



Two-step error-prone bypass of the (+)- and (–)-*trans-anti*-BPDE- N^2 -dG adducts by human DNA polymerases η and κ

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Abstract

Benzo[*a*]pyrene is a polycyclic aromatic hydrocarbon (PAH) associated with potent carcinogenic activity. Mutagenesis induced by benzo[*a*]pyrene DNA adducts is believed to involve error-prone translesion synthesis opposite the lesion. However, the DNA polymerase involved in this process has not been clearly defined in eukaryotes. Here, we provide biochemical evidence suggesting a role for DNA polymerase η (Pol η) in mutagenesis induced by benzo[*a*]pyrene DNA adducts in cells. Purified human Pol η predominantly inserted an A opposite a template (+)- and (–)-*trans-anti*-BPDE- N^2 -dG, two important DNA adducts of benzo[*a*]pyrene. Both lesions also dramatically elevated G and T mis-insertion error rates of human Pol η . Error-prone nucleotide insertion by human Pol η was more efficient opposite the (+)-*trans-anti*-BPDE- N^2 -dG adduct than opposite the (–)-*trans-anti*-BPDE- N^2 -dG. However, translesion synthesis by human Pol η largely stopped opposite the lesion and at one nucleotide downstream of the lesion (+1 extension). The limited extension synthesis of human Pol η from opposite the lesion was strongly affected by the stereochemistry of the *trans-anti*-BPDE- N^2 -dG adducts, the nucleotide opposite the lesion, and the sequence context 5' to the lesion. By combining the nucleotide insertion activity of human Pol η and the extension synthesis activity of human Pol κ , effective error-prone lesion bypass was achieved in vitro in response to the (+)- and (–)-*trans-anti*-BPDE- N^2 -dG DNA adducts.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAH) belong to a class of ubiquitous environmental pollutants and carcinogens. They are produced by incomplete combustion of organic materials. Benzo[*a*]pyrene is a

PAH compound associated with potent carcinogenic activity. In cells, benzo[*a*]pyrene is metabolized to water-soluble derivatives so that they can be excreted. Racemic *anti*-benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (*anti*-BPDE) are two such metabolites, which are active electrophiles that readily attack DNA. The (\pm)-*anti*-BPDE compounds react with DNA mainly at the N^2 position of guanine, forming stereoisomeric bulky adducts (+)-*trans-anti*-BPDE- N^2 -dG, (+)-*cis-anti*-BPDE- N^2 -dG, (–)-*trans-anti*-BPDE- N^2 -

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dG, and (–)-*cis-anti*-BPDE- N^2 -dG [1,2]. In cells, the major benzo[*a*]pyrene adduct is (+)-*trans-anti*-BPDE- N^2 -dG [2]. All four stereoisomers of the *anti*-BPDE- N^2 -dG DNA adducts are mutagenic in simian kidney cells, producing primarily G → T transversions in a defined sequence context [3,4]. Sequence context effect has also been observed on mutagenesis induced by *anti*-BPDE- N^2 -dG adducts in prokaryotic and eukaryotic cellular systems [3–8].

Mutagenesis induced by *anti*-BPDE- N^2 -dG DNA adducts is believed to be a key factor to benzo[*a*]pyrene-induced carcinogenesis. However, little is known about the biochemistry of mutagenesis induced by these adducts. Lesion bypass represents a major mechanism of damage-induced mutagenesis, because this cellular process directly copies the damaged DNA template during replication. Copying the damaged template requires a specialized DNA polymerase. Recent studies suggest that the Y family DNA polymerases are involved in lesion bypass of several types of DNA lesions (reviewed in [9–12]). Human DNA polymerase η (Pol η) and Pol κ are two members of the Y family polymerases [13]. Some lesions may be efficiently bypassed by a single polymerase, such as the bypass of TT dimers by human Pol η [14,15]. However, bypass of other lesions likely involves nucleotide insertion opposite the lesion by one polymerase followed by extension synthesis from opposite the lesion by another polymerase (the two-polymerase two-step mechanism) [16–18]. Therefore, nucleotide insertion opposite the lesion and extension synthesis from opposite the lesion constitute two important biochemical reactions during lesion bypass. Depending on whether the correct nucleotide or a wrong nucleotide is primarily incorporated opposite the lesion, the result of lesion bypass is error-free or error-prone, respectively. Whereas error-free lesion bypass would suppress mutagenesis, error-prone lesion bypass would promote mutagenesis. Thus, both modes of lesion bypass directly affect the mutagenic potential of DNA damage. Identifying the DNA polymerases involved in the bypass of the *anti*-BPDE- N^2 -dG DNA adducts is therefore a key step toward understanding the mutagenicity and carcinogenicity of benzo[*a*]pyrene.

We have identified human Pol κ as an effective error-free bypass polymerase in response to the template (–)- and (+)-*trans-anti*-BPDE- N^2 -dG adducts

[19,20]. Most recently, these findings were confirmed by Shen et al. [21] using purified *Escherichia coli* DNA polymerase IV, the bacterial counterpart of human Pol κ . The role of this bacterial polymerase in the bypass of the benzo[*a*]pyrene DNA adducts was further supported by the genetic results of Napolitano et al. [22]. Additionally, we found that human Pol κ can also function as the extension synthesis polymerase in vitro during lesion bypass by the two-polymerase two-step mechanism [18,20].

In our previous studies, we discovered that human Pol η is capable of error-prone translesion synthesis opposite a template (+)-*trans-anti*-BPDE- N^2 -dG adduct [23]. To further evaluate whether Pol η indeed plays an important role for benzo[*a*]pyrene-induced mutagenesis, it is important to examine additional DNA adducts of this carcinogen and to examine the extension synthesis step of human Pol η during translesion synthesis. In this report, we show error-prone translesion synthesis of purified human Pol η opposite the (–)-*trans-anti*-BPDE- N^2 -dG adduct, determine the biochemistry of extension synthesis by human Pol η , and demonstrate bypass of the (+)- and (–)-*trans-anti*-BPDE- N^2 -dG DNA adducts by the sequential actions of Pol η and human Pol κ . These results provide important biochemical evidence suggesting a role for Pol η in mutagenesis induced by the (+)- and (–)-*trans-anti*-BPDE- N^2 -dG DNA adducts in cells.

