A role for DNA polymerase β in mutagenic UV lesions bypass

Laurence Servant†, Christophe Cazaux†, Anne Bieth†, Shigenori Iwai*, Fumio Hanaoka§¶, and Jean-Sébastien Hoffmann¶.

From †Group “Genetic instability and cancer” at the Institut de Pharmacologie et Biologie Structurale, UMR CNRS 5089, 31077 Toulouse cédex 4, France, *Biomolecular Engineering Research Institute, 6-2-3 Furuedai, Suita, Osaka 565-0874, Japan, and § Graduate School of Frontier Biosciences, Osaka University and CREST, Japan Science and Technology Corporation, 1-3 Yamada-oka, Suita, Osaka 565-0871, Japan.

§¶corresponding authors, jseb@ipbs.fr and fhanaoka@imcb.osaka-u.ac.jp

Running title: Bypass of UV-photoproducts by Pol β

Key words: bypass, mutagenesis, DNA polymerases, UV lesions

Abbreviations: Pol, DNA polymerase; CPD, cis-syn cyclopyrimidine dimer; (6-4)TT, (6-4) photoproduct at TT site; 8-oxo-dG, 8-oxo-deoxyguanosine; HPRT, hypoxanthine-guanine phosphoribosyltransferase; TLS, translesion synthesis; XPV, xeroderma pigmentosum variant.
We report here that DNA polymerase β (Pol β), the base excision repair polymerase, is highly expressed in human melanoma tissues, known to be associated to UV radiation exposure. In order to investigate the potential role of Pol β in UV-induced genetic instability, we analysed the cellular and molecular effects of excess Pol β. We firstly demonstrated that mammalian cells overexpressing Pol β are resistant and hypermutagenic after UV irradiation and that replicative extracts from these cells are able to catalyse complete translesion replication of a thymine–thymine cyclobutane pyrimidine dimer (CPD). By using in vitro primer extension reactions with purified Pol β, we showed that CPD as well as, at a lesser extent, the 6-4 thymine–thymine pyrimidine–pyrimidone photoproduct (6-4PP) were bypassed. Pol β mostly incorporates the correct A opposite the 3' T of both CPD and 6-4PP, but can also misinsert C at a frequency of 32% and 26% respectively. In the case of CPD, efficient and error-prone extension of the correct A was found. These data support biological role of Pol β in UV lesion bypass and suggest that deregulated Pol β may enhance UV-induced genetic instability.
Introduction

Exposure of cells to UV light results in the formation of a variety of lesions in their DNA, the most common being cyclobutane pyrimidine dimers (CPD) and (6-4) pyrimidine–pyrimidone photoproducts ((6-4)PP) at adjacent pyrimidines (1). Unrepaired, these lesions can interfere with normal DNA metabolism including DNA replication, eventually resulting in mutations that lead to carcinogenesis and/or cell death. To maintain their genetic integrity, cells have evolved multiple pathways to repair various types of DNA damage, such as nucleotide excision and base excision repair pathways (1). However, all lesions on the genome cannot be repaired efficiently by these processes in time for DNA replication, and some types of lesions are repaired very inefficiently. To prevent cell death through arrested DNA replication at unrepaired lesions, cells have a mechanism, referred to as translesion synthesis, which allows DNA synthesis to proceed past lesions and employs specialized DNA polymerases for promoting continued nascent strand extension.

In human cells, recent genetic and biochemical studies suggest that translesion synthesis (TLS) past a CPD-TT or a (6-4)TT lesions could be facilitated by at least four DNA polymerases, Pol η, ζ, ι and κ. In the case of Pol η, this process appears to be efficient and largely accurate opposite a CPD (2), whereas it could be mutagenic and limited at the 3’T opposite a (6-4)TT (3). Overexpression of antisense mRNA of Rev3, one of the component of Pol ζ, leads to a dramatic drop in the extent of UV-induced mutagenesis (4), thereby implicating human Pol ζ as having a pivotal role in error-prone translesion replication in normal cells. Indeed Pol ζ can catalyse an efficient extension of nucleotides inserted opposite the 3’ T of both CPD and (6-4)TT lesions (5) (3). Another DNA polymerase, Pol κ, shows similar properties opposite the CPD (6). In the case of Pol ι, the in vitro incorporation of
nucleotides opposite the UV lesions and subsequent bypass can be highly error prone, but its physiological role in TLS is still controversial (5,7,8). Presumably, all these polymerases can compete for 3' primer terminus at the site of a lesion, and one would predict an effect on the quantitative and qualitative mutagenesis in UV-irradiated cells expressing differentially these enzymes. For example, mutations in the POLH (XPV/RAD30A) human gene that generate a severely truncated and inactive Pol η protein result in the Xeroderma pigmentosum variant (XP-V) phenotype characterized by UV-induced hypermutability (9,10) and a strong sunlight-induced skin cancer incidence (11-13).

The study reported here indicates that Pol β can now be added to the list of enzymes that can perform unassisted UV lesion bypass. Pol β is believed to function primarily in the repair of damaged bases in normal somatic cells (14). It is a monomeric protein of 335 amino acids (39 kDa) that lacks exonuclease activities and whose enhanced expression has been demonstrated by our laboratory to result in an increased mutation frequency (15) as well as chromosome instability and tumorigenesis (16). At the transcriptional level Pol β is overexpressed in many cancer cells (17). High levels of Pol β have also been detected at the protein level in ovarian tumors (18) as well as in prostate, breast and colon cancer tissues where the enzyme amount was respectively 11-fold, 286-fold, and 22-fold higher compared with adjacent normal tissues (19). Furthermore, Pol β level and activity are increased by ten fold in blood samples from chronic myelogenous leukemia (CML) patients and in tumor biopsies from non small cell lung tumors (unpublished data). The Pol β-dependent translesion replication that we observed here differs from that of the related DNA polymerases in the efficiency as well as in the accuracy of the reaction. These data may be relevant within the tumoral cellular context where Pol β is up-regulated, especially in melanomas, since several analysis showed a significant positive association between cutaneous melanoma incidence and high levels of intermittent solar exposure (20-24).
Experimental procedures

