellular mechanism underlying the error-free and error-prone translesion DNA syntheses, *in vitro* primer extension experiments using purified DNA polymerases and site-specific α -OH-PdG were conducted. The results suggest the involvement of pol η in the cellular error-prone translesion synthesis. Experiments with xeroderma pigmentosum variant cells, which lack pol η , confirmed this hypothesis. The *in vitro* results also suggested the involvement of pol ι and/or REV1 in inserting correct dCMP opposite α -OH-PdG during error-free synthesis. However, none of translesion-specialized DNA polymerases catalyzed significant extension from a dC terminus when paired opposite α -OH-PdG. Thus, our results indicate the following. (i) Multiple DNA polymerases are involved in the bypass of α -OH-PdG in human cells. (ii) The accurate and inaccurate syntheses are catalyzed by different polymerases. (iii) A modification of the current eukaryotic bypass model is necessary to account for the accurate bypass synthesis in human cells.

extensive sequence homology and comprise a new polymerase family designated the Y family (4). These polymerases are different from replicative polymerases in several aspects, *i.e.* they replicate more efficiently across altered bases and catalyze both accurate and inaccurate translesion DNA syntheses, they have more flexible and larger catalytic pockets (5–7) that give them the ability to tolerate damaged template bases, and they show reduced fidelity when copying unmodified DNA (8–14). Their ability to catalyze translesion synthesis has been studied extensively *in vitro* using various DNA lesions as substrates, but knowledge of their roles in translesion synthesis in mammalian cells is still very fragmentary. Among these polymerases, pol η , which is defective in cells of xeroderma pigmentosum variant (XPV) patients, was shown to catalyze accurate and efficient translesion synthesis across certain UV photoproducts (15), whereas human pol ζ (16) and REV1 (17) are involved in inaccurate syntheses across UV photoproducts. One recent study using pol κ -defective mouse cells has shown that the enzyme is involved in the error-free translesion synthesis across a benzo[*a*]pyrene-dG adduct(s) (18). Two eukaryotic translesion synthesis pathways have been proposed (19–23). In one pathway, both insertion and extension steps are catalyzed by one DNA polymerase. In the other pathway, extension is catalyzed by a DNA polymerase, such as pol ζ or pol κ , which is different from the one inserting a nucleotide opposite a DNA lesion.

In this research, we conducted translesion synthesis studies *in vitro* and *in vivo* to probe the cellular bypass mechanism for an acrolein-derived dG adduct. Acrolein, the simplest member of the α,β -unsaturated aldehyde family, is widely found in the environment and is also produced endogenously. It initiates urinary bladder carcinogenesis in rats (24) and is mutagenic in bacteria (25, 26) and cultured cells (27–29). Acrolein reacts with dG residues in DNA to form two pairs of stereoisomeric exocyclic propano adducts (Fig. 1), namely the 8*R* and 8*S* isomers of 3*H*-8-hydroxy-3-(β -D-2'-deoxyribofuranosyl)-5,6,7,8-tetrahydropyrido[3,2-*a*]purine-9-one (γ -OH-PdG) and the 6*R* and 6*S* isomers of 3*H*-6-hydroxy-3-(β -D-2'-deoxyribofuranosyl)-5,6,7,8-tetrahydropyrido[3,2-*a*]purine-9-one (α -OH-PdG). γ -OH-PdG predominates over α -OH-PdG (30–32) and has been detected in DNA isolated from human and animal tissue (30, 33). Lipid peroxidation is suspected to be the major endogenous source (30). Comparative genotoxic studies with a site-specific adduct in human cells have shown that γ -OH-PdG is less blocking than is α -OH-PdG (34) and is bypassed with high

During the last several years, many new DNA polymerases (pol)¹ have been discovered in prokaryotes and eukaryotes (1–3). Several of these polymerases, such as eukaryotic pol η , pol κ , pol ι , pol ζ , and REV1 and *Escherichia coli* pol IV and pol V, are thought to be involved in translesion DNA synthesis. With the exception of pol ζ , which belongs to the B family, they share

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¹ The abbreviations used are: pol, DNA polymerase; α -OH-PdG, the 6*R* and 6*S* isomers of 3*H*-6-hydroxy-3-(β -D-2'-deoxyribofuranosyl)-5,6,7,8-tetrahydropyrido[3,2-*a*]purine-9-one; BSA, bovine serum albumin; DTT, dithiothreitol; exo, 3'→5' exonuclease; γ -OH-PdG, the 8*R* and 8*S* isomers of 3*H*-8-hydroxy-3-(β -D-2'-deoxyribofuranosyl)-5,6,7,8-tetrahydropyrido[3,2-*a*]purine-9-one; PCNA, proliferating cell nuclear antigen; XPV, xeroderma pigmentosum variant; *mXPV*, mouse XPV cDNA.

