

The *p*-benzoquinone DNA adducts derived from benzene are highly mutagenic

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Abstract

Benzene is a human leukemia carcinogen, resulting from its cellular metabolism. A major benzene metabolite is *p*-benzoquinone (pBQ), which can damage DNA by forming the exocyclic base adducts pBQ-dC, pBQ-dA, and pBQ-dG in vitro. To gain insights into the role of pBQ in benzene genotoxicity, we examined in vitro translesion synthesis and in vivo mutagenesis of these pBQ adducts. Purified REV1 and Pol κ were essentially incapable of translesion synthesis in response to the pBQ adducts. Opposite pBQ-dA and pBQ-dC, purified human Pol ι was capable of error-prone nucleotide insertion, but was unable to perform extension synthesis. Error-prone translesion synthesis was observed with Pol η . However, DNA synthesis largely stopped opposite the lesion. Consistent with in vitro results, replication of site-specifically damaged plasmids was strongly inhibited by pBQ adducts in yeast cells, which depended on both Pol ζ and Pol η . In wild-type cells, the majority of translesion products were deletions at the site of damage, accounting for 91%, 90%, and 76% for pBQ-dA, pBQ-dG, and pBQ-dC, respectively. These results show that the pBQ-dC, pBQ-dA, and pBQ-dG adducts are strong blocking lesions, and are highly mutagenic by predominantly inducing deletion mutations. These results are consistent with the lesion structures predicted by molecular dynamics simulation. Our results led to the following model. Translesion synthesis normally occurs by directly copying the lesion site through base insertion and extension synthesis. When the lesion becomes incompatible in accommodating a base opposite the lesion in DNA, translesion synthesis occurs by a less efficient lesion loop-out mechanism, resulting in avoiding copying the damaged base and leading to deletion.

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1. Introduction

Benzene is widely used in industry and is a common environmental pollutant found in automobile exhaust and cigarette smoke. Its toxicity was recognized long ago from studies of exposed human populations [1]. Benzene is toxic to bone marrow and is a leukemia carcinogen (leukemogen) for humans [1,2]. In rodents, benzene is a carcinogen in multiple tissues [2]. Benzene itself is a stable compound. Its metabolism in cells, however, yields toxic metabolites. A major metabolite of benzene is *p*-benzoquinone (pBQ) [3,4]. In vitro, pBQ is able to form exocyclic adducts on DNA bases C, A, and G [5–9]. Furthermore, induced mutagenesis was observed following transfection of pBQ-damaged shuttle vector plasmid into cultured mouse and

human cells [10–12], suggesting that pBQ may contribute to the observed mutagenicity of benzene [13,14]. However, DNA adducts are not readily detected following benzene exposure [2]. Thus, the precise mechanism of benzene-mediated carcinogenesis remains unknown.

Several hypotheses have been proposed to account for the genotoxicity of benzene metabolites [2]. One hypothesis involves formation of benzene DNA adducts and subsequent mutagenesis induced by these lesions. Thus, understanding the mutagenic properties of pBQ-dC, pBQ-dA, and pBQ-dG could yield important insights into understanding the role of pBQ in benzene-induced carcinogenesis. In cells, error-prone translesion synthesis is the major mechanism of base damage-induced mutagenesis. Translesion synthesis is the cellular process that directly copies damaged sites of the template during DNA synthesis. It consists of nucleotide insertion opposite the lesion and extension synthesis from opposite the lesion. Recent studies indicate that Pol ζ and the Y family polymerases are

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important translesion polymerases in eukaryotes (reviewed in Refs. [15–19]). In the yeast *S. cerevisiae*, the Y family consists of Pol η and Rev1 [20]. Mammals contain two additional members of the Y family polymerases: Polk and Polt [15,20].

Translesion synthesis can be error-free or error-prone, depending on the specific type of DNA damage and the specific translesion polymerase [16]. Error-free translesion synthesis suppresses mutagenesis, and thus preventing carcinogenesis, as in the case of Pol η response to UV-induced TT dimers [21,22]. In contrast, error-prone translesion synthesis results in mutagenesis, and thus promoting carcinogenesis. If a DNA lesion is efficiently bypassed in cells by error-free translesion synthesis, this lesion is unlikely to make a significant contribution to carcinogenesis. Thus, studying translesion synthesis of a specific type of DNA damage would help assess its potential role in genotoxicity. Pol ζ is believed to be important in catalyzing extension synthesis from opposite the lesion [23–25]. It may also be involved in nucleotide insertion opposite some lesions [24]. Pol η , Polt, and Polk are capable of nucleotide insertions opposite many different types of DNA lesions. Extension synthesis from opposite certain types of lesions may also involve Pol η and Polk [26–29]. Rev1 is unique in that it is a template-dependent dCMP transferase in response to several types of DNA lesions [30–32]. In addition to its dCMP transferase activity, it is generally believed that REV1 plays another non-catalytic function in translesion synthesis [33,34]. It appears that there is no general rule as to what translesion polymerase is specific to what type of DNA damage. To understand translesion synthesis and mutagenesis requires experimental determination case by case.

To better understand a potential role of pBQ in benzene-induced carcinogenesis, we have examined translesion synthesis and mutagenesis of site-specific pBQ-dC, pBQ-dA, and pBQ-dG adducts. In this report, we show that (i) the pBQ-dC, pBQ-dA, and pBQ-dG exocyclic adducts are strong blocking lesions and (ii) these pBQ lesions are highly mutagenic by predominantly inducing deletion mutations in yeast cells. In addition, computational modeling was used to provide a possible rationale for the unusual mutagenic property of the pBQ lesions.

2. Materials and methods

2.1. Materials

T4 DNA ligase, the T4 gene 32 protein, T4 polynucleotide kinase, and yeast Pol η were obtained from Enzymax (Lexington, KY). Human Pol η , Polt, Polk, REV1, and yeast Rev1 were purified as previously described [32,35–38]. Oligonucleotides (25mer) containing a site-specific pBQ-dC, pBQ-dA, or pBQ-dG were prepared by automated DNA phosphoramidite methods as previously described [9,39]. The oligonucleotide sequence is 5'-CCGCTAGCGGGTACCGAGCTCGAAT-3', in which the pBQ-dA, pBQ-dG, and pBQ-dC lesion sites are indicated by the underlined A, G, and C, respectively. Undamaged DNA oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA).

2.2. Yeast strains

Yeast strains used are the wild-type BY4741 (*MATa his3 leu2 met15 ura3*) and its isogenic BY4741 Δ rad30 (*rad30* deletion mutant), BY4741 Δ rev3

(*rev3* deletion mutant), and BY4741 Δ rev3 Δ rad30 (*rev3 rad30* double deletion mutant). BY4741 was purchased from ATCC (Manassas, VA). BY4741 Δ rad30 (lacking Pol η) was purchased from Research Genetics (Huntsville, AL). BY4741 Δ rev3 (lacking Pol ζ) and BY4741 Δ rev3 Δ rad30 were constructed as previously described [40].