2. Materials and methods

2.1. Materials

A 33mer DNA template, 5'-CTCGATCGCTAACG-CTACCATCCGAATTCGCCC-3', containing a site-specific (+)-*trans-anti*-BPDE- N^2 -dG or (–)-*trans-anti*-BPDE- N^2 -dG adduct at the N^2 position of the underlined G was prepared as previously described [24–26]. The undamaged 33mer DNA template and the various DNA primers were synthesized via automated DNA phosphoramidite methods by Operon (Alameda, CA). Human Pol η was purified to near homogeneity as previously described [23]. Human Pol κ was purified to near homogeneity as a fusion protein containing the following peptide sequence at its N-terminus, MGSKNNQKSEPLIGRKKTGSKR-

RWKKNFIAVSAANRFKKISSSGALRS. This recombinant human Pol κ was expressed in *E. coli* cells and purified through a calmodulin affinity column (Stratagene, La Jolla, CA), a heparin Sepharose column (Amersham Pharmacia Biotech, Piscataway, NJ), and an FPLC Superdex 200 column (Amersham Pharmacia Biotech).

2.2. DNA polymerase 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 purified human Pol η . 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.

2.3. Kinetic analysis

Kinetic analysis of nucleotide insertion opposite benzo[*a*]pyrene DNA adducts or extension from opposite the lesion by human Pol η was performed as previously described [27,28]. Briefly, the assays were performed using 50 fmol of a primed DNA template, 0.8 ng (10 fmol) of purified Pol η , and increasing concentrations of dATP, dCTP, dTTP, or dGTP. The primer was labeled at its 5' end with ³²P. After incubation for 2–20 min at 30 °C under standard DNA polymerase assay conditions, reaction products were separated by electrophoresis on a 20% denaturing polyacrylamide gel. The percentage of primers extended by the polymerase was calculated following scanning densitometry of the extended DNA band(s) and the remaining primer band on the autoradiogram. Product formed (*P*) was derived from the calculation: *P* = percent of primer extension \times 50 fmol. The observed velocity (*v*) was obtained from the calculation: *v* = *P*/incubation time in min. Then, the observed velocity was plotted as a function of dNTP concentration. The plotted data was fitted by a nonlinear regression curve to the Michaelis–Menton equation:

$$v = (V_{\max} \times [\text{dNTP}]) / (K_m + [\text{dNTP}]),$$

using SigmaPlot software. *V*_{max} and *K*_m values for the insertion of the correct and the incorrect nucleotides were obtained from the fitted curves. The mis-incorporation error rate was calculated from the equation: *f*_{inc} = (*V*_{max}/*K*_m)_{incorrect} / (*V*_{max}/*K*_m)_{correct}.

3. Results

3.1. Effect of the (+)- and (–)-*trans-anti-BPDE-N*²-dG adducts on DNA synthesis by human Pol η

To examine the effect of a (+)- or (–)-*trans-anti-BPDE-N*²-dG adduct on DNA synthesis by human Pol η , we labeled a 19mer primer with ³²P at its 5' end and annealed it to the damaged DNA template right before the lesion (Fig. 1). DNA polymerase assays were then performed with increasing concentrations of purified human Pol η . As shown in Fig. 1, hu-

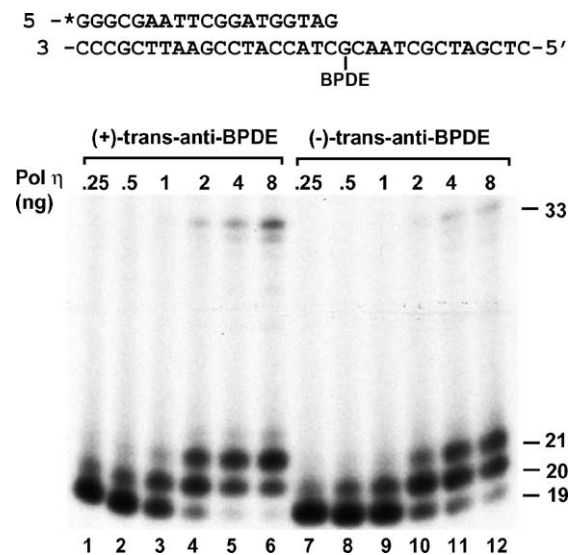


Fig. 1. DNA synthesis of human Pol η from templates containing a (+)- or (–)-*trans-anti-BPDE-N*²-dG adduct. A 19mer primer was labeled with ³²P (asterisk) at its 5' end and annealed right before the lesion as shown at the top. DNA synthesis assays were performed at 30 °C for 10 min with 0.25–8 ng (3.2–103 fmol) of purified human Pol η as indicated, using 50 fmol DNA substrate. DNA size markers in nucleotides are indicated on the right.

man Pol η inserted one nucleotide opposite the lesion in a protein concentration-dependent manner. However, translesion synthesis largely stopped opposite the lesion and at one nucleotide downstream of the lesion, as evidenced by the accumulation of the 19mer and the 20mer DNA bands, respectively (Fig. 1). Only a small fraction of the damaged DNA templates was copied by human Pol η to the end (Fig. 1). Bypass of the (+)-*trans-anti*-BPDE-*N*²-dG adduct by human Pol η was more efficient than that of the (-)-*trans-anti*-BPDE-*N*²-dG as indicated by the yield of the 31–33mer DNA bands (Fig. 1). These results show that human Pol η can effectively insert a nucleotide opposite a template (+)- and (-)-*trans-anti*-BPDE-*N*²-dG adduct, but the subsequent extension synthesis is strongly blocked by both lesions.

