Lesion Bypass Activities of Human DNA Polymerase μ*

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DNA polymerase μ (Polμ) is a newly discovered member of the polymerase X family with unknown cellular function. The understanding of Polμ function should be facilitated by an understanding of its biochemical activities. By using purified human Polμ for biochemical analyses, we discovered the lesion bypass activities of this polymerase in response to several types of DNA damage. When it encountered a template 8-oxoguanine, abasic site, or 1,N\textsuperscript{6}-ethenoadenine, purified human Polμ efficiently bypassed the lesion. Even bulky DNA adducts such as N\textsuperscript{2}-acetylaminofluorene-adducted guanine, (+)- and (-)-trans-anti-benz[a]pyrene-N\textsuperscript{2}-dG were unable to block the polymerase activity of human Polμ. Bypass of these simple base damage and bulky adducts was predominantly achieved by human Polμ through a deletion mechanism. The Polμ specificity of nucleotide incorporation indicates that the deletion resulted from primer realignment before translesion synthesis. Purified human Polμ also effectively bypassed a template cis-syn TT dimer. However, this bypass was achieved in a mainly error-free manner with AA incorporation opposite the TT dimer. These results provide new insights into the biochemistry of human Polμ and show that efficient translesion synthesis activity is not strictly confined to the Y family polymerases.

DNA polymerase μ (Polμ) \cite{1} is a newly discovered member of the X family polymerases \cite{2,3}. Additional members in this family include Polδ, Polα, and terminal deoxynucleotidyltransferase \cite{1–3}. During base excision repair in higher eukaryotes, Polβ is a major repair synthesis polymerase \cite{4–6}. Terminal deoxynucleotidyltransferase catalyzes nucleotide additions to DNA in a template-independent manner \cite{7,8}. This enzyme functions during V(D)J recombination of the immunoglobulin genes and T-cell receptor genes and is restricted to lymphoid tissues \cite{7–9}. Cellular functions of Polα and Polμ have not been clearly defined.

Although the biochemical activities of the X family DNA polymerases appear to be quite diverse, all of the Y family DNA polymerases share a common biochemical activity: synthesis of opposite DNA lesions \cite{reviewed in Refs. 10–13}. In eukaryotes, the Y family consists of REV1 and DNA polymerases \(\eta, \zeta,\) and \(\kappa\) \cite{14}. Therefore, it is generally believed that a major function of the Y family DNA polymerases is to copy damaged sites of DNA during replication, a cellular process referred to as lesion bypass or translesion synthesis. Genetic studies indicate that REV1 \cite{15–18} and Pol\(\eta\) \cite{19–22} are involved in lesion bypass in cells. Lesion bypass can be error-free as a result of insertion of the correct nucleotide opposite the lesion or error-prone as the result of insertion of an incorrect nucleotide opposite the lesion. Both error-free and error-prone nucleotide insertions have been observed with the Y family polymerases depending on the specific lesion and the specific polymerase \cite{reviewed in Refs. 10–12}.

Biochemical studies of purified human Polμ have uncovered a unique property that has never been observed with any other polymerases studied so far \cite{23}. Human Polμ is highly prone to frameshift DNA synthesis \cite{23}. At single-nucleotide repeat sequences, DNA synthesis by human Polμ is mediated mainly by a deletion mechanism because of primer-template realignment before synthesis \cite{23}. Furthermore, when the primer 3’ end contains one or a few mismatches, human Polμ can promote primer-template realignment such that the primer 3’ end can find its complementary sequences on the template several nucleotides downstream, achieving microhomology search and microhomology pairing \cite{23}. These striking biochemical properties led Zhang et al. \cite{23} to propose that Polμ may be involved in nonhomologous end joining (NHEJ) for double-strand DNA repair. The biochemical properties of human Polμ ruled out a significant role for this polymerase in somatic hypermutation during immunoglobulin development.