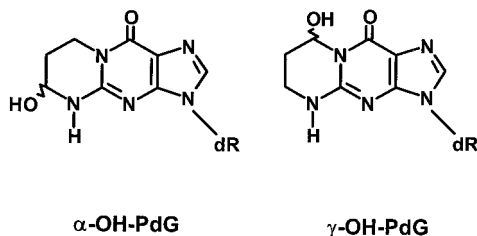


FIG. 1. Structures of acrolein-derived α - and γ -OH-PdG.

fidelity (34, 35). α -OH-PdG, on the other hand, miscodes substantially in human cells with a frequency of 10–12% per bypass synthesis, with G \rightarrow T being predominant (34). As α -OH-PdG strongly inhibits DNA synthesis (34), it is likely that the translesion polymerases are involved in bypassing this adduct. This leads to the following questions: (i) which translesion polymerase is responsible for the correct and incorrect syntheses; and (ii) whether these syntheses are catalyzed by one polymerase or by different polymerases. Here, we show the following. (i) Multiple DNA polymerases are involved in the bypass synthesis. (ii) Pol η participates in incorrect synthesis. (iii) The current eukaryotic bypass model (19–23) does not seem to account for the error-free bypass of this adduct.

EXPERIMENTAL PROCEDURES

Oligonucleotides—The procedures for the synthesis, purification, and characterization of oligonucleotides containing α -OH-PdG have been described (37). The 13-mer (5'-CTCCTCXATACCT-3') and 28-mer (5'-CTGCTCCTCXATACCTACACGCTAGAAC-3'), in which X represents α -OH-PdG, were the same oligonucleotides as those used in our previous study (34). The 13-mer and 28-mer were used in the translesion synthesis studies *in vivo* (human cells) and *in vitro*, respectively. The 16-mer (5'-GTTCTAGCGTGTAGGT-3'), 18-mer (5'-GTTCTAGCGTGTAGGTAT) and 19-mer (5'-GTTCTAGCGTGTAGGTATN-3', in which N stands for A, G, C, or T) were employed as primers in the experiments of read-through nucleotide incorporation opposite α -OH-PdG and primer extension from a terminus opposite α -OH-PdG, respectively. The 28-mer template contained the entire sequence of the 13-mer. All unmodified as well as modified oligonucleotides were purified by electrophoresis in denaturing 20% polyacrylamide gel and formed a single band following purification.

DNA Polymerases and Proliferating Cell Nuclear Antigen (PCNA)—Human Pol η (38), pol κ (39), pol ι (13), REV1 (40), calf thymus pol δ (41), and *Saccharomyces cerevisiae* pol ζ (19) were purified as described. The 3' \rightarrow 5' exonuclease (exo)-proficient Klenow enzyme was obtained from New England Biolabs (Beverly, MA); human PCNA was a gift from Paul A. Fisher (State University of New York, Stony Brook, NY).

Primer Extension Reaction—The 28-mer template and a 5'-³²P-end-labeled primer were mixed at a molar ratio of 1:2, heated at 70 °C for 5 min, and annealed by slow cooling. Reaction mixtures (10 μ l) contained 40 mM bis-Tris (pH 6.8), 6 mM MgCl₂, 10 mM dithiothreitol (DTT), 40 μ g/ml bovine serum albumin (BSA), and 14 ng/ μ l PCNA for pol δ ; 40 mM Tris-HCl (pH 8.0), 30 mM KCl, 5 mM MgCl₂, 10 mM DTT, and 250 μ g/ml BSA for pol η and pol κ (42); 25 mM KH₂PO₄ (pH 7.0), 5 mM MgCl₂, 5 mM DTT, 100 μ g/ml BSA and 10% glycerol for REV1 (43) and pol ζ (19); 40 mM Tris-HCl (pH 8.0), 5 mM MgCl₂, 10 mM β -mercaptoethanol (replacing DDT used in the original buffer), 250 μ g/ml BSA and 2.5% glycerol for pol ι (44); and 10 mM Tris-HCl (pH 7.5), 5 mM MgCl₂, and 7.5 mM DTT for the Klenow enzyme. The final concentration of dNTP was 10 μ M for incorporation experiments and 100 μ M each in extension and read-through experiments. A primed template was added at a concentration of 40 nM. The amounts of polymerases added are indicated in the legends to Figs. 3–7. Reactions with pol δ were incubated at 30 °C for 30 min, and those with the other enzymes were at 37 °C for 10 min. Following reaction, 7 μ l of a formamide dye mixture (95% formamide, 0.1% xylene cyanol, 0.1% bromophenol blue, and 20 mM EDTA) was added, and aliquots (4 μ l) were subjected to electrophoresis in denaturing (8 M urea) 20% polyacrylamide gel at 2300 V for 2.5 h. Radioactive bands were detected and, if necessary, quantified by a PhosphorImager and ImageQuant software (Amersham Biosciences).