2.3. Computer modeling of pBQ-dA and pBQ-dG by molecular dynamics simulation

The pBQ-dA and pBQ-dG adducts were placed opposite T and C bases, respectively, in the 25-mer duplexes. The force field parameters for the pBQ-dA and pBQ-dG adducts were obtained using quantum mechanical calculations as previously described [41,42]. Prior to unrestrained molecular dynamics simulation, potential energy minimization was used to eliminate direct steric overlaps between the adduct and the opposite base. Two different starting structures with the adducts displaced toward 5' or 3' ends, respectively, were used as initial structures in the simulations. A total of 200 ps of equilibration and 2 ns of unrestrained molecular dynamics were carried out on each of four systems. Equilibration and production runs were performed using explicit solvent with the TIP3P waters and 12 Å Lennard–Jones interaction distance cutoff according to the previously reported procedure [41,42]. All calculations were performed with SANDER module of AMBER 7.0 (Case et al., 2002, AMBER 7, University of California, San Francisco, CA) [43]. The CARNAL and ANAL modules of AMBER 7 were used to analyze the trajectories for features including root mean square deviations values, interatomic distances, and energies. All calculations were performed using Silicon Graphics Origin 200 server interfaced with the dual processor Octane workstation.

2.4. In vitro translesion synthesis assays

A standard DNA polymerase reaction mixture (10 μ l) contained 25 mM KH₂PO₄ (pH 7.0), 5 mM MgCl₂, 5 mM dithiothreitol, 100 μ g/ml bovine serum albumin, 10% glycerol, 50 μ M of dNTPs (dATP, dCTP, dTTP, and dGTP individually or together as indicated), 50 fmol of an indicated DNA substrate containing a ³²P-labeled primer, and a purified DNA polymerase as indicated. After incubation at 30 °C for 10 min, reactions were terminated with 7 μ l of a stop solution (20 mM EDTA, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol). The reaction products were resolved on a 20% polyacrylamide gel containing 8 M urea and visualized by autoradiography.

2.5. Kinetic analysis of in vitro translesion synthesis

Kinetic analysis of in vitro translesion synthesis was performed as previously described [37,44]. Briefly, the assays were performed using 50 fmol of a primed DNA template, 2 ng of purified human Pol η or 19 ng of purified human Polt, and increasing concentrations of each dNTP (dATP, dCTP, dTTP, or dGTP). The dNTP concentrations used for Polt assays were 3–1000 μ M dATP, dGTP, dCTP, or dTTP. The dNTP concentrations used for Pol η assays were 0.1–300 μ M dATP, 0.3–300 μ M dGTP, 3–1000 μ M dCTP and dTTP for pBQ-dA; 0.3–300 μ M dGTP, 3–1000 μ M dCTP, 1–1000 μ M dATP and dTTP for pBQ-dG; 0.1–300 μ M dATP and dCTP, 1–300 μ M dGTP, 1–1000 μ M dTTP for pBQ-dC. A 5' ³²P-labeled primer was annealed to the damaged 25-mer DNA template with the primer 3' end terminating right before the lesion. After incubation for 10 min at 30 °C under standard translesion synthesis assay conditions, reaction products were separated by electrophoresis on a 20% denaturing polyacrylamide gel and quantitated by scanning densitometry using the SigmaGel software (Sigma, St. Louis, MO) for analysis. The observed enzyme velocity (v) was plotted as a function of dNTP concentration. The plotted data were fitted by a non-linear regression curve to the Michaelis–Menton equation, $v = (V_{\max} \times [\text{dNTP}]) / (K_m + [\text{dNTP}])$, using the SigmaPlot software. V_{\max} and K_m values for the incorporation of the correct and the incorrect nucleotides were obtained from the fitted curves. Relative misinsertion frequency (f_{inc}) was calculated from the equation: $f_{\text{inc}} = (V_{\max}/K_m)_{\text{incorrect}} / (V_{\max}/K_m)_{\text{correct}}$.

2.6. Construction of plasmids containing a site-specific pBQ-dC, pBQ-dA, or pBQ-dG

Plasmids containing a site-specific pBQ adduct were constructed by Enzymax as previously described [29]. Briefly, single-stranded phagemid pELUf1 vector was annealed to a 20-mer DNA oligonucleotide, 5'-GTGCCCTCCATGGAAAAATC-3', at its unique *Nco*I restriction site within the *URA3* gene, and digested with the *Nco*I restriction endonuclease. Then, the linearized pELUf1 was annealed with a 54-mer DNA scaffold, 5'-CTGUGCCCUCCAUGATUCGAGCUCGGTAUCCGCUAGCGGGAAAAUCAGTCAAG-3', and a 5'-phosphorylated 25-mer oligonucleotide containing a site-specific pBQ adduct. While the mid region of the scaffold is complementary to the damaged oligonucleotide, its ends are complementary to the single-stranded pELUf1 ends. Ligation of the damaged oligonucleotide into the pELUf1 vector was performed with T4 DNA ligase at 16 °C for 20 h, and DNA was precipitated in ethanol. Finally, the complementary strand of pELUf1 was synthesized with T4 DNA polymerase in the presence of T4 gene 32 protein and 0.5 mM each of dATP, dCTP, dGTP, and dUTP, using the scaffold as the primer. The resulting construct was a double-stranded plasmid containing a site-specific pBQ adduct, in which the undamaged strand contained U in place of T. Formation of double-stranded plasmid pELUf1-PBQ was confirmed by electrophoresis on a 1% agarose gel.

2.7. In vivo translesion synthesis assays in yeast

In vivo translesion synthesis assays in yeast were performed as previously described [29]. Briefly, site-specifically damaged pELUf1 plasmid (2 µg) was transformed into yeast cells of various strains by the lithium acetate method as described [45]. Following transformation, yeast cells were collected by centrifugation (20 s at 5000 rpm) in a microcentrifuge. Cells were resuspended in 400 µl of sterile water and were plated onto two YNB minimal agar (0.17% yeast nitrogen base, 0.49% ammonium sulfate, 2% glucose, and 2% agar) plates lacking leucine but supplemented with 5 mM 5-fluoroorotic acid (5-FOA), 150 µM methionine, and 380 µM uracil to score for colonies containing replicated pELUf1-PBQ. Cells transformed by the undesired background plasmid pELUf1 without the damaged oligonucleotide insert remained *URA3* wild-type, and thus cannot grow on plates containing 5-FOA. After incubation at 30 °C for 3–4 days, yeast colonies were counted. In each experiment with each strain, transformation efficiency was determined by a parallel transformation using the undamaged and double-stranded pELUf1. Translesion synthesis was calculated as transformants per µg of the damaged plasmid per 10⁶ transformable cells with the undamaged plasmid (i.e., transformants per µg of the damaged plasmid × 10⁶/transformation efficiency expressed as transformants per µg of the undamaged plasmid). Relative translesion synthesis was obtained by comparing translesion synthesis in various mutant strains to that in the wild-type cells.

Replicated plasmid clones were individually recovered from yeast colonies on the 5-FOA plates by a zymolyase method essentially as described [46] and amplified in *E. coli* DH5α cells. Each plasmid clone was analyzed by digestion with the *Kpn*I restriction endonuclease. The damaged 25-mer oligonucleotide contained a *Kpn*I restriction site. Thus, *Kpn*I restriction analysis further eliminated undesired background transformants by the undamaged pELUf1. These background transformants escaped selection by the 5-FOA plates because they contained mutations somewhere in the vector *URA3* gene. Plasmid clones that did not contain this added *Kpn*I restriction site were excluded from further analysis and calculation. Finally, the precise specificity of translesion synthesis opposite the site-specific pBQ adduct was determined by DNA sequencing.