One important cause of DNA double-strand breaks is DNA damage. It is conceivable that some damaged sites may contain clustered lesions or that base damage may be contained near some double-strand DNA breaks. Under those circumstances, Polμ would encounter DNA base damage while performing microhomology search and pairing, as well as DNA synthesis, during NHEJ. Hence, we asked whether Polμ is capable of translesion synthesis. In this report, we demonstrate that human Polμ indeed possesses efficient lesion bypass activities in response to very different types of DNA damage, ranging from simple base modifications and baseless sites to bulky chemical DNA adducts and cis-syn TT dimer of UV radiation. Although in vitro bypass of a template TT dimer is achieved by human Pol\(\alpha\) in an error-free manner, bypass of the other tested lesions is mediated by a deletion mechanism that effectively avoids copying the damaged template base through primer realignment. These findings provide new insights into the biochemistry of human Polμ and show that efficient translesion synthesis activity is not strictly confined to Y family polymerases.
Lesion Bypass by Human Polμ

EXPERIMENTAL PROCEDURES

Materials—Human Polμ, human Polγ, human Polβ, yeast Polα, and the catalytic subunit of yeast Polα were purified to near homogeneity as previously described (23–27). The Klenow fragment of Escherichia coli DNA polymerase I was purchased from Invitrogen. Oligonucleotides were synthesized by Operon (Alameda, CA). N-Acetoxy-N-2-acetylaminofluorene (the activated form of N-2-acetylaminofluorene (AAF)) was obtained from the Midwest Research Institute (Kansas City, MO).

DNA Templates Containing a Site-Specific Lesion—The 30-mer DNA template, 5'-GGATGCTGACGGATCCGGAGCCCGG-3', contained an 8-oxoguanine at the underlined G. Four 36-mer templates, 5'-GAAGGAGTCTTAAAGATCTGCCTGTCGCGG-3', contained a tetrahydrofuran (AP site analog) at the 5' position and A, T, G, or C at the 3' position. The 29-mer DNA template, 5'-CCATCGTTAATGCTCTTCGTTTCCGTCCG-3', contained a trans-anti tetrahydrofuran (AP site analog) at the underlined A. These damaged DNA templates were synthesized via automated DNA phosphoramidite methods by Operon. A 33-mer DNA template containing either a (+)-trans-anti-benzyljopryrene (BPDE)-N2-dG or a (+)-trans-anti-BPDE-N2-dG was prepared as described previously (28–30). Its sequence is 5'-CTCTGATCGTAACGCTTTTCTTCTTCCC-3', with the modified guanine underlined. A 30-mer DNA template containing an AAF-adducted guanine was prepared as previously described (31). Its sequence is 5'-CCCTCTTTAATAQGGTATACCTCTTCTTCTTCC-3', with the modified guanine underlined. A 49-mer DNA template containing a cis-syn TT dimer or a TT (6–4) photoproduct was prepared as previously described (32). Its sequence is 5'-AGCTTACCATGCCTGCACGAATTAAGCAATTCGTAATC-3', with the modified TT underlined.

DNA Polymerase Assays—A standard DNA polymerase reaction mixture (10 μl) contained 25 mM KH2PO4, pH 7.0, 5 mM MgCl2, 5 mM dithiothreitol, 100 μM/ml bovine serum albumin, 10% glycerol, 50 μM dNTPs (dATP, dCTP, dTTP, and dGTP individually or together as indicated), 50 fmol of an indicated DNA substrate containing a 32P-labeled 17-mer primer that terminated just before the underlined A. These damaged DNA templates were quantitated by scanning densitometry with the SigmaGel software (Sigma) for analysis.