Cell Lines—The SV40-transformed human XPV cell lines CTag (45) and XP30RO(sv) (46) were obtained from M. Cordeiro-Stone (University of North Carolina, Chapel Hill, NC) and J. Cleaver (University of

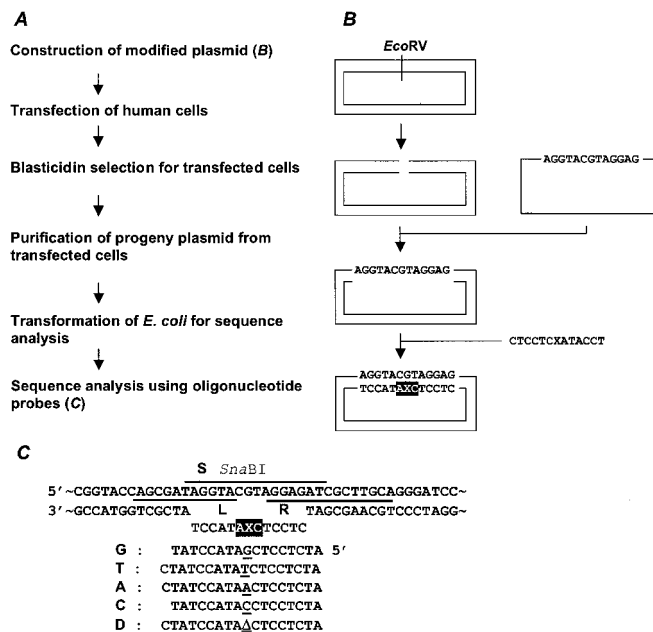


FIG. 2. Outline of experimental procedure (A), construction of modified plasmid (B), and oligonucleotide probes used for sequence analysis of progeny plasmid (C). In panels B and C, note mismatches at the sequence of 3'-AXC (highlighted). X represents α -OH-PdG. Probe S (overscored) hybridizes only to unmodified strand. Probes L and R detect plasmid containing a 13-mer insert. Probes G, T, A, C, and D determine targeted events. Δ , single base deletion.

California, San Francisco, CA), respectively. CTag and XP30RO(sv) were established from XP4BE and XP30RO (GM3617), respectively. XP4BE and XP30RO cells contain a four-nucleotide (positions 289–292) and a 13-nucleotide (positions 343–355) deletion, respectively, in the coding region of one allele of the *XPV* gene and produce severely truncated proteins due to the new stop codons generated (47, 48). The other allele is not transcribed in either cell line. Cells were cultured in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, penicillin (100 μ g/ml), and streptomycin (100 μ g/ml) at 37 °C in 5% CO₂. An expression vector containing a mouse *XPV* cDNA (*mXPV*) was constructed as follows. A *NotI* fragment containing *mXPV* was isolated from pGEM-*mXPV* (49) and cloned in the correct orientation into the *NotI* site of pIRESneo2 (Clontech), which has the G418 resistance gene. The construct, pIRES-*mXPV*, was introduced into CTag cells by the FuGENE6 method (Roche Molecular Biochemicals) according to a manufacturer's protocol. Transfected cells were selected for G418 (Mediatech, Herndon, VA) resistance at 500 μ g/ml medium. pIRESneo2 is designed to translate a cloned gene and the G418 resistance gene from the same transcript. As this transcript contains an internal ribosome entry site between the cloned gene and the G418 resistance gene, the *mXPV* gene and the G418 resistance gene are independently translated. Furthermore, translation of the G418 resistance gene is designed to be less efficient than that of the cloned gene. Therefore, all G418-resistant cells are expected to express *mXPV*. To further assure the collection of *mXPV*-expressing cells, G418-resistant cells were irradiated with UV at 2J/m² and then cultured in the presence of 1 mM caffeine (49). Almost all cells transfected with the empty pIRESneo2 vector died after 4 days, whereas cells transfected with pIRES-*mXPV* survived. Following two cycles of this phenotypic selection, surviving cells were used as the host for site-specific experiments. Finally, the transcription of the *mXPV* gene was confirmed by RT-PCR (reverse transcriptase-polymerase chain reaction) using RNeasy Mini Kit (Qiagen) and SuperScript One-Step RT-PCR Kit (Invitrogen).