**Western blotting**
Tissues from normal skin and metastatic melanoma, kindly given by Dr Voigt (ICR, Toulouse, France) were lysed. For analysis of Pol β, cell lysates (70µg of proteins) were electrophoresed in a 12% SDS-PAGE gel and transferred to PVDF membrane (Schleicher and Schuell). Blots were blocked in Tris buffered saline-Tween20 (0.1% Tween) with 5% non fat dry milk, incubated with anti-Pol β monoclonal antibody (1/200, DNA polymerase β Ab-1, clone 18S, Neomarkers, Interchim) followed by incubation with horseradish peroxidase-conjugated anti-mouse IgG, and revealed by using an enhanced chemiluminescence system (Amersham Pharmacia Biotech). Equal loading was determined using monoclonal antibody to actin (1/5000) (Chemicon, Euromedex, France).

**Clonogenic and mutagenic assays**
AA8 CHO cells were maintained in MEMα (Gibco) with 10% fetal calf serum, 4 mM glutamine, and antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin) at 37°C in a humidified 5% CO₂ atmosphere. CHO cell lines overexpressing Pol β were established previously after stable transfection of pUTPolβ plasmid (15). Control cells and cells overexpressing Pol β were plated in 6-well plates and allowed to attach overnight. Next, they were irradiated with a 254nm UV-C lamp at the fluence rate of 0.5J/m²/sec. Colonies were fixed and stained after 6 days of post-incubation and those >50 cells were scored. For the 6-thioguanine (6-TG) resistant tests, cells were first irradiated at 20J/m² and then exposed to 20µM 6-TG-containing media (10⁶ cells/14cm plate) in order to determine the number of
HPRT mutants. After one week, plates were stained, and colonies of >50 cells were counted. Mutant frequencies were corrected for plating efficiency and for UV cytotoxicity.

**Proteins, cells and substrates**

Rat Pol β was purified in *E.coli* as described (25). One unit of rat Pol β corresponds to 1 pmol of dNTP incorporated into acid-insoluble materials at 37°C in 60 min, by using as a substrate an activated calf thymus DNA pre-incubated with DNAse I. Human Pol β was provided by Trevigen (Gaithersburg, USA) and showed a 0.68µg/µl concentration and a 4U/µl activity. Calf Thymus Pol α and HIV-1 RT were purified as described previously (26,27). AA8 CHO Sh: : Pol β cells and AA8 CHO Sh cells were obtained after stable transfection of pUTPolβ and empty pUT687 vectors as reported previously (15). Briefly, Pol β cDNA was fused in-frame with the bacterial Sh: :ble gene conferring resistance to the broad-spectral zeocin xenobiotic of the phleomycin family. 30-mer UV-modified oligonucleotides and pBS-SV oriA/B vectors were prepared as described (28).

**Primer extension assay**

UV-modified 30-mer oligomers 5’-CTGTCAGCATCTCATCATACAGTCAGTG-3’ were chemically synthesized as described previously (29,30). They were hybridized to 5’-32P-labeled 16-mer (5’-CACTGACTGTATGATG-3’), 17-mer (5’-CACTGACTGTATGATG-3’) or 18-mer (5’-CACTGACTGTATGATGNN-3’) primers at a molar ratio of 1 :1 for 10 min. at 70°C in a buffer containing 10 mM Tris-HCl pH 7.5, 50 mM NaCl, and 10 mM MgCl₂, following by slow cooling to room temperature. Standard 15-µl reaction mixtures contained 14nM of the 5’-32P-labeled primer-template DNA and specific buffer as following : Pol β buffer contained 50mM Tris-HCl pH 8.8, 10 mM MgCl₂, 100 mM KCl, 0.4 mg/ml BSA, 1 mM DTT, 10% glycerol ; Pol α buffer contained 20 mM Hepès-KOH
pH 7.8, 3mM MgCl₂, 1mM DTT; HIV-RT buffer contained 60mM Tris-HCl pH 8.2, 7mM MgCl₂, 1 mM DTT, 0.5 mM EDTA, 10 mM KCl; buffer for cell extract reaction contained 45 mM Hepes-KOH pH 7.8, 7 mM MgCl₂, 1 mM DTT, 0.4 mM EDTA, 3.4% glycerol, 65 mM mono-K-glutamic acid, 1 mg/ml BSA. Reactions were performed at 37°C and terminated by adding 5µl of stopping buffer (90% formamide / 0.1% xylene cyanol / 0.1% bromophenol blue / 0.1 mM EDTA). Samples were denaturated for 10 min at 70°C and loaded to a 20% polyacrylamide/7M urea gel. CHO extract preparation was performed according to the previously described protocol (31). Competent replicative extracts from the melanoma cell lines were not feasible. An 11-mer unlabeled oligonucleotide (5’ – ATGCTGACGAG - 3’) was also used and annealed to the template at a molar ratio of 2 : 1 in order to saturate the 3’end of the template.

Two step SV40 DNA replication assay

PBS-SvoriA(CPD) or pBS-SvoriB(CPD) plasmids were generated as described (2). Replication reaction mixtures (25 µl) contained 30 mM HEPES, pH 7.8, 7 mM MgCl₂, 200 µM each CTP, GTP, and UTP, 4 mM ATP, 100 µM each dATP, dCTP, dTTP, 10 µM dGTP, 40 mM creatine phosphate (Sigma), 100 µg/ml creatine phosphokinase (Sigma), 100 ng of pBS-SvoriA(CPD) or oriB(CPD), 0.5 µg of SV40 large T-antigen (Molecular Biology Resources) and 400 µg Hela cell extract. After incubation at 37°C for 4 h, 0.012U rat Pol β and 1µCi (α³²P) dATP (4000 cpm/pmol; Amersham) were added to reaction mixtures and incubated for 1h. Reactions were quenched by adding an equal volume of «stop solution» (2% SDS, 2 mg/ml proteinase K, and 50 mM EDTA) and further incubation was done for 1 h at 55°C. DNA (0.5 µg pc-DNA II, In vitroGen, containing one BamHI site and multiple Dpn I sites) was added to each sample as internal purification controls. Reaction products were purified by extraction with phenol-chloroform-isoamyl alcohol followed by ethanol
precipitation. The DNA was resuspended in distilled water. The samples were then treated with BamH I and Dpn I (New England Biolabs) and the restriction digests were separated on a 1% agarose gel. After BET-staining of the gel, internal control DNAs were quantified. The gel was then dried, and autoradiography performed. Quantification analysis of the resolved radioactive bands on the gel was achieved by PhosphoImager Storm-system analysis using Imagequant software.
Results