RESULTS

Lesion Bypass of Simple Base Damage by Human Polμ—To determine whether a strong blocking lesion such as an AP site could block the frameshift synthesis of human Polμ, we annealed a 5'-32P-labeled 17-mer primer that terminated just before a template AP site and performed DNA synthesis assays with purified human Polμ. The template AP-T, in which the primer 3' A could pair with the template T 5' to the AP site by primer-template realignment, was examined first (Fig. 1A). Surprisingly, DNA synthesis was observed (Fig. 1A, lane 1). To determine which nucleotide was incorporated during translesion synthesis, DNA polymerase assays were performed in the presence of only one deoxyribonucleoside triphosphate at a time. As shown in Fig. 1A, lane 5, a G was incorporated. This result is consistent with realignment of the primer 3' A with the template T two nucleotides downstream before DNA synthesis, leading to G insertion opposite the next template C. To confirm this interpretation, purified human Polγ was used for extension DNA synthesis after a 10-min reaction of Polβ bypass of the AP-T template. Primer extension by Polγ alone from the undamaged DNA template was used as the control. The control reaction yielded the expected 36-mer DNA product (Fig. 1B, lane 3). In contrast, after DNA synthesis across from the AP site by human Polγ, Polγ extended the synthesis to the 34-mer DNA band (Fig. 1B, lane 1), indicating that a 2 deletion had occurred during DNA synthesis by human Polγ across from the AP site. To prove that this is a reliable method for analysis of DNA products of human Polμ, we digested the DNA with DpnII restriction endonuclease after 10-min translesion synthesis by Polμ and 10-min extension by Polγ. Indeed, the product of Polμ contained a 2 deletion (Fig. 1B, lane 2) as compared with the normal DNA synthesis of the control (Fig. 1B, lane 4). These results show that AP site bypass by human Polμ is mediated by a deletion mechanism as a result of primer realignment during translesion synthesis.

Lesion bypass by the deletion mechanism predicts that sequence context 5' to the lesion would significantly affect the specificity of nucleotide incorporation during translesion synthesis. When the template T 5' to the AP site was replaced by an A (AP-A template), T was also incorporated by human Polμ in addition to G incorporation (Fig. 1C, lanes 1–5). With a template G 5' to the AP site (AP-G template), C and G were preferentially incorporated (Fig. 1C, lanes 6–10). With a template C 5' to the AP site (AP-C template), only G was incorporated (Fig. 1C, lanes 11–15). These results were precisely predicted by −1 and −2 deletions as a consequence of a shift of the primer 3' end downstream by 1 or 2 nucleotides, respectively, by human Polμ before DNA synthesis.

To determine whether the unexpected lesion bypass activity of human Polμ is limited to an AP site, we analyzed two more examples of simple base damage in the template: 8-oxoguanine and 1,N2-ethenoadenine. The 8-oxoguanine template contained a 5'-32P-labeled 17-mer primer that terminated just before the lesion (Fig. 2A). As shown in Fig. 2A, lane 1, human Polμ performed translesion synthesis and predominantly incorporated
lead to primer realignment during translesion synthesis, which would indeed occurred, the products were extended by purified hu-
DNA synthesis assays were performed with 3 ng (53 fmol) of purified human Polα in the presence of a single dATP (A), dCTP (C), dTTP (T), or dGTP (G) or all four dNTPs (N). B, DNA synthesis was initiated with purified human Polα (3 ng) at 30 °C for 10 min. Next, purified human Polη (2 ng, 25 fmol) was added to the reaction, and the incubation was continued for another 10 min at 30 °C (lane 2). Lane 1, DNA synthesis by human Polη alone; lane 3, DNA synthesis by human Polη (2 ng) alone. DNA size markers in nucleotides are indicated on the sides.

T (Fig. 2A, lane 4). This result is predicted by the mechanism of primer realignment during translesion synthesis, which would lead to −1 deletion. To confirm that −1 frameshift synthesis indeed occurred, the products were extended by purified hu-
DNA synthesis was initiated with purified human Polα (3 ng) at 30 °C for 10 min. Next, purified human Polη (2 ng, 25 fmol) was added to the reaction, and the incubation was continued for another 10 min at 30 °C (lane 2). Lane 1, DNA synthesis by human Polη alone; lane 3, DNA synthesis by human Polη (2 ng) alone. DNA size markers in nucleotides are indicated on the sides.