3.2. Fidelity of human Pol η for nucleotide insertion opposite the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG DNA adducts

To identify the nucleotide inserted opposite the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG adducts, we performed DNA polymerase assays with only one deoxyribonucleoside triphosphate, using the damaged templates containing a ³²P-labeled 19mer primer right before the lesion (Fig. 2). The undamaged template G was also examined as a control. As expected, human Pol η predominantly inserted the correct C opposite the undamaged template G (Fig. 2, lane 3). Less frequently, T and A mis-insertions were also observed (Fig. 2, lanes 2 and 4), consistent with the low fidelity DNA synthesis of this polymerase [29]. In contrast, human Pol η most frequently inserted A opposite

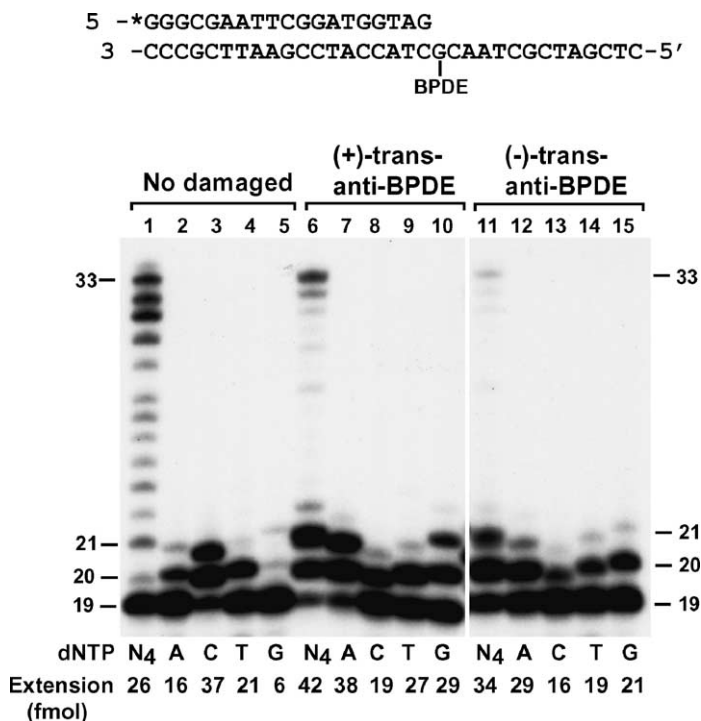


Fig. 2. Nucleotide insertion by human Pol η opposite the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG DNA adducts. A 19mer primer was labeled with ³²P (asterisk) at its 5' end and annealed right before the lesion as shown at the top. Polymerase reactions were performed with 0.2 ng (2.6 fmol) (lanes 1–5) or 2 ng (26 fmol) (lanes 6–15) of purified human Pol η in the presence of a single dATP (A), dCTP (C), dTTP (T), or dGTP (G), or all four dNTPs (N₄). Lanes 1–5, control experiments with undamaged template of the same sequence. Quantitation of extended primers is shown below the gel. DNA size markers in nucleotides are indicated on the sides.

Table 1

Kinetic measurement of nucleotide insertion opposite (+)- and (-)-*trans-anti*-BPDE-*N*²-dG adducts by human Polη

dNTP	V_{\max} (fmol/min; mean \pm S.D.)	K_m (μ M; mean \pm S.D.)	V_{\max}/K_m	f_{inc}^a
Undamaged G				
dATP	4.08 \pm 0.53	75.5 \pm 28.1	0.054	1.4×10^{-3}
dCTP	12.8 \pm 0.51	0.34 \pm 0.06	37.6	1
dTTP	7.62 \pm 0.58	53.3 \pm 12.4	0.14	3.8×10^{-3}
dGTP	0.26 \pm 0.03	9.32 \pm 3.19	0.028	7.4×10^{-4}
(+)- <i>trans-anti</i> -BPDE- <i>N</i> ² -dG				
dATP	2.62 \pm 0.13	6.62 \pm 1.28	0.40	1.9×10^0
dCTP	1.95 \pm 0.04	9.21 \pm 1.26	0.21	1
dTTP	2.93 \pm 0.18	36.3 \pm 9.36	0.081	3.9×10^{-1}
dGTP	2.13 \pm 0.09	16.6 \pm 2.90	0.13	6.2×10^{-1}
(-)- <i>trans-anti</i> -BPDE- <i>N</i> ² -dG				
dATP	3.46 \pm 0.37	43.1 \pm 14.8	0.080	3.6×10^0
dCTP	2.10 \pm 0.03	95.5 \pm 6.60	0.022	1
dTTP	2.85 \pm 0.12	184 \pm 31.0	0.015	6.8×10^{-1}
dGTP	1.99 \pm 0.12	51.6 \pm 9.20	0.039	1.8×10^0

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

either the (+)- or (-)-*trans-anti*-BPDE-*N*²-dG adduct (Fig. 2, lanes 7 and 12). Less frequently, G, T, and C were also inserted by human Polη opposite both lesions (Fig. 2, lanes 8–10 and 13–15).

To quantitatively determine the fidelity of nucleotide insertion by human Polη opposite the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG DNA adducts, we performed kinetic measurements using a previously described method [27]. The efficiency of nucleotide insertion is indicated by V_{\max}/K_m , and the mis-insertion frequency is determined by f_{inc} , which is calculated as $(V_{\max}/K_m)_{\text{incorrect}} / (V_{\max}/K_m)_{\text{correct}}$. As shown in Table 1, human Polη most efficiently inserted A opposite the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG DNA adducts. The lesions also dramatically elevated G and T mis-insertion error rates (Table 1). Nucleotide insertion of human Polη was five-fold (0.40/0.08) more efficient opposite the (+)-*trans-anti*-BPDE-*N*²-dG adduct than opposite the (-)-*trans-anti*-BPDE-*N*²-dG adduct (Table 1). Both lesions significantly reduced nucleotide insertion activity of human Polη, as indicated by a large reduction of the V_{\max}/K_m values as compared to the V_{\max}/K_m value for C insertion opposite the undamaged template G. These results show that human Polη performs error-prone nucleotide insertion opposite the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG adducts.

