

A mechanism for the exclusion of low-fidelity human Y-family DNA polymerases from base excision repair

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The human Y-family DNA polymerases, Pol ι , Pol η , and Pol κ , function in promoting replication through DNA lesions. However, because of their low fidelity, any involvement of these polymerases in DNA synthesis during base excision repair (BER) would be highly mutagenic. Mechanisms, therefore, must exist to exclude their participation in BER. Here, we show that although Pol ι , Pol η , and Pol κ are all able to form a covalent Schiff base intermediate with the 5'-deoxyribose phosphate (5'-dRP) residue that results from the incision of DNA at an abasic site by an AP endonuclease, they all lack the ability for the subsequent catalytic removal of the 5'-dRP group. Instead, the covalent trapping of these polymerases by the 5'-dRP residue inhibits their DNA synthetic activity during BER. The unprecedented ability of these polymerases for robust Schiff base formation without the release of the 5'-dRP product provides a means of preventing their participation in the DNA synthetic step of BER, thereby avoiding the high incidence of mutagenesis and carcinogenesis that would otherwise occur.

[*Keywords:* Y-family DNA polymerases; base excision repair; abasic site; 5'-doxyribose phosphate; 5'-dRP lyase]

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Abasic (AP) sites, resulting from spontaneous hydrolysis of the N-glycosylic bond, arise in DNA relatively frequently, as hydrolytic depurination causes the loss of ~9000 bases per day from the DNA of a mammalian cell (Nakamura et al. 1998). AP sites are also formed in DNA as intermediates in the removal of damaged bases by base excision repair (BER; Wallace 1997). A plethora of endogenous and exogenous agents yield modified or damaged bases. For example, the DNA bases cytosine and 5-methyl cytosine are subject to hydrolytic deamination to uracil and thymine, respectively, and oxygen-free radicals produced during normal cellular metabolism are an important source of endogenous damage to DNA bases that results in oxidized purines such as 8-hydroxyguanine, and ring-saturated derivatives of pyrimidines such as thymine glycol (Wallace 1994, 1997; Seeberg et al. 1995). The different types of modified or damaged bases are removed by specific DNA glycosylases by cleavage of the N-glycosylic bond between the base and the deoxyribose moiety of the nucleotide. The resulting AP site is then acted on by a class II AP endonuclease, which cleaves the phosphodiester backbone on the 5' side of the AP site, leaving a 3'-hydroxyl group and 5'-

baseless deoxyribose-5'-phosphate (5'-dRP) residue (Wallace 1994, 1997; Seeberg et al. 1995). The 5'-dRP residue is then removed by a dRP lyase, such as that contained in DNA polymerase (Pol) β (Matsumoto and Kim 1995; Piersen et al. 1996), and the resulting single nucleotide gap is filled in by Pol β (Wilson 1998). Because of the high frequency with which endogenous base modifications, base damages, and AP sites are generated, the repair synthesis step of BER needs to be fairly accurate to keep the incidence of mutations low.

Pol β is especially adapted to function in BER because it can efficiently catalyze both the removal of the 5'-dRP group via β -elimination and the subsequent single-nucleotide gap-filling reaction. Limited proteolysis studies have revealed two independently folded domains in Pol β , the 8-kD N-terminal portion, which contains the 5'-dRP lyase activity, and the 31-kD C-terminal portion, which contains the polymerase activity. Lys 72 in the conserved helix-turn-helix motif of the 8-kD domain acts as the nucleophile that initiates the 5'-dRP removal reaction (Prasad et al. 1998b; Deterding et al. 2000). First, Lys 72 initiates an attack on the C₁ position of the 5'-dRP group. Next, a transient covalent intermediate is formed in which the DNA substrate is linked covalently to the enzyme as a Schiff base that can be stabilized by reduction with sodium borohydride. Finally, the 5'-dRP group is removed from the DNA and released from Pol β (Matsumoto et al. 1998; Prasad et al. 1998a). Pol β -null

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mice cells are hypersensitive to the cytotoxic effects of the alkylating agent methyl methanesulphonate (MMS), indicating an *in vivo* requirement for Pol β in the repair of alkylated bases (Sobol et al. 1996). Only the 5'-dRP lyase activity of Pol β , however, is critical for resistance to MMS, as Pol β -null mice cells stably transfected with a Pol β minigene carrying mutations in the 5'-dRP lyase active site still retain their sensitivity to MMS, whereas transfection with a Pol β minigene carrying mutations in the DNA polymerase active site complement the MMS sensitivity of Pol β -null mice cells (Sobol et al. 2000). Thus, in mammalian cells, Pol β is primarily responsible for the rate-limiting removal of the 5'-dRP-group, whereas the other DNA polymerases can functionally substitute for the polymerase activity of Pol β in BER.

