# Ubiquitylation of Proliferating Cell Nuclear Antigen and Recruitment of Human DNA Polymerase $\eta$[†]

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ABSTRACT: This study investigated the requirement for ubiquitylation of PCNA at lysine 164 during polymerase $\eta$-dependent translesion synthesis (TLS) of site-specific *cis-syn* cyclobutane thymine dimers (T^T). The *in vitro* assay recapitulated origin-dependent initiation, fork assembly, and semiconservative, bidirectional replication of double-stranded circular DNA substrates. A phosphocellulose column was used to fractionate HeLa cell extracts into two fractions; flow-through column fraction I (CFI) contained endogenous PCNA, RPA, ubiquitin-activating enzyme E1, and ubiquitin conjugase Rad6, and eluted column fraction II (CFII) included pol $\delta$, pol $\eta$, and RFC. CFII supplemented with purified recombinant RPA and PCNA (wild type or K164R, in which lysine was replaced with arginine) was competent for DNA replication and TLS. K164R-PCNA complemented CFII for these activities to the same extent and efficiency as wild-type PCNA. CFII mixed with CFI (endogenous PCNA, E1, Rad6) exhibited enhanced DNA replication activity, but the same TLS efficiency determined with the purified proteins. These results demonstrate that PCNA ubiquitylation at K164 of PCNA is not required *in vitro* for pol $\eta$ to gain access to replication complexes at forks stalled by T^T and to catalyze TLS across this dimer.

Proliferating cell nuclear antigen (PCNA)[1] participates in different DNA metabolic pathways by interacting with protein factors involved in DNA replication, repair, and recombination (reviewed in ref *1*). In this study, we focused specifically on the need for post-translational modification of PCNA during translesion synthesis (TLS) of UV-induced *cis-syn* cyclobutane thymine dimers (T^T) by the specialized DNA polymerase (pol) $\eta$. Monoubiquitylation at lysine 164 (K164) of PCNA is thought to be involved in the recruitment of pol $\eta$ and/or other specialized DNA polymerases to DNA replication forks that are blocked at template lesions (reviewed in refs *2* and *3*). During the past decade, several of these specialized polymerases were discovered (e.g., pol $\zeta$, $\eta$, $\iota$, $\kappa$, Rev1p) and shown to be essential for bypass replication of a variety of DNA damage sites (reviewed in ref *4*).

Faithful duplication of eukaryotic genomes depends on very low error rates, which are achieved through the concerted operation of high-fidelity DNA polymerases, pre- and postreplicative DNA repair, and DNA damage checkpoint responses (see ref *5* for a review). The replicative polymerases are blocked by base modifications that cannot be accommodated in their catalytic sites or interfere with hydrogen bonding between the template base and the incoming nucleotide. Polymerases capable of replicating past template lesions are less discriminative (hence, more error-prone) but serve important biological functions by rescuing stalled replication forks and avoiding the potentially greater harm from fork collapse and induction of DNA double-strand breaks. Pol $\eta$ is one of the best-characterized bypass polymerases in terms of its biological functions (*6*), as well as structure (*7*); it plays an important role in the efficient and accurate catalysis of replication past UV-induced cyclobutane pyrimidine dimers. The absence of pol $\eta$ activity underlies the high susceptibility to skin cancers of xeroderma pigmentosum variant (XP-V) patients (*8–10*). In spite of intense research and a wealth of genetic and biochemical information (refs *3* and *11–13* and references therein), the

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[1] Abbreviations: 2D, two-dimensional; CF, column fraction; PCNA, proliferating cell nuclear antigen; $PCNA^{Ubi}$, monoubiquitylated PCNA at lysine 164; pol, polymerase; pUC, pUC19; PRR, postreplication repair; RFC, replication factor C; RPA, replication protein A; SCE, sister chromatin exchange; SUMO, small ubiquitin-related modifier; TLS, translesion synthesis; T^T, *cis-syn* cyclobutane thymine dimer; UV, ultraviolet light; XP-V, xeroderma pigmentosum variant; wt, wild type.

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mechanism by which pol $\eta$ is regulated to provide the maximum benefit to the organism remains to be elucidated.

Monoubiquitylation of PCNA at lysine 164 ($PCNA^{Ubi}$) was detected in yeast and human cells in response to inhibition of DNA synthesis by hydroxyurea, aphidicolin, or template lesions that block replication fork displacement (*14–18*). The biological importance of post-translational modifications of PCNA at K164 was firmly established with the discovery of their regulation by the *Saccharomyces cerevisiae* RAD6 pathway, which exerts genetic control on both error-prone and error-free mechanisms of DNA damage tolerance and/or avoidance (*14, 19, 20*). Hence, current TLS models envision a central role for $PCNA^{Ubi}$ in recruiting specialized polymerases to stalled replication forks and/or modulating the polymerase-switching steps required for TLS (*1–3*).

The ubiquitin residues in $PCNA^{Ubi}$ are located on the outside rim of the homotrimeric clamp, i.e., structurally favored positions for association with different factors of the DNA replication and repair machineries (*1*). Immunoprecipitation experiments indicated that monoubiquitylation of PCNA strengthens its binding to pol $\eta$ (*15, 21*). Whether $PCNA^{Ubi}$ plays a direct role in TLS by this polymerase, however, has not been completely resolved. *In vitro* systems relying on purified proteins and primer extension assays have provided conflicting results. The PCNA binding domain at the C-terminus of pol $\eta$ was important for the enhancement of its polymerase activity by RFC, PCNA, and RPA (*22, 23*). Recent results with the same assay indicated that monoubiquitylation of PCNA neither interfered with pol $\delta$ activity nor stimulated pol $\eta$; TLS of an abasic site was not stimulated by PCNA ubiquitylation (*24*), in contrast with the TLS stimulation effect observed by others (*25*). A recent report detected only a weak stimulation by RFC, PCNA, and RPA of the efficiency of pol $\eta$-dependent TLS on a template containing a T^T and little effect of these accessory proteins on pol $\eta$ fidelity (*26*). Another provided genetic evidence that in *S. cerevisiae* the binding of the ubiquitin residue in PCNA to a ubiquitin-binding motif in pol $\eta$ is not required for pol $\eta$-dependent TLS at replication forks stalled by UV-induced DNA damage (*27*).

The study reported here focused on the effect of PCNA monoubiquitylation on pol $\eta$-dependent TLS during *in vitro* replication of closed circular double-stranded DNA. Assay conditions recapitulated origin-dependent initiation, assembly of competent DNA synthetic machinery, and bidirectional replication through coupled synthesis of the leading and lagging strands of the newly replicated DNA (*28, 29*). In this assay system, replication past a T^T cannot be detected in the absence of pol $\eta$ (*30–32*). The bypass efficiency of a single T^T on the leading-strand template was measured with a fractionated hypotonic cell extract, which was depleted of endogenous RPA and PCNA and then supplemented with purified recombinant RPA and wild-type (wt) or mutant PCNA; in the latter, the acceptor site (K164) for monoubiquitylation was replaced with an arginine residue (K164R). Results demonstrated that ubiquitylation of PCNA at K164 is not necessary for pol $\eta$-dependent TLS during semiconservative DNA replication *in vitro*.

## EXPERIMENTAL PROCEDURES

*Materials.* Radioactive nucleotides and unlabeled deoxyribonucleoside triphosphates were purchased from GE Healthcare Inc. DNA modification enzymes were from New England Biolabs, Roche, Invitrogen, and Stratagene. Sources of primary antibodies were Sigma-Aldrich (anti-Flag M2 antibody and affinity gel), Santa Cruz Biotechnology (PC10, anti-PCNA; P4D1, anti-ubiquitin; PolH B7, anti-pol $\eta$; H-300, anti-pol $\delta$), Bethyl Laboratory, Inc. (anti-Rad6), Upstate Biotechnology (anti-human E1), and Calbiochem (anti-RPA). A. Sancar provided an aliquot of anti-RFC p37 (*33*). Secondary antibodies were from Amersham Biosciences. Sigma Aldrich was the source for 3× Flag peptide. The purified human proteins E1 and Rad6 (E2), His-ubiquitin, and ubiquitin were purchased from Boston Biochem, and Topo II was from Promega. SV40 large T antigen was purchased from CHIMERx.

*Preparation and Fractionation of Cell-Free Extracts.* HeLa cells were grown as monolayers in DMEM medium, or cell pellets were obtained either from the National Cell Culture Center (Minneapolis, MN) or from the UNC Lineberger Comprehensive Cancer Center Tissue Culture Facility. CTag (SV40-transformed XP-V fibroblasts) were grown as previously described (*30, 34*). Replication-competent cell-free hypotonic extracts were prepared as described previously (*35, 36*) and fractionated by subsequent phosphocellulose and hydroxylapatite chromatography into column fractions I and II (CFI and CFII, respectively), following the method of Wold et al. (*37*). CFI and CFII were dialyzed overnight against 30 mM HEPES-KOH (pH 7.8), 0.25% inositol, 0.25 mM EDTA, 1 mM DTT, 0.01% NP-40, and 15 mM KCl and concentrated using Amicon Ultra device (Millipore). The protein concentration was determined by the Bradford method (*38*).

*Protein Purification.* RPA was purified as described previously (*39, 40*). PCNA and Rad18 tagged with the Flag epitope at their N-termini were constructed and purified as follows.

*(1) PCNA.* The wt-PCNA coding sequence in pcDNA3-PCNA was PCR amplified using the following primers: 5′ttt*aagctt*ATGTTCGAGGCGCGCCTGGTCC and 5′ccc*aagctt*CTAAGATCCTTCTTCATCCTC. The PCR product was digested by*Hin*dIII and ligated into the pT7-Flag bacterial expression vector (Sigma). Positive clones were selected and sequenced.

The monoubiquitylation site in wt-PCNA was mutagenized *in vitro* by replacing the lysine in position 164 with arginine (K164R) using Pfu-ultra polymerase (Stratagene) and primers 5′GGAGATGCTGTTGTAATTTCCTGTGCAAgAGACGGAGTG and 5′CACTCCGTCTcTTGCACAGGAAATTACAACAGCATCTCC (mutagenized nucleotide in lowercase). The presence of the designed mutation was verified by DNA sequencing.

Expression plasmids (pT7-wt-PCNA and pT7-K164R-PCNA) were transformed into BL21 (DE3) *Escherichia coli* cells (Stratagene). Transformants were screened by immunoblotting with anti-Flag M2 antibodies to select a clone with a high level of expression of human PCNA. Expression of human wt- and K164R-PCNA in bacterial cells was induced with 1 mM IPTG for 4 h. Bacterial cells from a 200 mL culture were lysed using "CellLytic B Bacterial Cell Lysis

Extraction Reagent" (Sigma) in the presence of 1 mM PMSF and 5 $\mu$g/mL DNase; PCNA was purified using an anti-Flag M2 affinity gel, according to the manufacturer's instructions. Purified wt- and K164R-PCNA were dialyzed against hypotonic buffer [20 mM HEPES (pH 7.9), 5 mM KCl, 1.5 mM $MgCl_2$, and 1 mM DTT]. The purity was checked by Coomassie blue and silver staining (Figure S1 of the Supporting Information).

*(2) Rad18.* The Flag epitope sequence was fused with the open reading frame of human Rad18 through PCR amplification, using pcDNA3.1/NT-GFP-Rad18 (*41*) as the template, Pfu polymerase, and the primers 5′ggg*ggatcc*ATGGACTACAAGGACGACGATGACAAGgactccctggccgagtct and 5′ggg*ctcgag*aaagtcagcaaaagcccaca (Flag sequence in uppercase letters, *Bam*HI and *Xho*I restriction sites in italics). Digested PCR product was cloned into pcDNA3 (Invitrogen). Positive clones were selected and sequenced. pcDNA3-Flag-Rad18 was transfected into 293T cells; after incubation for 60 h, Flag-Rad18 in the cell extract was bound to anti-Flag M2 affinity gel, eluted with 200 $\mu$g/mL 3× Flag peptide (according to the manufacturer's instructions), and dialyzed against hypotonic buffer [20 mM HEPES (pH 7.9), 5 mM KCl, 1.5 mM $MgCl_2$, and 1 mM DTT]. The purity of the Rad18 preparation was checked by silver staining; coprecipitation of human Rad6 with Rad18 was verified by immunoblotting with anti-Rad6 antibodies (Figure S3 of the Supporting Information).

*Mass Spectrometry Methods.* Recombinant PCNA proteins were analyzed by mass spectrometry to verify their identity. Salts and other contaminants were removed from Flag-purified, full-length proteins using $C_4$ Zip Tips (Millipore), according to the manufacturer's instructions. Full-length protein masses were determined by nanoelectrospray analysis on an API Q-Star-Pulsar apparatus (Applied Biosystems Inc.). Wild-type and mutant forms of PCNA were digested with trypsin, and masses of the peptides were measured on an ABI4700 proteomics analyzer (Applied Biosystems) (Figure S2 of the Supporting Information).

*In Vitro Ubiquitylation of PCNA. In vitro* ubiquitylation of purified proteins was carried out for 1 h at 25 °C in 15 $\mu$L reaction mixtures containing 50 mM HEPES (pH 7.6), 0.5 mM DTT, 1 mM $MgCl_2$, 1 mM ATP, 0.1 mg/mL creatine phosphokinase, 50 mM creatine phosphate, wt- or K164R-PCNA (500 nM), 1 mM ubiquitin or 500 $\mu$M His-ubiquitin, 450 nM E1, and 120 nM Rad6/Rad18 (E2/E3). Reactions were terminated by the addition of equal volumes of 2× Laemmli buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, and 20% glycerol], and proteins were fractionated by SDS−PAGE and analyzed by immunoblotting with anti-PCNA antibody (Figure S4 of the Supporting Information). *In vitro* ubiquitylation with 50 $\mu$g of CFII was conducted as described above in the presence of 300 ng of wt-PCNA (3.3 pmol of PCNA trimeric clamp), 1 pmol of single-stranded m13mp2 with 1 pmol each of four different primers annealed around the circular DNA, and each dNTP at 0.2 mM with or without addition of 300 nM E1, 60 nM Rad18/Rad6, and 500 $\mu$M ubiquitin (Figure S5 of the Supporting Information).

*Western Immunoblot Analysis.* Samples for immunoblotting were subjected to SDS−polyacrylamide gel electrophoresis and proteins transferred to nitrocellulose membranes. The blots were blocked in TBST [100 mM Tris (pH 7.5), 150 mM NaCl, and 0.1% Tween 20] containing 5% nonfat dry milk for 1 h at room temperature and probed with the indicated antibodies (see Materials).

*In Vitro DNA Replication.* The two-step DNA *in vitro* replication assay, the preparation of the circular duplex DNA substrates, and the method of analysis of products of replication have been described previously (*30, 32*). Each 25 $\mu$L reaction mixture contained 50 $\mu$g of HeLa CFII supplemented with 250 ng of Flag-PCNA and 300 ng of RPA, or 40 $\mu$g of CFI (the indicated amounts of PCNA and RPA were determined to be equivalent to those present in 40 $\mu$g of CFI by immunoblotting different volumes of CFI in parallel to increasing masses of the purified proteins). The DNA substrates have the SV40 origin of replication either to the left (T^ToriL) or to the right (T^ToriR) of a single T^T; the location of the lesion results in blockage of leading- or lagging-strand synthesis, respectively. A mismatch in the nearby *Pst*I site allows for recognition of the replication products generated from either the damaged (resistant to digestion) or the undamaged (cut by *Pst*I) template strands. The corresponding control substrates (TToriL and TToriR) lack the dimer but include the mismatch in the *Pst*I recognition site (*32*). DNA replication in this *in vitro* assay requires the addition of SV40 large T antigen (1 $\mu$g per reaction) to direct the assembly of the origin−initiation complex.

DNA was purified from *in vitro* replication reactions after addition of an internal standard, $^{32}$P-end-labeled pUC19 (pUC), carrying a *Pst*I recognition site in the center of the linearized molecule. DNA recovered from each *in vitro* reaction mixture was divided into two equal-volume samples for incubation in the presence or absence of *Pst*I, followed by fractionation in 1% agarose gels containing 0.2 $\mu$g/mL ethidium bromide. Dried gels were exposed to a phosphor screen and scanned with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). The intensity of form IV DNA bands (closed circular duplex) before and after *Pst*I digestion was quantified and normalized to the internal control band. The fraction of form IV resistant to *Pst*I digestion was calculated by dividing the normalized amount of form IV measured in the digested sample by the same value in the parallel sample incubated in the absence of *Pst*I. Then, the amount of TLS products, relative to the overall DNA synthetic capacity under different reaction conditions (i.e., TLS efficiency), was calculated by dividing the fraction of *Pst*I-resistant form IV measured with the T^T-containing substrate by the equivalent fraction observed with the control substrate (*32, 42*).

*Two-Dimensional Agarose Gel Electrophoresis.* DNA replication products were purified from one-step *in vitro* replication reactions (30 min incubation) and digested with *Cla*I (*30*). Doubled reactions (50 $\mu$L) were used with CFII fractions supplemented with PCNA and RPA or CFI. First- and second-dimension electrophoreses were carried out as described previously (*30*).

## RESULTS

The main goal of this study was to determine whether monoubiquitylation of PCNA is a required step for pol $\eta$-dependent translesion synthesis of a cyclobutane thymine dimer during semiconservative replication of double-stranded DNA. *In vitro* assays are helpful tools for the investigation

of mechanistic details, in this case the need for post-translational modification of PCNA to allow switching of the blocked 3′ terminus from the replicative polymerase to the active site of pol $\eta$. To complete this task, it was necessary to set up experimental conditions for (i) the removal of endogenous PCNA from replication-competent, cell-free extracts containing DNA pol $\eta$, (ii) purification of functional recombinant PCNA with either lysine at position 164, the acceptor site for monoubiquitylation, or another amino acid with a side chain that cannot be similarly modified [in this study, arginine, the same amino acid substitution in PCNA expressed by the *S. cerevisiae* mutant *pol30-119* (*20*)], and (iii) reconstitution of DNA replication and TLS activity with the purified recombinant PCNA.

*TLS of a Site-Specific T^T on a Double-Strand Circular DNA Substrate Requires Pol η.* An assay was previously optimized for reliable detection of the TLS product (i.e., the daughter molecule generated from the template strand carrying a thymine dimer) during *in vitro* replication of circular duplex DNA substrates (*32, 42*). In this assay, replication-competent extracts prepared from XP-V fibroblasts, which lack pol $\eta$, do not generate detectable amounts of TLS products (*42*). Concordant with the observation that diploid XP-V fibroblasts recover normal responses to UV radiation upon complementation for pol $\eta$ expression (*43, 44*), addition of small amounts of purified human pol $\eta$ to XP-V extracts was necessary and sufficient to recover *in vitro* TLS across the T^T (Figure 1). Therefore, the assay illustrated in Figure 1 and used throughout this study provides a clear readout of DNA pol $\eta$-dependent TLS activity.

*Phosphocellulose Chromatography Separates PCNA and RPA from Other Essential Replication Factors.* Figure 2 illustrates how PCNA, RPA, and other proteins of interest to this study were distributed between CFI and CFII in the preparations used in the experiments reported here. As expected (*37*), PCNA and RPA did not bind to the phosphocellulose column and were recovered in CFI. CFII was also depleted of E1 and Rad6 and contained small amounts of ubiquitin. RFC and DNA polymerases $\delta$ and $\eta$ were detected primarily in CFII (Figure 2).

*DNA Replication and TLS Activities Are Reconstituted when RPA and PCNA Are Added to CFII.* Reactions with CFII alone or supplemented only with purified RPA did not support DNA replication *in vitro* (Figure 3A). Addition of purified RPA and PCNA to CFII was sufficient to replicate circular duplex DNA substrates with or without the T^T (Figure 3B; Figure S6C of the Supporting Information). Furthermore, when either wt- or K164R-PCNA was added to the reaction mixtures (see Figures S1 and S2 of the Supporting Information for characterization of the purified proteins), the relative efficiencies of *in vitro* DNA replication and TLS (*32*) were similar. We confirmed this observation in a separate experiment in which relative TLS efficiencies (*42*) were determined in parallel reaction mixtures containing CFII supplemented with purified RPA and PCNA, or its matched CFI fraction (Figure 4) (all reaction mixtures contained equivalent concentrations of RPA and PCNA). These results further established that (i) RPA and PCNA were the only essential DNA replication factors recovered in the CFI fraction and (ii) pol $\eta$-dependent synthesis across and beyond the site-specific T^T was not enhanced by the presence of proteins implicated in monoubiquitylation of PCNA (see below).

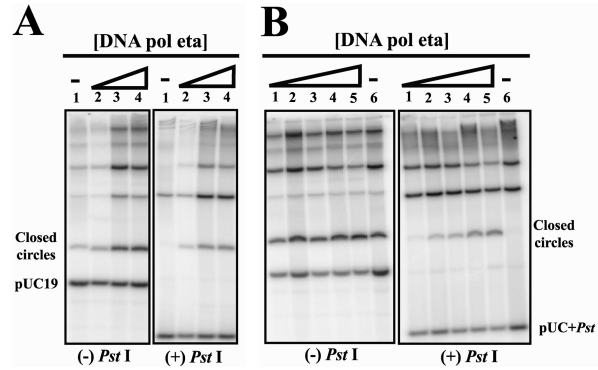


FIGURE 1: Addition of purified human pol $\eta$ to XP-V extracts is necessary and sufficient for recovery of TLS across a thymine dimer. Replication-competent extracts were prepared from XP-V fibroblasts and incubated with T^ToriL (A) or T^ToriR (B) in the absence (−) or presence of increasing concentrations of purified human DNA pol $\eta$. Equal amounts of linearized $^{32}$P-labeled internal standard (pUC19) were added to each reaction mixture before purification to control for DNA recovery and efficiency of *Pst*I digestion. Each sample was divided into two equal-volume aliquots, which were incubated in the absence (−) or presence (+) of *Pst*I. Undigested and digested samples were fractionated in 1% agarose gels in the presence of ethidium bromide. The positions of replicated form IV (Closed circles) and the bands for full-length (pUC19) and *Pst*I-digested (pUC+*Pst*I) internal standard are indicated. (A) Each reaction mixture (12.5 $\mu$L) included 6 fmol of T^ToriL and 0 (lane 1), 0.13 (lane 2), 0.31 (lane 3), or 0.63 nmol (lane 4) of DNA pol $\eta$; these reaction mixtures were incubated for 2.5 h without a preincubation step. (B) Replication reaction mixtures (12.5 $\mu$L) included 5 fmol of T^ToriR and 0.12 (lane 1), 0.6 (lane 2), 1.2 (lane 3), 6 (lane 4), 12 (lane 5), or 0 fmol (lane 6) of DNA pol $\eta$; these reaction mixtures were incubated for 65 min after a 20 min preincubation step without dNTPs. Note the absence of closed circles from reaction mixtures lacking DNA pol $\eta$ once the replication products were digested with *Pst*I. TLS products were detected in reaction mixtures supplemented with DNA pol $\eta$, even when the enzyme was added at one-tenth the concentration of DNA template.

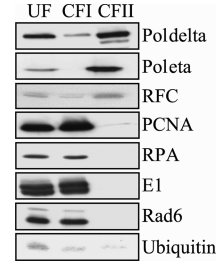


FIGURE 2: Distribution of proteins in HeLa cell extracts into CFI and CFII phosphocellulose column fractions. Western blot analyses with antibodies specific for the indicated proteins (see Materials) were carried out after aliquots from unfractionated extracts (UF), CFI, and CFII had been pooled from three independent fractionations of cell extracts. Equal amounts of protein (50 $\mu$g; except that 200 $\mu$g was used for the pol $\eta$ immunoblot) were separated by electrophoresis in 6% (pol $\eta$), 7% (pol $\delta$ and E1), 10% (PCNA, RPA, and RFC) or 18% (ubiquitin and Rad6) SDS−polyacrylamide gels. After being transferred to nitrocellulose membranes, the indicated proteins were probed with specific antibodies.

*2D Gel Electrophoresis Confirmed TLS across the T^T in Reaction Mixtures Supplemented with K164R-PCNA.* The duplex DNA substrate was T^ToriL (Figure 5A); thus, the dimer was on the template to the leading strand when the first fork approached the lesion. Reaction mixtures were incubated for 30 min with CFII supplemented with CFI

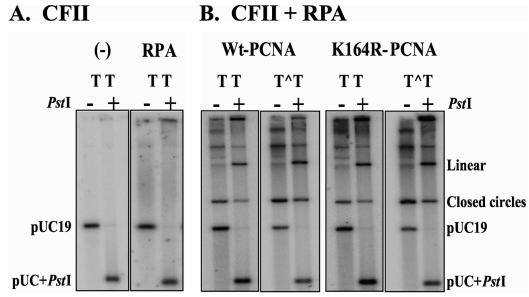


FIGURE 3: K164R-PCNA supports replication of circular duplex DNA molecules and TLS across a site-specific T^T. Replication reactions were carried out for 2 h after a 20 min preincubation step without dNTPs. Equal amounts of linearized $^{32}$P-labeled internal standard (pUC19) were added to each reaction mixture before purification to control for DNA recovery and efficiency of *Pst*I digestion. One-half of each sample was treated with *Pst*I. Undigested and digested samples were fractionated in 1% agarose gels. The positions of replicated forms IV (Closed circles) and III (linear) and the bands for full-length (pUC19) and *Pst*I-digested (pUC+*Pst*I) internal standard are indicated. (A) *In vitro* reaction mixtures with the control TToriL substrate included CFII as the only source of replication factors (−) or with supplementation with RPA. (B) Replication of the control (TT) and dimer-containing (T^T) substrates in reaction mixtures with CFII+RPA that were supplemented with wt- or K164R-PCNA. Both panels shows results for paired samples incubated in the absence (−) or presence (+) of *Pst*I prior to gel electrophoresis.

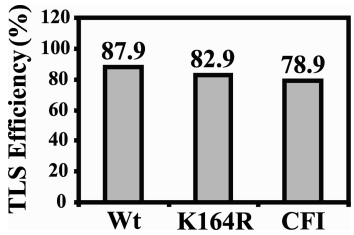


FIGURE 4: Estimated efficiency of *in vitro* TLS across a site-specific T^T by human replication factors. The amounts of replicated closed circles detected before (total form IV) and after *Pst*I digestion (resistant form IV, product of the strand carrying the T^T) in an experiment similar to the one illustrated in Figure 3B were quantified and normalized to the internal control. TLS efficiencies in the presence of recombinant RPA and wild-type (wt) or mutant (K164R) PCNA, and CFI (endogenous RPA, PCNA, and other proteins), were estimated from the fraction of *Pst*I-resistant replication products (resistant form IV/total) derived from the strand with the T^T, relative to the identical strand in the control substrate (TT in place of the dimer).

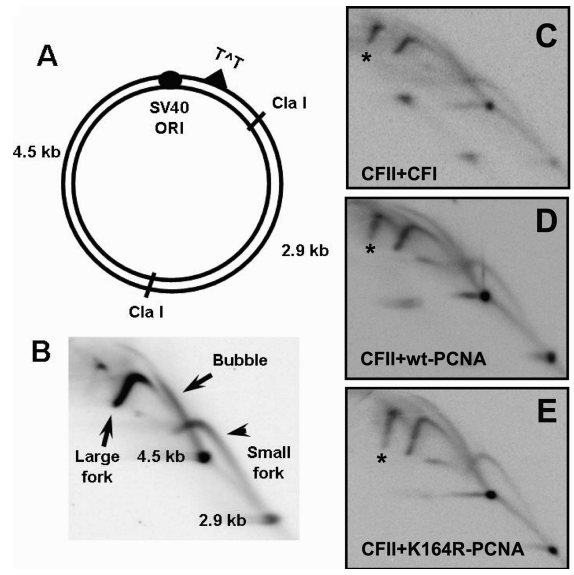


FIGURE 5: Two-dimensional gel analysis of intermediates of *in vitro* DNA replication. Closed circular DNA substrates without (control) or with (A) a single cyclobutane thymine dimer (T^T) were incubated for 30 min under defined conditions for *in vitro* replication (*30*). The T^T was positioned 385 bp from the center of the SV40 origin of replication (ORI). *Cla*I digestion of these DNA substrates results in two fragments of 4.5 and 2.9 kb. (B) Typical pattern of separation by 2D gel electrophoresis of restriction fragments from intermediates of replication of the control substrate. The 4.5 kb fragment can be associated with either a replication bubble or a simple-fork structure (see arrows); only a simple replication fork can be found associated with the 2.9 kb fragment (arrowhead), given the positions of ORI and the *Cla*I restriction sites in the circular duplex molecules (see panel A). (C−E) When the substrate contained a T^T on the template to the leading strand, the appearance of fork structures in the 2.9 kb fragment indicated the capacity of the replication machinery to replicate across the dimer. Molecules in which TLS failed cannot be digested at the *Cla*I site closest to the dimer (*30*) and migrate as a larger fragment (asterisk). Mixing CFII containing pol $\eta$ with CFI obtained from the same (C) or different extracts (Figure S6 of the Supporting Information) reconstituted *in vitro* replication and TLS. Mixing HeLa CFII with purified RPA and with wt-PCNA (D) or K164R-PCNA (E) also recovered DNA replication and TLS activities.

(Figure 5C) or with purified RPA and wt- or K164R-PCNA (Figure 5D,E). Replication intermediates were digested with *Cla*I and analyzed by 2D gel electrophoresis (*30*). A fraction of the forks reaching the dimer was able to replicate across the lesion, continue through the downstream *Cla*I site, and move into and through the 2.9 kb fragment, as revealed by the appearance of the smaller simple-fork arc (indicated by the arrowhead in Figure 5B). The appearance of this fork arc in association with the 2.9 kb fragment is diagnostic of TLS by pol $\eta$, as previously described (*30*). It was not expected to be seen in the reaction mixtures supplemented with K164R-PCNA (Figure 5E), if this mutant protein could not recruit pol $\eta$ to the replication fork that was arrested by the dimer. Instead, both wt-PCNA (Figure 5D; Figure S6C of the Supporting Information) and K164R-PCNA (Figure 5E) supported the generation of DNA replication intermediates, yielding 2D gel electrophoresis patterns that were indistinguishable from each other and from that observed when CFII was supplemented with CFI (Figure 5C; Figure S6B of the Supporting Information). Therefore, K164R-PCNA substitutes well for wt-PCNA to promote pol $\eta$-dependent TLS during *in vitro* replication, in the absence of enzymes required to conjugate ubiquitin to K164 of PCNA (Figure 2).

*Does PCNA Ubiquitylation Improve the Efficiency of Pol $\eta$-Dependent TLS of Thymine Dimers.* The data reported above demonstrate that ubiquitylation of PCNA was not required for pol $\eta$-dependent TLS *in vitro*. Time course experiments were performed to verify whether the initial rate of pol $\eta$-dependent TLS could be enhanced by ubiquitylation of PCNA at K164. Such an effect would be in line with reports that unmodified PCNA interacts with pol $\eta$, stimulating its activity *in vitro* (*23*), and that pol $\eta$ bound to chromatin of irradiated cells interacts preferentially with ubiquitylated PCNA (*15, 21*) (presumably favoring polymerase switching and enhancing TLS). In the experiment illustrated in Figure 6, the accumulation of total and *Pst*I-resistant duplex circular DNA from the T^T-containing substrate was followed from 30 to 120 min. Side-by-side reaction mixtures included the same CFII preparation

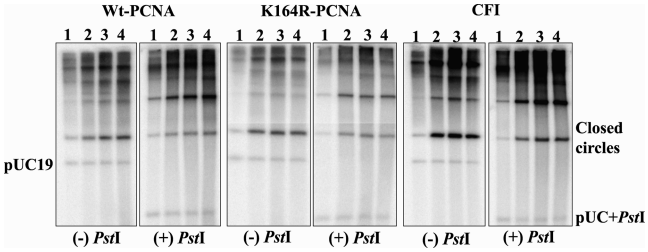


FIGURE 6: Time course of replication of T^ToriL by CFII supplemented with RPA and with wild-type or mutant PCNA. *In vitro* replication reactions with the T^T substrate were performed under the conditions described in the legend of Figure 3, except that after the 20 min preincubation the reactions proceeded for 30, 60, 90, and 120 min (lanes 1–4, respectively). Closed circles resistant to *Pst*I digestion correspond to TLS products; they were produced in reaction mixtures that included endogenous PCNA (CFI) and also in those supplemented with recombinant wild-type or mutant PCNA (in addition to RPA).

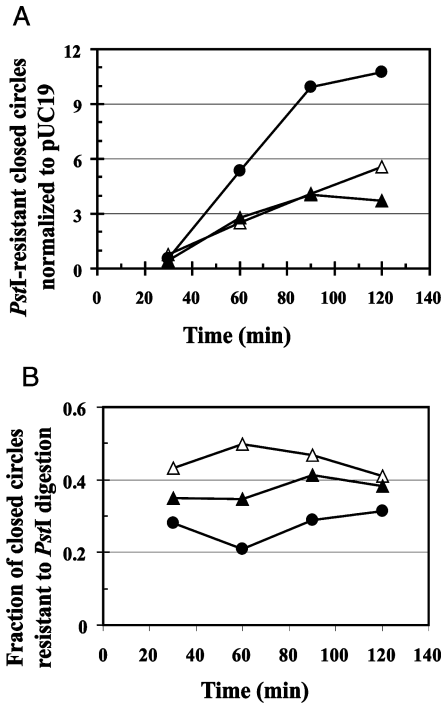


FIGURE 7: Time course of generation of daughter molecules derived from the DNA strand containing the T^T dimer. (A) Accumulation with time of *Pst*I-resistant closed circles produced from the dimer-containing strand of T^ToriL, when CFII was supplemented with CFI (●) or purified RPA with wt-PCNA (△) or K164R-PCNA (▲). (B) *Pst*I-resistant closed circles as a fraction of the total amount of form IV DNA detected in the absence of restriction digestion. Under all three experimental conditions (symbols as indicated in panel A), the relative fraction of daughter molecules containing the T^T remained constant as the total amount of replication and TLS products increased with time.

supplemented with purified RPA and PCNA or with its matched CFI (Figure 6). The radioactivity signals associated with closed circular duplex DNA that was resistant to digestion by *Pst*I (TLS product) were normalized to the internal standard (pUC19) and plotted against incubation time (Figure 7A). Very similar results were observed for the reaction mixtures supplemented with wt- or K164R-PCNA. Recalling that E1 and Rad6 bound poorly to phosphocellulose and were recovered with CFI (Figure 2), we were not surprised to observe apparently similar rates of TLS, regardless of the PCNA variant added to the mixture. Without the components of the PCNA ubiquitylation system in CFII, wild-type PCNA would not be modified, thus explaining the similarity in bypass kinetics. The apparent TLS activity was higher when CFII was supplemented with its corresponding CFI; these results were very similar to those published earlier with unfractionated HeLa cell extracts (*32*). The slopes of the lines defined by the first three points in the curves depicted in Figure 7A suggest that in the presence of purified RPA and PCNA the initial rate of accumulation of bypass product was 35–40% of that observed when the replication activity in CFII was restored by addition of CFI. It is important, however, to consider also the fraction of *Pst*I-resistant molecules in relationship to the total amount of radiolabeled duplex circular DNA produced in these reactions. The total amount of replicated molecules was greater in the samples containing CFI, as compared to those in which CFII was supplemented with RPA and PCNA; however, the fraction resistant to *Pst*I (bypass product) was approximately the same (Figure 7B). As illustrated in Figure 4, when the fraction of bypass product (closed circles derived from the strand carrying the T^T) is normalized to the fraction of replication of the same strand in the control substrate, the estimated TLS efficiency is not higher in the CFII + CFI reaction mixtures. These observations suggest that in addition to RPA and PCNA, CFI included factors that enhanced the overall rate of DNA replication, thus allowing for more bypass events to take place, but these same factors did not contribute to a higher efficiency of TLS across the T^T.

## DISCUSSION

The mechanisms by which the structure of DNA replication forks is protected and associated activities (e.g., replication, repair, damage tolerance, and checkpoint activation) are regulated have important biological implications for the maintenance of genomic stability. Translesion synthesis by DNA polymerase $\eta$ must be preceded by its recruitment to the 3′-end of the nascent DNA strand that cannot be elongated across a thymine dimer by the replicative DNA polymerase. The sequence of signaling events and protein interactions that underlie the required exchange of DNA pol $\epsilon$ or $\delta$ for pol $\eta$ must take place amidst many other DNA–protein transactions involved in DNA damage responses (*45*). Therefore, it is quite a challenge to demonstrate that a specific reaction (e.g., monoubiquitylation of PCNA) observed when cultured human cells are exposed to UV radiation is necessary for one and not another biochemical pathway activated by a stalled replication fork. *In vitro* assays that reliably report on a biochemical end point can be very useful in the dissection of mechanistic details of complex pathways.

The results reported here add to the evidence that pol $\eta$-dependent TLS across a T^T does not require monoubiquitylation of PCNA at K164. They extend the findings of Haracska et al. (*24*) that PCNA ubiquitylation is not necessary for stimulation of DNA synthetic and TLS activities of pol $\eta$. The new data show that K164R-PCNA supports pol $\eta$-dependent TLS during *in vitro* replication of a circular duplex DNA by human proteins. The assay used requires origin initiation and DNA polymerase switching events; when the substrate contains a site-specific thymine dimer, TLS in this assay is completely dependent on pol $\eta$

activity (Figure 1; Figure S6A of the Supporting Information). Therefore, the absence of monoubiquitylation of PCNA did not interfere with its interactions with DNA replication factors, including the replicative polymerases and pol $\eta$, in agreement with the observations that unmodified PCNA and $PCNA^{Ubi}$ can carry out the same replicative functions (*25*). Both wt- and K164R-PCNA supported full replication of the DNA substrates, regardless of the presence or absence of the T^T (Figure 3), and with similar TLS efficiencies (Figure 4), even in the absence of E1, Rad6, and presumably also Rad18 (Figure 2).

Pol $\eta$-dependent TLS of the site-specific T^T was carried out during elongation of the leading strand and before completion of replication of the circular duplex DNA substrate (Figure 5). The 2D gel electrophoresis patterns observed in the presence of wt-PCNA (Figure 5D), K164R-PCNA (Figure 5E), or CFI (Figure 5C) were very similar, suggesting comparable TLS kinetics in the presence (Figure 5C) or absence (Figure 5D,E) of the enzyme system involved in ubiquitylating PCNA. This conclusion is supported also by the time course of accumulation of the true product of replication across the T^T (*Pst*I-resistant form IV) (Figure 6). Although the rate of accumulation of the TLS product was higher when CFII was complemented by CFI (Figure 7A), the fraction of circular duplex DNA generated from the T^T-containing strand, relative to the total amount of form IV, remained approximately the same at each time point under the three reaction conditions (Figure 7B). Thus, the results in Figure 7A could be explained simply by the total DNA replication capacity of *in vitro* reactions being lower when CFII was supplemented only with purified RPA and PCNA. It is plausible that other proteins present in CFI would boost the total replication activity (a requirement for TLS) without having a direct impact on TLS catalysis. Note that when variations in DNA replication activity among the different reactions were taken into account, the estimated TLS efficiencies were similar when CFII was complemented with CFI or with purified RPA and PCNA (Figure 4). If $PCNA^{Ubi}$ were formed in sufficient amounts in reaction mixtures containing CFII and CFI and the modified clamp were to provide a significant kinetic advantage for TLS catalysis by pol $\eta$, the assays used in this study would have detected differences in TLS efficiency (*32, 42*). This issue could be further examined in future experiments by determining the efficiency of pol $\eta$-dependent TLS when CFII is supplemented only with unmodified PCNA (K164R) or with PCNA already containing a monoubiquitin residue attached to K164.

Genetic studies in *S. cerevisiae* have demonstrated that postreplication repair (PRR, used here to denote completion of genome replication in the presence of template lesions) is controlled by the RAD6 epistasis group, which includes RAD18, RAD5, MMS2, UBC13, RAD30 (pol $\eta$), REV1, and REV3-REV7 (pol $\zeta$) (*46–48*). *S. cerevisiae* strains with mutations in RAD6 or RAD18 display a low rate of survival, are defective in PRR, and are not mutagenized after exposure to UV (*46, 49*). The K164R mutation in PCNA (pol30-119) leads to defective PRR and does not increase further the sensitivity to the toxic effects of UV radiation when combined with *rad6*, rev3$\Delta$ (lacks pol $\zeta$), rad30$\Delta$ (lacks pol $\eta$), and *rad5*$\Delta$ (defective in damage avoidance) mutations (*20*). These epistatic relationships reveal that in *S. cerevisiae* PCNA plays important roles in the REV3, RAD30, and RAD5 pathways. SUMO (small ubiquitin-related modifier) is also conjugated to K164 in yeast PCNA. Interestingly, the *pol30-119* mutation eliminates UV-induced mutagenesis (*19, 20*) but also partially suppresses the UV hypersensitivity of *rad6* and *rad18* mutants (*20*). This genetic interaction was interpreted as evidence of the elimination of suppression of the Rad52-dependent recombination pathway by $K164\text{-}PCNA^{SUMO}$ (*20*). These findings indicate that both ubiquitylation and SUMOylation at K164 of PCNA cooperate *in vivo* [instead of competing (*50, 51*)] to enhance TLS and inhibit replication fork-associated recombination. In the absence of post-translational modification at K164, the replication fork is more likely to collapse and require the Rad52 recombination pathway for its repair and restart.

Dissecting genetic interactions into specific mechanistic details is difficult, and translating them to higher eukaryotes is even more challenging. Monoubiquitylation of PCNA has been well-documented in human cells (see the introductory section), but SUMOylated forms of this clamp have not been identified (*52*). Polyubiquitylation of PCNA is not immediately apparent but has been detected in UV-irradiated human cells (*53*). Expression of K63R-ubiquitin to interfere with formation of K63-linked polyubiquitin chains did not affect proliferation of A549 cells or their survival after UV exposure. However, it resulted in an increase in the UV-induced mutation frequency, consistent with the interpretation that an error-free, damage-tolerance pathway was inhibited, thus resulting in error-prone TLS at a higher fraction of photoproducts (*53*). Such interpretation is in agreement with previously published evidence that human MMS2, like its yeast homologue, participates in a damage-avoidance pathway involving homologous recombination between two alleles or between sister duplexes in UV-irradiated cells (*54*). Emerging evidence suggests that the protein encoded by *WRN*, a DNA helicase-exonuclease (RecQ family) whose loss of function underlies premature aging and cancer predisposition (Werner syndrome), interacts functionally with TLS polymerases, including pol $\eta$ (*55*). As for Srs2 helicase in yeast (*50*), the published data suggest that interaction of WRN and error-prone polymerases might promote replication fork progression and suppress recombination events at stalled forks, despite the risk for an increased level of mutagenesis (*55*).

The members of the RAD6 pathway that have been clearly implicated in damage-induced mutagenesis in mammalian cells are REV1 and REV3/REV7 (*56–59*). Curiously, the effects of UV on survival and PRR in $RAD18^{-/-}$ mouse ES cells were mild relative to those observed in yeast; induced mutability was reduced but not abolished, and levels of homologous recombination events, such as sister chromatid exchanges (SCEs) and gene targeting frequencies, were elevated 2−3-fold (*60*). The effects of UV radiation on the colony-forming efficiency and the rate of DNA strand growth were also mild in SV40-transformed and neoplastic human cells that were transfected with an expression vector carrying the C28F mutant allele of human Rad18; this mutant protein failed to interact with Rad6 but did localize to the nucleus in the irradiated cells (*61*). On the other hand, the absence of pol $\eta$ in XP-V fibroblasts leads to the accumulation of daughter-strand gaps and enhanced mutagenesis but does not enhance the frequency of SCEs, unless these fibroblasts are

first transformed by viral oncoproteins (*62*). Also puzzling is the finding that despite the higher frequency of stalled replication forks in UV-irradiated XP-V fibroblasts (*63*) the fraction of monoubiquitylated PCNA in these cells is not any higher than in HeLa cells (Figure S7 of the Supporting Information), normal fibroblasts (*15*), or XP-V fibroblasts complemented for pol $\eta$ expression (Figure S8 of the Supporting Information).

DNA polymerase switching is part of normal DNA replication; competition of different replication factors for RPA is thought to mediate the switch between pol $\alpha$ and $\delta$ (*64*). The switch from pol $\delta$ to pol $\eta$ and back again appears to be mediated at least in part by differences in the binding affinities of these enzymes for the undamaged template versus the one containing a thymine dimer (*65, 66*). Recently, the evidence that leading strand synthesis in eukaryotes is catalyzed by pol $\epsilon$ (*67, 68*) has been strengthened by mutagenesis studies in *S. cerevisiae* strains expressing a pol $\epsilon$ mutant with reduced replication fidelity (*69*). These findings suggest that TLS across a T^T on the template to the leading strand could involve switching between pol $\epsilon$ and pol $\eta$.

In summary, the data presented in this report suggest that PCNA monoubiquitylation is not a requirement for polymerase switching or any other step involved in the catalysis of pol $\eta$-dependent TLS across a thymine dimer during *in vitro* replication of a double-stranded DNA substrate. The role of $PCNA^{Ubi}$ *in vivo* cannot be addressed by the approach used in this study. $PCNA^{Ubi}$ is generated under different conditions that cause DNA replication stress in mammalian cells, but the significance of this post-translational modification in the overall response of mammalian cells to the inhibition of fork movement remains unclear. It is possible that the more complex chromatin structure *in vivo* (not replicated in replication assays *in vitro*) requires specific modifications of PCNA to facilitate the many different interactions among proteins and biochemical reactions needed for checkpoint signaling, for stability of stalled replication forks, and for tolerance and/or avoidance pathways that allow completion of DNA replication despite the presence of template damage.

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## SUPPORTING INFORMATION AVAILABLE

Characterization by SDS−PAGE, immunoblotting, and mass spectrometry of purified PCNA, SDS−PAGE and immunoblotting analyses of Rad18/Rad6 used in this study, evidence that CFII lacks PCNA monoubiquitylation activity, 2D gel electrophoresis results showing that pol $\eta$ activity is found in CFII, results with independent preparations demonstrating that RPA and PCNA complement CFII for pol $\eta$-dependent TLS across a thymine dimer, and UVC-induced monoubiquitylation of PCNA in HeLa cells and an isogenic pair of diploid XP-V fibroblasts with or without complementation for pol $\eta$ expression. This material is available free of charge via the Internet at http://pubs.acs.org.

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