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DNA Polymerases β and λ Bypass Thymine Glycol in Gapped DNA Structures[†]

Ekaterina A. Belousova,[‡] Giovanni Maga,[§] Yang Fan,[∥] Elena A. Kubareva,[∥] Elena A. Romanova,[∥] Natalia A. Lebedeva,[‡] Tatiana S. Oretskaya,[∥] and Olga I. Lavrik*[,‡]

[‡]*Institute of Chemical Biology and Fundamental Medicine, SB RAS, Novosibirsk, Russia,* [§]*Istituto di Genetica Molecolare, CNR, Pavia, Italy, and* [∥]*Chemistry Department and A. N. Belozersky Institute of Physico-Chemical Biology, M. V. Lomonosov Moscow State University, Moscow, Russia*

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ABSTRACT: Here we investigated the ability of the human X-family DNA polymerases β and λ to bypass thymine glycol (Tg) in gapped DNA substrates with the damage located in a defined position of the template strand. Maximum velocities and the Michaelis constant values were determined to study DNA synthesis in the presence of either Mg^{2+} or Mn^{2+}. Additionally, the influence of hRPA (human replication protein A) and hPCNA (human proliferating cell nuclear antigen) on TLS (translesion synthesis) activity of DNA polymerases β and λ was examined. The results show that (i) DNA polymerase λ is able to catalyze DNA synthesis across Tg, (ii) the ability of DNA polymerase λ to elongate from a base paired to a Tg lesion is influenced by the size of the DNA gap, (iii) hPCNA increases the fidelity of Tg bypass and does not influence normal DNA synthesis catalyzed by DNA polymerase λ, (iv) DNA polymerase β catalyzes the incorporation of all four dNTPs opposite Tg, and (v) hPCNA as well as hRPA has no specific effect on TLS in comparison with the normal DNA synthesis catalyzed by DNA polymerase β. These results considerably extend our knowledge concerning the ability of specialized DNA polymerases to cope with a very common DNA lesion such as Tg.

DNA is constantly affected by a spectrum of damaging agents. They come from inside the cell, such as reactive oxygen species and free radicals, or from exogenous sources, including UV light, chemicals, and ionizing radiation. Additionally, DNA lesions can arise spontaneously through hydrolysis.

One of the most common oxidation products of thymine is thymine glycol (Tg;[1] 5,6-dihydro-5,6-dihydroxythymine). About 400 Tgs are formed per cell per day (*1*, *2*), and the presence of Tg in DNA has been used as a marker of oxidative stress (*1*, *3*). Unlike normal DNA bases, Tg is nonplanar and induces strongly pronounced distortion in regular DNA structure. Moreover, it dramatically changes the thermodynamic properties of the DNA duplex (*4*, *5*). Tg is a poor mutagenic lesion because it generally pairs with correct A (*6*). However, it was shown to be a very effective block of DNA replication (*7*, *8*). When Tg is encountered as a template base by replicative DNA polymerases (pols), it leads to immediate termination of primer extension past the damaged site coupling with A. It has been observed that Tg strongly blocks the action of repair and replicative DNA pols *in vitro* (*7*, *8*) and that it is a lethal lesion *in vivo* (*6*, *9*, *10*).

Several repair pathways that restore the genomic DNA exist in the cell (*11*), but in some cases, they are deficient or incomplete. Replication of damaged DNA is likely to cause stalling of replication forks and accumulation of potentially dangerous mutations. In this case, the cells will need to restart replication progression by directly recruiting the specific DNA pols to pass the lesion, introducing TLS. Lesion bypassing will thus occur on the extended DNA templates. This mechanism might be favored on the leading strand. Alternatively, the fork can be arrested and later restarted downstream of the template lesion leaving a gap. In this situation, TLS will be postreplicative. This mechanism can be suggested for replication on the lagging strand where discontinuous Okazaki fragment synthesis will more readily lead to gap formation (reviewed in ref *12*). It should be noted that TLS pols simply associate with normal replicases through protein−protein interactions, for example, with PCNA (*p*roliferating *c*ell *n*uclear *a*ntigen), and accumulate into foci whenever a fork is blocked (reviewed in ref *13*).

TLS is usually mediated by one or more members of the Y-family of DNA pols (*14*). However, recent observations suggest that DNA pols from other structural families can support DNA synthesis across the lesion (*15*−*19*). Among them are the human X-family DNA pols β (*20*−*24*) and λ (*25*−*28*).

DNA pol β is 39 kDa monosubunit protein, endowed with two catalytic activities, a DNA polymerase and a 5′-deoxyribose phosphate lyase activity, but it lacks 3′−5′ exonuclease activity (*29*). DNA pol β is the main DNA pol of the base excision repair (BER) process (*30*, *31*), but it also participates in meiosis and in neurogenesis (*32*, *33*). DNA pol β displays maximum accuracy on DNA substrates containing a one-window gap with a phosphate group on the 5′-end and acts as a processive enzyme on DNA substrates containing a gap up to six nucleotides long (*34*).

DNA pol λ is a recently identified 67 kDa eukaryotic DNA-dependent DNA polymerase. It has been suggested that it participates in BER (*35*, *36*), meiotic recombination (*37*), nonhomologous end joining process (*38*), and TLS (*39*). This enzyme has

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*Corresponding author: telephone, +7 (383) 3635195; fax, +7 (383) 3635153; e-mail, lavrik@niboch.nsc.ru.

[1]Abbreviations: hRPA, *h*uman *r*eplication *p*rotein *A*; hPCNA, *h*uman *p*roliferating *c*ell *n*uclear *a*ntigen; TLS, *t*rans*l*esion *s*ynthesis; Tg, thymine glycol; pol, DNA polymerase.

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Table 1: Substrate Constructs Used in the Present Study[a]

DNA substrates	scheme	name
5′-GGCTTCATCGTTGTC**X**CAGACCTGGTGGATACCG 3'-CCGAAGTAGCAp GTCTGGACCACCTATGGC*		DNAgap5
5′-GGCTTCATCGTTGTC**X**CAGACCTGGTGGATACCG 3'-CCGAAGTAGCAACAp GTCTGGACCACCTATGGC*		DNAgap2
5′-GGCTTCATCGTTGTC**X**CAGACCTGGTGGATACCG 3'-CCGAAGTAGCAACAGp GTCTGGACCACCTATGGC*		DNAgap1

[a]Key: **X**, Tg, T, or A; p, phosphate group; *, ^{32}P-radioactive label.