3. Results

3.1. The *p*-benzoquinone DNA adducts

Three *p*-benzoquinone DNA adducts, pBQ-dA, pBQ-dG, and pBQ-dC, were chemically synthesized in the form of phosphoramidites, which were subsequently incorporated into oligonucleotide DNA at a defined position via automated DNA

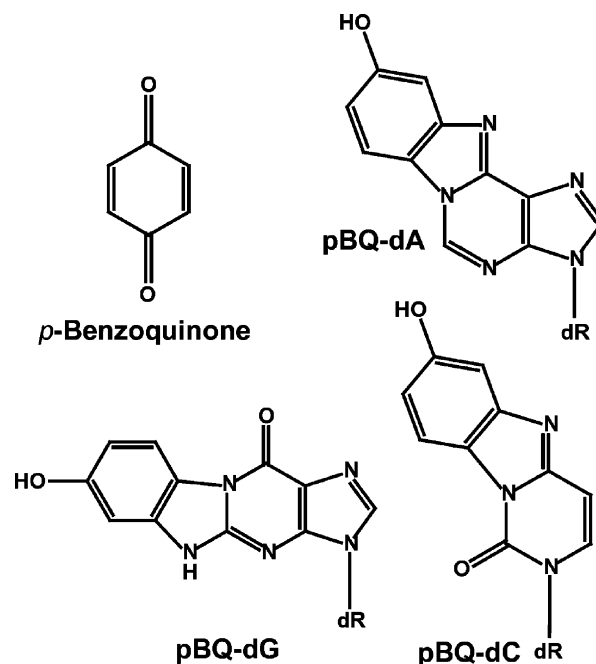


Fig. 1. Chemical structures of pBQ DNA adducts. A major metabolite of benzene, *p*-benzoquinone (pBQ) and its exocyclic adducts on DNA bases A (pBQ-dA: 1,*N*⁶-benzetheno-dA), G (pBQ-dG: 1,*N*²-benzetheno-dG), and C (pBQ-dC: 3,*N*⁴-benzetheno-dC) are shown.

phosphoramidite methods [9,39]. Chemical structures of *p*-benzoquinone and its DNA adducts are shown in Fig. 1.

To gain insights into the potential effects of *p*-benzoquinone DNA adducts on DNA structure, we performed molecular dynamics simulations of a 25-mer DNA duplex containing either a pBQ-dA or pBQ-dG adduct opposite T or C bases, respectively. The pBQ-dA or pBQ-dG occupied over 55% more space than did the corresponding unmodified base (compare Fig. 2A and B, and data not shown). The starting van der Waals energies for the lesion and flanking base-pairs, even after initial minimization, were 818 kcal/mol Å for the pBQ-dA duplex and 834 kcal/mol Å for the pBQ-dG duplex. The van der Waals energy for the rest of the structures was around -230 kcal/mol Å. After the first 30 ps of unrestrained molecular dynamics, we observed displacement of the adduct or the opposite base toward extrahelical position. This displacement resulted in the immediate drop in the van der Waals energy to -120 kcal/mol Å. The final structures produced by the molecular dynamics simulation indicate that the adduct is significantly displaced toward the major groove (pBQ-dA duplex) (Fig. 2C), or the opposite base has to rotate out of the duplex into an extrahelical conformation (pBQ-dG duplex) (Fig. 2D). Analysis of pBQ-dC by molecular dynamics simulation suggested displacement of the adduct toward the major groove, as compared to the normal Watson-Crick base pair [47]. Therefore, the simulation predicts that these pBQ adducts cause significant structural alterations at the lesion site.

3.2. In vitro translesion synthesis of *p*-benzoquinone DNA adducts by the Y family DNA polymerases

The unusually large van der Waals space occupied by *p*-benzoquinone DNA adducts and the structural alterations

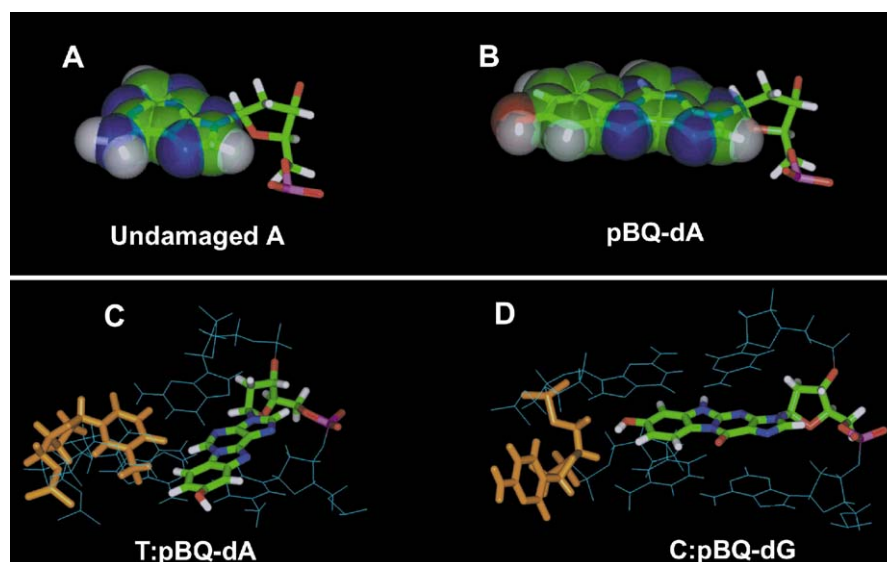


Fig. 2. Computer modeling of pBQ-dA and pBQ-dG adducts by molecular dynamics simulation. (A) van der Waals surfaces for undamaged dA. (B) van der Waals surfaces for the pBQ-dA adduct. (C) Side view from the major groove for 3 bp of the 25-mer duplexes containing a pBQ-dA. (D) Side view from the major groove for 3 bp of the 25-mer duplexes containing a pBQ-dG adduct. The pBQ-dA and pBQ-dG adducts are colored by atom, and the opposite bases are shown in dark orange. Average minimized structures were produced by 2 ns molecular dynamics simulations. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

induced by these adducts, as predicted by the molecular dynamics simulations, suggest that copying these adduct sites during replication likely requires specialized translesion polymerases. To examine how these *p*-benzoquinone DNA adducts might be replicated, we performed in vitro translesion synthesis with purified Y family DNA polymerases. A DNA primer was 5'-labeled with ^{32}P and annealed to the damaged DNA template with the primer 3' end terminating right before the lesion (Fig. 3A). Then, DNA synthesis assays were performed using a purified DNA polymerase. As shown in Fig. 3B, DNA synthesis from the undamaged control templates was readily detected with the purified human Pol η . In contrast, synthesis from the damaged templates was greatly inhibited by *p*-benzoquinone adducts, as indicated by undetectable or barely detectable full-length synthesis products even at a higher Pol η concentration (25-mer DNA band) (Fig. 3C). Nevertheless, human Pol η was able to insert a nucleotide opposite pBQ-dA, pBQ-dG, and pBQ-dC. One nucleotide extension from opposite the lesion occurred to a limited extent, but further extension beyond the 5' undamaged template base was greatly inhibited by the lesion (Fig. 3C).