The human Y-family DNA polymerases, Pol η , Pol ι , and Pol κ , promote replication through DNA lesions that present a block to the normal replication machinery. Pol η replicates through UV-induced thymine-thymine (TT) dimers with the same efficiency and accuracy as it replicates through undamaged Ts (Johnson et al. 1999b, 2000c; Washington et al. 2000), and genetic studies in yeast have implicated Pol η in the error-free bypass of cyclobutane dimers formed at TC and CC sites (Yu et al. 2001). Because of the involvement of Pol η in the error-free bypass of cyclobutane pyrimidine dimers, its mutational inactivation in humans causes an increase in the incidence of UV mutagenesis and leads to the cancer-prone syndrome, the variant form of xeroderma pigmentosum (Johnson et al. 1999a; Masutani et al. 1999). Pol η can efficiently replicate through other DNA lesions as well such as 7,8-dihydro-8-oxoguanine (8-oxoG) and O⁶-methyl guanine (m6G; Haracska et al. 2000a,b). In contrast to Pol η , the ability of which to replicate through DNA lesions derives both from its ability to proficiently incorporate nucleotides opposite the lesion site and to proficiently extend from the inserted nucleotide, the bypass of certain DNA lesions requires the sequential action of two DNA polymerases, wherein one inserts the nucleotide opposite the lesion site and the other extends from the inserted nucleotide (Prakash and Prakash 2002). Pol ι is able to incorporate nucleotides opposite the 3'-T of a (6-4) T-T photoproduct or opposite an AP site; but it does not extend from the nucleotide opposite these lesions sites (Johnson et al. 2000b). Pol κ , on the other hand, is unable to insert nucleotides opposite DNA lesions, as, for example, opposite the 3'-T of a TT dimer or opposite an m6G lesion, but it can efficiently extend from the nucleotides opposite these lesion sites (Haracska et al. 2002a; Washington et al. 2002). The Y-family polymerases possess an active site that is more tolerant of geometric distortions in the DNA than the replicative DNA polymerases that are unable to bypass DNA lesions. The cost of the ability of Pol η , Pol ι , and Pol κ to promote lesion bypass, however, is borne by their low fidelity. Steady-state kinetic analyses have indicated that Pol η and Pol κ misincorporate nucleotides with a frequency of $\sim 10^{-2}$ to 10^{-3} and $\sim 10^{-3}$ to 10^{-4} , respectively (Washington et al. 1999; Johnson et al. 2000a,c). The fidelity of Pol ι is much poorer than that of Pol η , particu-

larly for incorporating nucleotides opposite the template pyrimidines (Johnson et al. 2000b; Tissier et al. 2000).

Previously, a role for human Pol ι has been implicated in BER (Bebenek et al. 2001). However, considering that Pol ι is an extremely low-fidelity DNA polymerase, DNA synthesis by this enzyme in BER would be very highly mutagenic. Here, we address whether the human Y-family DNA polymerases Pol η , Pol ι , and Pol κ can, in fact, play a role in BER, or whether these low-fidelity polymerases are somehow excluded from the DNA synthesis step of BER. Interestingly, we found that although each of these human polymerases can form a covalent Schiff base complex with the 5'-dRP group in DNA, they are unable to carry out the subsequent removal of the 5'-dRP group. Furthermore, and in striking contrast to Pol β , the DNA synthetic activity of Pol ι , Pol η , and Pol κ is inhibited upon their binding to the 5'-dRP group. From these and other observations presented here, we conclude that nonproductive binding to the 5'-dRP group represents a means to exclude these DNA polymerases from the DNA synthetic step of BER.

Results

Human DNA polymerases ι , η , and κ form a covalent complex with the 5'-dRP group

We purified human Pol ι , Pol η , and Pol κ as native as well as N-terminal GST-fusion proteins (Fig. 1A), and first examined their DNA synthesis activities. As shown in Figure 1B, the DNA synthesis activity of the GST-fusion polymerases were nearly identical to the corresponding native enzymes. Thus, the GST-fusion does not interfere with the activity of Pol ι , Pol η , or Pol κ .

Next, we examined whether Pol ι , Pol η , and Pol κ can form a Schiff-base covalent complex with the 5'-dRP-containing DNA substrate. A Schiff base can be formed between a properly positioned N-terminal α NH₂ group (Dodson et al. 1993) or an ϵ NH₂ group (Matsumoto et al. 1998; Prasad et al. 1998a) of a protein and the -CHO group of an open abasic residue in DNA, and the covalent linkage between DNA and protein can be trapped by the reduction of this Schiff base with sodium borohydride (Piersen et al. 1996). As outlined in Figure 2A, we generated a DNA substrate containing a 5'-dRP residue at an internal nick by the sequential treatment of a single-uracil-containing DNA duplex with uracil DNA glycosylase and a class II AP endonuclease. Figure 2B shows that, similarly to Pol β , Pol ι , Pol η , and Pol κ are active in the borohydride trapping assay. Because of the cross-linking of the polymerase to the 5'-dRP-containing DNA, the trapped products exhibit a slower mobility on an SDS-polyacrylamide gel than the unmodified polymerases (cf. Figs. 2B and 1A). We were able to cross-link the GST-fusion proteins as well as the native polymerases to the DNA, which indicates that the GST-fusion part is not required for the Schiff base formation. In conclusion, Pol ι , Pol η , and Pol κ are able to react with the 5'-dRP residues in DNA to form a Schiff-base covalent complex.