Translesion Synthesis Studies in Human Cells—The shuttle vector, pBTE, was described previously (35). This vector is stably maintained in human cells and confers blasticidin S resistance to host human and *E. coli* cells. Expression of the resistance gene is driven by the SV40 early promoter in human cells and the EM7 bacterial promoter in *E. coli*. The construction of double-stranded DNA plasmid containing site-specific α -OH-PdG has been described (34) and is shown in Fig. 2 together with the experimental strategy. α -OH-PdG was incorporated into the leading strand template. An important feature of this construct is that the adduct was inserted opposite a unique *Sna*BI site (5'-

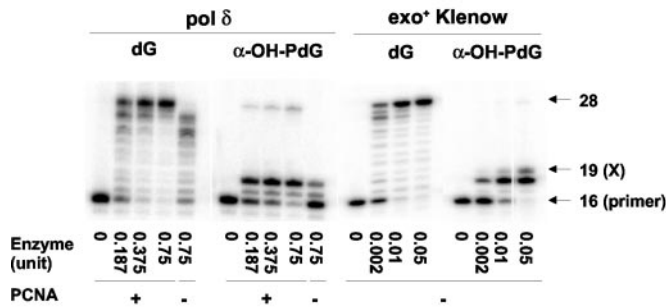


FIG. 3. **Translesion synthesis catalyzed by pol δ /PCNA or exo⁺ Klenow enzyme.** ³²P-5'-end-labeled 16-mer primer/28-mer template complex (40 nM) was incubated with various amounts of calf thymus pol δ or exo⁺ Klenow enzyme in the presence of 100 μ M each of four dNTPs at 30 °C for 30 min for pol δ and 37 °C for 10 min for Klenow. Where indicated, PCNA (140 ng) was added to 10- μ l reaction mixture. Reaction products were analyzed in denaturing 20% polyacrylamide gel. X indicates the position of α -OH-PdG. The activity of pol δ decreased during prolonged storage but was not re-determined, and the original unit value was used.

TACGTA-3') with mismatches on both sides of the adduct (Fig. 2); thus, only the unmodified complementary strand contains the *Sna*BI site. Progeny plasmids derived from the unmodified strand and excision repair events are sensitive to *Sna*BI digestion, whereas those derived from translesion synthesis are not. Hence, progeny derived from translesion synthesis can be selectively collected for fidelity analysis by digesting with *Sna*BI prior to *E. coli* transformation.

CTag/pIRES and CTag/pIRES-*mXPV* cells were seeded at 1×10^6 cells/25-cm² flask, cultured overnight, then transfected overnight with 1 μ g of a DNA construct by the FuGENE6 method. Where indicated, cells were treated with mitomycin C at 1 μ g/ml medium for 50 min in an incubator, after which the medium was replaced with a fresh medium, and transfection was begun immediately. The next day, cells were detached by treating with trypsin-EDTA and replated in a 75-cm² flask. The following day, blasticidin S (Invitrogen) was added to the culture medium at 5 μ g/ml. Resistant cells were collected after 5 or 6 days. The progeny plasmid was purified by the method of Hirt (50) and treated with *Dpn*I (2 units) for 1 h to remove residual input DNA.

To establish the apparent efficiency of translesion DNA synthesis, *Dpn*I-treated plasmid was used to transform *E. coli*. To determine coding events at the site of α -OH-PdG, the *Dpn*I-treated plasmid was digested with *Sna*BI prior to transformation. One-tenth to one-fifth of the recovered plasmid was electroporated into *E. coli* DH10B ElectroMAX (25 μ l) (Invitrogen) by an *E. coli* Pulsar (Bio-Rad), after which 975 μ l of YT (2 \times) medium (36) was added, and the bacteria were cultured for 40 min at 37 °C. Portions of the transformation mixture were plated onto YT (1 \times) plates containing blasticidin S (50 μ g/ml) and ampicillin (100 μ g/ml). After overnight incubation, *E. coli* transformants were subjected to differential oligonucleotide hybridization (51, 52) to analyze for mutations in the adducted region. This method permits the detection of specific sequences using oligonucleotide probes. G, T, A, C, and D probes (Fig. 2C) determine coding specificity at the site of α -OH-PdG. The S probe hybridizes to the complementary *Sna*BI-containing strand. L and R probes confirm the presence of the 13-mer insert. Automated DNA sequence analysis was performed as necessary.