Overexpression of Pol β in melanoma cells compared with normal skin tissues

We and others previously found that Pol β was overexpressed at the protein level in many cancer tissues compared with normal tissues (17-19). Here, we analyzed 4 independent melanomas protein extracts and we compared their Pol β content relative to normal skin tissues (Fig. 1). More than 10-fold increase in Pol β level was observed in all the melanomas tested while a slight detection of the enzyme was discernible only after a long time exposure in normal skin (data not shown). In this work, we hypothesized that excess Pol β in skin cells exposed to UV-light may predispose these cells to initiation and/or progression into tumoral melanomas by rising the UV-induced genetic instability.

Decreased sensitivity to UV-radiation and enhanced induced mutagenesis in CHO-Polβ cells

To investigate if high level of Pol β can affect genetic stability after UV irradiation in mammalian cells, we examined UV sensitivity as well as UV-induced mutagenesis in two independent transfected CHO cell lines which overproduce the enzyme by 3.2 and 2.4-fold (AA8 Pol β2::Sh cells and AA8 Pol β3::Sh cells ) (16). Firstly, we conducted clonogenic experiments after treatment with increasing doses of UV-C irradiation concommitantly with the isogenic control AA8 Sh cells. In at least three separate experiments performed in duplicate, we demonstrated a significant 1.5 to 2-fold resistance of cells up-regulating the enzyme compared with the control cells (Fig. 2A). To compare the mutation frequency in the surviving irradiated cells, we used the conventional methodology testing the appearance of mutational events leading to a resistance phenotype at the locus encoding the purine salvage enzyme hypoxanthine guanine phosphoribosyl transferase (HPRT). After irradiation, cells
were allowed to grow for 1 week before plating in 6-thioguanine-supplemented medium, grown for one additional week, and 6-TG\textsuperscript{R} mutant colonies were counted. A 2.6- to 50-fold increase in mutagenesis for the Pol \( \beta::\text{Sh} \) cells relative to the Sh cells was observed in three independent experiments after a 20 J/m\(^2\) UV dose (Fig. 2B). The lack of correlation between pol beta expression level and UV resistance as well as hypermutability may be due to the mutator phenotype induced by pol beta overexpression (15). It is possible that the higher expression of Pol \( \beta \) may cause deleterious side effects and may affect other genes that would interfere with cell viability after UV treatment.

In order to investigate the molecular bases for these \textit{in vivo} phenotypes, we tested a potential translesion ability of UV lesions of the replicative extracts from these cell lines. We performed \textit{in vitro} primer extension reactions with a 30-mer template containing a CPD adduct, annealed to a 5\(^\prime\)-\( ^{32}\)P-labeled 16-mer primer (Fig. 2C upper part). The primer was localized at a position so that the two first nucleotides were always incorporated opposite the lesion. In the presence of replicative extracts prepared from the control cells and one Pol \( \beta::\text{Sh} \) cell line, we found that the Pol \( \beta::\text{Sh} \) cell extracts could replicate past the CPD more efficiently as compared with the control extracts (Fig 2C), demonstrating that excess Pol \( \beta \) facilitated the bypass process. Addition of purified Pol \( \beta \) to the control extracts also increased a bypass synthesis capability of the CPD lesion (data not shown). In contrast, we did not observed any TLS of the heavy distorting (6-4)TT lesion with either cell extracts (data not shown). These results suggest that bypass synthesis of CPD damage by excess Pol \( \beta \) may contribute to the \textit{in vivo} resistance and hypermutagenesis towards UV irradiation in the cells overexpressing Pol \( \beta \).

\textbf{Ability of purified Pol \( \beta \) to bypass \textit{in vitro} CPD and (6-4)TT adducts}
To investigate deeply the specific ability of Pol β to bypass UV-photoproducts, we performed a kinetic study on the 30-mer template containing either CPD or (6-4)TT adduct, annealed to a 5’-^{32}P-labeled 16-mer primer (Fig. 3A). We used purified human and rat Pol β and we compared their behaviour to Pol α, which was previously reported as unable to incorporate nucleotides opposite the CPD or the (6-4)TT (2). As can be seen in Fig. 3B, by using amounts of enzymes allowing efficient and complete primer extension on undamaged template (right part of the fig 3B), Pol β was able to incorporate nucleotides opposite both the CPD and the (6-4)TT lesions (17-mer and 18-mer products) as well as to perform extension beyond the adducts (products with a size larger than 18-mer) in a time-dependent manner while Pol α is not, as expected. Some discrete radioactive fragments were also observed as 24-mer products; the mechanism involved in the generation of these products will be addressed deeply later in the manuscript when describing fig 4. We also reported here that the HIV-1 RT, which shares structural and inaccuracy features with Pol β, catalyzed efficient translesion synthesis of UV photoproducts (Fig. 3B). To better visualize and quantify the Pol β - dependent bypass process, the 30-mer template was annealed in presence of the 16-mer labeled-primer at a ratio 1/1 and an excess of 11-mer oligonucleotide complementary to the 3’end of the template in order to generate a 3 nucleotides gapped DNA, a preferential substrate for Pol β which offers the possibility to analyse the incorporation opposite the lesion and further extension of one nucleotide (Fig. 3A). Primer extension reactions were performed in presence of 0.05U or 0.5U of human Pol β, leading respectively to a 1/1 or 10/1 molar ratio compared with the primer-template. We found a more efficient Pol β-mediated bypass in both a time and dose dependent manner on this gapped DNA as compared with the non-gapped template (Fig. 3C). The higher efficiency for nucleotides incorporation opposite the lesions may be favoured by the ability of Pol β 8 kDa domain, which binds to the downstream 5’ terminus, to promote processive extension of misinserted nucleotides on undamaged gapped
DNA (32). In presence of 0.05 U and 0.5 U Pol β, the efficiency of the bypass of CPD into the 3 nucleotide-gap represented respectively 20% and 75% extension of the primer for 60 min incubation time (Fig. 3C). A minor bypass product showed also full-size synthesis in the presence of higher polymerase concentration, probably resulting from the previously reported *in vitro* strand-displacement activity by Pol β of the 11-mer oligonucleotide (18). In the case of the (6-4)TT adduct, the presence of the 11-mer oligonucleotide allowed a 3-fold increase of nucleotide incorporation opposite the 3’T of the adduct by 0.05 U Pol β (Fig. 3C). A complete 3 nt-gap filling reaction was achieved in the presence of 0.5 U human Pol β and bypass products represented more than 55% of the extended oligonucleotides after 20 min incubation (Fig. 3C). Taken together, these results demonstrated that purified Pol β can bypass CPD and (6-4)TT adducts during *in vitro* primer extension.