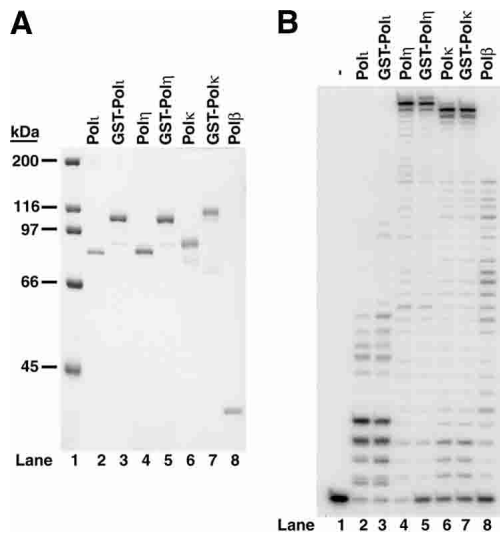


Figure 1. Purity and DNA synthetic activity of human Pol ι , Pol η , Pol κ , and Pol β . (A) Purified native and N-terminal GST-fusion Pol ι , Pol η , Pol κ , and native Pol β were analyzed on an 8% denaturing polyacrylamide gel and stained with Coomassie blue. (Lane 1) Molecular weight standards containing 500 ng of protein in each band; (lane 2) 300 ng of Pol ι ; (lane 3) 500 ng of GST-Pol ι ; (lane 4) 300 ng of Pol η ; (lane 5) 500 ng of GST-Pol η ; (lane 6) 500 ng of Pol κ ; (lane 7) 500 ng of GST-Pol κ ; (lane 8) 300 ng of Pol β . (B) The DNA synthetic activity of purified GST-fusion and native Pol ι , Pol η , Pol κ , and native Pol β was assayed using a 75-nt template oligonucleotide primed with a 40-nt 5'-³²P-labeled oligonucleotide (S1 substrate) in the presence of all four deoxynucleotides (100 μ M) in buffer A containing 40 mM Tris-HCl (pH 7.5), 8 mM MgCl₂, 1 mM dithiothreitol, 100 μ g/mL bovine serum albumin, and 10% glycerol. The DNA substrate (20 nM) was incubated with 2 nM of DNA polymerase at 37°C for 10 min. The reaction products were analyzed on an 8 M urea, 8% polyacrylamide gel, and the DNA bands were visualized by autoradiography.

Lack of 5'-dRP lyase activity in human DNA polymerases ι , η , and κ

Although Schiff base formation is a prerequisite for a nonhydrolytic dRP lyase reaction, it does not necessitate a strong subsequent β -elimination reaction. To test whether the Schiff base formed by Pol ι , Pol η , and Pol κ with DNA was an intermediate in the 5'-dRP excision reaction, we examined whether Pol ι , Pol η , and Pol κ could excise the 5'-dRP group. We directly compared the 5'-dRP lyase (Fig. 2C) and DNA polymerase (Fig. 2D) activities of Pol ι , Pol η , and Pol κ at different enzyme concentrations, and as a control, we used Pol β . Whereas Pol β displayed a strong activity for removing the 5'-dRP group from the AP endonuclease-incised oligonucleotide (19 nt + dRP band), thereby generating the 19-nt product that lacks the 5'-dRP group and therefore has a faster mobility (Fig. 2C, lanes 10,11), we were unable to detect any 5'-dRP lyase activity associated with Pol ι , Pol η , or Pol κ (Fig. 2C, lanes 4–9). Despite the fact that at 2 nM concentration, Pol ι , Pol η , and Pol κ had as robust a DNA polymerase activity as Pol β (Fig. 2D), neither Pol ι , Pol η , nor Pol κ removed any 5'-dRP group even at as high a

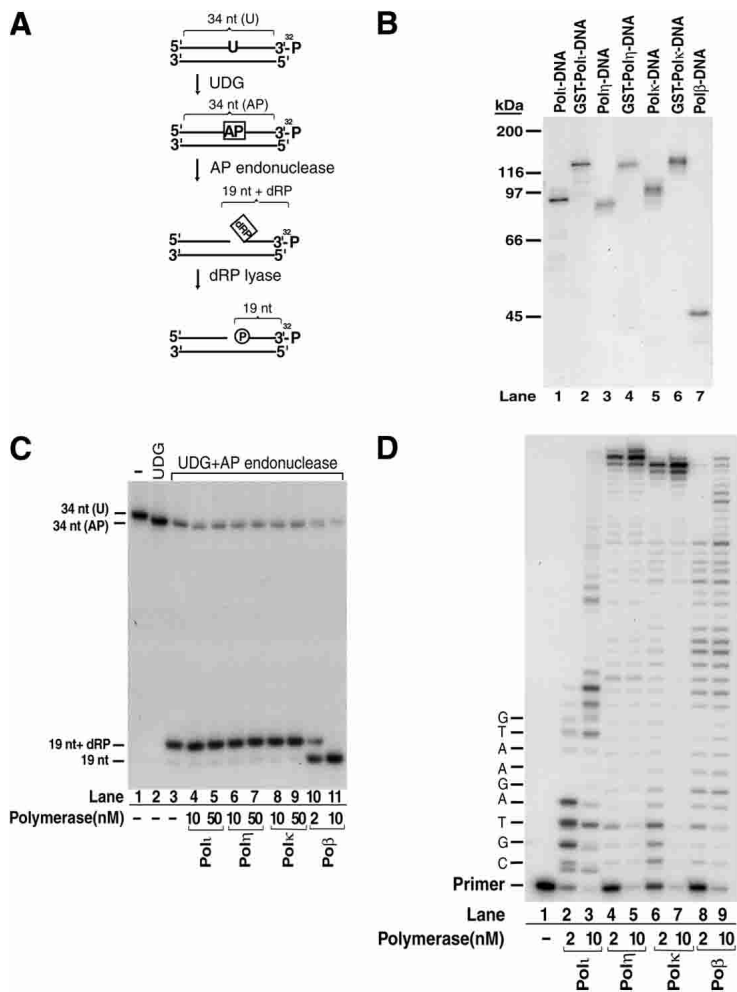
concentration as 50 nM. With Pol β , however, as little as 2 nM enzyme removed 70% of the 5'-dRP group, and 10 nM Pol β could remove all of the 5'-dRP groups from the DNA. Thus, although the human Y-family DNA polymerases are all able to form a covalent complex with the 5'-dRP-group in DNA, the resulting polymerase–DNA complexes do not act as intermediates for the 5'-dRP excision reaction.