RESULTS

To understand the mechanism of the translesion synthesis across α -OH-PdG in human cells, we first conducted *in vitro* experiments to select candidate polymerases whose translesion synthesis activity and fidelity are consistent with the *in vivo* results, and we then examined the role of one (pol η) of the candidates in human cells.

Pol δ -catalyzed Translesion Synthesis—A running start experiment (Fig. 3) using a 16-mer primer and a 28-mer template showed that pol δ bypassed α -OH-PdG very weakly only in the presence of PCNA. Extended products were not observed opposite the adduct, and the majority of the extension was terminated at one base before the adduct site. These results suggest that nucleotide insertion opposite α -OH-PdG and the subsequent extension are poor. When the read-through experiment

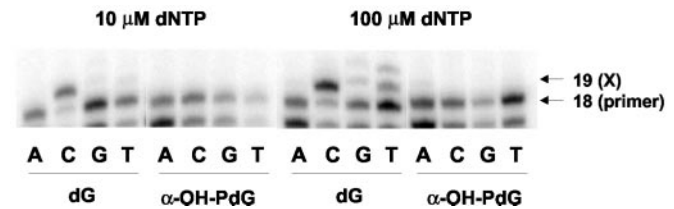


FIG. 4. **Incorporation of a nucleotide opposite α -OH-PdG by pol δ /PCNA.** ³²P-5'-end-labeled 18-mer primer/28-mer template complex (40 nM), 0.75 units of pol δ , 140 ng of PCNA, and 10 μ M (left) or 100 μ M (right) dNTP were used. The other conditions were the same as those in Fig. 3.

was catalyzed by exo⁺ Klenow enzyme, full-length products were rarely observed, and some extended products were observed opposite the adduct. These results suggest that the full-length products observed in the pol δ -catalyzed reaction were generated by true bypass synthesis across the adduct. No stable insertion of a nucleotide opposite α -OH-PdG by pol δ was confirmed by nucleotide incorporation experiments using 10 and 100 μ M dNTP (Fig. 4). The results of these experiments indicate that pol δ /PCNA catalyzes bypass of α -OH-PdG very weakly. At this time the fidelity of this bypass synthesis is not known.

In subsequent experiments designed to determine the nucleotide distance between the adduct and the primer terminus at which pol δ /PCNA recovered efficient synthesis, we found that exonucleolytic proofreading prevailed over polymerization when the primer terminus was located three nucleotides or less 5' to the adduct (Fig. 5). When the terminus was five nucleotides away, net polymerization efficiency increased. At seven nucleotides, proofreading became marginal, and polymerization was predominant. Therefore, if a translesion polymerase catalyzes DNA synthesis ≥ 7 nucleotides past α -OH-PdG, the subsequent synthesis can be performed efficiently by pol δ .

Translesion DNA Polymerase-catalyzed Bypass Synthesis—As α -OH-PdG inhibits DNA synthesis strongly, it is conceivable that translesion polymerases participate in bypassing this adduct. To determine which polymerase(s) plays a role in the accurate and inaccurate bypass syntheses, we first examined the abilities of pol η and pol κ to catalyze a bypass synthesis. The running start experiments revealed that both polymerases could bypass this adduct (Fig. 6). Qualitative nucleotide incorporation experiments (Fig. 7A) showed that pol η inserted predominantly dAMP and, weakly, dGMP and dTMP opposite α -OH-PdG, whereas it preferentially inserted dCMP and, moderately, dAMP and dTMP opposite dG. The extension experiments (Fig. 7B) with the 19-mer primer revealed that dA and dG but not dC or dT termini were extended from opposite α -OH-PdG. These results suggest that the bypass synthesis catalyzed by pol η predominantly results in a G \rightarrow T transversion, which is the major miscoding event observed in human cells (34). A similar analysis with pol κ (Fig. 7A) showed that this polymerase inserted dGMP weakly, dAMP and dTMP marginally, and no dCMP opposite α -OH-PdG, whereas it predominantly inserted correct dCMP opposite dG. Extension experiments (Fig. 7B) showed that dA, dG, and dT termini, but not a dC terminus, were extended weakly. These results suggest that pol η - and pol κ -catalyzed bypass syntheses are inaccurate and do not account for the accurate synthesis in human cells.