DNA polymerase activity and lacks 3′−5′ exonuclease activity. DNA pol λ is able to carry out template-independent synthesis utilizing single-stranded DNA (*40*). It displays high efficacy with DNA substrates containing a one- or two-nucleotide gap, including a 5′-end phosphate, in contrast to extended DNA templates (*36*, *40*).

It has been found that both DNA pols β and λ are able to interact with hPCNA (*25*, *41*, *42*). Moreover, the influence hPCNA and hRPA (*h*uman *r*eplication *p*rotein *A*) on the normal and translesion activity of these enzymes has been shown previously (*28*, *42*−*44*). However, in spite of the massive amount of data, the biological role of DNA pols β and λ, including TLS specificity, remains unclear.

In the present study we investigated TLS activity of the human X-family DNA pols β and λ on gapped DNA substrates. As lesion Tg, located in the +1 position of the template strand relative to the 3′-end of the primer, was used. The effect of hPCNA and hRPA on TLS in this system was studied. Reactions were carried out in the presence of either Mg^{2+} or Mn^{2+}. Maximum velocities (V_{max}) and the Michaelis constant values (K_m) in reaction of DNA synthesis were determined.

EXPERIMENTAL PROCEDURES

Materials. Synthetic oligonucleotides were obtained from GenSet (Switzerland). Reagents for electrophoresis and the basic components of buffers were from Sigma (USA). [γ-^{32}P]ATP (with specific activity 5000 Ci/mmol) was from the Laboratory of Biotechnology (Institute of Chemical Biology and Fundamental Medicine, Novosibirsk, Russia). T4 polynucleotide kinase was from Biosan (Novosibirsk, Russia). Ultrapure dNTPs were from Promega (USA).

Proteins. Human recombinant DNA pol λ and human recombinant DNA pol β were purified from *Escherichia coli* BL21(DE3) RP cells as described in refs *45* and *46*, respectively. Proteins hPCNA and hRPA were purified according to refs *47* and *48*, respectively.

Oligonucleotide Substrates. The synthesis of the 34-mer oligodeoxynucleotide containing thymidine glycol was performed as described (*4*, *5*, *49*). The automatic oligonucleotide synthesis technique was applied to incorporate the thymidine glycol residue into a certain position of the oligonucleotide strand. Thymidine glycol synthon 5′-*O*-dimethoxytrityl-2′-deoxy-(5*R*,6*S*)-5,6-bis(*tert*-butyldimethylsilyloxy)-5,6-dihydrothymidine 3′-[(2-cyanoethyl)-(*N*,*N*-diisopropyl)]phosphoramidite was purchased from Glen Research (USA). After the oligonucleotide synthesis and deprotection step, the purity of modified oligonucleotides was confirmed by reverse-phase high-performance liquid chromatography, polyacrylamide gel electrophoresis, and MALDI-TOF MS. Epimerization was not observed. Studies on the stability of Tg-containing oligonucleotides have been previously reported (*4*). Modified oligonucleotides were not degraded under the conditions used for their synthesis and purification. Oligodeoxynucleotides were 5′-^{32}P-phosphorylated with T4 polynucleotide kinase as described (*50*). Unreacted [γ-^{32}P]ATP was removed by passing the mixture over a MicroSpin G-25 column (Amersham Pharmacia Biotech, GE Healthcare, USA) using the manufacturer's protocol. Complementary oligodeoxynucleotides were annealed in equimolar amounts by heating a water solution to 80 °C (for Tg-containing oligos) and to 95 °C (for native DNA substrates) for 10 min, followed by slow cooling to room temperature. The stability of Tg-containing oligos at this temperature was investigated previously (*4*). The oligonucleotide substrates used in the study are presented in the Table 1.

DNA Pol Assay. DNA synthesis by DNA pols β and λ was performed in reaction buffer (TDB buffer) containing 50 mM Tris-HCl (pH 7.5), 0.5 mM dithiothreitol, 0.25 mg/mL bovine serum albumin, and 1 mM $MgCl_2$ or $MnCl_2$. All mixtures (final volume 10 μL) included 10 nM 5′-^{32}P-phosphorylated DNA substrate, 10 nM DNA pol β or λ, and 5 μM dNTP. All reactions were incubated at 37 °C for 20 min. The reactions were terminated by adding the gel loading solution (90% formamide, 0.1% xylene cyanole, and 0.1% bromophenol blue) and heated at 97 °C for 5 min. The mixtures were resolved on a 20% polyacrylamide gel containing 7 M urea in TBE buffer (*50*). The gels were dried and subjected to autoradiography and/or phosphorimaging for quantitation using the Molecular Imager Pro FX+ and "Quantity One" software (Bio-Rad, USA) and analyzed using Origin-Pro7.5 (Microcal Software, USA).

The Michaelis constants, K_m, and the maximum velocities, V_{max}, of DNA synthesis catalyzed by DNA pols β and λ were determined in two steps. In the first step, kinetics of dNMP incorporation into different DNA substrates by DNA pols β and λ were estimated. The reaction was performed in 90 μL of a mixture containing 10 nM DNA pol β or λ, 5 μM dNTP, 10 nM 5′-^{32}P-phosphorylated DNA substrate, and 1 mM $MgCl_2$ or $MnCl_2$ in TDB buffer at 37 °C. Aliquots (10 μL) were taken after selected time periods in the range 0−40 min. The reaction was terminated by placement on ice. The reaction products were separated as described above. In the next step the Michaelis constants, K_m, and the maximum velocities, V_{max}, of DNA synthesis catalyzed by DNA pols β and λ were determined by the variation of dNTP concentration from 0.05 to 7 μM dNTP, and reaction products were analyzed as described above. The data were fitted according to the Michaelis−Menten kinetic equation (*51*).