To further verify the absence of a 5'-dRP lyase activity in DNA polymerases ι , η , and κ , we examined the kinetics of borohydride cross-linking with these enzymes. Depending on whether the polymerase–5'-dRP–DNA complex is an intermediate in the dRP lyase reaction or not, the borohydride cross-linking should occur with different kinetics, and therefore, the amount of the cross-linked product should change if the polymerase were first preincubated with the 5'-dRP substrate for varied intervals before the addition of borohydride. For an enzyme such as Pol β , which has a robust 5'-dRP lyase activity, the cross-linked protein–DNA complex would diminish following preincubation with the enzyme because the enzyme would have removed the 5'-dRP group from the DNA. However, for an enzyme that has no 5'-dRP lyase activity, as our results indicate for Pol ι , Pol η , and Pol κ , preincubation of these enzymes with the 5'-dRP substrate should cause no decrease in the amount of cross-linked protein–DNA complex. Whereas Pol ι , Pol η , and Pol κ , as well as Pol β , rapidly formed a complex with the 5'-dRP substrate, as even without any preincubation, we were able to trap their complex with borohydride (Fig. 3, lane 1), striking differences were observed between Pol β and the other polymerases upon their preincubation with the 5'-dRP substrate before borohydride addition. Thus, after preincubation for 10 min, the intensity of the Pol β –5'-dRP complex diminished, while the intensity of the Pol ι –5'-dRP, Pol η –5'-dRP, and Pol κ –5'-dRP–DNA complexes increased (Fig. 3, cf. lanes 1 and 2). With a 30-min preincubation, the amounts of Pol ι , Pol η , and Pol κ covalent complexes showed a further increase, but no cross-linked product could be seen with Pol β (Fig. 3, lane 3). These results show that in contrast to Pol β , which has a potent 5'-dRP lyase activity, Pol ι , Pol η , and Pol κ did not excise the 5'-dRP group after complex formation with the 5'-dRP in DNA, and instead, with these DNA polymerases, the amount of cross-linked product increased with the increase in preincubation time. These are the results expected for an enzyme that has no 5'-dRP lyase activity but can form a covalent complex with the 5'-dRP group in DNA.

Exclusion of DNA Polymerases ι , η , and κ from DNA synthesis at an incised abasic site

The ability of Pol ι , Pol η , and Pol κ to form a covalent complex with the 5'-dRP group without carrying out the subsequent lyase reaction raised the possibility that binding to the 5'-dRP group precludes these polymerases from carrying out the DNA synthesis step during BER. To test this possibility, first we compared the DNA synthetic activity of Pol ι and Pol β on two DNA substrates,

Figure 2. Covalent binding of human Y-family DNA polymerases to the 5'-dRP residue in DNA. (A) Schematic representation of reactions to generate a 5'-dRP-group-containing DNA substrate. The initial substrate (S2 substrate) was generated by annealing a single-uracil-containing 3'-end-labeled 34-nt oligonucleotide [34 nt (U)] to its complementary oligonucleotide. Treatment with UDG generated an abasic-site-[34 nt (AP)]-containing DNA, which was incised at the 5' side of the abasic site by a class II AP endonuclease, generating the 5'-dRP-containing substrate (19 nt + dRP). Removal of the 5'-dRP residue by dRP lyase will result in a 3'-³²P-labeled 19-nt DNA fragment. (B) Trapping of polymerase-DNA covalent complexes with sodium borohydride (NaBH₄). In 10 μ L of reaction mixture, the 3'-³²P-labeled 5'-dRP-containing DNA (100 nM), shown in A, was incubated with 50 nM each Pol ι (lane 1), GST-Pol ι (lane 2), Pol η (lane 3), GST-Pol η (lane 4), Pol κ (lane 5), GST-Pol κ (lane 6), or Pol β (lane 7) in buffer B containing 50 mM HEPES (pH 7.5), 10 mM MgCl₂, 20 mM KCl, and 2 mM dithiothreitol, in the presence of 20 mM NaBH₄ for 30 min on ice. Reactions were terminated by the addition of SDS-containing loading buffer, samples were resolved on an 8% SDS-PAGE gel, and products were visualized by autoradiography. (C) 5'-dRP lyase activity. The 5'-dRP-containing DNA substrate (20 nM) generated as shown in A was incubated in buffer B at 37°C for 10 min with Pol ι (lanes 4,5), Pol η (lanes 6,7), Pol κ (lanes 8,9), and Pol β (lanes 10,11), which were present at two different concentrations as indicated, followed by stabilization of the reaction product by incubation in the presence of 340 mM NaBH₄ at 0°C for 30 min. The products were analyzed by a denaturing 16% polyacrylamide gel and visualized by autoradiography. (D) DNA synthesis activity. S1 DNA substrate (20 mM) containing a 5'-³²P-labeled primer was incubated with the DNA polymerase added at two different concentrations (2 nM or 10 nM), as indicated, in the presence of all four deoxynucleotides (100 μ M) in buffer B at 37 °C for 10 min.