### Specificity of Pol β-dependent incorporation opposite the CPD ant (6-4)TT

A steady-state “single hit” gel kinetic assay (33) was performed using primed unmodified or UV-modified 30-mer DNA templates in order to quantitatively determine the specificity of nucleotide incorporation opposite the 3’T of CPD and (6-4)TT. For damaged templates, the concentration of incoming dNTP varied from 5 to 1000 µM and incubation time was 1 h in the presence of 0.5 U Pol β. Regarding the undamaged templates, the concentration of incoming dNTP varied from 1 to 500 µM and incubation time was 15 min in the presence of 0.001 U Pol β when using dATP and 30 min with 0.5 U Pol β when using dCTP or dGTP. All the data we obtained are summarized in Table 1 and revealed that the A represented 55% and 71% of the inserted nucleotides opposite the CPD and the (6-4)TT lesions respectively, leading to an error-free insertion. Insertion of C opposite the 3’T of the lesion represented the major error-prone insertion with 32% and 26% of the inserted
nucleotides opposite the CPD and the (6-4)TT lesions respectively. Some Gs can be inserted opposite the 5’T but at a lesser extend.

Interestingly, when comparing kinetic parameters, we found that Pol β ability to insert the incorrect dGTP nucleotide opposite the 3’T of an undamaged template was only 4 to 16-fold higher as compared with the Pol β efficiency to misinsert dATP or dCTP opposite the 3’T of the CPD or the (6-4)TT, signifying the high capacity of Pol β to incorporate nucleotide opposite distorting lesions. Finally, Pol β inserted dATP opposite the 3’T of the CPD or the (6-4)TT lesion with an efficiency 3500-8000 less as compared with the insertion of dATP opposite the 3’T of the undamaged template.

**Pol β-dependent efficiency of extending primers with one base opposite the CPD and the (6-4)TT**

As it could be of biological significance to determine if the incorporated nucleotide can be extended, 17-mer primers in which each of the four bases was paired to the 3’T of each adduct were annealed to damaged templates. Extension of these primers was assayed after 1h reaction in the presence of 0.5U Pol β and either the all four dNTP (200µM) (Fig. 4A) or a unique dNTP (Fig. 4B and 4C). The most efficient extension to the full-size product of a primer annealed to the CPD-containing template occurred with the correctly paired A in presence of all 4 dNTPs (Fig. 4A). We analysed the 5’T incorporation specificity at the A.T primer and found that, while A was incorporated majoritary, all the other dNTPs could be also incorporated with a slightly lower efficiency (Fig. 4B). Indeed, there was a significant misincorporation of T, C, G opposite the 5’T of the CPD, and after G incorporation, an incorporation opposite the adjacent undamaged C in the template occurred, leading to a complete lesion bypass. With the G.T primer in the presence of all 4 dNTPs, the obtention of full-size product was as efficient as compared with the correctly paired A.T primer (Fig. 4A),
probably initiated by dATP incorporation (Fig. 4B). In contrast, Polβ-dependent extension of C.T and T.T mispairs was inefficient since aborted after incorporation of one nucleotide (Fig. 4A). Extension reactions of primers annealed to the (6-4)TT-containing template revealed 1 nt incorporation but no further extension as shown in Fig. 4A and 4C. Extension of A.T, C.T and G.T mispairs occurred only in presence of dTTP, rendering this weak process highly mutagenic (Fig. 4C). A discrete radioactive fragment product migrating as a 24-mer product was observed when the C.T mispair was extended on both the CPD and the (6-4)TT templates (Fig. 4A); this seems likely to correspond to a 6nt synthesis resulting from an annealing event of the microsequence ATGC at 3’-terminus of the 17-mer primer that can pair to an homologous sequence TACG at position 20-23 of the template, generating a template loop. Such misalignment incorporation mechanism facilitated by Pol β has already been described during TLS of an abasic site (34,35), a 8-oxo-dG (36), and propano-deoxyguanosine lesions (37). This specific ability to catalyse template misalignment by searching microhomology sequence is shared by Pol µ, another member of the DNA polymerase X family (38).