The 1,N²-ethenoadenine template contained a 5',32P-labeled 20-mer primer that terminated just before the lesion (Fig. 3). Purified human Polη synthesized DNA from both the damaged and the undamaged templates (Fig. 3A, lanes 1 and 6). However, the specificity of nucleotide incorporation was quite different. From the undamaged template, Polη incorporated nucleotides in the order of T>G>C (Fig. 3A, lanes 2–5). This specificity is consistent with T incorporation opposite the template A, −2 template shift, and −3 template shift, respectively, as predicted by the unique Polη property of highly frequent frameshift DNA synthesis (23). From the damaged template, G was preferentially incorporated (Fig. 3, lane 10). To determine whether deletion had occurred during translesion synthesis, the products were extended by the purified catalytic subunit of yeast Polα. Polα was chosen for extension synthesis because of its better activity in copying the last nucleotide of the template 5’ end (Fig. 3B, lane 2). Indeed, the lesion bypass products of human Polη contained −1 and −2 deletions, respectively (Fig. 3B, lane 1), as compared with the normal synthesis control of Polα alone (Fig. 3B, lane 2). The −2 deletion is consistent with a shift of the primer 3’ end downstream by two nucleotides by Polη before DNA synthesis. The −1 deletion may have resulted from C misincorporation opposite the lesion before shift of the primer 3’ end downstream by one nucleotide. Supporting this interpretation, Polη significantly incorporated C in response to the template 1,N²-ethenoadenine (Fig. 3A, lane 8). These results show that human Polη efficiently bypasses a template 1,N²-ethenoadenine by a deletion mechanism.

**Lesion Bypass of Bulky DNA Adducts by Human Polη**—To determine whether human Polη can respond to bulky adducts in DNA, we examined synthesis from a template containing an AAP-adducted guanine and a (+) or (−)-trans-anti-BPDE-N²-dG adduct. The AAP-damaged template contained a 5',32P-labeled 17-mer primer that terminated just before the lesion (Fig. 4). As shown in Fig. 4A, DNA synthesis from the damaged template was observed (Fig. 4A, lane 1), and G was most frequently incorporated by human Polη (Fig. 4A, lane 5). Less frequently, A was also incorporated, and C was rarely incorporated (Fig. 4A, lanes 2 and 3). G incorporation is consistent with a −1 deletion mechanism resulting from a shift of the primer 3’ end downstream by one nucleotide and copying of the undamaged template C 5’ to the lesion. A incorporation is consistent with a −2 template shift synthesis and copying of the undamaged template T two nucleotides downstream. Supporting this conclusion, Polη bypass followed by Polη extension resulted in bypass products of 28-mer and 29-mer DNA bands, as compared with the 29-mer and 30-mer bypassed DNA bands by Polη alone (Fig. 4B, compare lanes 2 and 3).

For DNA synthesis from templates containing the (+)- and (−)-trans-anti-BPDE-N²-dG adducts, a 32P-labeled 19-mer primer that terminated right before the lesion was annealed. As shown in Fig. 5, lanes 1–5, human Polη predominantly incorporated C opposite the undamaged template G. Minor T incorporation was also observed, probably as a result of realignment of the primer 3’ G to pair with the template C two nucleotides downstream (−2 template shift). In the presence of
the BPDE adducts, DNA synthesis was observed, although Polɛ was more active on the template containing the (+)-trans-anti-BPDE-N²-dG adduct (Fig. 5A, lanes 6 and 11). In contrast to the undamaged template, C incorporation was barely detectable, whereas T incorporation became predominant during bypass of the BPDE adducts (Fig. 5A, lanes 6–15). These results are precisely predicted by the −2 deletion mechanism that resulted from a shift of the primer 3′ end downstream by two nucleotides before DNA synthesis. The bypass products of human Polɛ indeed contained −2 deletion as analyzed by the Klenow extension after Polɛ translesion synthesis (Fig. 5B, compare lanes 1 and 3 with lanes 2 and 4). The Klenow DNA polymerase was chosen for extension synthesis because of its better activity in copying the last nucleotide of the template 5′ end (Fig. 5B, lanes 2 and 4).