one of which was incised at the AP site and the other had a single nucleotide gap (Fig. 4A). To examine DNA synthesis, and to determine which nucleotide is inserted opposite the template residue T, which is situated opposite the 5'-incised AP site or opposite the single nucleotide gap, we monitored the extension of the 5'-³²P-labeled 15-nt standing start primer, the 3'-end of which is situated right before the gap or the AP site, in the presence of all four deoxynucleotides. We compared the electrophoretic mobilities of the products of DNA synthesis with 16-nt oligonucleotide markers containing the 15-nt primer plus a G or an A residue at position 16, on a 20% polyacrylamide gel (Fig. 4A, lanes 1,2). As expected, whereas Pol β inserted an A opposite the template T (Fig. 4A, lanes 6,7), Pol ι inserted predominantly a G opposite the template T (Fig. 4A, lanes 4,5). In striking contrast to Pol β , which displayed nearly identical DNA synthetic activity on the two DNA substrates (Fig. 4A, lanes 6,7), the DNA synthetic activity of Pol ι was severely inhibited on the nicked AP-site-containing DNA (Fig. 4A, cf. lanes 4 and 5). Thus, not only does Pol ι have an extremely low fidelity in single nucleotide gap filling, it is

also very poor in carrying out the DNA synthesis reaction on the 5'-nicked AP-site-containing DNA substrate.

To test if the 5'-dRP group inhibits the DNA synthesis activity of all three Y-family DNA polymerases, we compared the activity of Pol ι , Pol η , Pol κ , and Pol β on two DNA substrates containing a 5'-C or a 5'-dRP residue at the nick (Fig. 4B). Compared with DNA synthesis on the nicked DNA substrate without the 5'-dRP residue (Fig. 4B, lanes 2–7), the DNA synthesis activities of Pol ι , Pol η , and Pol κ were severely inhibited on the DNA substrate containing the 5'-dRP moiety (Fig. 4B, lanes 10–15), whereas no such inhibition was observed with Pol β (Fig. 4B, cf. lanes 8,9 and 16,17). Thus, relative to primer extension on the nicked DNA substrate lacking the 5'-dRP residue, the inhibition of the DNA synthetic activity on the 5'-dRP-containing DNA substrate was ~10-fold for Pol ι , ~5-fold for Pol η , and ~10-fold for Pol κ , whereas Pol β was ~20% more efficient at synthesizing DNA on the 5'-dRP substrate than on the nicked DNA substrate lacking this lesion. In conclusion, binding of Pol ι , Pol η , and Pol κ to the 5'-dRP group in DNA severely inhibits their DNA synthetic activity.

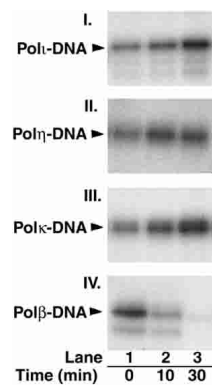


Figure 3. Time course of polymerase–DNA cross-linking by borohydride. Pol ι (panel I), Pol η (panel II), Pol κ (panel III), or Pol β (panel IV; each at 10 nM) were mixed at 0°C with the internal 5'-dRP-containing 3'- 32 P-labeled DNA (50 nM) shown in Figure 2A, followed by incubation at 37°C for 0, 10, or 30 min (lanes 1–3, respectively) before the addition of 20 mM NaBH $_4$ and further incubation at 0°C for 30 min. After the addition of SDS-containing loading buffer, the samples were resolved on an 8% SDS–polyacrylamide gel, and the cross-linked polymerase–DNA products, indicated by arrows on the *left* of panels, were analyzed by autoradiography.

Discussion

Translesion DNA synthesis is one of the main roles of human Pol η , Pol ι , and Pol κ (Prakash and Prakash 2002). These DNA polymerases, however, replicate DNA with a low fidelity, and the fidelity of Pol ι is the poorest, particularly for incorporating nucleotides opposite pyrimidine templates. Therefore, to prevent increased mutagenesis, it is critical that the DNA synthetic activity of these polymerases be restricted. Because of the preponderance of a variety of base damages, and because of the high incidence of AP sites, which are generated as a result of spontaneous base loss and as intermediates in BER, it is particularly important that high-fidelity DNA synthesis prevail during BER.

We show here that Pol η , Pol ι , and Pol κ can each form a Schiff base with 5'-dRP-containing DNA. These enzyme–DNA complexes, however, are not intermediates of a 5'-dRP lyase reaction, as we found no 5'-dRP lyase activity in our purified Pol ι , Pol η , and Pol κ preparations, which are fully active in the DNA synthesis reaction. Thus, in contrast to Pol β , where as little as 2 nM of the enzyme removed ~70% of the 5'-dRP group from the substrate, Pol ι , Pol η , and Pol κ had no 5'-dRP lyase activity, even at a concentration as high as 50 nM. The absence of a 5'-dRP lyase activity from Pol ι , Pol η , and Pol κ was further supported from studies in which these enzymes were preincubated with the 5'-dRP DNA substrate prior to borohydride reduction of the polymerase–DNA complexes. Although preincubation with Pol β before the addition of borohydride decreased the Pol β –DNA cross-linked product, consistent with a robust 5'-dRP lyase activity in Pol β , such preincubation with Pol ι , Pol η , and Pol κ resulted in an increase in the amount of Pol–DNA cross-linked product. The persistence of 5'-

dRP residues, even after prolonged preincubation with Pol ι , Pol η , and Pol κ , has provided unambiguous evidence for the lack of any 5'-dRP lyase activity in these enzymes.