3.3. Extension DNA synthesis by human Polη from opposite the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG adducts

Lesion bypass consists of two steps: (a) nucleotide insertion opposite the lesion; and (b) extension DNA synthesis from opposite the lesion. To further examine extension synthesis by human Polη from opposite a template (+)- or (-)-*trans-anti*-BPDE-*N*²-dG adduct, we labeled four 20mer primers with ³²P at their 5' ends and separately annealed each to the damaged DNA template (Fig. 3). The four primers ended with a C, A, T, or G, respectively, opposite the lesion (Fig. 3). DNA synthesis assays were then performed with purified human Polη at a high enzyme concentration (128 fmol). Opposite the (+)-*trans-anti*-BPDE-*N*²-dG adduct, significant extension synthesis was detected when the primer 3' end was either an A or a G opposite the lesion (Fig. 3, lanes 1 and 4). However, significant amount of DNA synthesis stopped after extending only one nucleotide from opposite the lesion, as evidenced by the accumulation of the 21mer DNA band (Fig. 3, lanes 1 and 4). DNA synthesis from the T-terminated primer largely stopped after extending one nucleotide from opposite the lesion (Fig. 3, lane 3). Remarkably, extension DNA synthesis from the primer 3' C opposite the lesion was the least efficient

5 -*GGGCGAATTCGGATGGTAGX
 3 -CCCCTTAAGCCTACCATCGCAATCGCTAGCTC-5'
 BPDE

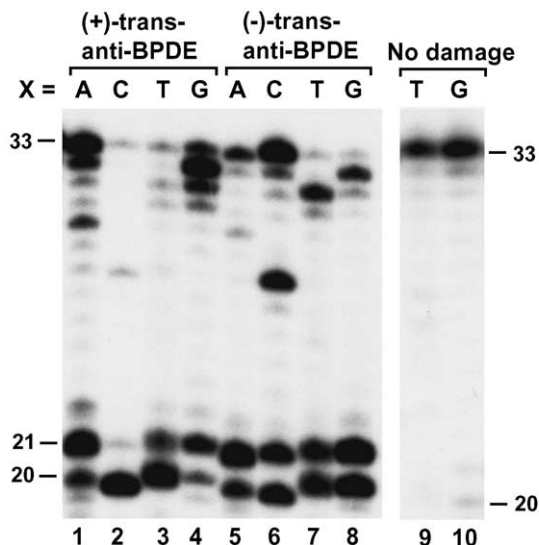


Fig. 3. Extension DNA synthesis of human Pol η from opposite the (+)- and (-)-*trans-anti-BPDE-N²-dG* DNA adducts. Four 20mer ³²P-labeled primers were separately annealed to the damaged DNA template with the primer 3' end opposite the lesion as shown at the top. Each primer differed only at the 3' end as indicated by X. DNA synthesis assays were then performed with 10 ng (128 fmol) of purified human Pol η in the presence of a single dATP (A), dCTP (C), dTTP (T), or dGTP (G), or all four dNTPs (N₄). Lanes 9–10, control experiments with undamaged template of the same sequence. DNA size markers in nucleotides are indicated on the sides.

and was mostly refractory to extension by human Pol η (Fig. 3, lane 2).

Opposite the (-)-*trans-anti-BPDE-N²-dG* adduct, extension synthesis by human Pol η was observed with all four DNA primers (Fig. 3, lanes 5–8), although the most efficient extension was detected when the primer 3' C was paired with the lesion. Again, strong synthesis stop was observed after extending only one nucleotide from the lesion, as indicated by the prominent 21mer DNA band (Fig. 3, lanes 5–8). These results indicate that extension DNA synthesis by human Pol η from opposite the (+)- and (-)-*trans-anti-BPDE-N²-dG* adducts is significantly affected by the adduct stereochemistry and by the nucleotide opposite the lesion, and suggest that ex-

tension beyond the undamaged template base 5' to the lesion is a rate limiting step.

3.4. Specificity of nucleotide insertion by human Pol η during extension synthesis from opposite the (+)- and (-)-*trans-anti-BPDE-N²-dG* adducts

Among the products beyond one nucleotide extension, the major extension product from the G-terminated primer was one nucleotide shorter (Fig. 3, lanes 4 and 8). The major extension product from the T-terminated primer opposite the (-)-*trans-anti-BPDE-N²-dG* adduct was two nucleotides shorter (Fig. 3, lane 7). Additionally, a strong DNA band six nucleotides shorter was formed during extension from the C-terminated primer opposite the (-)-*trans-anti-BPDE-N²-dG* adduct (Fig. 3, lane 6), and a significant DNA band four nucleotides shorter was observed from extending the A-terminated primer opposite the (+)-*trans-anti-BPDE-N²-dG* adduct (Fig. 3, lane 1). These products could be explained by a mechanism of primer–template realignment prior to DNA synthesis, due to base-pairing between the primer 3' end and the complementary template base downstream. Consequently, -4, -6, -2, and -1 deletion products were generated (Fig. 3, lanes 1, 6–8, respectively). This primer–template realignment mechanism would predict that the nucleotide added to the primer 3' end is determined by the next template base following the realignment, not by the undamaged template C 5' to the lesion. To test this prediction, we identified the nucleotide that extended the primer from opposite the lesion.