**Accurate Translesion Synthesis by Human Polɛ Opposite a Template cis-syn TT Dimer—Cyclobutane pyrimidine dimers and (6–4) photoproducts are the major DNA lesions of UV radiation.** The cis-syn TT dimer is a widely studied cyclobutane pyrimidine dimer. To determine whether human Polɛ is able to bypass a cis-syn TT dimer or a TT (6–4) photoproduct, we annealed a 5′-³²P-labeled 15-mer primer to the template; the primer 3′ end terminated just before the lesion (Fig. 6). Next, DNA synthesis assays were performed. A higher Polɛ concentration (15 ng, 263 fmol) was needed to achieve DNA synthesis of four nucleotides or longer from the undamaged template at this sequence context (Fig. 6, A and B, lane 1). Purified human Polɛ was unable to bypass the template TT (6–4) photoproduct (Fig. 6A, lane 3). In contrast, Polɛ catalyzed DNA synthesis to a similar extent in the absence or presence of the template TT dimer (Fig. 6, A, lanes 1 and 2, and B, lanes 1 and 6). With the undamaged template, AA was incorporated by Polɛ opposite the template TT sequence (Fig. 6B, lane 2). T was also significantly incorporated, probably as a result of −2 frameshift synthesis (Fig. 6B, lane 4). With the damaged template, surprisingly, T incorporation was barely detectable (Fig. 6B, lane 9). Instead, AA was predominantly incorporated during translesion synthesis across from the TT dimer (Fig. 6B, lane 7). Less frequently, C was also incorporated (Fig. 6B, lane 8), which could be explained by a realignment of the primer 3′ TT to pair with the template AA four nucleotides downstream to copy the next template G (−4 deletion).

To confirm that AA was indeed predominantly incorporated by human Polɛ opposite the TT dimer, we extended the bypassed products by purified yeast Polζ. As the control, primer extension by Polζ was performed by using the undamaged DNA template. As shown in Fig. 6C, lane 1, Polζ alone extended the primer near the end of the undamaged template, forming a 35-mer DNA band. Extension of the Polɛ-bypassed products also yielded the 35-mer DNA band (Fig. 6C, lane 2). Furthermore, the mobility of the various DNA bands between the

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**Fig. 4. DNA synthesis by human Polɛ from templates containing a site-specific AAF-guanine.** A ³²P-labeled 17-mer primer was annealed to the damaged template with the primer 3′ end terminating right before the lesion as shown at the top. A, DNA synthesis assays were performed with purified human Polɛ (3 ng, 53 fmol) in the presence of a single dATP (A), dCTP (C), dTTP (T), or dGTP (G) or all four dNTPs (N), by using 50 fmol of the damaged template. Quantitation of extended primers is shown below the gel. B, after initial DNA synthesis by human Polɛ (3 ng) at 30 °C for 10 min, purified human Polη (2 ng, 25 fmol) was added to the reaction, and the incubation was continued for another 10 min (lane 2). Lane 1, DNA synthesis by human Polɛ alone; lane 3, DNA synthesis by human Polη alone. DNA size markers in nucleotides are indicated on the sides.

**Fig. 5. DNA synthesis by human Polɛ from templates containing a site-specific (+)- or (−)-trans-anti-BPDE-N²-dG adduct.** A ³²P-labeled 19-mer primer was annealed to the damaged template with the primer 3′ end terminating right before the lesion as shown at the left. A, DNA synthesis assays were then performed with 4 ng (70 fmol) of purified human Polɛ in the presence of a single dATP (A), dCTP (C), dTTP (T), or dGTP (G) or all four dNTPs (N), by using the undamaged or damaged templates as indicated. B, DNA synthesis was initiated with human Polɛ (10 ng) at 30 °C for 10 min. Next purified Klenow DNA polymerase (1 unit) was added to the reaction, and the incubation was continued for another 10 min at 30 °C (lanes 1 and 3). Lanes 2 and 4, DNA synthesis from the undamaged template by the Klenow polymerase (1 unit) alone. Lane 1, the (+)-trans-anti-BPDE-N²-dG adduct; lane 3, the (−)-trans-anti-BPDE-N²-dG adduct. DNA size markers in nucleotides are indicated on the left.
primer and the 35-mer DNA was identical between DNA synthesis from the undamaged template by Polα alone and the Polα-extended Polα products (Fig. 6C). Because the 3’ T of the TT dimer completely blocks yeast Polα (25), the 35-mer DNA band could only result from extension of the Polα-synthesized bypass products. These results show that human Polα possesses error-free lesion bypass activity in response to a template cis-syn TT dimer.