The ability of Pol ι , Pol η , and Pol κ to form a covalent protein–DNA Schiff base intermediate, but not to promote the subsequent 5'-dRP lyase reaction, could arise

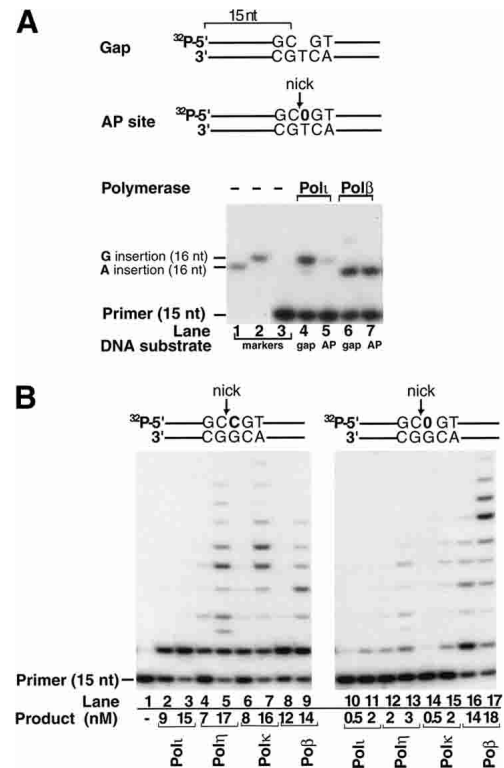


Figure 4. Exclusion of Y-family DNA polymerases from DNA synthesis on a 5'-dRP-containing DNA substrate. (A) The DNA synthetic activity of Pol ι and Pol β on a DNA substrate containing a single nucleotide gap or a 5'-dRP residue at the nick. Pol ι (2 nM; lanes 4,5) or Pol β (2 nM; lanes 6,7) was incubated with the DNA substrate (20 nM) at 37°C for 10 min in the presence of all four deoxynucleotides in buffer B. On the schematic representation of DNA substrates (shown on *top*), the AP site is indicated by a 0. The gapped substrate (S3–; lanes 4,6) and the substrate nicked at the AP site (S4–; lanes 5,7) contained a template T opposite both the gap and the AP site, and the 15-nt primer was 5'- 32 P-labeled. The nucleotide inserted opposite the T was determined by comparing the products of DNA synthesis reactions to 16-nt 5'- 32 P-labeled oligonucleotide markers containing the 15-nt primer (lane 3) plus an A (lane 1) or a G (lane 2) at their 3'-end. The reaction products were resolved on an 8 M urea, 20% polyacrylamide gel, and the DNA bands were visualized by autoradiography. (B) DNA synthetic activity of Pol η , Pol κ , and Pol β on DNA substrates containing a nick 5' to a C or a nick at an AP site. DNA polymerases, each at 2 nM or 5 nM concentrations, were incubated with the DNA substrate (20 nM) at 37°C for 20 min in the presence of all four deoxynucleotides in buffer B. The DNA substrates nicked 5' to a C (S5 substrate; lanes 1–9) or nicked 5' to an AP site (S6 substrate; lanes 10–17) are shown schematically. The amount of reaction product was calculated from the intensity of bands generated by DNA synthesis divided by the intensity of all the bands.

from the differences in the chemistry for the two reactions. When the environment of a particular -NH_2 group in a protein lowers its pK_a , it becomes a good nucleophile at physiological pH and is able to form a Schiff base with the -CHO group at the C_1 position of an AP site. However, for the protein to display a strong 5'-dRP lyase activity, at this intermediate stage, the pK_a of the imine group needs to be high enough for it to be protonated and to carry out an efficient β -elimination reaction; otherwise, the 5'-dRP group removal will be highly inefficient, occurring via a spontaneous β -elimination reaction. Thus, Schiff base formation is a prerequisite for the lyase activity, but it is not sufficient for the subsequent β -elimination reaction to occur.

Covalent binding to the 5'-dRP residue in DNA inhibits the DNA synthetic activity of Pol ι , Pol η , and Pol κ . This inhibition of synthesis may arise because covalent binding to the 5'-dRP group results in a physical orientation so that the polymerase can no longer access the 3'-primer end; alternatively, a transient conformational change may occur in the polymerase that inhibits its binding to the 3'-primer end or inhibits its catalytic activity. Because the covalent complexes of Pol ι , Pol η , and Pol κ with the 5'-dRP-containing DNA can be stabilized by borohydride, structural studies of such complexes could help unravel the mechanism of inhibition of their DNA synthetic activity.

Previously, a 5'-dRP lyase activity has been reported for human Pol ι (Bebenek et al. 2001; Prasad et al. 2003). This observation is inconsistent with our results. In these studies, however, no convincing evidence was provided to establish that the lyase activity is, in fact, intrinsic to Pol ι . The dRP lyase activity was inferred to be intrinsic to Pol ι because a covalent Pol ι -DNA Schiff-base intermediate could be trapped by reduction with sodium borohydride (Bebenek et al. 2001), and this site in Pol ι has been recently mapped to a 40-kD domain spanning residues Met 79 to -460 (Prasad et al. 2003). Our results confirm the formation of a covalent Pol ι -DNA Schiff-base intermediate, but we have found no evidence for a 5'-dRP lyase activity in Pol ι . We have examined many different highly purified native Pol ι and GST-Pol ι preparations that support robust DNA synthesis, but even with long incubation periods and with high enzyme concentrations, we have found no evidence of any significant 5'-dRP lyase activity in Pol ι . Moreover, as we show here, Pol η and Pol κ also form a Schiff base intermediate, but they have no 5'-dRP lyase activity. And, for Pol η and Pol κ also, we expect the region corresponding to Pol ι 's 40-kD domain to be involved in Schiff base formation. In view of our observations, the formation of a Schiff base intermediate cannot be taken as evidence to support the existence of a 5'-dRP lyase activity in Pol ι . Furthermore, in the absence of additional supporting evidence, the coelution of Pol ι 's polymerase activity with 5'-dRP lyase cannot be considered definitive, as that could be fortuitous (Prasad et al. 2003). Although we cannot be sure of the source of the 5'-dRP lyase activity that has been reported for Pol ι , any number of factors, as, for example, the presence of an extraneous peptide or a pro-

tein in the Pol ι preparation, could have been the source of that activity. Because of the extreme lability of a 5'-terminal abasic site, the removal of a 5'-dRP group can occur spontaneously in the absence of β -elimination catalysts (Bailly and Verly 1989), and basic proteins such as histones and polyamines stimulate this reaction by inducing β -elimination (Bailly and Verly 1988). Moreover, even short basic peptides such as Lys-Trp-Lys and Lys-Tyr-Lys, can promote this reaction (Helene et al. 1982).

In summary, with respect to their interaction with a 5'-dRP group in DNA, all three human polymerases, Pol ι , Pol η , and Pol κ , behave very similarly. They all form a covalent Schiff base intermediate with the 5'-dRP group, but have no detectable 5'-dRP lyase activity (Fig. 5). To our knowledge, such robust Schiff base formation without the release of any significant 5'-dRP product is unprecedented, as all the other DNA polymerases that yield a covalent Schiff base intermediate also exhibit 5'-dRP lyase activity (Matsumoto et al. 1998; Pinz and Bogenhagen 2000; Garcia-Diaz et al. 2001). Our observation that the DNA synthetic activity of Pol ι , Pol η , and Pol κ is greatly reduced on a 5'-dRP-containing DNA substrate strongly supports the inference that covalent binding to the 5'-dRP residue represents a means by which these low-fidelity polymerases are excluded from the DNA synthetic step of BER (Fig. 5), thereby avoiding the high incidence of mutagenesis and carcinogenesis that would otherwise result.

Materials and methods

DNA substrates

Oligonucleotides were synthesized by Midland Certified Reagent Co. The S1 substrate, used for the DNA synthesis reactions shown in Figures 1B and 2D, was generated by annealing a 75-nt oligonucleotide template, 5'-AGCTACCATGCCTGCC TCAAGAATTTCGTAAGATGCCTACACTGGAGTACCGGAGCATCGTCGTGACTGGGAAAAC-3' to the 5'- ^{32}P -labeled, 40-nt oligonucleotide primer, 5'-GTTTTCCCAGTCACGAC GATGCTCCGGTACTCCAGTGTAG-3'. For the single-uracil-containing S2 substrate, shown schematically in Figure 2A, the 34-nt 3'- ^{32}P -labeled oligonucleotide, 5'-CTGCAGCTGATGC GCUGTACCGATCCCCGGGTAC-3', was annealed to the 34 nt template oligonucleotide, 5'-GTACCCGGGGATCCGTAC GGCGCATCAGCTGCAG-3'. The 5'-dRP-containing DNA substrate, used for the experiments shown in Figures 2B and C and 3, was generated by treatment of the S2 substrate with uracil DNA glycosylases (UDG; Trevigen) followed by treatment with human APE1, a class II AP endonuclease (Trevigen), as described below. For the DNA polymerase reactions shown in Figure 4A, the DNA substrates were generated by annealing three oligonucleotides together; the 34-nt template oligonucleotide, 5'-GTACCCGGGGATCCGTACTGCGCATCAGCTGCAG-3'; the 5'- ^{32}P -labeled 15-nt oligonucleotide primer, 5'-CTGCAGCTGATGCGC-3'; and either the 18-nt oligonucleotide, 5'-pGTACGGATCCCCGGGTAC-3' (S3 substrate); or the 19-nt oligonucleotide, 5'-pUGTACGGATCCCCGGGTAC-3' (S4 substrate). The DNA substrates shown in Figure 4B were generated by annealing three oligonucleotides together, the 34-nt template oligonucleotide used also for generating the S2 sub-