The effect of hPCNA and hRPA on the polymerization reaction catalyzed by DNA pols β and λ was studied as follows. The reaction mixtures (final volume 10 μL) contained 10 nM 5′-^{32}P-labeled DNA substrates, 10 nM DNA pol β or λ, dATP or dGTP

in defined concentrations, and hPCNA or hRPA in TDB buffer. The hPCNA concentration was 100 or 200 nM. The hRPA concentration was 100 or 200 nM in the reactions with $DNA_{Tg}1$ and $DNA_{Tg}2$ (in a ratio hPCNA:hRPA = 1:1) and 5, 10, or 20 nM in the reactions with $DNA_{Tg}5$ and $DNA_{Tg}2$ (in a ratio DNA: hRPA = 2:1, 1:1, and 1:2). The combined effect of hPCNA and hRPA was investigated by setting the hPCNA concentration at 100 nM. In this case the hRPA concentration was varied as described above. All reactions were incubated at 37 °C for 15 min. The reaction products were analyzed as outlined above.

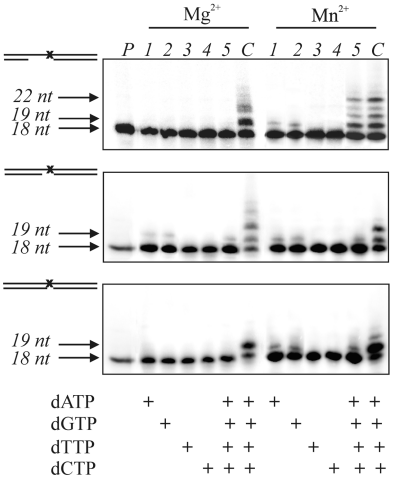

FIGURE 1: The capacity of DNA pol λ to possess the TLS activity on $DNA_{Tg}1$, $DNA_{Tg}2$, and $DNA_{Tg}5$ substrates. Lane P, initial position of the 5′-^{32}P-labeled DNA substrate (18 nt); lane C, DNA synthesis catalyzed by DNA pol λ in the presence of all four dNTPs using undamaged DNAgap1, DNAgap2, or DNAgap5 substrates; 19 nt and 22 nt, length of reaction products. Reaction mixture contained 10 nM 5′-^{32}P-phosphorylated DNA substrate, 10 nM DNA pol λ, and 5 μM each dNTP.

RESULTS

In present study we investigated the TLS process catalyzed by the human X-family DNA pols β and λ on gapped DNA substrates with the damage located in a definite position of the template strand. These DNA structures imitate the intermediates of the replication process on the lagging strand of the damaged DNA. We used a thymine glycol, oxidated pyrimidine base, as a lesion, which impedes DNA synthesis (*52*). DNA substrates were constructed as partial DNA duplexes with a one- ($DNA_{Tg}1$), two- ($DNA_{Tg}2$), or five-nucleotide ($DNA_{Tg}5$) gap containing 3′-hydroxyl and 5′-phosphate groups (see Experimental Procedures). The lesion was located in the +1 position of the template strand relative to the 3′-end of the primer. In all cases the reactions were carried out in the presence of either Mg^{2+} or Mn^{2+}, i.e., in optimal polymerization conditions or to support high efficiency but low fidelity of enzymatic synthesis, respectively. Control experiments were performed on undamaged DNA substrates. Maximum velocities (V_{max}) and the Michaelis constant values (K_m) in the reaction of DNA synthesis were determined.

DNA Pol λ Can Bypass the Tg Lesion on the Template Strand of Gapped DNA Substrates. First, we tested the capacity of DNA pol λ to perform the TLS activity on gapped DNA substrates containing Tg as lesion. We found that DNA pol λ was able to incorporate dAMP (as a complementary nucleotide to Tg) as well as dGMP (via template slippage according with the template sequence), independently of DNA structure (Figure 1, Table 2). Moreover, it was strongly possible in the Mn^{2+}-dependent manner using $DNA_{Tg}1$ or $DNA_{Tg}5$ substrates

Table 2: Michaelis Constants (K_m), Turnover Rates (k_{cat}), and Incorporation Efficiencies (k_{cat}/K_m) of dNMP Incorporation into $DNA_{Tg}1$, $DNA_{Tg}2$, and $DNA_{Tg}5$ Catalyzed by DNA Pol λ in the Presence of Mg^{2+} or Mn^{2+} [a]

	Mg^{2+}			Mn^{2+}		
substrate and dNTP	K_m, μM	k_{cat}, s^{-1}	k_{cat}/K_m, μM^{-1} s^{-1}	K_m, μM	k_{cat}, s^{-1}	k_{cat}/K_m, μM^{-1} s^{-1}
			Tg			
$DNA_{Tg}5$						
dATP	–	–	–	0.065	0.016	0.24
dGTP	–	–	–	0.221	0.028	0.12
$DNA_{Tg}2$						
dATP	0.091	0.0063	0.069	0.012	0.027	2.3
dGTP	1.42	0.011	0.008	0.083	0.043	0.52
$DNA_{Tg}1$						
dATP	–	–	–	0.048	0.019	0.39
dGTP	–	–	–	0.51	0.016	0.031
			Undamaged			
DNAgap5 (T)						
dATP	6.44	0.026	0.0041	0.144	0.060	0.42
dGTP	–	–	–	–	–	–
DNAgap2 (T)						
dATP	nm	nm	nm	0.102	0.19	1.9
dGTP	nm	nm	nm	1.54	0.035	0.023
DNAgap1 (T)						
dATP	nm	nm	nm	0.008	0.082	10
dGTP	nm	nm	nm	0.148	0.0095	0.064

[a](–), no incorporation was observed under conditions which were used for the investigation. nm, not measured. Note: the results are presented as the average value of three independent experiments. Standard error was estimated as 10%.

but not with $DNA_{Tg}2$. In all cases dTTP and dCTP could not serve as substrates for DNA pol λ. It should be noted that the efficiency and fidelity of the TLS reaction depended on the size of the DNA gap; however, the most efficient incorporation of both dAMP and dGMP was obtained using $DNA_{Tg}2$ (Table 2). In addition, independently of the DNA substrate the incorporation of complementary nucleotide dAMP was carried out more effectively (for example, for $DNA_{Tg}5$ k_{cat}/K_m(dA) 0.24 μM^{-1} s^{-1} and k_{cat}/K_m(dG) 0.12 μM^{-1} s^{-1}, respectively) and characterized by lower K_m value (K_m(dA) 0.065 μM^{-1} s^{-1} and K_m(dG) 0.221 μM, respectively). Interestingly, in the absence of a lesion, DNA pol λ catalyzed only the incorporation of complementary dAMP independently of the metal ion using DNAgap5 substrates (Table 2). In contrast to TLS activity, synthesis using undamaged DNA was more efficient and characterized by lower K_m values in the presence of Mn^{2+}.

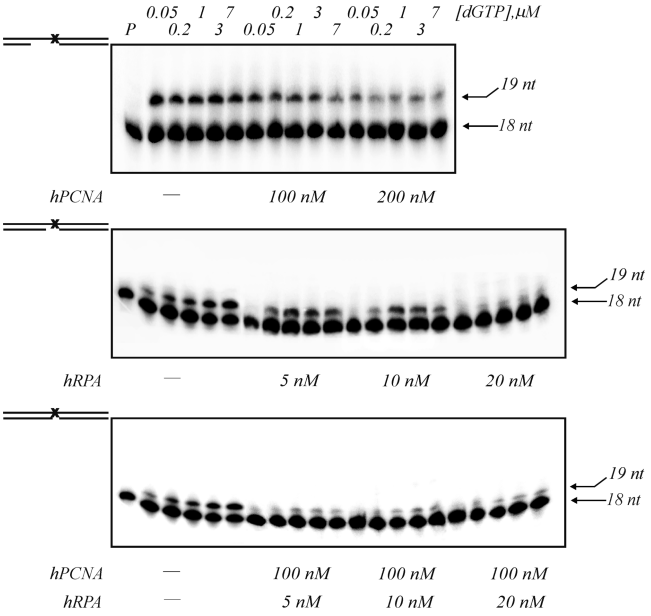

FIGURE 2: Influence of hPCNA (upper panel), hRPA (middle panel), or hPCNA and hRPA (bottom panel) on the incorporation of "incorrect" dGMP catalyzed by DNA pol λ using $DNA_{Tg}5$ substrates in the presence of Mn^{2+} ions. The final concentrations of dGTP are indicated on the top of the gels; the final concentrations of hPCNA and hRPA are indicated under the gels.

DNA Pol λ Can Bypass the Tg Lesion on the Template Strand of Gapped DNA Substrates Assisted by hRPA and hPCNA. The effect of the DNA replication proteins hPCNA and hRPA on TLS catalyzed by DNA pol λ was then investigated (Figure 2). The influence was estimated by comparing the different parameters (K_m, k_{cat}, k_{cat}/K_m) for incorporation of complementary dAMP or "incorrect" dGMP whether hPCNA and/or hRPA was present in the reaction mixture or not. The K_m and V_{max} were evaluated from these experiments, and the k_{cat} and k_{cat}/K_m values were calculated (see Tables 1−3 in Supporting Information). Figure 3 is the illustration of changing in V_{max}

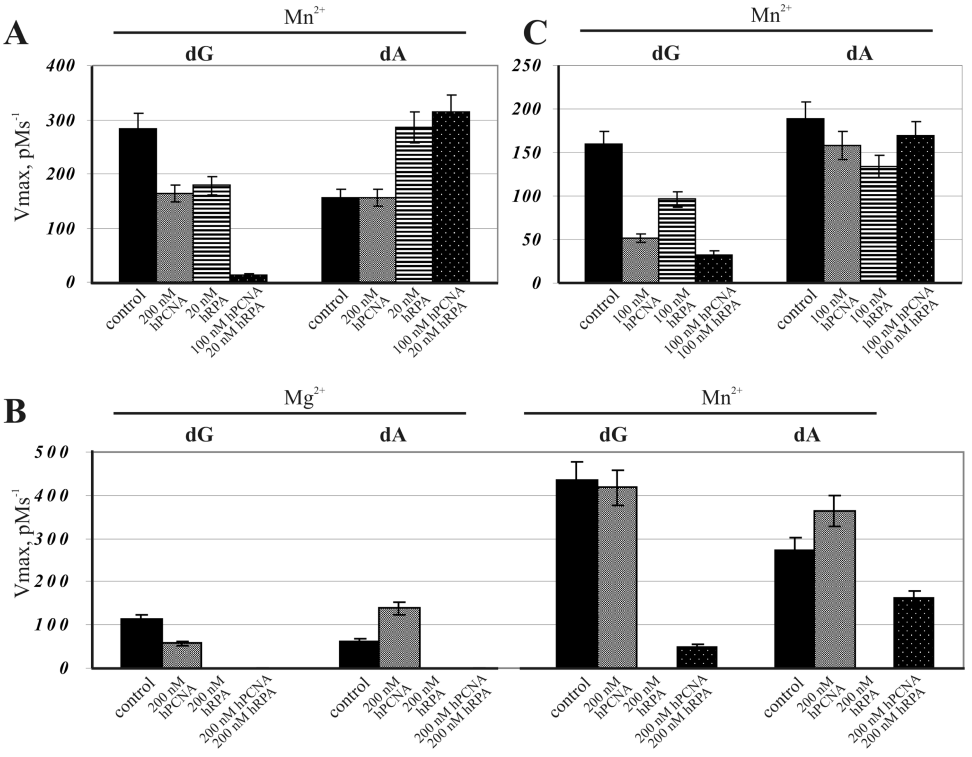

FIGURE 3: Influence of hPCNA and hRPA on the incorporation of dAMP or dGMP catalyzed by DNA pol λ using $DNA_{Tg}5$ (A), $DNA_{Tg}2$ (B), and $DNA_{Tg}1$ (C) substrates in different buffer conditions. The *Y*-axis presents the maximum velocities of DNA synthesis, in picomolar per second. The final concentrations of hPCNA (gray rectangle), hRPA (striped rectangle), or both hPCNA and hRPA (dotted rectangle) in the reaction mixture are indicated under the diagrams. Control experiments were performed in the same reaction conditions (black rectangle).

parameter of the dAMP and dGMP incorporation into DNA_{Tg} by DNA pol λ upon the addition of hPCNA and/or hRPA to the reaction mixtures.

DNA pol λ was able to bypass Tg assisted by hPCNA and hRPA in five-window gap structures (Figure 3A). Since the presence of hPCNA in the reaction mixture decreased the efficiency of incorporation of "incorrect" dGMP in Mn^{2+}-containing buffer (Table 1 in Supporting Information), this can indicate that hPCNA increased the fidelity of TLS catalyzed by DNA pol λ. This suggestion is confirmed by the misinsertion ratio which was based on the incorporation efficiencies of incorrect compared to correct nucleotide: misinsertion ratio = $(k_{cat}/K_m)_{incorrect\ dNTP}/(k_{cat}/K_m)_{correct\ dNTP}$. In this case the misinsertion ratio decreased from 0.54 ± 0.11 to 0.32 ± 0.06 upon hPCNA addition. At the same time, hPCNA had no significant effect on the incorporation of the complementary nucleotide dAMP (Figure 3A). hRPA provided "high-fidelity" TLS catalyzed by DNA pol λ to a greater extent than hPCNA, due to inhibition of the incorporation of the "incorrect" dGMP (misinsertion ratio in the presence of hRPA was 0.29 ± 0.06, approximately 1.5 times smaller than 0.54 ± 0.11 which was calculated for the reaction in the absence of the replication protein). The same effect on dGMP incorporation was observed when hPCNA and hRPA were added simultaneously (Table 3 in Supporting Information). Moreover, in this last case increase in reaction efficiency and k_{cat} value for dAMP incorporation were observed (Table 3 in Supporting Information).

Interestingly, only hRPA led to an increase of DNA pol λ fidelity during normal DNAgap5 synthesis in the presence of Mn^{2+} (Table 2 in Supporting Information). hPCNA alone had no sufficient influence on the different parameters under various reaction conditions (Table 1 in Supporting Information), and in combination with hRPA it led to a decrease in the fidelity of DNA synthesis in the presence of Mn^{2+} (Table 3 in Supporting Information).

hPCNA increased the TLS fidelity of DNA pol λ in the presence of Mn^{2+} or Mg^{2+} using $DNA_{Tg}2$ substrates in the following way (Figure 3B, Table 3 in Supporting Information). In the presence of Mg^{2+} ions hPCNA addition leads to an increase in the incorporation level of correct dAMP and to a decrease in the incorporation level of "incorrect" dGMP (Figure 3B, left panel). In this case the misinsertion ratio goes down to 0.07 ± 0.01 from 0.12 ± 0.02 which was calculated without hPCNA. After the substitution of Mg^{2+} to Mn^{2+} ions the same influence of hPCNA on the dAMP incorporation was observed, i.e., the increase in the incorporation level of correct nucleotide (Figure 3B, right panel). At the same time, hPCNA had no sufficient effect on the incorporation of dGMP in Mn^{2+}-dependent conditions (Figure 3B, right panel). hRPA completely suppressed the incorporation of correct dAMP or "incorrect" dGMP opposite the Tg (Table 2 in Supporting Information) in the presence of Mn^{2+} (Figure 3B, right panel) or Mg^{2+} (Figure 3B, left panel). hPCNA and hRPA when added simultaneously considerably decreased (in Mn^{2+}-containing buffer conditions) or totally inhibited (in Mg^{2+}-containing conditions) the TLS activity of DNA pol λ (Figure 3B, Table 3 in Supporting Information).

It was observed that hPCNA increased the accuracy of DNA pol λ in Tg bypassing on $DNA_{Tg}1$ (Figure 3C) in the presence of Mn^{2+} by decreasing the incorporation level of "incorrect" dGMP (Table 1 in Supporting Information). At same time, hPCNA had no sufficient effect on incorporation level of dAMP (Table 1 in Supporting Information). hRPA alone inhibited the incorporation of both correct and "incorrect" nucleotides by DNA pol λ using $DNA_{Tg}1$ (Figure 3C, Table 2 in Supporting Information). The combination of both hRPA and hPCNA leads to inhibition of TLS activity of DNA pol λ resulted in -1 deletion (Figure 3C, Table 3 in Supporting Information).

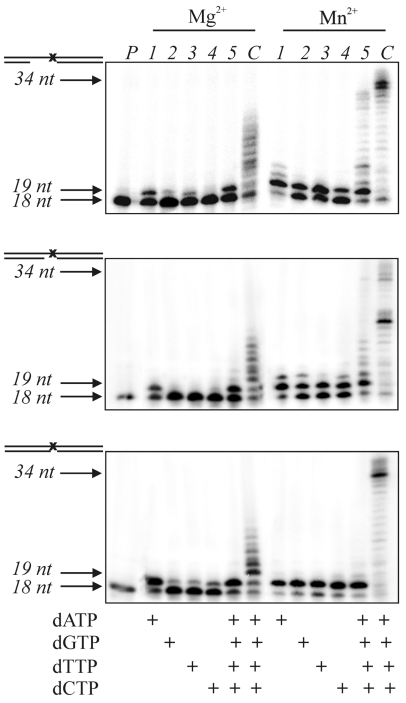

FIGURE 4: The capacity of DNA pol β to possess the TLS activity on $DNA_{Tg}1$, $DNA_{Tg}2$, and $DNA_{Tg}5$ substrates. Lane P, initial position of the 5′-^{32}P-labeled DNA substrate (18 nt); lane C, DNA synthesis catalyzed by DNA pol β in the presence of all four dNTPs using undamaged DNAgap1, DNAgap2, or DNAgap5 substrate; 19 nt, length of reaction products; 34 nt, size of the full-length reaction products. The reaction mixture contained 10 nM 5′-^{32}P-phosphorylated DNA substrate, 10 nM DNA pol β, and 5 μM each dNTP.

It should be noted that the presence of hRPA only (Table 2 in Supporting Information) or in combination with hPCNA (Table 3 in Supporting Information) reduced the fidelity of DNA synthesis catalyzed by DNA pol λ on undamaged DNAgap1 (misinsertion ratios go up from 0.006 ± 0.001, which was calculated without replication proteins, to 0.44 ± 0.09 and 0.35 ± 0.07 under addition of hRPA alone or combined with hPCNA). At the same time, hPCNA alone had no influence on the synthesis catalyzed by DNA pol λ (Table 1 in Supporting Information).

DNA Pol β Can Bypass the Tg Lesion on the Template Strand of Gapped DNA Substrates. DNA pol β was able to catalyze the incorporation of dAMP (as a complementary nucleotide to template T-base) and the incorporation of dGMP, dCMP, and dTMP during DNA synthesis across Tg using $DNA_{Tg}1$, $DNA_{Tg}2$, and $DNA_{Tg}5$ substrates in the presence of both Mg^{2+} and Mn^{2+} (Figure 4); however, it performed TLS activity more efficiently in the presence of Mn^{2+} (Figure 4, left panel). Since DNA pol β catalyzes the incorporation of all four dNTPs opposite Tg, it can be concluded that it has an error-prone bypassing of the lesion.

Here we only investigated DNA synthesis in the presence of dATP and dGTP (Table 3). Independently of the metal cofactor, DNA synthesis using complementary dATP as substrate was more efficient than using "incorrect" dGTP (for example, for $DNA_{Tg}5$ k_{cat}/K_m(dA, Mn^{2+}) 1.2 $\mu M^{-1}\ s^{-1}$ and k_{cat}/K_m(dG, Mn^{2+}) 0.018 $\mu M^{-1}\ s^{-1}$, respectively). Moreover, in the presence of Mn^{2+} ions strand displacement synthesis was observed. In the

Table 3: Michaelis Constants (K_m), Turnover Rates (k_{cat}), and Incorporation Efficiencies (k_{cat}/K_m) of dNMP Incorporation into $DNA_{Tg}1$, $DNA_{Tg}2$, and $DNA_{Tg}5$ Catalyzed by DNA Pol β in the Presence of Mn^{2+} or Mg^{2+} [a]

	Mg^{2+}			Mn^{2+}		
substrate and dNTP	K_m, μM	k_{cat}, s^{-1}	k_{cat}/K_m, μM^{-1} s^{-1}	K_m, μM	k_{cat}, s^{-1}	k_{cat}/K_m, μM^{-1} s^{-1}
			Tg			
$DNA_{Tg}5$						
dATP	11.3	0.038	0.0034	0.07	0.084	1.2
dGTP	12.5	0.0064	0.0005	3.51	0.064	0.018
$DNA_{Tg}2$						
dATP	0.322	0.068	0.21	0.043	0.093	2.2
dGTP	0.162	0.0073	0.045	0.653	0.083	0.13
$DNA_{Tg}1$						
dATP	0.371	0.090	0.24	0.006	0.092	15
dGTP	61.73	0.070	0.0011	0.337	0.076	0.23
			Undamaged			
DNAgap5 (T)						
dATP	0.019	0.084	4.4	0.085	0.078	0.92
dGTP	−	−	−	5.99	0.059	0.0098
DNAgap2 (T)						
dATP	nm	nm	nm	0.183	0.037	0.21
dGTP	nm	nm	nm	2.28	0.021	0.0090
DNAgap1 (T)						
dATP	nm	nm	nm	0.003	0.097	32
dGTP	nm	nm	nm	0.343	0.076	0.22

[a](−), no incorporation was observed under conditions which were used for the investigation. nm, not measured. Note: the results are presented as the average value of three independent experiments. Standard error was estimated as 10%.

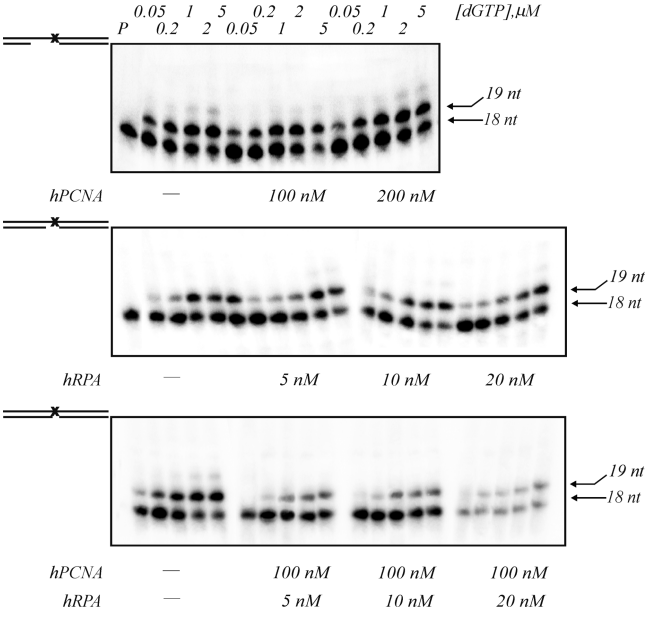

FIGURE 5: Influence of hPCNA (upper panel), hRPA (middle panel), or hPCNA and hRPA (bottom panel) on the incorporation of "incorrect" dGMP catalyzed by DNA pol β using $DNA_{Tg}5$ substrates in the presence of Mn^{2+} ions. The final concentrations of dGTP are indicated on the top of the gels; the final concentrations of hPCNA and hRPA are indicated under the gels.

absence of the lesion DNA pol β catalyzed only error-free DNA synthesis using all DNA substrates (data not shown).

DNA Pol β Can Bypass the Tg Lesion on the Template Strand of Gapped DNA Substrates Assisted by hRPA and hPCNA. We tested the effect of hPCNA and hRPA on TLS and normal synthesis catalyzed by DNA pol β (Figure 5; see Tables 4−6 in Supporting Information). hPCNA increased the fidelity of DNA synthesis across the Tg in $DNA_{Tg}5$ catalyzed by DNA pol β in the presence of Mg^{2+} (Figure 6A, left panel) by both increasing the efficiency of incorporation of the complementary nucleoside dAMP and decreasing the efficiency of the reaction and the catalytic rate k_{cat} for the incorporation of "incorrect" dGMP (Table 4 in Supporting Information). In this case in the absence of hPCNA the misinsertion ratio was 0.147 ± 0.030, while in the presence of hPCNA this value was 0.017 ± 0.003, approximately 10 times smaller. However, in Mn^{2+}-containing buffer conditions (Figure 6A, right panel) hPCNA increased the efficiency of incorporation of "incorrect" dGMP as well as correct dAMP by DNA pol β (Table 4 in Supporting Information). Therefore, the presence of hPCNA in the reaction mixture increased the fidelity of DNA synthesis catalyzed by DNA pol β under optimal polymerization conditions and had no specific effect when the metal cofactor was changed.

hRPA significantly decreased the efficiency and the level of incorporation of complementary dAMP and completely inhibited the incorporation of "incorrect" dGMP opposite Tg in the presence of Mg^{2+} into $DNA_{Tg}5$ (Figure 6A, left panel, Table 5 in Supporting Information). At the same time, the high concentrations of hRPA had no sufficient effect on DNA synthesis using dATP or dGTP as substrate in the presence of Mn^{2+} (Figure 6A, right panel, Table 5 in Supporting Information). Thus, it can be concluded that hRPA had no specific influence on DNA synthesis catalyzed by DNA pol β on $DNA_{Tg}5$ substrates.

The combined effect of the replication proteins depended on the hRPA concentration in the reaction mixture (Figure 6A, Table 6 in Supporting Information): (i) low concentrations of hRPA (hRPA:$DNA_{Tg}5$ = 1:2 or 1:1) led to inhibition of incorporation of complementary dAMP as well as of "incorrect" dGMP; (ii) when hRPA was added at the high concentrations

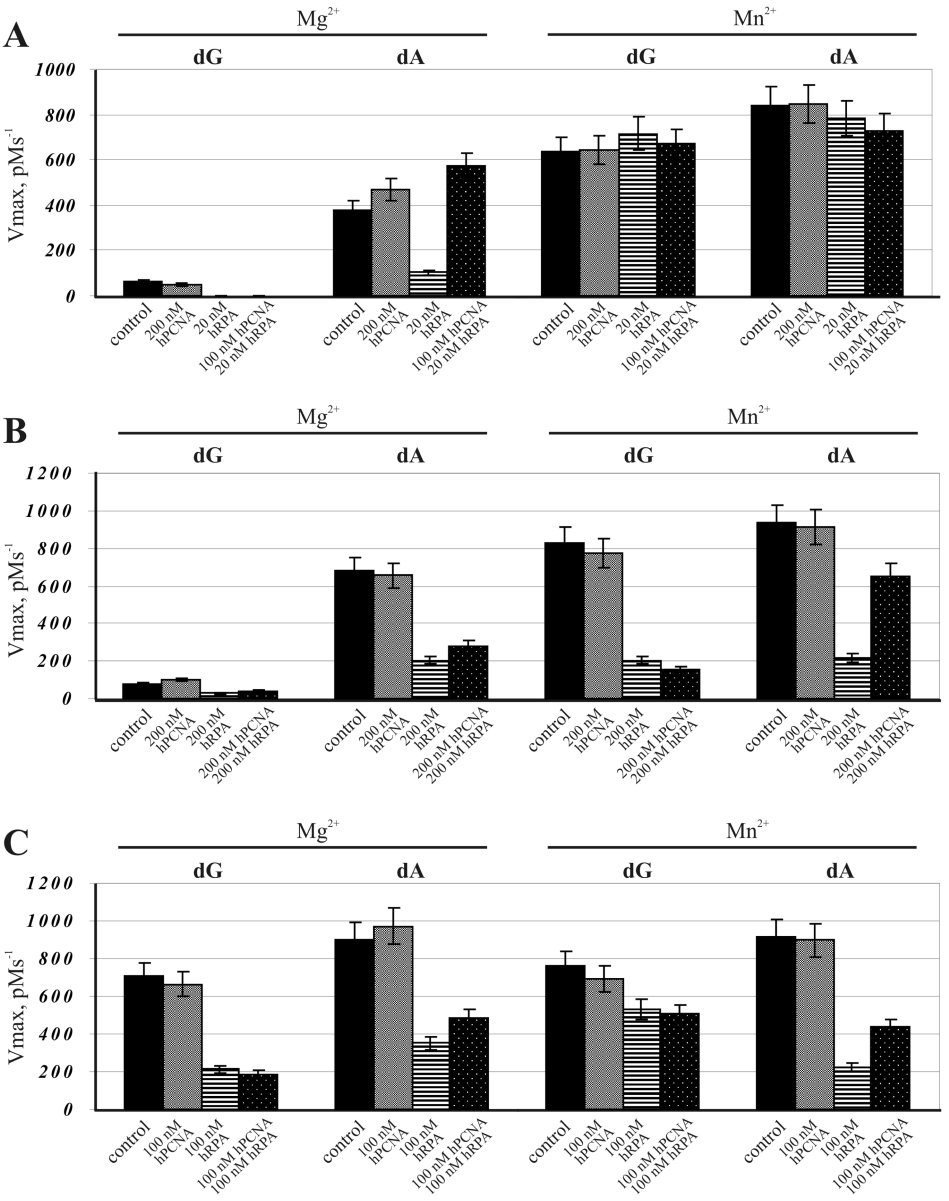

FIGURE 6: Influence of hPCNA and hRPA on the incorporation of dAMP or dGMP catalyzed by DNA pol β using $DNA_{Tg}5$ (A), $DNA_{Tg}2$ (B), or $DNA_{Tg}1$ (C) substrates in different buffer conditions. The *Y*-axis presents the maximum velocities of DNA synthesis in picomolar per second. The final concentrations of hPCNA (gray rectangle), hRPA (striped rectangle), or both hPCNA and hRPA (dotted rectangle) in the reaction mixture are indicated under the diagrams. Control experiments were performed in the same reaction conditions (black rectangle).

(hRPA:$DNA_{Tg}5$ = 2:1), an increase in the incorporation level of complementary dAMP and full decrease in DNA synthesis with dGTP as substrate were observed in the presence of Mg^{2+}. The reverse effect was observed in the presence of Mn^{2+} ions.

The presence of hPCNA or hRPA or both in the reaction mixture with undamaged DNAgap5 led to inhibition of normal polymerase activity of DNA pol β for the incorporation of complementary dAMP as well as "incorrect" dGMP under different buffer conditions (Tables 4−6 in Supporting Information). Based on these results it can be concluded that hPCNA and hRPA had no specific combined effect on DNA synthesis catalyzed by pol β on undamaged DNAgap5.

DNA pol β can bypass the Tg assisted by hPCNA and hRPA in two-window gap DNA structures (Figure 6B). It was found that hPCNA had no sufficient effect on the incorporation level of dAMP or dGMP opposite Tg in the presence of Mg^{2+} or Mn^{2+} (Table 4 in Supporting Information). At the same time, hRPA alone (Table 5 in Supporting Information) or present together with hPCNA (Table 6 in Supporting Information) significantly suppressed the incorporation of correct dAMP or "incorrect" dGMP under different reaction conditions.

The same effect of hPCNA on TLS catalyzed by DNA pol β was shown using $DNA_{Tg}1$ (Figure 6C). hPCNA had no significant influence on TLS in the presence of Mn^{2+} or Mg^{2+} (Table 4 in Supporting Information). hRPA decreased the incorporation efficiency of "incorrect" dGMP opposite Tg in the presence of Mn^{2+} when it was added alone (Table 5 in Supporting Information).