Human Polβ Is Unable to Bypass a Variety of DNA Lesions—To evaluate whether the lesion bypass activity of human Polβ is unique among X family DNA polymerases, we performed translesion synthesis assays with purified human Polβ using the same DNA templates under identical reaction conditions as in the Polα experiments. Purified human Polβ was active in copying the undamaged template (Fig. 7, lane 2). In contrast, human Polβ was unable to perform translesion synthesis opposite a template AP site regardless of the sequence context 5’ to the lesion, even when a large excess of the polymerase (520 fmol) was used (Fig. 7, lanes 3–6). Similarly, human Polβ was completely unresponsive to a template 1,N6-ethenoadenine, an AAF-adducted guanine, a TT dimer, or a TT (6–4) photoprotein (Fig. 7, lanes 7–13). Purified human Polβ was also unable to perform translesion synthesis opposite a template (+) or (−)-trans-anti-BPDE-N2-dG adduct, as we demonstrated recently (26). Thus, lesion bypass activity is not a common feature among the X family DNA polymerases.

DISCUSSION

Previously, we proposed that Polμ may be involved in NHEJ of double-strand DNA breaks through its microhomology searching and pairing activities (23). Most recently, Mahajan et al. (33) reported that cellular levels of human Polμ protein are increased by ionizing radiation, and that Polμ is associated with the NHEJ proteins Ku and XRCC4-ligase IV, further supporting a role of this polymerase in NHEJ. In this study, we found that human Polμ possesses DNA lesion bypass activities in response to various types of DNA damage. Thus, efficient translesion synthesis activity is not strictly limited to the Y family of DNA polymerases. Under similar experimental conditions, we did not detect any lesion bypass activities of purified human Polβ (Fig. 7), except for 8-oxoguanine (34), which is a miscoding rather than a strong blocking lesion (35). Because Polμ and Polβ share sequence homologies and they both belong to the X family of DNA polymerases (1, 2), the lesion bypass activity of human Polμ appears to be unique among X family members.

In response to the template AP site 8-oxoguanine, 1,N6-ethenoadenine, AAF-adducted guanine, and (+) and (−)-trans-anti-benz(a)pyrene-N2-dG, human Polμ bypasses the lesion predominantly by a deletion mechanism. The specificity of nucleotide incorporation during translesion synthesis indicates that deletion is a result of primer realignment. Because these
DNA lesions differ dramatically in structure, we propose that bypass of these lesions by human Polθ may be achieved by looping out the template lesion, thus avoiding a direct copying of the damaged template base. The exact deletion size appears to depend on the sequence context of the lesion. For example, if the primer 3’ end can pair with a template base 5’ to the lesion, such realignment would be preferred by human Polθ. Thus, when human Polθ encounters a lesion, if the coding capacity of the modified base is lost or significantly altered, Polθ simply realigns the primer-template strands to continue DNA synthesis by skipping the lesion. Most recently, it was reported that human cell extracts supplemented with purified human Polθ are able to extend a primer from opposite an AAF-adducted guanine by adding a ladder of as many as 15 guanines in an apparent nontemplated reaction (36). This activity is different from the lesion bypass activity of human Polθ reported here, and its functional significance remains unknown.

On the basis of mutation spectra of several DNA lesions, base substitutions rather than deletions appear to be the major product during NHEJ even in the presence of base lesions. After microhomology search, microhomology pairing, and DNA synthesis by skipping the lesion. Most recently, it was reported that human cell extracts supplemented with purified human Polθ lack Polθ activity are sensitive to and hypermutable by UV radiation (19–21,42); thus they establish an important role for this polymerase in error-free bypass of UV lesions. However, it is not known how Polθ would respond to other cyclobutane pyrimidine dimers such as the C-containing dimers. Therefore, it is unknown to what extent loss of the TT dimer bypass by Polθ contributes to UV-induced sensitivity and mutagenesis in xeroderma pigmentosum variant cells. With this uncertainty, we are unable to assess at the present time the importance of the error-free TT dimer bypass by Polθ. Nevertheless, our results raised the possibility that Polθ may participate in the error-free bypass of TT dimers in cells, especially when the Polθ function is compromised, as is the case of the xeroderma pigmentosum variant cells.

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