**Pol β-dependent extension of primers with two bases opposite the CPD or the (6-4)TT**

To investigate if Pol β-dependent incorporated nucleotides opposite the two damaged bases could be extended, we used 18-mer primers whose termini were located directly opposite the CPD or (6-4)TT (Fig. 5). We focused on a set of 11 primers, 8 primers representing the best incorporations opposite the dimers (primers ending with AA, AG, AT, AC, GA, GT, CA, CT for the CPD ; primers ending with AT, CT, GT for the (6-4)TT ) and 3 primers randomly chosen (primers ending with GG, CG, GC). Pol β was able to extend efficiently a primer with two As opposite the CPD, generating a full-size product. Interestingly, efficient extension was also observed with the ‘AG’, ‘GA’, ‘AC’, and ‘CA’ primers with a decreasing efficiency (AG>AC>GA>CA). None of the primers randomly chosen were extended by Pol β opposite
the CPD. Taken together, this shows that the best efficiencies were obtained with the nucleotides specifically incorporated opposite the CPD by Pol β. Discrete radioactive fragments were also observed as 21-mer and 24-mer products when we used the ‘AC’ and ‘CT’ primers opposite either lesions, reflecting probably a misalignment incorporation mechanism facilitated by Pol β, between TGAC or ATGCT at the 3-terminus of the 18-mer primers and the homologous sequence ACTG (position 24-27) or TACGA (position 20-24) of the 30-mer template respectively. In the case of the (6-4)TT-containing template, we did not detect any significant primer extension with all the primers tested (Fig. 5 – right part). Taken together, these results suggest that Pol β is able to extend efficiently mutagenic as well as correct nucleotides incorporated opposite the CPD.

**Recruitment of excess Pol β during *in vitro* SV40 replication to bypass the CPD**

In order to investigate if excess Pol β could interfere with the replicative machinery during replication of UV-damaged duplex DNA, we performed a two-step *in vitro* SV40 replication assay. This assay can be used to observed CPD bypass as demonstrated for Pol η in HeLa cell extracts (2). We used two covalently closed circular templates containing the SV40 origin of DNA replication with a single CPD located on each side of the SV40 origin (Fig. 6A). Replication forks encounter the lesion during lagging strand synthesis in the case of pBS-SvoriA(CPD) and in the course of the leading strand synthesis in the case of pBS-SvoriB(CPD). These plasmids were first incubated for 4h with 400µg Hela extracts in the reaction buffer, then (α-32P)dATP and purified Pol β were added for an additional hour. During the first incubation period, DNA replication machinery stalled at the lesion on the damaged strand and during the shorter period of the second incubation in the presence of radioactive dATP and purified Pol β, radioactivity will be incorporated preferentially into products of damage bypass replication. Then, DNA was purified, linearized by BamH1 and
DpnI, and subjected to electrophoresis onto a 1% agarose gel. Ethidium bromide staining and autoradiography of the gel are shown in Fig. 6B. DpnI digestion was done to visualize only the DNA population replicated once. Additionally, we verified that addition of up to 0.024U Pol β in reaction mixtures replicating undamaged DNA did not result in an increase of the radioactive replication signal (39). As observed in Fig. 6B, radioactivity incorporation during DNA replication is lower with the pBS-SVoriA DNA as compared with the pBS-SVoriB DNA. As suggested in a previous report, SV40 replication of a UV lesion-containing plasmid could be synchronous between the two parental strands in the case of pBS-SVoriA(CPD) and asynchronous in the case of pBS-SVoriB(CPD): during lagging strand synthesis, the replication fork moves past the lesion and re-initiation occurs at the next Okasaki fragment, leaving a small single-stranded gap; during the leading strand replication, the progression of the fork is inhibited and uncoupling of leading and lagging strand occurs: the replication machinery continues to synthesize the lagging strand (40,41).

We found that, in the presence of 0.012U rat Pol β, DpnI-resistant products increased by 4-fold with pBS-SVoriA(CPD) and by 2-fold with pBS-SVoriB(CPD) as compared with the control reactions without Pol β (Fig. 6B, right tracks). For the global replication products (without digestion by DpnI; Fig. 6B, left tracks), a Pol β–dependent increase was also detected. When Pol β and the radioactive nucleotide were added at the beginning of the reaction (one-step reaction), a 2-, 3.5-, and 5-fold signal increase was observed with pBS-SVoriB(CPD) in the presence of 0.0048, 0.012 and 0.024U rat Pol β respectively (data not shown). Taken together, these results suggest that when DNA synthesis during replication of duplex DNA is stopped by a CPD, excess Pol β can be notably recruited to overcome the lesion.
Discussion

We showed here that Pol β, an enzyme required in somatic cells for the BER pathway (14), can facilitate translesion replication of a CPD as well as, at a lesser extent, a 6-4 thymine–thymine pyrimidine–pyrimidone photoproduct (6-4PP). Such a result was obtained by using the well-calibrated primer extension assay using site-specific UV-modified oligonucleotides as well as the SV40 replication assay, which reconstitutes the mammalian DNA replication fork, using CPD-modified duplex DNA. Pol β mostly incorporated the correct A opposite the 3’ T of the CPD and the 6-4PP, but could also misinsert C. For the CPD, we found that the 5’T incorporation specificity by Pol β at the A.T and C.T primers was highly mutagenic. Whether the nucleotides were correctly or incorrectly inserted opposite the CPD, some of them were efficiently extended by Pol β and this extension is highly error-prone, supporting that Pol β could compete with Pol ζ (5) or Pol κ (6) in order to extend nucleotides incorporated opposite the 3’ T of the CPD adduct. Opposite the (6-4)TT lesion, the incorporation by Pol β opposite the 3’T, essentially A like Pol ι, is poorly efficient and is most of the time aborted, probably because of the strong distortion of DNA. This low extension capability of Pol β is shared with Pol η (3) and Pol ι (5,7). It has been proposed that Pol ζ is responsible for the subsequent extension of the nucleotide incorporated opposite the 3’T of the (6-4)TT damage (3,5). This suggests that in vivo, an efficient, mostly accurate but potentially error-prone, TLS of the (6-4)TT lesion may result from the combined activities of Pol β and Pol ζ.