The same effects of hPCNA and hRPA were observed using undamaged DNAgap1 structures. hPCNA had no significant influence on the dNMP incorporation efficiency provided by DNA pol β in the presence of Mn^{2+} (Table 4 in Supporting Information). The presence of hRPA only (Table 5 in Supporting Information) or in combination with hPCNA (Table 6 in Supporting Information) reduced the incorporation efficiency of dTMP as well as dGMP catalyzed by DNA pol β.

DISCUSSION

TLS is one of the DNA damage tolerance strategies that evolved to enable organisms to replicate their genome despite the presence of unrepaired damage. TLS employs a specialized set of DNA pols that are characterized by more open active sites in comparison to replicative DNA pols and by lack of 3′−5′ exonuclease proofreading activity. Such DNA pols mainly belong to the structural Y-family. However, recent observations suggest that DNA pols from other families are able maintain DNA synthesis across the lesion (*15*−*18*, *20*). Here, we investigated the TLS activity of the human X-family DNA pols β and λ on DNA duplexes containing a Tg residue as lesion in the template strand.

Tg, the most common oxidation product of thymine, which is produced by endogenous or exogenous factors, is nonplanar unlike normal DNA bases. This lesion strongly blocks repair and replicative DNA pols.

Here we used DNA substrates with the damage located in the +1 position of the template strand with respect to the 3′-end of the primer. They were constructed as mono-, di-, or pentanucleotide gapped DNA duplexes with 3′-hydroxyl and 5′-phosphate groups inside the gap ($DNA_{Tg}1$, $DNA_{Tg}2$, or $DNA_{Tg}5$). Such DNAs can serve as DNA intermediates of the lagging strand during normal genome replication or of the leading strand under fork arrest and restart downstream of the template lesion leaving a gap (reviewed in ref *12*). We determined the maximum velocities (V_{max}) and the Michaelis constants (K_m) of dNMP incorporation in the model systems under optimal and "low fidelity" polymerization conditions (in the presence of Mg^{2+} or Mn^{2+}, respectively).

Both DNA pols β and λ can process $DNA_{Tg}1$, $DNA_{Tg}2$, or $DNA_{Tg}5$ substrates but in the different ways. DNA pol λ was able to incorporate dAMP (as complementary nucleotide to Tg) and dGMP (via template slippage according with the template sequence) on $DNA_{Tg}1$, $DNA_{Tg}2$, or $DNA_{Tg}5$ (Figure 1). Moreover, DNA pol λ can perform TLS activity strongly in a Mn^{2+}-dependent manner (except in the case of $DNA_{Tg}2$). Interestingly, after passing the lesion DNA pol λ was not able to elongate the 3′-end of the primer paired with Tg in one- and two-window gap structures. However, the same 3′-end of the primer was extended by up to four nucleotides when the $DNA_{Tg}5$ structure was used. In contrast, undamaged DNAgap1, DNAgap2, and DNAgap5 were processed normally, and the newly synthesized strand did not exceed the length of the gap; i.e., strand displacement synthesis was not observed. These results are in agreement with published data obtained on undamaged DNA substrates (*36*, *40*).

As opposed to DNA pol λ, DNA pol β catalyzed DNA synthesis opposite Tg using all four dNTPs, and its fidelity did not depend on the metal cofactor. Additionally, DNA pol β was able to conduct strand displacement synthesis in "low fidelity" buffer conditions. In the absence of lesion DNA pol β provided only error-free DNA synthesis. The results obtained using undamaged DNAs correlated with previous results (*53*−*55*).

These data have a promising consequence. If Tg is encountered in a template by replicative or repair pols, termination of primer extension occurs immediately past the lesion site with the base inserted opposite the Tg, even in the case of dA (*7*, *8*, *55*). Hence the observation that DNA pol λ was able to support DNA synthesis on substrates containing template Tg paired with the 3′-terminal base of the primer is very important and suggests that DNA pol λ is a candidate in Tg bypassing.

Since TLS pols could be constitutively associated with the replication machinery at the progressing replication fork, they could interact with replication and repair proteins such as hPCNA and hRPA (*56*). hPCNA has been proposed to coordinate switching from replicative DNA pols to specialized TLS enzymes (reviewed in refs *57* and *58*). It has been shown that DNA pol λ interacts both functionally and physically with hPCNA (*25*, *41*). DNA pol λ activity is modulated in various ways by hRPA (*27*, *28*, *43*, *44*, *59*, *60*). Moreover, a specific role of hRPA and hPCNA in bypassing of the 8-oxoguanine and 2-hydroxyadenine lesion by DNA pol λ was demonstrated (*27*, *28*, *43*). The influence of hRPA on DNA pol β activity was examined by others (*61*, *62*). Kedar et al. (*42*) observed that DNA pol β is able to bind to hPCNA. For this reason, we decided to investigate the influence of hPCNA and hRPA on DNA pols β and λ catalyzed processing of gapped DNA substrates bearing Tg.

Based on the data obtained, it can be concluded that hPCNA has a positive effect on TLS activity and has no influence on normal DNA synthesis catalyzed by DNA pol λ on gapped DNAs. hRPA did not display a specific effect on the functioning of DNA pol λ. At the same time, hPCNA as well as hRPA showed no difference in their effect on TLS and normal DNA synthesis catalyzed by DNA pol β using gapped DNA substrates. Thus, in spite of the fact that DNA pol β is more efficient in Tg bypassing in gapped DNAs, DNA pol λ seems to be the preferential candidate for the translesion process on such types of DNA structures.

The actual model for lesion bypass during TLS comprises the following steps. Meeting with damage site can lead to replication fork stalling. To complete genome duplication high-fidelity replicative DNA pols should give the place to specialized TLS pols on damaged DNA template. The main trigger which might serve for such rearrangements is PCNA (*13*). Damage-induced modification of PCNA would increase the affinity for the TLS pols, resulting in its gaining access to the 3′-OH primer terminus to successfully restore the replication fork moving. Our data suggest that DNA pol λ could be such TLS pol in the case of a lesion being Tg.

Combining the present results with already published data, we can propose the hypothesis that DNA pol λ in combination with hPCNA and hRPA plays an important role in TLS across oxidated bases such as 2-hydroxyadenine, 8-oxoguanine, and Tg during genomic replication on the lagging strand *in vivo*.

SUPPORTING INFORMATION AVAILABLE

Six tables as referenced in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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