In experiments using pol ι , REV1 and pol ζ (Fig. 7, A and B), REV1 exclusively inserted correct dCMP, and pol ι incorporated dCMP and dTMP opposite α -OH-PdG (Fig. 7A). However, no extension from these termini was observed (Fig. 7B), suggesting that these two polymerases require another DNA polymerase for the subsequent extension to complete accurate

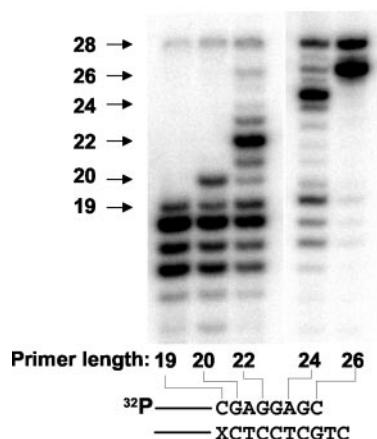


FIG. 5. Resumption of DNA synthesis by pol δ /PCNA. 32 P-5'-end-labeled primers of various lengths (19–26) were annealed to a modified 28-mer template, and the primer extension reaction was performed using pol δ (0.75 units) and PCNA (140 ng) as described in the legend to Fig 3. X represents α -OH-PdG.

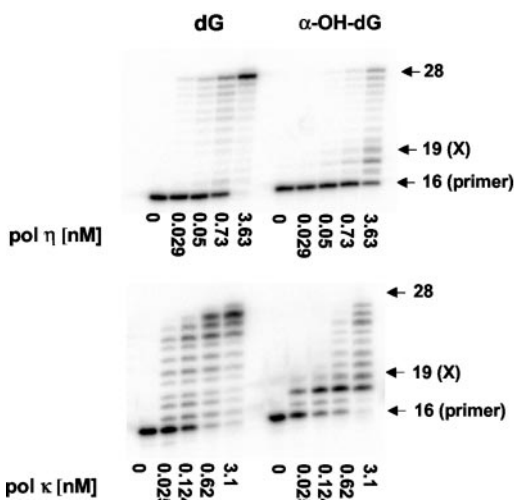


FIG. 6. Translesion syntheses catalyzed by pol η and pol κ . 32 P-5'-end-labeled 16-mer primer/28-mer template complex (40 nM) was incubated with various amounts of a DNA polymerase in the presence of 100 μ M each of four dNTPs at 37 $^{\circ}$ C for 10 min. Reaction products were analyzed in denaturing 20% polyacrylamide gel. X indicates the position of α -OH-PdG.

translesion synthesis. As pol ζ is known to have this capability, *i.e.* extension of a primer from a mismatched terminus and from a terminus opposite DNA lesions (19, 53–56), we examined a pol ζ -catalyzed extension from four termini opposite α -OH-PdG. Although this polymerase catalyzed extension from all four termini opposite dG, with a dC terminus being most efficiently extended, the extension from a dC terminus opposite α -OH-PdG was much less efficient than that from the other three (dA, dG, and dT) termini (Fig. 7B). This result suggests that pol ζ does not efficiently complete the accurate synthesis initiated by pol ι or REV1. Rather, it may contribute to error-prone syntheses by extending from dA, dG, and dT termini generated by other polymerases. No efficient extension was observed as expected when REV1 and pol ζ were simultaneously added to a reaction mixture (data not shown). Pol ζ did not efficiently insert any nucleotide opposite α -OH-PdG (Fig. 7A). These results suggest that the combination of pol ι and pol ζ or REV1 and pol ζ does not account for the accurate translesion synthesis observed in cells.

The Role of Pol η in Mutagenic Bypass in Human Cells—The results of the *in vitro* experiments have suggested that pol η

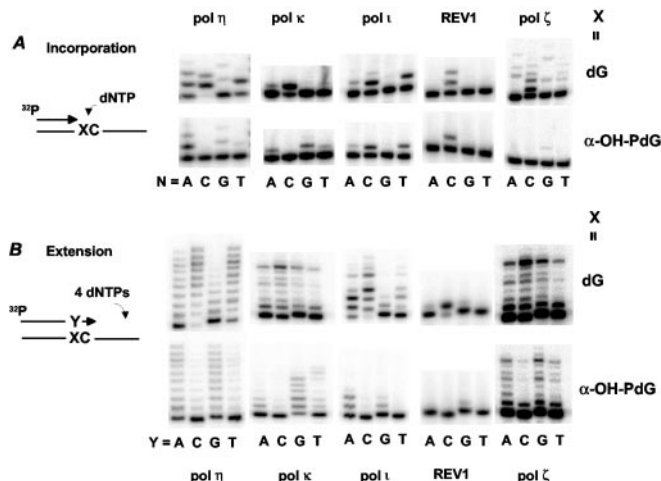


FIG. 7. Translesion DNA polymerase-catalyzed nucleotide incorporation opposite α -OH-PdG (A) and extension from termini opposite α -OH-PdG (B). A, 32 P-5'-end-labeled 18-mer primer/28-mer template complex (40 nM) was incubated with a DNA polymerase in the presence of 10 μ M of one dNTP at 37 $^{\circ}$ C for 10 min. Concentrations of polymerases were 3.63 nM pol η , 3.1 nM pol κ , 2.5 nM pol ι , 14.4 nM REV1, and 5.4 nM pol ζ in a 10- μ l reaction mixture. B, 5' 32 P-labeled 19-mer primer/28-mer template complex (40 nM) was incubated with a DNA polymerase in the presence of four dNTPs (100 μ M each) at 37 $^{\circ}$ C for 10 min. Concentrations of DNA polymerases were the same as those used in panel A.

and pol κ contribute to the cellular miscoding events. Using the XPV cell line CTag, we found a lower miscoding frequency of 1.1% (Table I) than in XPA cells (10–12%) (34). However, as this XPV cell line and the XPA cell line do not have an isogenic background, it may not be appropriate to compare the results directly. To address this issue, we introduced into CTag cells an expression plasmid containing *mXPV*, which was previously shown to complement the defect in human XPV cells (49). The introduction of the *mXPV* plasmid significantly (2.4-fold, $p < 0.001$) increased the miscoding frequency, which was largely ascribed to the increase in the number of G \rightarrow T transversions. A similar enhancing effect (2.3-fold, $p < 0.001$) of *mXPV* was noted when control and *mXPV*-transfected cells were pretreated with mitomycin C. Pretreatment of cells with mitomycin C appears to cause a slight increase in miscoding frequencies in these engineered XPV cells, though the increases were not statistically significant. The fractions of progeny derived from the modified strand were 24 and 23% for CTag/pIRES and CTag/pIRES-*mXPV*, respectively, without mitomycin pretreatment, and 27 and 22% for CTag/pIRES and CTag/pIRES-*mXPV*, respectively, with pretreatment, showing no significant differences between these two cell lines. Subsequently, another XPV cell line, XP30RO(sv), was used to confirm the result with CTag; a very low miscoding frequency was also noted in this cell line (Table I). Taken together, our results indicate the following. (i) Pol η does not play a major role in translesion synthesis across α -OH-PdG. (ii) Pol η is not critical to error-free bypass. (iii) Pol η is primarily responsible for inaccurate translesion synthesis (α -OH-PdG \rightarrow T). The latter two ideas are supported by the results of *in vitro* experiments (Fig. 7, A and B).

DISCUSSION

Acrolein is a bifunctional agent that reacts with the 1 and N^2 positions of dG to form two exocyclic propano adducts. The exocyclic rings are formed in the region involved in Watson-Crick hydrogen bonding to dC. Both adducts inhibit DNA synthesis, and α -OH-PdG miscodes in human XPA cells (34). To investigate the cellular translesion synthesis mechanism for α -OH-PdG, we conducted experiments *in vitro* with purified

TABLE I
Coding events induced by α -OH-PdG in XPV and complemented XPV cells

Host/plasmid ^a	Treatment	Coding events				MF ^b
		G	T	A	C	
CTag	None	371	2	2	0	1.1
XP30RO(sv)	None	232	0	0	0	<0.5
CTag/pIRES	None	351	7	2	1	2.8
CTag/pIRES-mXPV	None	316	17	4	2	6.8 ^d
CTag/pIRES	Mitomycin C ^c	335	11	3	0	4.0
CTag/pIRES-mXPV	Mitomycin C ^c	322	22	8	2	9.0 ^d

^a XPV cells (CTag) were transfected with a mouse XPV gene expression vector (pIRES-mXPV) or an empty vector (pIRES) and selected for G418 resistance. pIRES-mXPV-transfected, G418-resistant cells were further selected for resistance to 2 J/m²UV plus 1 mM caffeine treatment. The selected cells were used as hosts.

^b Miscoding frequency of targeted, single point mutation.

^c Cells were treated with 1.0 μ g of mitomycin C per milliliter of medium for 50 min in an incubator, then the medium was replaced with fresh medium, and transfection was immediately started using 1 μ g of DNA construct.

^d $p < 0.001$.

eukaryotic DNA polymerases and compared the results with the previous *in vivo* data to deduce a likely *in vivo* mechanism.

The Role of Pol η in Inaccurate Synthesis in Human Cells—We showed that pol η bypassed α -OH-PdG (Fig. 6), incorporated dAMP but not dCMP opposite this adduct (Fig. 7A), and extended the primer efficiently from this dA terminus (Fig. 7B). These results suggest that pol η -catalyzed synthesis can be highly inaccurate, resulting in α -OH-PdG \rightarrow T transversions and that pol η does not contribute to error-free translesion synthesis. The miscoding frequencies obtained in the two XPV cell lines (Table I) were significantly lower than those obtained in XPA cells (34), and the lowered frequencies were complemented, though not perfectly, by the introduction of mXPV (Table I). The mXPV did not affect translesion synthesis efficiency. These results indicate that pol η plays a minor role in the overall process of translesion synthesis but is largely responsible for the inaccurate synthesis. The involvement of pol η in inaccurate replication was also reported recently for γ -OH-PdG (57). In *S. cerevisiae*, pol η has been shown to be responsible for the accurate synthesis past 8-oxo dG (58), the inaccurate synthesis past (6–4) thymine-thymine dimers (59), and both accurate and inaccurate syntheses past acetylaminofluorene dG adducts (59).

The Role of Other Translesion Polymerases in Inaccurate Synthesis in Human Cells—We observed miscoding events in XPV (CTag) cells, though at reduced frequencies, suggesting that another polymerase(s) catalyzes inaccurate translesion synthesis in the absence of pol η . Among the polymerases examined, we found that pol κ and pol ι incorporate incorrect nucleotides opposite α -OH-PdG (Fig. 7A); pol κ bypassed this adduct (Fig. 6) and extended from the dA, dG, and dT termini (Fig. 7B); and pol ι inserted dCMP and dTMP opposite α -OH-PdG (Fig. 7A), but no further extension was observed from these termini (Fig. 7B). This extension may be catalyzed by pol ζ (Fig. 7B), as has been observed *in vitro* for abasic sites (53, 55, 56) and (6-4) thymine-thymine dimers (19, 54, 55).

What Mechanism Operates in Error-free Translesion Synthesis?—The experiments confirm and extend our previous work (34), demonstrating that accurate translesion synthesis of α -OH-PdG is the major event in human cells, accounting for ~90% of the products. It appears unlikely that pol η or pol κ contribute to a substantial degree for the following reasons. (i) Neither polymerase inserted correct dCMP opposite α -OH-PdG (Fig. 7A), and neither catalyzed extension from a dC terminus opposite this adduct (Fig. 7B). (ii) XPV cells conducted error-free translesion synthesis (Table I) with a substantial level of translesion synthesis. (iii) The introduction of mXPV did not enhance the level of translesion synthesis. In contrast to pol η and pol κ , pol ι and REV1 incorporated dCMP relatively effi-

ciently opposite α -OH-PdG (Fig. 7A), but extension from this dC terminus was not observed with either polymerase. Extension may be catalyzed by other polymerases such as pol ζ and pol κ , as has been observed for (6-4) thymine-thymine dimers (19, 54, 55) and abasic sites (53, 55, 56). Pol ζ , however, catalyzed limited extension from a dC terminus opposite α -OH-PdG as compared with that from the other three termini (Fig. 7B). We did not observe any fully extended products by the simultaneous addition of REV1, which exclusively inserted dCMP, and pol ζ to a reaction mixture. Thus, the 3'-terminal dC paired to α -OH-PdG was very resistant to extension by pol ζ as well as by pol κ . It is likely, then, that pol ζ is not involved in the accurate synthesis, but rather may play a role in inaccurate synthesis. With all the translesion polymerases examined, extension from purine (dA and dG) termini appears to be more efficient than it is from pyrimidine (dT and dC) termini, and a dC terminus is most resistant to such extension (Fig. 7B). In conclusion, pol ι and REV1 can serve to insert dCMP, but a polymerase that can catalyze a ≥ 7 nucleotide extension is required to propose a two polymerase-catalyzed bypass mechanism (19–23). Thus, the mechanism for this error-free synthesis is currently unknown.

Pol δ is possibly responsible for the accurate synthesis, though its *in vitro* bypass ability does not seem sufficient to account for the *in vivo* bypass synthesis. Another possibility is that other DNA polymerases, such as pol θ (60), pol λ (60), and pol μ (60) catalyze this accurate translesion synthesis in cells. For example, our preliminary experiments have shown that pol β , unlike translesion polymerases, extends a primer efficiently from a dC terminus (data not shown). We should also consider, however, that the current *in vitro* system lacks critical accessory factors that mediate the activity of these polymerases. In conclusion, our results indicate that multiple DNA polymerases are involved in the translesion synthesis across α -OH-PdG and that accurate and inaccurate translesion syntheses are catalyzed by different polymerases.

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