Todate, among the 12 eukaryotic DNA polymerases that have been identified, only Pol η, Pol ζ, Pol κ and Pol ι have been shown to exhibit such potential involvement in CPD and (6-4)TT photoproduc
replication is normally Pol η–dependent since in XP-V cell extracts, in which Pol η is inactive, only 10% of the lesion bypass activity of normal cell extracts is observed (2,42). So what could be the biological significance of such Pol β-dependent bypass? Analysis of the mutagenic spectra observed after exposing human cells to UV light suggests that most mutations are, in fact, targeted to the 3’ site of a di-pyrimidine containing a C (at CC and TC) (1,43). However, some minor mutations T→A and T→C targeted to the 5’ site of TT can be also observed (10,44) and these match to the Pol β–dependent mutations that we observed here in vitro, suggesting a role of Pol β in some of the UV-induced mutations in somatic cells. The frequency of this kind of mutations increases strongly up to 45% in XP-V cells (1,10,45), supporting that Pol β, like Pol ι, may be involved in the TLS process at the TT sites in the absence of Pol η.

Moreover, situations where imbalance of Pol β expression in cells occurs may be of interest in such translesion process of UV lesions. Interestingly, we observed in this work that high levels of Pol β can been found in various melanomas tumours, which are known to be associated to UV radiation exposure. We recently showed that Pol β can interfere in vitro with duplex DNA replication when up-represented, rendering the process inaccurate (39). The data presented here suggest strongly that interference of excess Pol β at the replication forks not only can affect the accuracy of the process, but can also modulate the genotoxicity of UV lesions when present on the genomic DNA. Although the mutagenic translesion replication experiments reported here were performed entirely in vitro, we believe that they shed light on the mutagenic process in vivo in melanoma cells and that excess Pol β may enhance CPD translesion in a mutagenic manner by competing with Pol η. By using isogenic CHO cells, we found that the sole Pol β overexpression event resulted in a resistant phenotype towards UV treatment and can dramatically enhance the induced mutagenesis. Both phenotypes may result
from the TLS catalyzed by Pol β during the elongation of the replication forks. Overexpression of Pol β could be therefore identified as a host risk factor that may potentiate the genetic instability in cells exposed to UV and may consequently affect melanomas risk.
References

1. Friedberg, E., Walker, G., and Siede, W. (1995) *DNA repair and mutagenesis*

2. Masutani, C., Araki, M., Yamada, A., Kusumoto, R., Nogimori, T., Maekawa, T., Iwai, S., and Hanaoka, F. (1999) *Embo J* 18, 3491-3501

3. Johnson, R. E., Haracska, L., Prakash, S., and Prakash, L. (2001) *Mol Cell Biol* 21, 3558-3563.

4. Gibbs, P. E., McGregor, W. G., Maher, V. M., Nisson, P., and Lawrence, C. W. (1998) *Proc Natl Acad Sci U S A* 95, 6876-6880

5. Johnson, R. E., Washington, M. T., Haracska, L., Prakash, S., and Prakash, L. (2000) *Nature* 406, 1015-1019.

6. Washington, M. T., Johnson, R. E., Prakash, L., and Prakash, S. (2002) *Proc Natl Acad Sci U S A* 99, 1910-1914.

7. Tissier, A., Frank, E. G., McDonald, J. P., Iwai, S., Hanaoka, F., and Woodgate, R. (2000) *Embo J* 19, 5259-5266.

8. Zhang, Y., Yuan, F., Wu, X., Taylor, J. S., and Wang, Z. (2001) *Nucleic Acids Res* 29, 928-935.

9. Maher, V. M., Ouellette, L. M., Curren, R. D., and McCormick, J. J. (1976) *Nature* 261, 593-595.

10. McGregor, W. G., Wei, D., Maher, V. M., and McCormick, J. J. (1999) *Mol Cell Biol* 19, 147-154.

11. Johnson, R. E., Kondratick, C. M., Prakash, S., and Prakash, L. (1999) *Science* 285, 263-265.

12. Masutani, C., Kusumoto, R., Yamada, A., Dohmae, N., Yokoi, M., Yuasa, M., Araki, M., Iwai, S., Takio, K., and Hanaoka, F. (1999) *Nature* 399, 700-704
13. Broughton, B. C., Cordonnier, A., Kleijer, W. J., Jaspers, N. G., Fawcett, H., Raams, A., Garritsen, V. H., Stary, A., Avril, M. F., Boudsocq, F., Masutani, C., Hanaoka, F., Fuchs, R. P., Sarasin, A., and Lehmann, A. R. (2002) Proc Natl Acad Sci U S A 99, 815-820.

14. Sobol, R., Horton, J., Kühn, R., Gu, H., Singhal, R., Prasad, R., Rajewsky, K., and Wilson, S. (1996) Nature 379, 183-186

15. Canitrot, Y., Cazaux, C., Frechet, M., Bouayadi, K., Lesca, C., Salles, B., and Hoffmann, J. (1998) Proc. Natl. Acad. Sci. USA 95, 12586-12590

16. Bergoglio, V., Pillaire, M., Lacroix-Tricki, M., Raynaud-Messina, B., Bieth, A., Canitrot, Y., Garès, M., Wright, M., Delsol, G., Loeb, L. A., Cazaux, C., and Hoffmann, J. S. (2002) Cancer Res. in press

17. Scanlon, K., Kashani-Sabet, M., and Miyachi, H. (1989) Cancer Invest. 7, 581-587

18. Canitrot, Y., Hoffmann, J. S., Calsou, P., Hayakawa, H., Salles, B., and Cazaux, C. (2000) Faseb J 14, 1765-1774.

19. Srivastava, D., Husain, I., Arteaga, C., and Wilson, S. (1999) Carcinogenesis 20, 1049-1054

20. Elwood, J., Gallagher, R., Hill, G., and Pearson, J. (1985) Int J Cancer 35, 427-443

21. Elwood, J., and Jopson, J. (1997) Int. J. Cancer 73, 198-203

22. de Gruijl, F. R. (1999) Eur J Cancer 35, 2003-2009.