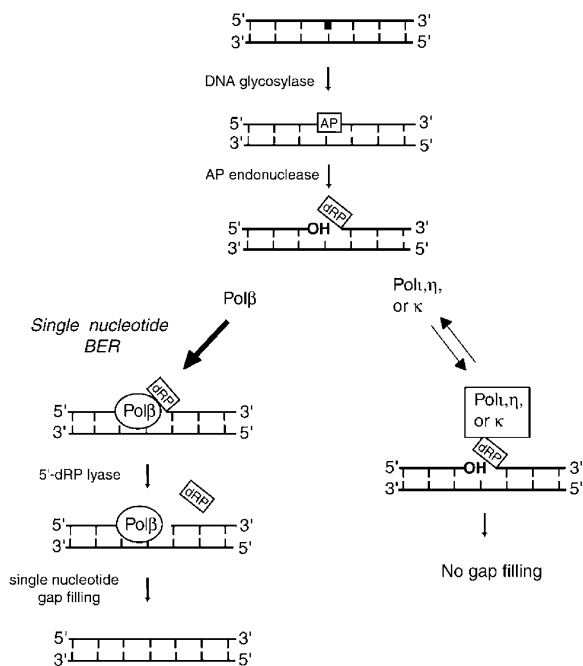


Figure 5. A model for the exclusion of Y-family polymerases from DNA synthesis during BER. An AP site is generated in DNA either by the action of a DNA glycosylase on the damaged base (shown in bold) or via spontaneous hydrolysis of purines (data not shown). Nicking of the phosphodiester backbone on the 5'-side of the AP lesion by a class II AP endonuclease generates a 3'-OH terminus and a 5'-dRP moiety, which is removed by the potent 5'-dRP lyase activity of Pol β , followed by single nucleotide gap filling by this enzyme (*left*). The thick arrow on the *left* denotes that because of its high affinity for the lesion site, Pol β would be the predominant enzyme to carry out the reactions shown. The low-fidelity Y-family polymerases, however, would be excluded from the DNA synthesis reaction during BER because of their unproductive binding to the 5'-dRP residue, which inhibits their ability to synthesize DNA on such a substrate (*right*). The bidirectional thin arrows on the *right* signify the less efficient and transient binding of Y-family polymerases to the lesion site. Dissociation of these polymerases from the 5'-dRP residue would free up the residue for removal by the 5'-dRP lyase activity of Pol β and for repair synthesis, as is shown on the *left*.

strate; the 5'-³²P-labeled 15-nt oligonucleotide primer used also for generating the S3 and S4 substrates; and either the 19-nt oligonucleotide 5'-pCGTACGGATCCCCGGGTAC-3' (S5 substrate), or the 19-nt oligonucleotide used for generating the S4 substrate (S6 substrate). The AP site in the S4 and S6 substrates was generated by the removal of uracil by UDG treatment as described below.

Purification of DNA polymerases

Human Pol ι , Pol η , and Pol κ in fusion with glutathione S-transferase (GST) were expressed in yeast strain BJ5464 and bound to a glutathione-Sepharose 4B column as described (Haracska et al. 2001a,b, 2002b). GST-Pol ι , GST-Pol η , and GST-Pol κ were eluted from one-half of the glutathione-Sepharose 4B beads with 20 mM reduced glutathione in elution buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM DTT, 0.01% Nonidet

P-40, and 10% glycerol. The other half of the glutathione-Sepharose 4B beads were incubated with PreScission protease (Amersham), which cleaves the GST fusion from the native proteins, releasing the native Pol ι , Pol η , and Pol κ in elution buffer. Purified proteins were concentrated by using a Microcon 30 (Amicon) and frozen at -70°C . Human Pol β purified as described (Rajendran et al. 1998) was kindly provided by W. Bujalowski (University of Texas Medical Branch, Galveston, TX).

DNA polymerase assays

The DNA polymerase reaction mixture (10 μL) contained 20 nM DNA substrate, 100 μM dGTP, dATP, dCTP, and dTTP, in either buffer A containing 40 mM Tris-HCl (pH 7.5), 8 mM MgCl₂, 1 mM dithiothreitol, 100 $\mu\text{g}/\text{mL}$ bovine serum albumin, and 10% glycerol (Fig. 1B); or in buffer B containing 50 mM HEPES (pH 7.5), 10 mM MgCl₂, 20 mM KCl, and 2 mM dithiothreitol (Figs. 2D, 4). Polymerase reactions were started by the addition of Pol ι , Pol η , Pol κ , or Pol β in concentration as indicated in the figure legends. After incubation at 37°C for 10 or 20 min as indicated in the figure legends, the reactions were terminated by the addition of 40 μL of formamide containing loading buffer (95% formamide, 20 mM EDTA, 0.3% bromphenol blue, and 0.3% cyanol blue). The reaction products were resolved on 8% or 20% polyacrylamide gels containing 8 M urea, as indicated in the figure legends. Quantitation of the products was done using a Molecular Dynamics STORM PhosphorImager and ImageQuant software.

Preparation of 5'-dRP-containing DNA

The incised AP-site-containing DNA was generated by first pre-treating the DNA duplex, which contained a single U in the 3'-³²P-labeled DNA strand, with UDG (2 units/1 pmole DNA) in 50 mM HEPES (pH 7.5) at 30°C for 10 min, followed by treatment with human APE1, a class II AP endonuclease (2 units/1 pmole DNA) in buffer B at 30°C for 10 min, which incises the phosphodiester backbone on the 5'-side of the AP site, leaving a 3'-OH and a 5'-dRP residue. Owing to the labile nature of the AP site, the 5'-dRP-containing DNA substrate was prepared just before use.

Trapping of polymerase-DNA complexes by sodium borohydride

DNA polymerases were mixed on ice in buffer B with DNA substrate containing a 5'-dRP residue followed by immediate addition of 20 mM sodium borohydride. The reaction with sodium borohydride was continued for 30 min on ice. In the experiment shown in Figure 3, the polymerase was first preincubated with the DNA substrate at 37°C for the indicated time before the addition of sodium borohydride. The reactions were terminated by the addition of sodium dodecyl sulfate (SDS)-containing loading buffer, and the cross-linked polymerase-3'-³²P-labeled DNA complexes were resolved on an 8% SDS-polyacrylamide gel. Products were visualized by autoradiography.

5'-dRP lyase assay

Reaction mixtures (10 μL) contained the DNA polymerases at two different concentrations, as indicated in the figure legends, and the 5'-dRP-containing DNA substrate (20 nM) in buffer B. Reactions were incubated at 37°C for 10 min. Thereafter, the reaction product was stabilized by the addition of 2M sodium borohydride to a final concentration of 340 mM, followed by incubation at 0°C for 30 min. Formamide-containing gel loading

buffer (40 μ L) was then added, and the reaction products were resolved on 16% polyacrylamide gels containing 8 M urea followed by visualization by autoradiography.

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