To determine what nucleotide was inserted opposite the lesion by human Pol η , we further performed the translesion synthesis assay with each of the four deoxyribonucleoside triphosphates: dATP, dCTP, dGTP, or dTTP. Opposite pBQ-dA and pBQ-dG, an A or a G was most frequently inserted, while C and T were also inserted but at a lower frequency (Fig. 4A, lanes 2–5 and 7–10). Opposite pBQ-dC, A, G, C, and T were inserted with decreasing frequencies (Fig. 4A, lanes 12–15). To quantitatively determine the specificity of nucleotide insertion by human Pol η opposite the lesion, we performed kinetic analysis. In agreement with results of Fig. 4A, studies on catalysis efficiency, as indicated by the V_{\max}/K_m values, showed nucleotide insertions from the most to the least frequent: A > G > C > T for pBQ-dA and pBQ-dC,

Table 1

Kinetic measurement of nucleotide insertion opposite pBQ-dA, pBQ-dG, and pBQ-dC by human Pol η

dNTP	V_{\max} (fmol/min; mean \pm S.D.)	K_m (μM ; mean \pm S.D.)	V_{\max}/K_m	f_{inc}^a
pBQ-dA				
dATP	2.05 \pm 0.11	0.89 \pm 0.22	2.30	1.9×10^1
dCTP	2.26 \pm 0.10	8.62 \pm 2.01	0.26	2.2×10^0
dTTP	2.29 \pm 0.04	19.8 \pm 1.64	0.12	1
dGTP	2.24 \pm 0.16	1.76 \pm 0.58	1.27	1.1×10^1
pBQ-dG				
dATP	3.36 \pm 0.05	11.3 \pm 0.77	0.30	3.7×10^0
dCTP	2.25 \pm 0.15	27.9 \pm 8.10	0.081	1
dTTP	2.23 \pm 0.16	13.5 \pm 4.72	0.17	2.1×10^0
dGTP	2.27 \pm 0.04	1.16 \pm 0.09	1.96	2.4×10^1
pBQ-dC				
dATP	3.05 \pm 0.09	3.21 \pm 0.42	0.95	1.4×10^0
dCTP	3.03 \pm 0.22	6.26 \pm 2.07	0.48	7.1×10^{-1}
dTTP	2.70 \pm 0.05	24.4 \pm 2.05	0.11	1.6×10^{-1}
dGTP	2.75 \pm 0.10	4.04 \pm 0.76	0.68	1

$$^a f_{\text{inc}} = (V_{\max}/K_m)_{\text{incorrect}} / (V_{\max}/K_m)_{\text{correct}}$$

and G > A > T > C for pBQ-dG (Table 1). Furthermore, fidelity of nucleotide insertion, as indicated by the f_{inc} values, showed that a wrong base was predominantly inserted opposite each *p*-benzoquinone adduct (Table 1). Therefore, nucleotide insertion by human Pol η opposite the *p*-benzoquinone DNA adducts is highly error-prone.

To examine whether the translesion synthesis activity of Pol η is conserved in other organisms, we performed in vitro assays using purified yeast Pol η . As shown in Fig. 3D, nucleotide insertion opposite pBQ-dA, pBQ-dG, and pBQ-dC was also observed with yeast Pol η . Following one nucleotide insertion opposite the lesion, yeast Pol η was unable to perform extension synthesis

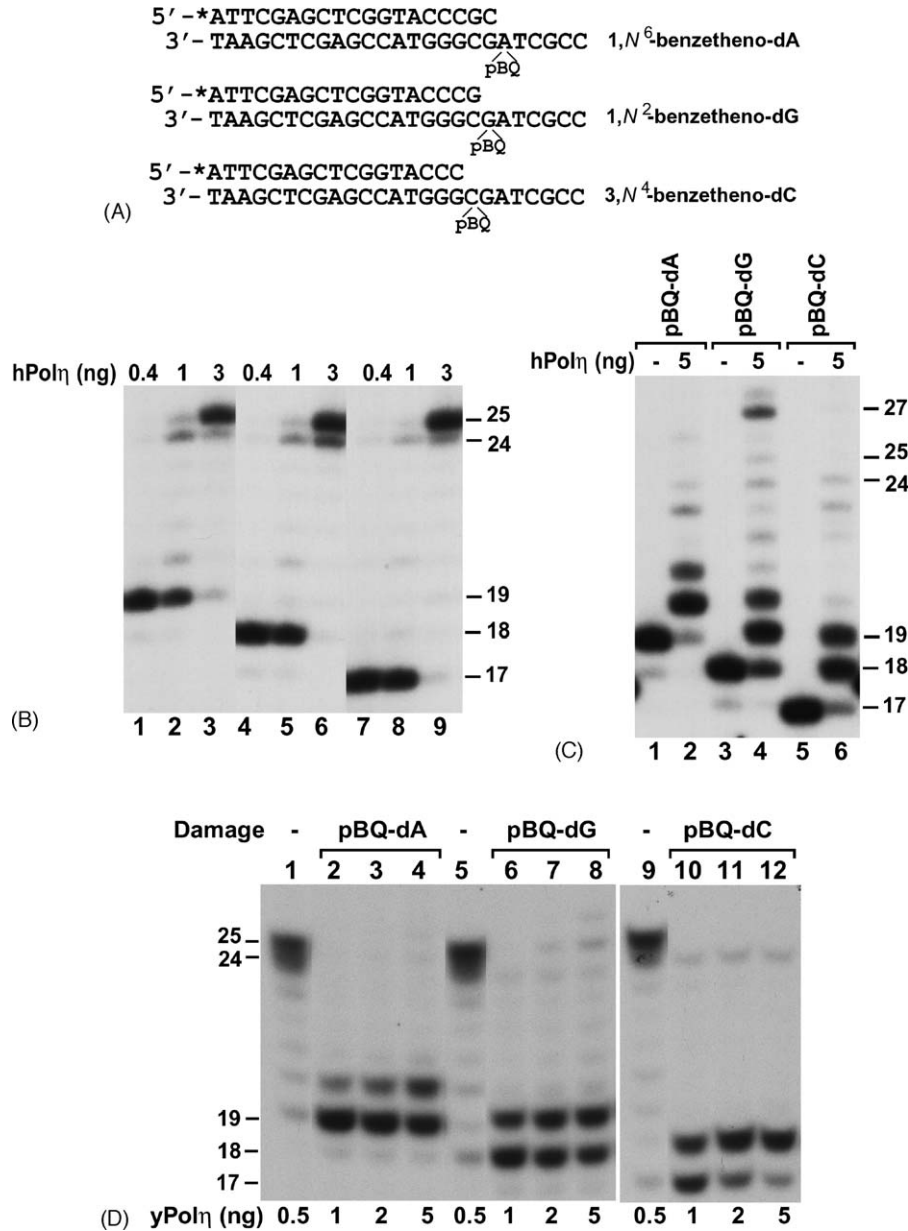


Fig. 3. Translesion synthesis of pBQ DNA adducts by Polη. (A) Damaged DNA templates for translesion synthesis. Three primers, 17 mer, 18 mer, and 19 mer, were labeled with ^{32}P at their 5' ends as indicated by an asterisk and separately annealed to the damaged templates (25 mer) with the primer 3' end terminating right before the lesion. (B) DNA synthesis assays were performed with increasing amounts of purified human Polη (hPolη) using undamaged templates. (C) DNA synthesis assays were performed with 5 ng of human Polη (lanes 2, 4, and 6) using damaged templates as indicated. Lanes 1, 3, and 5, control reactions without Polη. (D) DNA synthesis assays were performed with increasing amounts of purified yeast Polη (yPolη) as indicated in the presence of all four dNTPs. Lanes 1, 5, and 9, control reactions with undamaged DNA templates and 0.5 ng (7 pmol) of yeast Polη. Products of DNA synthesis were separated by 20% denaturing polyacrylamide gel and visualized by autoradiography of the gel. DNA size markers in nucleotides are indicated on the sides.

past the lesion (Fig. 3D, lanes 2–4, 6–8, and 10–12). In control reactions with undamaged templates, yeast Polη efficiently copied the template to its 5' end at a lower polymerase concentration (25-mer DNA band) (Fig. 3D, lanes 1, 5, and 9). G was most frequently inserted opposite pBQ-dA (Fig. 4B, lane 10) and pBQ-dG (Fig. 4B, lane 20), while G and A were inserted with similar frequencies and more frequently than C or T opposite pBQ-dC (Fig. 4B, lanes 27–30). In control reactions, yeast Polη inserted predominantly the correct nucleotide opposite the undamaged template A (lane 4), G (lane 13), and C (lanes 25).

Using purified human Polη, significant nucleotide insertion activity was observed opposite pBQ-dA and pBQ-dC (Fig. 5, lanes 1 and 5). This polymerase, however, was unable to extend DNA synthesis from opposite the lesion (Fig. 5, lanes 1 and 5). Opposite pBQ-dG, human Polη performed only a low level of nucleotide insertion opposite the lesion (Fig. 5, lane 3). DNA synthesis by purified human Polκ from the pBQ-dG template was barely detectable (Fig. 5, lane 4). Opposite both pBQ-dA and pBQ-dC, human Polκ was essentially unable to perform translesion synthesis (Fig. 5, lanes 2 and 6). Using kinetic

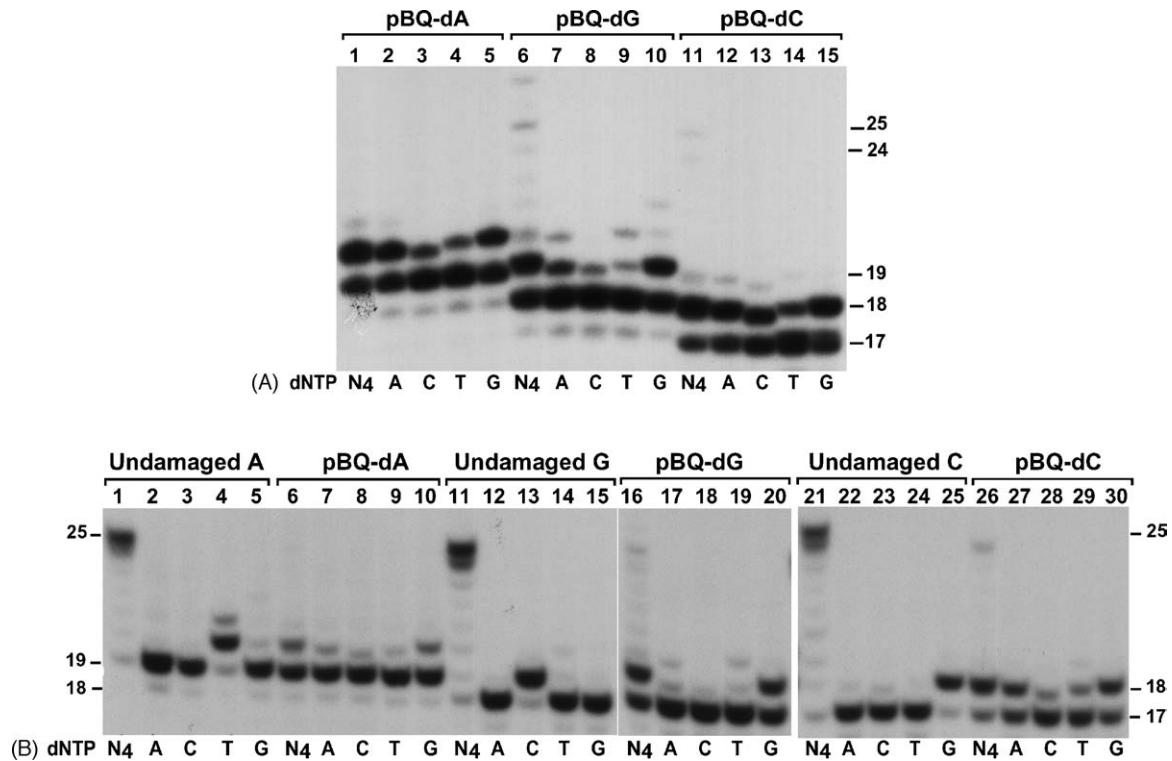


Fig. 4. Specificity of nucleotide insertion by Pol η opposite the pBQ DNA adducts. Damaged DNA templates (25 mer) containing a site-specific pBQ-dA (lanes 1–5), pBQ-dG (lanes 6–10), or pBQ-dC (lanes 11–15) were annealed with 5' 32 P-labeled primers (19 mer, 18 mer, and 17 mer, respectively) terminating right before the lesion (Fig. 3A). (A) Translesion synthesis assays were performed with 2 ng (26 fmol) of human Pol η in the presence of a single deoxyribonucleoside triphosphate dATP (lanes 2, 7, and 12), dCTP (lanes 3, 8, and 13), dTTP (lanes 4, 9, and 14), or dGTP (lanes 5, 10, and 15), or all four dNTPs (lanes 1, 6, and 11). (B) Translesion synthesis assays were performed with 2 ng (28 fmol) of yeast Pol η in the presence of a single deoxyribonucleoside triphosphate dATP (A), dCTP (C), dTTP (T), or dGTP (G), or all four dNTPs (N4). DNA synthesis assays using undamaged DNA templates were performed with 0.5 ng (7 fmol) of yeast Pol η as controls. Products of DNA synthesis were separated by 20% denaturing polyacrylamide gel and visualized by autoradiography of the gel. DNA size markers in nucleotides are indicated on the sides.

analysis, we determined the specificity of nucleotide insertion by human Pol η opposite both pBQ-dA and pBQ-dC. As shown in Table 2, A and G was inserted with similar efficiency opposite pBQ-dA. Opposite pBQ-dC, the insertion efficiency was G>T>A>C. As indicated by the f_{inc} values, the correct G insertion was only 1.3-fold more efficient than the incorrect T insertion (Table 2).

REV1 is a template-dependant dCMP transferase [31,32]. While purified human REV1 was active in response to undam-

aged template A, G, and C (Fig. 6, lanes 1, 7, and 13), it was inactive to pBQ-dA (Fig. 6, lanes 2–6) and essentially inactive to pBQ-dG and pBQ-dC (Fig. 6, lanes 8–12 and lanes 14–18). Similarly, purified yeast Rev1 was essentially incapable of translesion synthesis in response to pBQ-dA, pBQ-dG, and pBQ-dC DNA adducts (data not shown).

3.3. *In vivo* translesion synthesis of *p*-benzoquinone DNA adducts in yeast cells

Using an *in vivo* genetic assay [29], we examined translesion synthesis of *p*-benzoquinone adducts in yeast cells. This assay is based on transformation of site-specifically damaged plasmid into yeast cells. The damaged and single-stranded plasmid was converted into double-stranded form prior to transformation, replacing T with U during *in vitro* synthesis of the complementary strand. Use of double-stranded plasmid ensures efficient transformation. Upon entering into cells, the complementary strand was degraded as a result of the extensive and sequential actions of a uracil-DNA glycosylase and an AP endonuclease due to replacement of T by U in the sequence, thus, converting the plasmid DNA back into single-stranded form containing a site-specific lesion [29]. Therefore, this assay is expected to specifically reflect translesion synthesis without interference by DNA repair and template switching mechanisms [29], both of

Table 2
Kinetic measurement of nucleotide insertion opposite PBQ-dA and PBQ-dC by human Pol η

DNTP	V_{max} (fmol/min; mean \pm S.D.)	K_m (μ M; mean \pm S.D.)	V_{max}/K_m	f_{inc}^a
PBQ-dA^b				
dTTP	1.34 \pm 0.21	42.6 \pm 21.8	0.031	1
dGTP	1.13 \pm 0.08	42.5 \pm 11.0	0.027	8.7 $\times 10^{-1}$
PBQ-dC				
dATP	2.18 \pm 0.11	104.7 \pm 17.9	0.021	2.9 $\times 10^{-1}$
dCTP	1.91 \pm 0.10	236.1 \pm 36.0	0.0081	1.1 $\times 10^{-1}$
dTTP	2.76 \pm 0.07	48.8 \pm 4.87	0.057	7.8 $\times 10^{-1}$
dGTP	2.60 \pm 0.09	35.6 \pm 4.60	0.073	1

^a $f_{inc} = (V_{max}/K_m)_{incorrect} / (V_{max}/K_m)_{correct}$.

^b dATP and dCTP incorporations are undetectable.

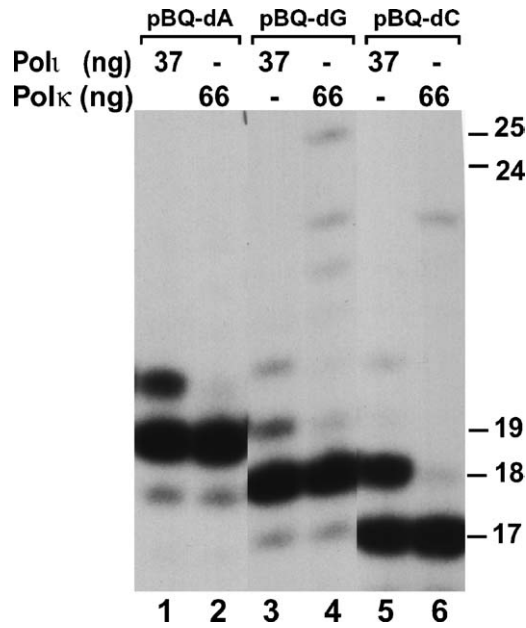


Fig. 5. Translesion synthesis of pBQ DNA adducts by human Polt and Polk. Damaged DNA templates (25 mer) containing a site-specific pBQ-dA (lanes 1 and 2), pBQ-dG (lanes 3 and 4), or pBQ-dC (lanes 5 and 6) were separately annealed with 5' ³²P-labeled primers (19 mer, 18 mer, and 17 mer, respectively) terminating right before the lesion (Fig. 3A). Translesion synthesis assays were performed with 37 ng (463 pmol) of purified human Polt (lanes 1, 3, and 5) or 66 ng (667 pmol) of purified human Polk (lanes 2, 4, and 6) in the presence of all four dNTPs. Products of DNA synthesis were separated by 20% denaturing polyacrylamide gel and visualized by autoradiography of the gel. DNA size markers in nucleotides are indicated on the right.

which require double-stranded DNA. Transformation efficiency was determined by using undamaged and double-stranded plasmid in the same experiment. After correcting for differences in transformation efficiency, translesion synthesis efficiency in various cells relative to that in the wild-type cells was calculated. Replicated plasmid clones were individually isolated from yeast colonies and subsequently amplified in *E. coli* for DNA sequencing.

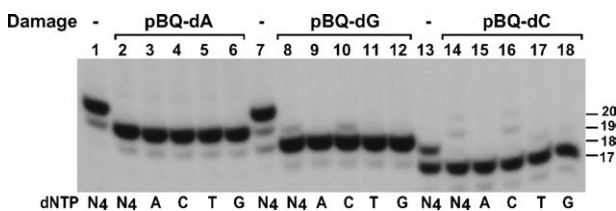


Fig. 6. Response of human REV1 to pBQ DNA adducts. Damaged DNA templates (25 mer) containing a site-specific pBQ-dA (lanes 2–6), pBQ-dG (lanes 8–12), or pBQ-dC (lanes 14–18) were separately annealed with 5' ³²P-labeled primers (19 mer, 18 mer, and 17 mer, respectively) terminating right before the lesion (Fig. 3A). Translesion synthesis assays were performed with 10 ng (72 pmol) of purified human REV1 in the presence of a single deoxyribonucleoside triphosphate dATP (lanes 3, 9, and 15), dCTP (lanes 4, 10, and 16), dTTP (lanes 5, 11, and 17), or dGTP (lanes 6, 12, and 18), or all four dNTPs (lanes 2, 8, and 14). Lanes 1, 7, and 13, control reactions with undamaged DNA templates. Products of DNA synthesis were separated by 20% denaturing polyacrylamide gel and visualized by autoradiography of the gel. DNA size markers in nucleotides are indicated on the right.

Table 3

Relative translesion synthesis of pBQ DNA adducts in various yeast strains

Strain ^a	pBQ-dA (%)	pBQ-dC (%)	pBQ-dG(%)
WT	100 ± 2	100 ± 11	100 ± 8
<i>rev3</i>	9 ± 1	51 ± 1	28 ± 1
<i>rad30</i>	ND ^b	ND ^b	59 ± 3
<i>rev3 rad30</i>	<2 ^c	<2 ^c	ND ^b

^a WT, wild-type; *rev3*, lacking Polζ; *rad30*, lacking Polη.

^b Not determined.

^c Limit of detection. No translesion synthesis product was recovered.

Site-specifically damaged plasmid DNA was constructed by ligating the 25-mer oligonucleotide containing pBQ-dA, pBQ-dG, or pBQ-dC into the yeast vector pELUf1. As a control, the corresponding undamaged 25-mer oligonucleotide was also similarly ligated into the vector. Transformation of the undamaged control plasmid into yeast cells yielded a relative replication efficiency of 100%, 98%, and 102% in the wild-type, *rev3* mutant, and *rev3 rad30* double mutant strains, respectively. Hence, replication of the undamaged control plasmid in yeast cells was not affected by Polζ (missing in *rev3* mutant) or Polη (missing in *rev3 rad30* double mutant), as expected. Transformation of pBQ-dA and pBQ-dC into the wild-type cells reduced the replication efficiency by ~17-fold, indicating that these adducts are strong blockers to DNA replication. Compared to the wild-type cells, translesion synthesis of pBQ-dA was greatly reduced in the *rev3* mutant cells, translesion synthesis of pBQ-dC was reduced in *rev3* mutant cells, and translesion synthesis of pBQ-dG was also reduced in either *rev3* or *rad30* mutant cells (Table 3). In the *rev3 rad30* double mutant cells, translesion synthesis of pBQ-dA and pBQ-dC was further reduced to an undetectable level (Table 3). These results show that both Polζ and Polη are involved in translesion synthesis of *p*-benzoquinone DNA adducts and suggest that contribution by one polymerase to the bypass of these lesions cannot be completely substituted by the other polymerase in yeast.

3.4. Specificity of in vivo translesion synthesis opposite *p*-benzoquinone DNA adducts

To determine the specificity of in vivo translesion synthesis products in various yeast strains, we recovered the replicated plasmids from yeast clones and individually amplified them in *E. coli* for DNA sequencing. In wild-type cells, the majority of translesion products were deletions, which accounted for 91%, 76%, and 90% for pBQ-dA, pBQ-dC, and pBQ-dG, respectively (Table 4). Among the deletion products, the majority bypassing pBQ-dA and pBQ-dC were small deletions (–1 and –2), whereas the majority bypassing pBQ-dG were bigger deletions (–3 to –23) (Table 4). Complex deletion products were also recovered. These products were composed of a small deletion at the lesion site plus additional mutations 3' or 5' of the lesion site. The remaining 9%, 24%, and 10% of translesion products for pBQ-dA, pBQ-dC, and pBQ-dG, respectively, were base incorporations (Table 4). Complex base incorporations were composed of a base incorporation at the lesion site plus additional mutations 3' or 5' of the lesion site. For example, five

Table 4
Specificity of translesion synthesis opposite pBQ DNA adducts in various yeast strains

Strain ^a	Clones sequenced ^b	Base incorporation					Deletion				
		A	G	T	Complex ^c	Total	–1	–2	–3 to –23	Complex ^d	Total
PBQ-dA											
WT	22	–	–	1 (5%)	1 (5%)	2 (9%)	9 (41%)	2 (9%)	–	9 (41%)	20 (91%)
<i>rev3</i>	3	–	–	–	–	–	2 (67%)	–	1 (33%)	–	3 (100%)
PBQ-dC											
WT	34	–	3 (9%)	–	5 (15%)	8 (24%)	22 (64%)	2 (6%)	–	2 (6%)	26 (76%)
<i>rev3</i>	24	–	–	–	–	–	21 (88%)	3 (13%)	–	–	24 (100%)
PBQ-dG											
WT	30	1 (3%)	2 (7%)	–	–	3 (10%)	9 (30%)	2 (7%)	15 (50%)	1 (3%)	27 (90%)
<i>rev3</i>	23	–	–	–	–	–	7 (30%)	–	11 (48%)	5 (22%)	23 (100%)
<i>rad30</i>	31	–	–	–	1 (3%)	1 (3%)	11 (36%)	–	13 (42%)	6 (19%)	30 (97%)

^a WT, wild-type; *rev3*, lacking Polζ; *rad30*, lacking Polη.

^b Number of independent clones sequenced following in vivo translesion synthesis assays using damaged pELUf1 plasmids containing a site-specific pBQ DNA adducts.

^c Complex base incorporations were composed of a base incorporation at the lesion site plus additional mutations 3' or 5' of the lesion site.

^d Complex deletion products were composed of a small deletion at the lesion site plus additional mutations 3' or 5' of the lesion site.

out of the eight complex base incorporations recovered from replication of pBQ-dC in wild-type cells were tandem base substitutions: 5'-CCAGTA opposite the template 3'-GGCGAT where the damaged C is underlined. This type of bypass product most likely resulted from the following mechanism: insertion of A by Polη opposite pBQ-dC → realigning primer → G insertion by Polη opposite pBQ-dC → extension by Polζ. The majority of base incorporations were error-prone for pBQ-dA, pBQ-dC, and pBQ-dG (Table 4).

In *rev3* mutant cells lacking Polζ, all translesion synthesis products detected were deletions (Table 4). When pBQ-dG plasmid was replicated in *rad30* mutant cells lacking Polη, only one base incorporation clone was recovered, in which a C was incorporated opposite the lesion plus a T misincorporation opposite the template G located four nucleotides 5' to the lesion site. The remaining bypass products were all deletions (Table 4). The pBQ-dA and pBQ-dC plasmids were additionally tested for in vivo translesion synthesis in *rev3 rad30* double mutant cells lacking both Polη and Polζ. However, these damaged plasmids were unable to replicate in this double mutant strain (Table 3).

Taken together, these results show that translesion synthesis of *p*-benzoquinone DNA adducts are mediated mainly by deletion DNA synthesis at the lesion sites in yeast cells. We further conclude that Polζ and Polη together are required for translesion synthesis by the base incorporation mechanism, and that translesion synthesis through deletion is mediated by a Polζ-dependent mechanism and another Polη-dependent mechanism in response to *p*-benzoquinone DNA adducts in yeast.

4. Discussion

Using both in vitro and in vivo methods, we have examined translesion synthesis of three *p*-benzoquinone DNA adducts: pBQ-dA, pBQ-dG, and pBQ-dC. We found that both purified human and yeast Polη possess significant translesion synthesis activity opposite these adducts. However, in vitro translesion synthesis by Polη was limited to nucleotide insertion opposite the lesion. Subsequent extension synthesis was mostly blocked.

In vitro translesion synthesis of pBQ-dG by human Polη yielded a minor product of 27-mer DNA, which is two nucleotides longer than the template (Fig. 3C, lane 4). The precise mechanism for generating this product is not known. We speculate that it may have resulted from the following mechanism. First, a G was inserted opposite the lesion, as this was the most frequently inserted nucleotide by Polη opposite pBQ-dG (Fig. 4A and Table 1). Secondly, the primer was realigned such that the primer 3' G was paired to the template C 3' of the lesion. Thirdly, this process of G insertion followed by primer realignment was repeated one more time before translesion synthesis to the end of the template, resulting in a product of two nucleotides longer than the template. As for human Polι, it exhibited some activity of in vitro translesion synthesis. Similar to Polη, Polι was limited to one nucleotide insertion opposite the lesion and was incapable of further extension synthesis. Nucleotide insertions by both Polη and Polι opposite these lesions were error-prone. The other two human Y family DNA polymerases, Polκ and REV1, were essentially incapable of translesion synthesis in response to the *p*-benzoquinone adducts in vitro. Our in vitro biochemical results suggest that Polη likely plays a role in bypassing the *p*-benzoquinone DNA adducts in cells. This prediction was confirmed in the yeast model organism using an in vivo genetic assay that measures replication of site-specifically damaged and single-stranded plasmid DNA in cells.

The wild-type level of replication of plasmid DNA containing a site-specific pBQ-dA, pBQ-dG, or pBQ-dC depended on both Polη and Polζ. Most dramatically, a pBQ-dA- or pBQ-dC-containing plasmid was unable to replicate in the *rev3 rad30* double mutant strain lacking both Polζ and Polη. In contrast, translesion synthesis was reduced but not abolished in mutant cells lacking Polζ. Similarly, translesion synthesis of pBQ-dG was reduced but not abolished in mutant cells lacking Polη. Thus, translesion synthesis of *p*-benzoquinone DNA adducts in yeast cells likely involves at least two different pathways, one depending on Polζ and the other depending on Polη. Analysis of the specificity of in vivo translesion products further supports this conclusion. Deletion constituted the predominant mechanism

of translesion synthesis in wild-type cells and remained as the exclusive or nearly exclusive mechanism of lesion bypass in cells lacking Pol ζ or Pol η , respectively. Since the overall levels of translesion synthesis in these mutant cells were reduced from the wild-type level (Table 3), lesion bypass through deletion at the adduct site is reduced but not completely eliminated in the absence of either polymerase. Complete loss of this mode of translesion synthesis requires inactivation of both Pol ζ and Pol η .

Base incorporation at the lesion site constituted only a minor mechanism of translesion synthesis of *p*-benzoquinone DNA adducts in yeast cells. This mode of lesion bypass was eliminated in *rev3* mutant cells lacking Pol ζ and nearly eliminated in *rad30* cells lacking Pol η . Thus, unlike the deletion mechanism, translesion synthesis through base incorporation could be explained by one pathway requiring both Pol η and Pol ζ . This interpretation is consistent with the two-polymerase two-step hypothesis of DNA lesion bypass [17]. Indeed, Pol η was capable of nucleotide insertion (most frequently A or G) opposite *p*-benzoquinone DNA adducts in vitro, but was unable to catalyze extension synthesis. It is likely that Pol ζ catalyzes such an extension synthesis. In such a bypass pathway, the insertion step would not occur without Pol η and the extension step would not occur without Pol ζ , thus explaining the observed dependence on both Pol η and Pol ζ for base incorporation mode of translesion synthesis.

Most types of DNA lesions predominantly induce base substitutions. Deletion is only rarely observed as a major mutagenesis mechanism, e.g., frameshift mutations induced by AAF-dG DNA adducts in which deletion most frequently occurs at G or GC repeats [48–51]. It was proposed that C insertion opposite the AAF-dG adduct can lead to realignment of the inserted C with the downstream undamaged template G, leading to slipped translesion synthesis [51]. The pBQ-dA, pBQ-dG, and pBQ-dC DNA adducts in our studies, however, are not located in a nucleotide repeat sequence. Therefore, it is surprising that these *p*-benzoquinone DNA adducts primarily induce deletions. It is likely that a different mechanism than the slipped translesion synthesis is involved in generating the observed deletion bypass. We propose that a major deletion mechanism for *p*-benzoquinone adducts is translesion synthesis by looping out the lesion. In this mechanism, -1 , -2 , or -3 deletions would be generated when the lesion, the lesion plus one undamaged template base, or the lesion plus two undamaged template bases, respectively, is looped out during translesion synthesis. This template loop-out model has been proposed for in vitro translesion synthesis by human Pol μ , [52,53].

Structural analysis of *p*-benzoquinone DNA adducts by molecular dynamics simulation provided important insights into understanding translesion synthesis of these lesions. Our molecular modeling suggests that these adducts do not pair with the opposite bases and they cannot be accommodated concurrently inside the DNA duplex, due to unusually large van der Waals surface occupied by *p*-benzoquinone adducts, as compared to normal bases. Thus, these adducts could create a great steric barrier for the incoming base. These predicted structural features of *p*-benzoquinone DNA adducts may provide an explanation as to

why translesion synthesis by base incorporation opposite these adducts is a minor mechanism.

The pBQ DNA adducts strongly inhibited extension synthesis from opposite the lesion by Pol η . Thus, it is not apparent whether a deletion mechanism was involved during translesion synthesis by Pol η in vitro (Figs. 3 and 4). Given the in vivo results showing deletion as the major mechanism for translesion synthesis, it is likely that the in vitro results with purified Pol η reflected both mechanisms of nucleotide insertion opposite the lesion and deletion by copying the undamaged template base 5' to pBQ adducts. For example, translesion synthesis of pBQ-dC by Pol η showed significantly more C insertion as compared to those of pBQ-dA and pBQ-dG (Fig. 4 and Table 1). A portion of the C insertion products may have resulted from copying the undamaged template G 5' of the pBQ-dC adduct through lesion loop-out, which represents a -1 deletion mechanism of translesion synthesis. It is possible that other protein factor(s) may also affect translesion synthesis mechanisms of specific lesions in cells. Our studies with pBQ adducts underscore the importance of performing in vivo translesion synthesis analyses to understand the bypass mechanism and the mutagenic specificity of a specific DNA lesion.

During initial repair studies of pBQ-dC, it was surprisingly discovered that this DNA lesion is recognized and cleaved by an AP endonuclease [54]. In fact, pBQ-dA and pBQ-dG are also substrates for DNA strand cleavage 5' to the lesion by endonucleases [55]. Molecular modeling of these adducts [47] (this study) are consistent with the notion that the large size of the modified base may lead to significant base displacement from its normal Watson–Crick base pairing position. Interestingly, we found that the specificity of nucleotide insertion by human and yeast Pol η opposite pBQ-dA, pBQ-dG, and pBQ-dC are similar to that by the respective polymerase opposite an AP site [23,35], and that nucleotide insertion opposite pBQ-dC by human Pol κ follows the same specificity as its response to an AP site: G > T > A > C [56]. However, the structure of *p*-benzoquinone DNA adducts is significantly different from that of an AP site. Thus, *p*-benzoquinone DNA adducts are not as good substrates as AP sites for AP endonucleases [57], and they are not responded to by Pol κ and REV1 that are responsive to AP sites.

Based on our yeast genetic assay, pBQ-dA, pBQ-dG, and pBQ-dC adducts are highly mutagenic. Remarkably, 95%, 91%, and 100% of pBQ-dA, pBQ-dG, and pBQ-dC plasmid clones, respectively, contained mutations following their replication in wild-type cells. Since this assay is not interfered by DNA repair [29], our results reflect the potent intrinsic mutagenicity of these *p*-benzoquinone adducts. Thus, if these DNA adducts are formed in cells and are not completely removed, deletion mutations would be induced. Using the *supF* shuttle vector system, it was reported that treatment of plasmid DNA with *p*-benzoquinone and another benzene metabolite, hydroquinone, results in increased mutations following plasmid replication in human cells [10–12]. Base substitutions accounted for the majority of mutations in this system. Nevertheless, significant levels of deletions were also observed [10–12]. Unlike our yeast system, the shuttle vector system is subject to DNA repair following

transfection into cells. Therefore, repair of pBQ-dA, pBQ-dG, and pBQ-dC by APE1 and possibly nucleotide excision repair may have reduced the mutagenic contributions by these adducts. Our results support the notion that *p*-benzoquinone DNA adducts contribute to mutagenesis and carcinogenesis of benzene. At the whole organism level, mechanisms of benzene mutagenesis are undoubtedly more complex. Other benzene metabolites, such as hydroquinone and trans, trans-muconaldehyde, likely contribute to mutagenesis as well [11,12]. Furthermore, oxidative DNA damage may also make a significant contribution to benzene mutagenesis, which has been suggested to result from metabolism of hydroquinone and the redox cycling between semiquinone radical and *p*-benzoquinone [11,58].

In addition to the insights we gained in understanding the mutagenic properties of *p*-benzoquinone DNA adducts, our studies led to the following conceptual model of translesion synthesis in eukaryotes. Translesion synthesis normally occurs by directly copying the lesion site through base insertion opposite the lesion followed by extension synthesis due to Pol ζ and the Y family DNA polymerases. When the lesion becomes incompatible in accommodating a base opposite the lesion in the duplex DNA, translesion synthesis occurs by a less efficient lesion loop-out mechanism, resulting in avoiding copying the damaged base, and thus leading to deletion. According to this concept, translesion synthesis would be a versatile cellular process in that even when a lesion is so bulky or distorting to allow translesion synthesis by base incorporation to occur, such a lesion can still be bypassed by the alternative template loop-out translesion synthesis.

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