23. Holman, C. D. J., Armstrong, B. K., and Heenan, P. J. (1986) J. nat. Cancer Inst. 76, 403-414

24. Osterlind, A., Tucker, M. A., Stone, B. J., and Jensen, O. M. (1988) Int. J. Cancer 42, 319-324

25. Kumar, A., Widen, S., Williams, K., Kedar, P., Karpel, R., and Wilson, S. (1990) J. Biol. Chem. 265, 2124-2131
26. Hoffmann, J. S., Fry, M., Ji, J., Williams, K. J., and Loeb, L. A. (1993) Cancer Res 53, 2895-2900
27. Hoffmann, J. S., Pillaire, M. J., Garcia-Estefania, D., Lapalu, S., and Villani, G. (1996) J Biol Chem 271, 15386-15392.
28. Masutani, C., Kusumoto, R., Iwai, S., and Hanaoka, F. (2000) Embo J 19, 3100-3109.
29. Murata, T., Iwai, S., and Ohtsuka, E. (1990) Nucleic Acids Res 18, 7279-7286.
30. Iwai, S., Shimizu, M., Kamiya, H., and Ohtsuka, E. (1996) J Am Chem Soc 118, 7642-7643
31. Hoffmann, J., Pillaire, M., Lesca, C., Burnouf, D., Fuchs, R., Defais, M., and Villani, G. (1996) Proc. Natl. Acad. Sci. USA 93, 13766-13769
32. Osheroff, W. P., Jung, H. K., Beard, W. A., Wilson, S. H., and Kunkel, T. A. (1999) J Biol Chem 274, 3642-3650.
33. Creighton, S., Bloom, L. B., and Goodman, M. F. (1995) Methods Enzymol 262, 232-256
34. Efrati, E., Tocco, G., Eritja, R., Wilson, S., and Goodman, M. (1997) J. Biol. Chem. 272, 2559-2569
35. Daube, S. S., Arad, G., and Livneh, Z. (2000) Biochemistry 39, 397-405.
36. Efrati, E., Tocco, G., Eritja, R., Wilson, S. H., and Goodman, M. F. (1999) J Biol Chem 274, 15920-15926.
37. Hashim, M. F., Schnetz-Boutaud, N., and Marnett, L. J. (1997) J Biol Chem 272, 20205-20212.
38. Zhang, Y., Wu, X., Yuan, F., Xie, Z., and Wang, Z. (2001) Mol Cell Biol 21, 7995-8006.
39. Servant, L., Bieth, A., Hayakawa, H., Cazaux, C., and Hoffmann, J. S. (2002) J Mol Biol 315, 1039-1047.
40. Svoboda, D. L., and Vos, J. M. (1995) *Proc Natl Acad Sci U S A* **92**, 11975-11979
41. Cordonnier, A. M., and Fuchs, R. P. (1999) *Mutat Res* **435**, 111-119.
42. Cordonnier, A. M., Lehmann, A. R., and Fuchs, R. P. (1999) *Mol Cell Biol* **19**, 2206-2211
43. Sage, E. (1993) *Photochem Photobiol* **57**, 163-174.
44. Lichtenauer-Kaligis, E. G., Thijssen, J., den Dulk, H., van de Putte, P., Giphart-Gassler, M., and Tasseron-de Jong, J. G. (1995) *Mutat Res* **326**, 131-146.
45. Wang, Y. C., Maher, V. M., Mitchell, D. L., and McCormick, J. J. (1993) *Mol Cell Biol* **13**, 4276-4283.

**Acknowledgements**

This work was exclusively supported financially by “La Ligue Nationale contre le Cancer “ (Equipe labellisée). Laurence Servant is an ARC fellowship recipient. We thank Dr. T. Kunkel for the pBS-SV oriA and oriB vectors.
Figure Legends

Figure 1 : Analysis of expression of Pol β protein in normal skin and melanoma tissues.
Cell extracts were analysed by Western Blot using monoclonal antibody to Pol β protein. Actin was used as internal control for loading. Cell lysates were prepared from skin tissues of 2 normal patients (samples 1 and 2), ganglion metastatic melanoma cells of 3 patients (samples 3, 4 and 5) and skin melanoma cells maintained in culture aseptic conditions (sample 6).

Figure 2 : Phenotypic comparison of AA8 Sh and Pol β : :Sh cells after UV-treatment
A) Sensitivity of Sh and Pol β : :Sh cell lines to UV-radiation. Survival is expressed as the relative plating efficiency of treated cells to untreated cells. Results are the mean +/- SD at least three separate experiments performed in duplicate. B) UV-induced mutation frequency at the HPRT locus in Sh and Pol β : :Sh cell lines. Cells were exposed at 20J/m², allowed to grow for 1 wk before plating at 10⁶ cells in 6-TG-supplemented medium and grown for an additional week. Next, plates were stained, and 6-TG R mutants colonies counted. C) In vitro translesion synthesis of CPD adduct by AA8 Sh and Pol β : :Sh cell extracts. The 30-mer template was annealed to a 16-mer primer and used to perform primer extension reaction. Undamaged and damaged templates were replicated for 1h by 5µg of indicated cell extracts. Arrows indicate the position of the primer (16), the products resulting from one nucleotide incorporation opposite the CPD (17), the products resulting from two nucleotides incorporation opposite the CPD (18) and the full-size product (30).

Figure 3 : Pol β translesion synthesis activity on CPD and (6-4)TT containing templates.
Reactions were performed as described in Materials and Methods for the times noted above
each track. **A)** Primed UV-modified 30/16 and 30/16/11 templates used for the primer extension assays. **B)** Primer extension assays with 0.012 U rat Pol β, 1U Calf Thymus Pol α and 1U HIV-1 RT with the UV-modified or undamaged templates. **C)** Primer extension assay by human Pol β with the UV-modified 30/16 and 30/16/11 templates.