Extension synthesis by human Pol η from opposite the lesion was assayed in the presence of only one deoxyribonucleoside triphosphate at a time (Fig. 4). When A was opposite the lesion, all four nucleotides were inserted, although A and G were inserted more efficiently than T and C (Fig. 4A). When C was opposite the lesion, G was predominantly inserted (Fig. 4B), consistent with the mechanisms of extension without deletion and extension with -6 primer–template realignment. When G was opposite the lesion, T was predominantly inserted (Fig. 4D), consistent with the predominant mechanism of -1 primer–template realignment. When T was opposite the (-)-*trans-anti-BPDE-N²-dG* adduct, T was predominantly inserted (Fig. 4C, lanes 6–10),

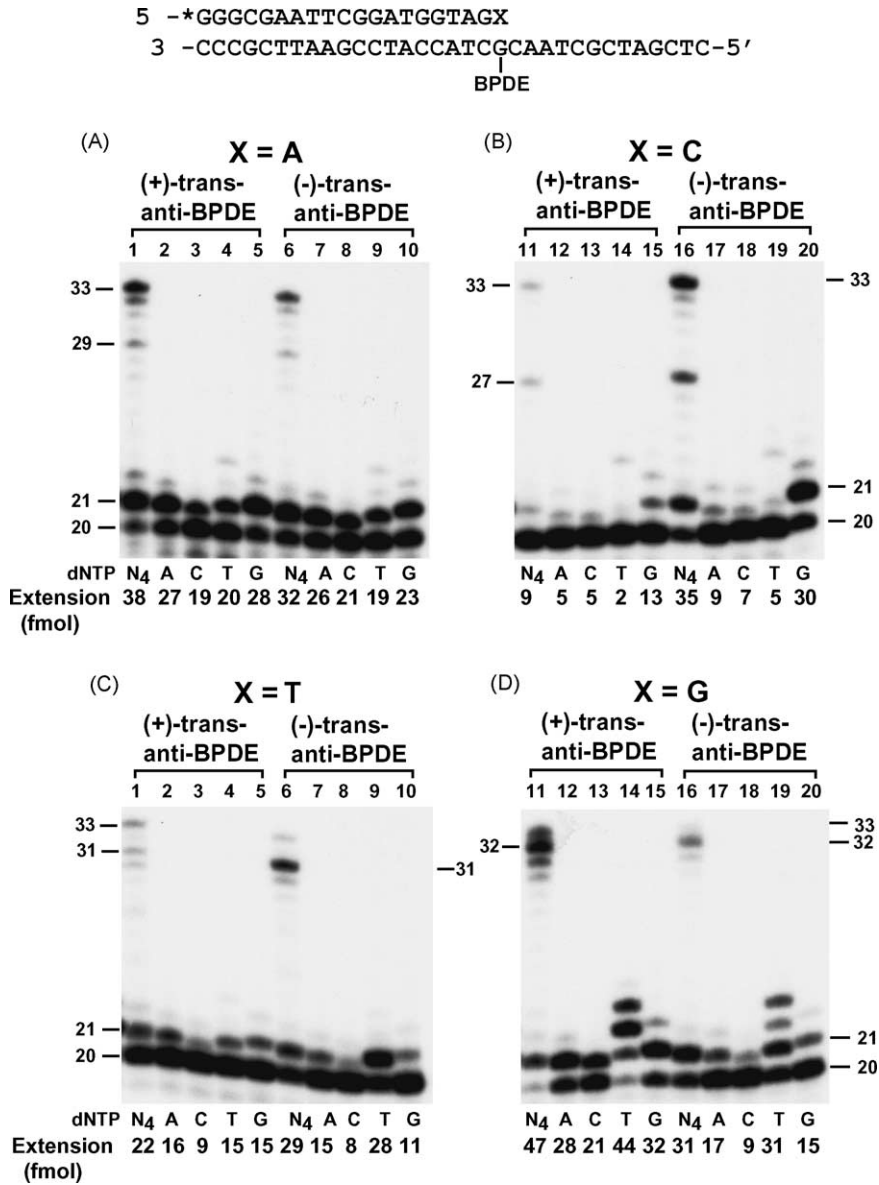


Fig. 4. Nucleotide insertion by human Pol η opposite the 5' undamaged template base during +1 extension synthesis. Four 20mer ³²P-labeled primers were separately annealed to the DNA template with the primer 3' end (X) opposite a (+)- or (-)-*trans-anti*-BPDE-*N*²-dG adduct as shown at the top. Each primer differed only at the 3' end. DNA synthesis assays were then performed with 5 ng (64 fmol) of purified human Pol η in the presence of a single dATP (A), dCTP (C), dTTP (T), or dGTP (G), or all four dNTPs (N₄). (A) A at the primer 3' end; (B) C at the primer 3' end; (C) T at the primer 3' end; and (D) G at the primer 3' end. Quantitation of extended primers is shown below the gels. DNA size markers in nucleotides are indicated on the sides.

consistent with the predominant mechanism of -2 primer-template realignment. These results suggest that the extension mechanisms from opposite the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG adducts are signif-

icantly affected by the sequence context 5' to the lesion.

To quantitatively determine the effect of the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG adducts on the

Table 2

Kinetic measurement of extension DNA synthesis by human Pol η from the mis-paired primer 3' A opposite (+)- and (-)-*trans-anti*-BPDE- N^2 -dG adducts

dNTP	V_{\max} (fmol/min; mean \pm S.D.)	K_m (μ M; mean \pm S.D.)	V_{\max}/K_m	f_{ext}^a
Undamaged G				
dATP	2.71 \pm 0.09	26.8 \pm 3.19	0.10	1.9 $\times 10^{-2}$
dCTP	2.07 \pm 0.11	21.6 \pm 4.36	0.096	1.8 $\times 10^{-2}$
dTTP	1.51 \pm 0.04	40.7 \pm 3.60	0.037	6.9 $\times 10^{-3}$
dGTP	3.50 \pm 0.05	0.65 \pm 0.04	5.38	1
(+)- <i>trans-anti</i> -BPDE- N^2 -dG				
dATP	2.14 \pm 0.04	37.9 \pm 2.70	0.056	7.3 $\times 10^{-1}$
dCTP	1.54 \pm 0.15	91.0 \pm 32.6	0.017	2.2 $\times 10^{-1}$
dTTP	2.01 \pm 0.07	132 \pm 19.4	0.015	1.9 $\times 10^{-1}$
dGTP	1.82 \pm 0.03	23.6 \pm 1.94	0.077	1
(-)- <i>trans-anti</i> -BPDE- N^2 -dG				
dATP	1.80 \pm 0.03	26.8 \pm 1.92	0.067	5.6 $\times 10^{-1}$
dCTP	2.00 \pm 0.04	90.1 \pm 7.32	0.022	1.8 $\times 10^{-1}$
dTTP	1.35 \pm 0.11	75.3 \pm 17.4	0.018	1.5 $\times 10^{-1}$
dGTP	1.10 \pm 0.12	8.98 \pm 2.40	0.12	1

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

fidelity of nucleotide insertion during extension from the primer 3' A opposite the lesion, we performed kinetic analysis of the extension reaction. As shown in Table 2, while nucleotide insertion during extension from the mis-paired primer 3' A followed the order G > A > C > T with or without the lesion, the presence of the lesion increased the mis-insertion error rates of human Pol η by more than 10-fold. The lesions also greatly reduced the efficiency of extension synthesis by human Pol η , as indicated by the V_{\max}/K_m values (Table 2).

During extension synthesis from the primer 3' A opposite the lesion, the discrimination by human Pol η between the correct G and the incorrect A was less than two-fold (Table 2). To determine which insertion would lead to a better substrate for further extension synthesis, we first extended the primer 3' A by one nucleotide from opposite the lesion with human Pol η in the presence of 50 μ M dATP or dGTP. After 10 min reaction, all four dNTPs were then added and the DNA syntheses were continued for another 10 min. As shown in Fig. 5, extension synthesis beyond the undamaged template C 5' to the lesion was more efficient when the correct G had been inserted first, regardless of the stereochemistry of the lesion (Fig. 5, lanes 2 and 4).

Taking into account of nucleotide insertion opposite the lesion (Table 1), the +1 extension (Table 2), and

+2 extension (Fig. 5), we conclude that the fully bypassed products by human Pol η in Fig. 1 (the 33mer DNA) mainly resulted from AG incorporations opposite the template 3'-G^{BPDE}C sequence.

3.5. Bypass of the (+)- and (-)-*trans-anti*-BPDE- N^2 -dG DNA adducts by the sequential actions of Pol η insertion and Pol κ extension

Human Pol η alone was inefficient in bypassing the (+)- and (-)-*trans-anti*-BPDE- N^2 -dG DNA adducts, as DNA synthesis mostly stopped opposite the lesion or one nucleotide beyond the lesion (Fig. 1). Therefore, if human Pol η indeed participates in the bypass of the (+)- and (-)-*trans-anti*-BPDE- N^2 -dG adducts in cells, the extension synthesis step would require another DNA polymerase. To test whether a replication polymerase such as Pol δ could perform extension synthesis during lesion bypass, we separately annealed six primers to the damaged template, with the primer 3' ends extending one to six nucleotides, respectively, beyond the lesion (Fig. 6). The purified catalytic subunit of yeast Pol δ was then added under standard DNA synthesis conditions. As shown in Fig. 6 (lanes 1–9), when the primer 3' end was annealed one to three nucleotides beyond the (+)- or (-)-*trans-anti*-BPDE- N^2 -dG adduct, no extension was observed. The primer was degraded from its 3' end by

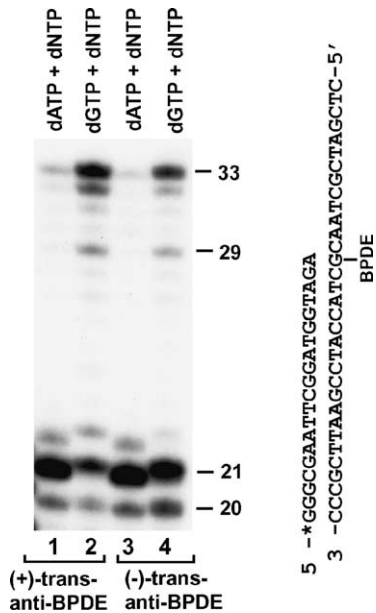


Fig. 5. Effect of +1 nucleotide incorporation on extension synthesis of +2 and beyond. A 20mer ³²P-labeled primer was annealed to the DNA template with the primer 3' A opposite a (+)- or (-)-*trans-anti*-BPDE-*N*²-dG adduct as shown on the right. The primer was then extended with 10 ng (128 fmol) of purified human Polη by one nucleotide in the presence of 50 μM dATP (lanes 1 and 3) or dGTP (lanes 2 and 4). After 10 min reaction at 30 °C, 50 μM of all four dNTPs were added to the reaction for further extension synthesis. DNA size markers in nucleotides are indicated on the right.

the 3' → 5' proofreading exonuclease activity of Polδ to a position right before the lesion (the 19mer DNA band). With the primer 3' end annealed further downstream of the lesion, decreased proofreading activity was observed. Concomitantly, increasing polymerase activity was detected (Fig. 6, lanes 10–15). The proofreading activity of Polδ became inactive only when the primer 3' end was at least six nucleotides beyond the lesion (Fig. 6, lanes 16–18). These results show that Polδ cannot perform extension synthesis during lesion bypass. Thus, a specialized DNA polymerase is required to carry out the extension synthesis step following nucleotide insertion by Polη opposite the (+)- or (-)-*trans-anti*-BPDE-*N*²-dG adduct.

Polκ is a candidate for performing extension synthesis following Polη action in response to the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG adducts [18,20]. To test this possibility, we performed lesion bypass ex-

periments with the combined activities of purified human Polη and human Polκ. Following a 10 min reaction with Polη (51 fmol) opposite the (+)- or (-)-*trans-anti*-BPDE-*N*²-dG adduct (50 fmol), Polκ (50 fmol) was added and the reaction was continued for another 10 min. Even after 20 min, the majority of DNA synthesis by Polη remained opposite (the 20mer DNA band) and one nucleotide beyond the lesion (the 21mer DNA band) (Fig. 7, lanes 1 and 3). Human Polκ, however, fully extended 52% and 70% of the Polη-aborted 20mer and 21mer DNA bands from the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG templates, respectively (Fig. 7, lanes 2 and 4). These results show that the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG DNA adducts can be effectively bypassed by the sequential actions of Polη insertion and Polκ extension in vitro.

4. Discussion

We have demonstrated two-step error-prone bypass of the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG DNA adducts by the sequential actions of Polη insertion and Polκ extension in vitro. Human Polη prefers A incorporation opposite the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG DNA adducts, and is additionally highly prone to G and T mis-insertions. Most recently, Chiapperino et al. [30] also observed a similar pattern of error-prone nucleotide insertion with human Polη opposite these two DNA lesions in two sequence contexts different from that in our studies.

The (+)- and (-)-*trans-anti*-BPDE-*N*²-dG adducts differ only in the stereochemistry [2,31]. Not surprisingly, the response of human Polη to the template (-)-*trans-anti*-BPDE-*N*²-dG adduct is similar to its response to the (+)-*trans-anti*-BPDE-*N*²-dG adduct. That is, error-prone DNA synthesis is largely aborted opposite the lesion or after only one nucleotide extension from opposite the lesion. On the other hand, NMR studies of the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG adducts in a 13mer DNA containing a 9mer primer annealed right before the lesion clearly indicate that the conformations of these two adducts can be quite different at the template–primer junction [31,32]. Although whether and how these conformations are modulated by the polymerase is unknown, the different adduct orientations may provide a structural basis for the observed effect of the adduct

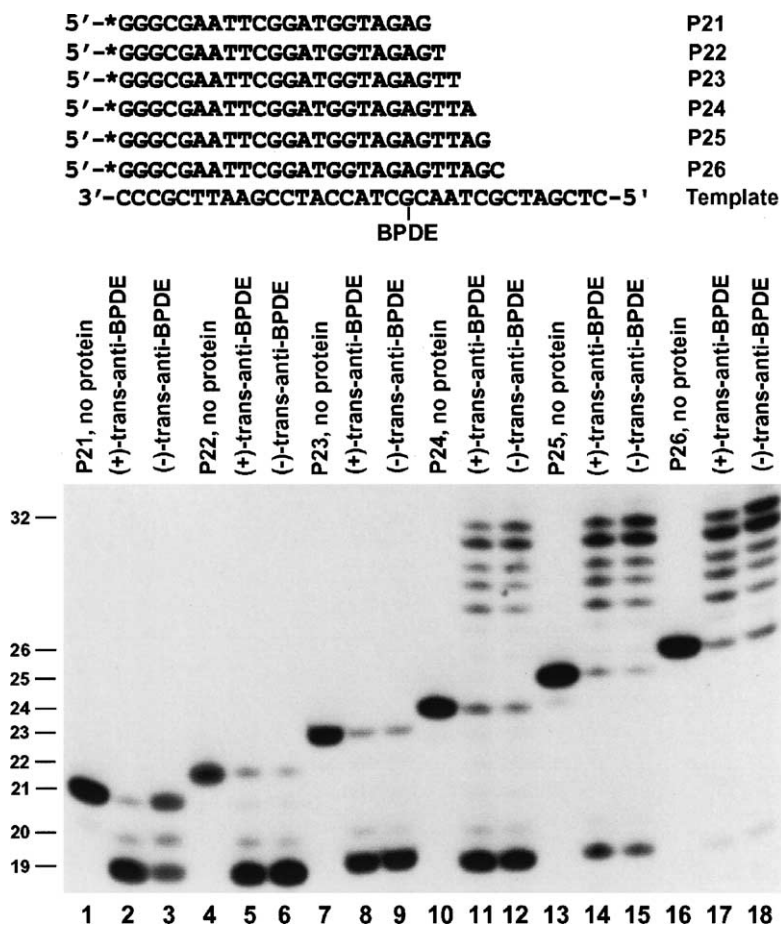


Fig. 6. Response of Pol δ to primers with the 3' ends annealed at various positions beyond the template (+)- or (-)-*trans-anti*-BPDE- N^2 -dG adduct. Six 32 P-labeled primers were separately annealed to the damaged DNA template, with the primer 3' end extending one to six nucleotides beyond the lesion as shown at the top. The purified catalytic subunit of yeast Pol δ (3 ng, 24 fmol) was then incubated with the DNA substrates under standard DNA synthesis conditions in the presence of all four dNTPs. Reactions were at 30 °C for 10 min. DNA size markers in nucleotides are indicated on the left.

stereochemistry on nucleotide insertion and +1 extension by human Pol η . Thus, human Pol η is more efficient in inserting a nucleotide opposite the (+)-*trans-anti*-BPDE- N^2 -dG adduct than opposite its (-)-stereoisomeric adduct. Human Pol η extends the purine-terminated primers more efficiently from opposite the (+)-*trans-anti*-BPDE- N^2 -dG adduct, but extends the C-terminated primer more efficiently from opposite the (-)-*trans-anti*-BPDE- N^2 -dG adduct, at least at the sequence context examined.

Structures of the (+)- and (-)-*trans-anti*-BPDE- N^2 -dG adducts are important to understand the interaction between the adduct and the polymerase

during translesion synthesis. However, the structure alone cannot predict the outcome of translesion synthesis, thus the outcome of mutagenesis. This is most dramatically demonstrated by the response of human Pol η and human Pol κ to the (+)- and (-)-*trans-anti*-BPDE- N^2 -dG adducts. Whereas Pol η predominantly inserts an A opposite the lesions leading to a mutagenic outcome, Pol κ predominantly inserts a C opposite the lesions leading to error-free outcome. It is therefore the unique interaction between the lesion and the specific polymerase that determines the outcome of the translesion synthesis. This underscores the biological significance of

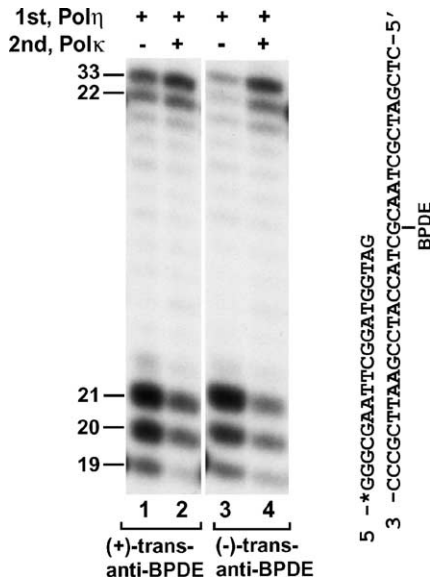


Fig. 7. Lesion bypass of the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG adducts by the combined activities of human Polη and human Polκ. A 19mer ³²P-labeled primer was annealed to the DNA template right before the lesion as shown on the right. Standard DNA polymerase assays were performed with 4 ng (51 fmol) of purified human Polη in the presence of all four dNTPs. After 10 min reaction at 30 °C, 5 ng (50 fmol) of purified human Polκ was then added to the reaction and the incubation continued for another 10 min. Lanes 1 and 3 show reactions with human Polη alone for 20 min at 30 °C. DNA size markers in nucleotides are indicated on the left.

evolving multiple DNA polymerases, notably the Y family of polymerases [13], in responding to various DNA damage during translesion synthesis.

So far, Polκ and Polη are the most efficient polymerases for nucleotide insertion opposite the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG adducts in eukaryotes [19,20,23,33]. The purified yeast Polζ is very inefficient in responding to these two lesions [33]. Since Polκ alone is largely error-free in bypassing these lesions [19,20,33], it is likely that Polη may contribute to error-prone bypass, thus mutagenesis, of the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG adducts in cells. If so, effective DNA synthesis beyond the +1 extension would involve another DNA polymerase. Such extension synthesis could not be served by the replication polymerases Polδ and Pole, since their 3' → 5' proofreading activity would degrade the primer back to a position before the lesion. Indeed,

this proofreading activity of Polδ was observed even when the primer 3' end was annealed five nucleotides beyond the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG DNA adducts (Fig. 6). Therefore, it is most likely that replication cannot resume until the primer is extended at least several nucleotides beyond the lesion by a lesion bypass polymerase. Recently, an extension synthesis activity has been observed with human Polκ [18,20,34]. Indeed, by combining the Polη insertion activity with the Polκ extension activity, the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG DNA adducts were effectively bypassed in vitro according to the two-polymerase two-step mechanism. Since Polη insertion opposite these two lesions is error-prone, the bypass by the two-step reactions of Polη and Polκ is also error-prone, thus mutagenic. Consistent with the preferential A insertion by human Polη opposite the lesions, the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG DNA adducts induced predominantly G → T mutations in simian kidney cells at the sequence contexts examined [3,4].

We observed that the (-)-*trans-anti*-BPDE-*N*²-dG DNA adduct was bypassed more efficiently by human Polκ in an error-free manner [20], whereas the (+)-*trans-anti*-BPDE-*N*²-dG DNA adduct was responded to by human Polη more efficiently in an error-prone manner (this study). If this observation is valid for most sequence contexts, the (+)-*trans-anti*-BPDE-*N*²-dG DNA adduct would be predicted to be more mutagenic than the (-)-*trans-anti*-BPDE-*N*²-dG adduct. Supporting this prediction, Moriya et al. [3] reported that the (+)-*trans-anti*-BPDE-*N*²-dG DNA adduct is indeed more mutagenic than the (-)-*trans-anti*-BPDE-*N*²-dG DNA adduct in a defined sequence context in simian kidney cells. Furthermore, it was also reported that the (+)-*anti*-benzo[*a*]pyrene diolepoxide is more mutagenic than the (-)-*anti*-benzo[*a*]pyrene diolepoxide in the hamster V79 cells [35].

At a high enzyme concentration, some extension DNA syntheses from opposite the (+)- and (-)-*trans-anti*-BPDE-*N*²-dG adducts were observed with human Polη. The extension mechanism, however, is influenced by the nucleotide residing opposite the lesion and the sequence context 5' to the lesion. When the nucleotide opposite the lesion is complementary to a template base downstream, primer–template realignment could occur prior to

DNA synthesis, resulting in deletion. This is most striking when the primer 3' G or T was opposite the (–)-*trans-anti*-BPDE-*N*²-dG adduct in the sequence context 3'-G^{BPDE}CAAT, which resulted in –1 and –2 deletions by human Polη, respectively. The primer–template realignment by human Polη was detected as far as six nucleotides away from the lesion. Such prominent primer–template realignment, however, was not observed with human Polη from mismatched primer end in the absence of the lesion (Fig. 3, lanes 9 and 10 and data not shown). Therefore, the realignment is likely a result of the strong inhibition on extension synthesis by the lesion and the ability of human Polη to search complementary template bases downstream of the lesion. A similar deletion mechanism during extension synthesis from opposite the BPDE adducts was also observed earlier by Zhuang et al. [36] with the Klenow fragment of the *E. coli* DNA polymerase I.

It has been demonstrated that Polη is capable of error-free translesion synthesis opposite a template *cis-syn* TT dimer and AAF-guanine adduct [14–16,37,38]. Masutani et al. [38] proposed that extension synthesis by human Polη from a correctly paired primer 3' end opposite the lesion, but not from a mismatched primer end, is a key mechanism to ensure accurate translesion synthesis. In this study, we surprisingly found that extension synthesis by human Polη from the primer 3' C opposite the (+)-*trans-anti*-BPDE-*N*²-dG adduct is much less efficient compared to that from the mismatched primer 3' A or G. Hence, it appears that there is not a strict rule governing the fidelity of translesion synthesis by human Polη at the extension synthesis step. Due to its capability of error-prone nucleotide insertion opposite some lesions such as the *trans-anti*-BPDE-*N*²-dG adducts and the 3' T of the TT (6–4) photoproduct [23], Polη may play an important role in mutagenesis induced by these lesions in cells. Indeed, recent genetic experiments indicate that Polη is involved in mutagenesis induced by TT photoproducts, likely the TT (6–4) photoproducts, in yeast cells [39]. Two-step error-prone bypass of the (+)- and (–)-*trans-anti*-BPDE-*N*²-dG DNA adducts provided a biochemical basis and revealed a potential mechanism for the possible involvement of Polη in benzo[*a*]pyrene-induced mutagenesis in human cells.

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