**Figure 4:** Extension of primers opposite the 3’T of the CPD or (6-4)TT by human Pol β. The sequence of each primer is shown above each group of experiments. A primer containing one nucleotide opposite the 3’T of each adduct was annealed to the templates depicted above each figure. **A)** Reactions were performed in the presence of 0.5U human Pol β and 4 dNTP (200µM) for 1h. **B)** Reactions were performed in the presence of 0.5U human Pol β for 1h. O, A, T, C, G indicate reactions in the absence of nucleotides or in presence of dATP, dTTP, dCTP or dGTP respectively.

**Figure 5:** Extension of primers with various dinucleotides opposite the CPD or the (6-4)TT by human Pol β. The 3’ dinucleotide sequence of each primer is given above each panel. Each reaction was performed for 1h in the presence of 0.5U Pol β and all 4 dNTPs. The arrow shows the starting position of the 18-mer primer.

**Figure 6:** Excess Pol β-mediated translesion synthesis of CPD during SV40 replication. **A)** Possible implications of excess Pol β in the bypass of the CPD adduct during the two-step SV40 replication assay. SV40-DNA constructs are shown here during bidirectionnal semi-conservative SV40 replication, that began at the origin (OriA or OriB for pBS-SV(CPD) oriA or ori B respectively). **B)** 100 ng pBS-SV ori A and ori B were replicated by 400µg cell-free extracts from human Hela cells in T-antigen dependent manner in presence or in absence of 0.012U Pol β, then they were linearized by BamH I (one unique site) or digested by BamH I
and Dpn I (multiple sites). The two step SV40 DNA replication and analysis of the products are described in Materials and Methods.

Table

Table 1: Steady-state kinetic parameters of incorporation opposite the 3’T of the CPD or (6-4)TT by human Pol β.
Figure 1
Figure 2

(A) Survival of AA8 cell lines: Sh Pol beta 2 vs. Sh Pol beta 3 vs. Sh.

(B) Mutation frequency (10^-4) in AA8 cell lines: Sh, Pol beta 2 :: Sh, and Pol beta 3 :: Sh.

(C) Image showing DNA adducts: Adduct, None, CPD.
A

\[
\begin{align*}
*5'\text{-CACTGACTGTATGATG}^\wedge & \\
3'\text{-GTGACTGACATACTACTTCTACGACTGCTC - 5'} & \\
\end{align*}
\]

11-mer

\[
\begin{align*}
*5'\text{-CACTGACTGTATGATG}^\wedge & \quad \text{ATGCTGACGAG} - 3' \\
3'\text{-GTGACTGACATACTACTTCTACGACTGCTC - 5'} & \\
\end{align*}
\]

B

| Time (min) | Pol α | Pol β | HIV-RT |
|------------|-------|-------|--------|
| 30         | 10    | 10    | 10     |
| 60         | 20    | 20    | 20     |
| 120        | 60    | 60    | 60     |

C

| Damage | None | CPD | (6-4) TT |
|--------|------|-----|----------|
| 11-mer oligo | -    | +   |          |
| Human Pol β (Unit) | 0.05 | 0.5 |          |
| Time (min) | 10    | 10   |          |

Figure 3
## Figure 4

### A

| *5'-TGN| 3' - ACTTCT | CPD | *5'-TGN| 3' - ACTTCT | CPD |
|---|---|---|---|---|---|
| A | T | C | G | A | T | C | G |

### B

| *5'-TGA| 3' - ACTTCT | (6-4)TT | *5'-TGC| 3' - ACTTCT | (6-4)TT | *5'-TGG| 3' - ACTTCT | (6-4)TT |
|---|---|---|---|---|---|---|---|---|
| A | T | C | G | A | T | C | G | A | T | C | G |

---

* 17
* 18

---

* A T C G

---

* 0 A T C G

---

* 0 A T C G

---

* 0 A T C G

---

* 0 A T C G

---

* 0 A T C G
**Figure 5**

```
| AA AG GA GG CG GC | AT AC GT CT CA |
|-------------------|----------------|
| *5'-'TGNN         | 3'- ACTTCT     |
|                   | CPD            |
| *5'-'TGNN         | 3'- ACTTCT     |
|                   | (6-4)TT        |
```
Figure 6

A

Lagging strand synthesis opposite the CPD adduct

Leading strand synthesis opposite the CPD adduct

B

| pBS-SV(CPD) | BamHI | BamHI+DpnI |
|-------------|-------|------------|
| 0.012U Polβ| oriA  | oriB      |
|             | -     | -         |
|             | +     | +         |
|             | -     | -         |
|             | +     | +         |

Full length replication products (linearized DNA)

Internal Control (undamaged pc-DNA II)
| adduct | dNTP added | $K_m$ (µM) | $V_{max}$ (%/min) | $V_{max}/K_m$ | Insertion (%) | $f_{inc}$ |
|--------|------------|------------|-------------------|---------------|--------------|----------|
| CPD    | dATP       | 171        | 0.28              | 0.00164       | 55.1         | 1        |
|        | dCTP       | 376        | 0.354             | 0.00094       | 31.7         | 1/1.77   |
|        | dGTP       | 372        | 0.146             | 0.00039       | 13.2         | 1/4.2    |
| (6-4)TT| dATP       | 150        | 0.57              | 0.00380       | 70.8         | 1        |
|        | dCTP       | 371        | 0.51              | 0.00138       | 25.7         | 1/2.75   |
|        | dGTP       | 1411       | 0.27              | 0.00019       | 3.5          | 1/20     |
| none   | dATP       | 183        | 2500              | 13            | 1            | 1        |
|        | dCTP       | 113        | 5.8               | 0.0514        | 1/252        |          |
|        | dGTP       | 25         | 1.3               | 0.0149        | 1/872        |          |

Table 1
A role for DNA polymerase beta in mutagenic UV lesions bypass
Laurence Servant, Christophe Cazaux, Anne Bieth, Shigenori Iwai, Fumio Hanaoka and Jean-Sébastien Hoffmann

J. Biol. Chem. published online October 17, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M207101200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts