

Proliferating Cell Nuclear Antigen Promotes Translesion Synthesis by DNA Polymerase ζ *

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DNA polymerase ζ (Pol ζ), a heterodimer of Rev3 and Rev7, is essential for DNA damage provoked mutagenesis in eukaryotes. DNA polymerases that function in a processive complex with the replication clamp proliferating cell nuclear antigen (PCNA) have been shown to possess a close match to the consensus PCNA-binding motif QxxLxxFF. This consensus motif is lacking in either subunit of Pol ζ , yet its activity is stimulated by PCNA. In particular, translesion synthesis of UV damage-containing DNA is dramatically stimulated by PCNA such that translesion synthesis rates are comparable with replication rates by Pol ζ on undamaged DNA. PCNA also stimulated translesion synthesis of a model abasic site by Pol ζ . Efficient PCNA stimulation required that PCNA was prevented from sliding off the damage-containing model oligonucleotide template-primer through the use of biotin-streptavidin bumpers or other blocks. Under those experimental conditions, facile bypass of the abasic site was also detected by DNA polymerase δ or η (Rad30). The yeast DNA damage checkpoint clamp, consisting of Rad17, Mec3, and Ddc1, and an ortholog of human 9-1-1, has been implicated in damage-induced mutagenesis. However, this checkpoint clamp did not stimulate translesion synthesis by Pol ζ or by DNA polymerase δ .

During the S phase of the eukaryotic cell cycle, when DNA damage may form a block for the high fidelity DNA polymerases such as DNA polymerase δ (Pol δ)¹ or Pol ϵ , several complex pathways are activated. The *Saccharomyces cerevisiae* *RAD6* and *RAD18* genes control a bifurcating pathway that activates translesion synthesis (TLS) by error-prone DNA polymerases as well as a recombinational damage avoidance pathway (reviewed in Ref. 1). Pol η is largely responsible for the relatively error-free bypass of *cis-syn* pyrimidine dimers in the template strand (2, 3). In contrast, most other types of damage are bypassed by a set of error-prone DNA polymerases, and this bypass forms the molecular basis for damage-induced mutagenesis in the cell. One of these translesion polymerases is Pol ζ , a heterodimer of the Rev3 and Rev7 subunits, and the

second enzyme is the Rev1 deoxycytidyl transferase (4, 5). The replicative polymerase Pol δ has also been shown to be required for damage-induced mutagenesis, primarily through mutational studies of its Pol32 subunit (3, 6–8). Consequently, a model has evolved in which Pol δ inserts a nucleotide across template damage, whereas Pol ζ and Rev1 are responsible for extension of that inserted nucleotide. In analogy to bacterial mutagenesis systems, this model has been named the two-polymerase model for TLS (9, 10). Biochemically, this model has mainly been studied by measuring bypass rates and efficiencies of the catalytic cores of these enzymes without the inclusion of processivity factors such as the proliferating cell nuclear antigen (PCNA) (11, 12).

Most DNA polymerases possess a unique consensus motif, QxxLxxFF, that is required for binding of the polymerase to PCNA and for PCNA-mediated processive DNA synthesis (reviewed in (13)). For the Y-family DNA polymerases, Pol η , Pol ι , and Pol κ , that function in TLS, inactivation of this motif greatly reduced binding of the enzyme to PCNA and PCNA-dependent bypass of damage (14–18). In yeast, mutation of the consensus motif in Pol η results in a phenotype equivalent to that of a null mutant (15). This consensus motif cannot be found in either the Rev3 or the Rev7 subunit of Pol ζ possibly suggesting that this DNA polymerase does not interact with PCNA or that Pol ζ uses a different motif if it does interact with PCNA. Alternatively, Pol ζ may interact with a modified form of PCNA or with a different clamp altogether. Currently, no genetic data exist that establish a specific link between PCNA and Pol ζ .

In response to DNA damage, PCNA is mono-ubiquitinated on Lys¹⁶⁴ by Rad6/Rad18, which is the initiating step for post-replicative DNA repair (19, 20). However, although ubiquitination of PCNA is essential for the initiation of this pathway, and therefore also for mutagenesis, its biochemical role remains obscure. Ubiquitinated PCNA may function in fork remodeling in preparation for TLS or as a chaperone to localize translesion polymerases to sites of damage (21). It is not known whether translesion synthesis by any of the TLS DNA polymerases requires ubiquitinated PCNA as a processivity factor. In fact, efficient TLS by several Y-class polymerases in a complex with native PCNA has been demonstrated (15–18).

DNA damage also activates the DNA damage checkpoint pathway that proceeds through a PCNA related heterotrimeric clamp, Rad17/Mec3/Ddc1 (Rad17/3/1), the ortholog of the human 9-1-1 complex, and the replication factor C (RFC)-related Rad24-RFC clamp loader (Rad17 in human; reviewed in Ref. 13). In yeast, efficient mutagenesis depends on the Rad17/3/1 clamp and its loader. However, when irreparable damage is present in the cell because of the inactivation of nucleotide excision repair, mutagenesis is almost entirely dependent on an intact checkpoint clamp-clamp loader system (22). This dependence has raised the possibility that the Rad17/3/1 clamp

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¹ The abbreviations used are: Pol ζ , η , and δ , DNA polymerases ζ , η , and δ ; TLS, translesion synthesis; PCNA, proliferating cell nuclear antigen; RFC, replication factor C; Rad24-RFC, complex of Rfc2-5 and Rad24; Rad17/3/1, complex of Rad17, Mec3, and Ddc1; RPA, replication protein A; GST, glutathione S-transferase; nt, nucleotide.

may actually function as a processivity factor for a TLS polymerase such as Pol ζ .

While studying the properties of Pol ζ during DNA damage bypass synthesis, we found, surprisingly, that the synthetic efficiency of Pol ζ is profoundly stimulated by PCNA when the template DNA contains damage. However, under the same conditions and with the same type of DNA damage, we failed to observe any indication for a functional interaction of the checkpoint clamp with Pol ζ . Our conclusion is that Pol ζ interacts with PCNA *via* an as yet unidentified motif.

MATERIALS AND METHODS

Strains and Plasmids—The protease deficient yeast strain BJ2168 (*MATa: ura3-52, trp1-289, leu2-3,112, prb1-1122, prc1-407, pep4-3*) was used for overproduction. Plasmids pWS1-REV7 and pGST-REV3 were a gift from Christopher Lawrence (4). Removal of the glutathione *S*-transferase (GST) purification tag by thrombin in the purified Pol ζ preparation proceeded only up to ~50% completion, and therefore, the REV3 gene from the pGST-REV3 plasmid was re-cloned into the pRS426-GALGST vector (2 μ M ori, *URA3, GAL1-10*) to yield a GST-REV3 fusion clone (pBL811) containing a PreScission protease cleavage site. *RAD30* was similarly cloned into pRS425-GALGST (2 μ M ori, *LEU2, GAL1-10*) to give plasmid pBL810. Plasmids and sequences are available upon request.

DNA Substrates—All oligonucleotides were obtained from Integrated DNA Technologies and purified by polyacrylamide electrophoresis or high performance liquid chromatography before use. The 107-nt 5'- and 3'-biotinylated template Bio-V5 (Bio-5'-AGTGGGTTGGTTGGGGT₃₀-CTCCCTTCTTCTCCCTCCCTTCCCT₃₁-Bio) was prepared by hybridizing two half-oligonucleotides to a bridging primer C12 (5'-AGGGAAGGGAGAGGGAGGAGAAGAAGGGAG) followed by ligation with T4 DNA ligase and purification by preparative urea-PAGE. The template was primed with C12 labeled at the 5'-end using T4 polynucleotide kinase (New England Biolabs) and [γ -³²P]ATP. After hybridization and priming, streptavidin was added in 2-fold molar excess. The DNA substrate was irradiated where indicated with 750 J/m² UV light (254 nm) at 0 °C.

Bio-V9 (Bio-5'-CCTTTGCGAATCT₂₅GCGGCTCCCTTCTTCTCCCTCCCTCCCTCCCT₃₁-Bio) and Bio-V9AP1 (Bio-5'-CCTTTGCGAATCT₂₅GCGOCTCCCTTCTTCTCCCTCCCTCCCTCCCT₃₁-3'-Bio), where the boldface "O" represents a tetrahydrofuran residue, were prepared in a similar fashion, primed with 5'-end-labeled C12-4 (5'-AGGGGAAGGGAGAGGGAGGAGAAGAAG) and incubated with 2-fold molar excess streptavidin.

Enzymes—Pol δ , Rad17/3/1, and Rad24-RFC were purified from yeast overproduction strains as described previously (23, 24). Replication protein A (RPA), PCNA, and RFC were purified from *Escherichia coli* overproduction strains (25, 26).

Expression and Purification of Pol ζ and Pol η —BJ2168 containing pBL811 and pWS1-REV7 was grown as described previously. The cells (about 150 g wet weight) were resuspended in 30 ml of water and frozen as kernels in liquid nitrogen. They were disrupted in a blender with dry ice at final concentrations of 50 mM Hepes, pH 7.8, 10% glycerol, 2 mM EDTA, 1 mM EGTA, 0.05% Nonidet P-40, 1 M NaCl, 5 mM dithiothreitol, and protease inhibitors as described (27). All further steps were carried out at 0–4 °C. Polymin P was added to the lysate to 0.4% and the mixture stirred for 10 min. After preclearing at 18,000 rpm for 30 min, 0.28 g/ml ammonium sulfate was added to the supernatant. After stirring for 30 min, the precipitate was spun at 18,000 for 30 min. The pellet was dissolved in A₀ buffer (30 mM Hepes, pH 7.4, 10% glycerol, 2 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.1% ampholytes 3.5–10, 5 mM dithiothreitol, and protease inhibitors) until the conductivity was equal to that of A₅₀₀ (subscripts refer to mM NaCl in the buffers). After batch binding with 2 ml of glutathione-Sepharose beads equilibrated in A₅₀₀ buffer, the beads were washed with 100 ml of A₅₀₀, 20 ml of A₅₀₀ + 1 mM ATP and 5 mM MgAc₂, 20 ml of A₅₀₀, and 30 ml of A₃₀₀. The protein was eluted in A₃₀₀ buffer containing 20 mM glutathione, pH = 8.0. Peak fractions were combined and digested overnight at 4 °C with 15 units of PreScission protease (Amersham Biosciences). The protein was diluted 2-fold with buffer B₀ (30 mM Hepes, pH 7.4, 10% glycerol, 2 mM EDTA, 1 mM EGTA, 0.1% ampholytes 3.5–10) and loaded onto a 1-ml heparin-agarose column in B₁₅₀ + 0.5% Triton X-100. After washing the column with B₃₀₀ + 0.5% Triton X-100, the protein was eluted with B₇₅₀ buffer containing 0.5% Triton X-100.

Pol η was similarly overexpressed in yeast using plasmid pBL810. Extract preparation and purification were similar, except that 150 mM

NaCl was used during extraction, and buffer A₁₅₀ was used during glutathione elution from the glutathione-Sepharose column. After elution the protein was diluted with A₀ to an equivalence of A₈₀, loaded on a heparin agarose column, and eluted using a gradient from A₈₀ to A₅₀₀.

DNA Replication/Bypass Assays—Standard 20- μ l assays contained 40 mM Tris-HCl, pH 7.8, 0.2 mg/ml bovine serum albumin, 8 mM MgAc₂, 100 μ M each dNTPs, 0.05 mM ATP, 100 fmol of DNA substrate, 1 pmol of RPA, 300 fmol PCNA or Rad17/3/1, 200 fmol of RFC or Rad24-RFC, respectively, and 100 mM NaCl (final concentration) unless otherwise indicated. The DNA was preincubated with RPA, PCNA, or Rad17/3/1 and RFC or Rad24-RFC, respectively, for 30 s at 30 °C, and the reaction was started by adding the relevant DNA polymerase (200 fmol). Incubations were at 30 °C for 5 min unless otherwise indicated. Reactions were stopped by the addition of 2 μ l of 100 mM EDTA and 3% SDS. After incubating at 50 °C for 10 min, 45 μ l of precipitation solution (2.5 M ammonium acetate, 20 μ g/ml sonicated salmon sperm DNA, 1 mM EDTA, and 0.05 mg/ml linear acrylamide (Ambion Technologies)) was added, followed by ethanol precipitation. Yields of pure DNA recovered by this method varied from 70 to 100%. Products were analyzed by electrophoresis on a 12% polyacrylamide 7 M urea gel. Gels were dried, quantitated by phosphorimaging analysis, and contrast enhanced for visualization purposes.

RESULTS AND DISCUSSION

Purification of Pol ζ —Purification and activity studies of Pol ζ showed a capricious behavior by this enzyme. Preparations with a consistent high specific activity could only be obtained when very high salt was included in the extraction buffer as described in the original publication (4). However, proteolytic removal of the GST purification tag by thrombin proceeded only up to ~50% completion regardless of the amount of thrombin added, suggesting the presence of two distinct enzyme populations. We, and others (4), have noticed that the enzyme still containing the GST tag had reduced enzymatic activity (data not shown). Therefore, we changed the thrombin-cleavable linker between GST and Rev3 with a linker that is cleaved by the rhinovirus protease (see "Materials and Methods"). This modification allowed complete removal of the GST tag and, therefore, a more homogeneous enzyme preparation. Final preparations of Pol ζ still contained 10–20% unidentified impurities and varied by about 2-fold in specific activity. We were concerned about the possibility that our preparations could be contaminated by Pol η because both enzymes function in the same pathway and might conceivably interact and therefore co-purify. However, Pol ζ overproduced in a *RAD30*- Δ strain had an activity comparable with that isolated from the isogenic wild-type strain (data not shown). In addition, preparations of Pol ζ lacking Rev7, because it was not overexpressed, were inactive (data not shown). Therefore, while we do not yet have a clear understanding of the variation in specific activity of Pol ζ , we are certain that all observed polymerase activity and the stimulation of this activity by PCNA originates from Rev3/Rev7.

PCNA Stimulates Pol ζ -mediated TLS Past UV Damage—To study the role of PCNA in DNA damage translesion synthesis we used a linear oligonucleotide-based system. The template strand contains a stretch of 29 T residues for the facile generation of dimer-containing DNA by UV irradiation. As PCNA tends to slide off linear DNA substrates, we used an anchoring method with biotin-streptavidin blocks (Fig. 1A) (26). In our standard replication and translesion synthesis assays, the template-primer containing the streptavidin blocks was coated with the yeast single-stranded binding protein, RPA. Subsequently, PCNA was loaded by the clamp loader RFC, and DNA synthesis was initiated at $t = 0$ by the addition of the DNA polymerase. In this assay system, DNA replication by Pol δ was greatly stimulated by PCNA (Fig. 1B). The role of the biotin-streptavidin blocks and of RPA in maintaining PCNA on the DNA and promoting translesion synthesis will be discussed in more detail below.

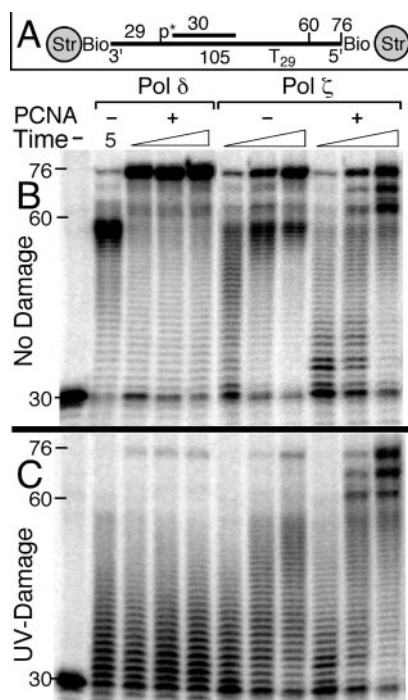


FIG. 1. PCNA stimulates UV damage translesion synthesis. *A*, schematic of the DNA substrate. The poly(dT) tract terminates at position 59, measured as primer extension product (see “Materials and Methods” for sequences). *Str*, streptavidin; *Bio*, biotin. *B*, replication of undamaged DNA under standard assay conditions containing 75 mM NaCl, for either 5 min or for 30 s, 90 s, and 5 min. *C*, template-primer was irradiated with 750 J/m² of UV₂₅₄ prior to assays as described for *B*. Pausing between positions 60 and 76, measured as primer extension product, reflects the presence of G-rich template stretches. Full-length replication products should be 76 nt, but 1–2 nt shorter products were mainly observed because the biotin-streptavidin block inhibited progression of the polymerase (see also Fig. 4).

When this replication system was used to study DNA replication by Pol ζ , we measured no significant stimulation by PCNA in the generation of full-length replication products (Fig. 1*B*). Next, the poly(dT) template stretch was damaged by irradiation with 750 J/m² of UV₂₅₄ light which produces ~5 UV dimers per template molecule (28). Remarkably, whereas poor but detectable TLS by Pol ζ alone was observed, a dramatic stimulation of bypass synthesis was detected in the presence of PCNA, suggesting that PCNA can function as a processivity factor for Pol ζ on damaged DNA (Fig. 1*C*). In fact, DNA synthesis by the PCNA-Pol ζ complex past UV damage was nearly as efficient as that of undamaged DNA. Control reactions showed that PCNA had to be properly loaded onto the DNA as stimulation was abolished in the absence of RFC (see Fig. 3). Under the same conditions, PCNA-mediated TLS by Pol δ was not observed. The very small percentage (~3%) of completely replicated products synthesized by the PCNA-Pol δ complex on this substrate is consistent with the estimated percentage of DNA that escapes T-T dimerization. In support of this conclusion, the percentage of fully replicated products did not increase from 30 s to 5 min (Fig. 1*C*).

The PCNA stimulation data for Pol ζ suggest that PCNA forms a complex with Pol ζ ; however, its stimulatory effect was only observed on the UV-damaged template where barriers to elongation were present. We reasoned that if progression by Pol ζ on undamaged DNA were also impeded by unfavorable reaction conditions, this stimulation by PCNA should be observable. Indeed, when DNA binding by Pol ζ was reduced by increasing the salt concentration in the assay, the stabilizing influence of PCNA on processive DNA synthesis on undamaged

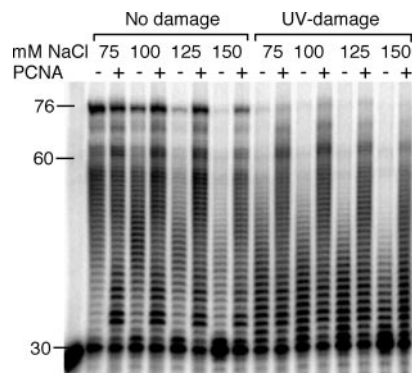


FIG. 2. PCNA confers salt resistance on Pol ζ -mediated DNA synthesis. Replication assays as in Fig. 1 were carried out for 5 min with the indicated final concentrations of NaCl.

DNA also became evident (Fig. 2).

Only PCNA and Not the Checkpoint Clamp Stimulates Pol ζ —Given the known role of the DNA damage checkpoint system in mutagenesis, we asked whether the Rad17/3/1 checkpoint clamp would stimulate DNA synthesis by Pol δ or Pol ζ on undamaged or damaged DNA (22). The Rad17/3/1 is efficiently loaded onto primed DNA by its loader Rad24-RFC; however, sliding of the clamp away from the loading site is slow, particularly when the single-stranded DNA is coated with RPA (24). In addition, loading can occur at either the 3'- or the 5'-primer/template junction, and there is disagreement in the literature regarding the relative efficiency of these loading sites (reviewed in Ref. 13). The substrate used in this study should permit clamp loading at either loading site.

In the experiment in Fig. 3, the Rad17/3/1 clamp was loaded by the Rad24-RFC clamp loader and its effect on Pol δ - or Pol ζ -mediated DNA synthesis assessed. However, rather than stimulation, a minor inhibition was observed that was dependent on the presence of both Rad17/3/1 and Rad24-RFC (compare lanes 7 with lanes 6 and 12 for Pol δ and lanes 9 with lanes 8 and 15 for Pol ζ). As a similar pattern and degree of inhibition was also observed for Klenow DNA polymerase and for Sequenase (data not shown), we reason that the occupancy of the template-primer junction by the checkpoint clamp limits accessibility to all these DNA polymerases. Increased salt, up to 125 mM NaCl did not reveal a stimulatory function for the Rad17/3/1 checkpoint clamp (data not shown).

Two recent studies with the human 9-1-1 checkpoint clamp, the ortholog of yeast Rad17/3/1, have indicated that 9-1-1 stimulates the activity of the FEN1 nuclease and of Pol β but not that of Pol δ (29, 30). However, whether the 9-1-1 clamp needed to be loaded onto DNA by its loader was not investigated. It is also possible that the observed stimulation by 9-1-1 is mediated by direct protein-protein interactions and not through the function of 9-1-1 as a sliding clamp. Therefore, it is problematic to make a direct comparison between our results and those obtained in the human system.

Bypass Synthesis of a Model Abasic Site—To determine which synthetic steps in TLS are accelerated by PCNA, we studied bypass of a unique model abasic site (Fig. 4*A*). The tetrahydrofuran moiety in the template DNA that we used qualitatively resembles an abasic site *in vivo* (31). Previously, we have shown that full bypass synthesis of this model abasic site required the sequential action of Pol δ and Pol ζ ; however, these studies were carried out in the absence of PCNA (12). In other studies, it was shown that PCNA stimulated insertion by Pol η opposite the abasic site, although extension past the abasic site was not observed (15). Because in preliminary studies we observed efficient extension past the abasic site by PCNA-Pol η , we reexamined the experimental conditions that

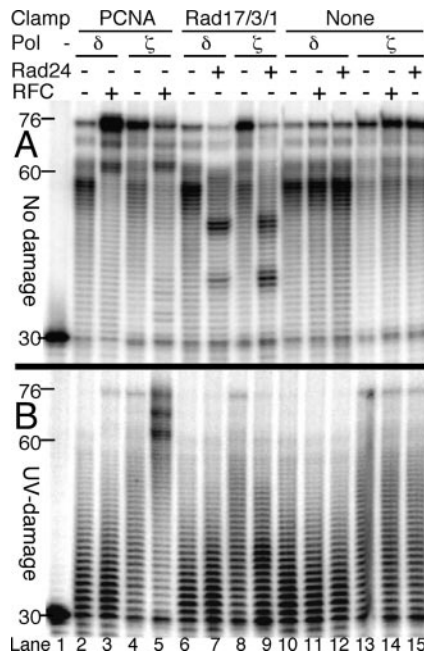


FIG. 3. Pol ζ is stimulated by PCNA but not by the checkpoint clamp Rad17/3/1. Assays were for 2 min under standard reaction conditions (see “Materials and Methods”). *A*, undamaged DNA. *B*, UV-irradiated DNA (750 J/m²). Rad24 designates Rad24-RFC.

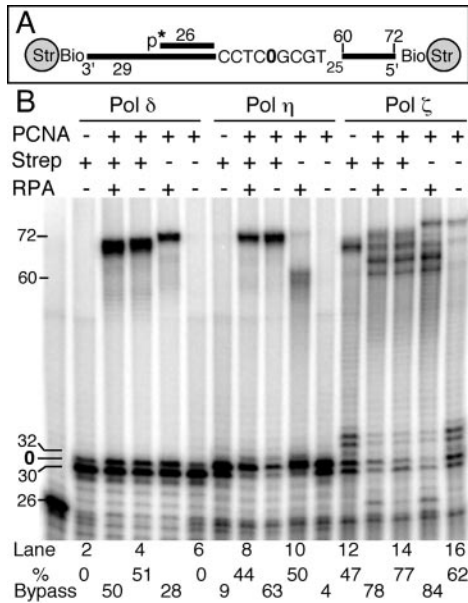


FIG. 4. PCNA-mediated TLS requires its confinement on the DNA. *A*, schematic of the DNA substrate. The poly(dT) tract terminates at position 59, measured as primer extension product. The tetrahydrofuran site (**boldface 0**) is position 31, and complete replication produces a 72-nt product (see “Materials and Methods” for sequences). *B*, assays were for 5 min under standard reaction conditions (see “Materials and Methods”). The sum of the products 32 nt in length and longer were taken to calculate percent of bypass.

permit PCNA-dependent bypass by Pol δ , Pol η , and Pol ζ .

In clamp-clamp loader studies of the bacteriophage T4 replication system on linear template-primer substrates, it was observed that biotin-streptavidin moieties serve as effective blocks at the ends of the primer-template, thus preventing sliding of the clamp away from the loading site and off the ends of the DNA (32). Subsequent replication studies with yeast PCNA confirmed the necessity of these biotin-streptavidin bumpers, particularly when the PCNA-polymerase complex was stalled, *e.g.* at DNA nicks (26). We reasoned that such

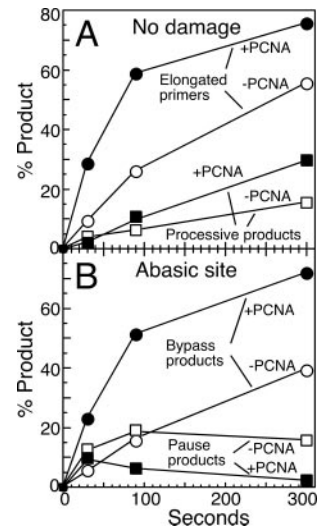


FIG. 5. PCNA increases primer utilization, processivity, and lesion bypass by Pol ζ . Substrates were as described in the legend to Fig. 4*A*. Aliquots were taken from standard replication assays at 30, 90, and 300 s. Quantitation was by phosphoimaging analysis. *A*, replication of undamaged DNA. The template contained a G instead of the tetrahydrofuran residue. The percent of elongated primers is 100% minus the percent of 26-mer primer remaining; the percent of processive products identifies those products longer than 59 nt. *B*, replication of abasic site-containing DNA. The percent of pause products is the combined percent of products 30 and 31 nt in length; the percent of bypass is the combined percent of products 32 nt in length and longer.

terminal bumpers might also be important when PCNA-polymerase complexes stall at sites of damage. On naked DNA, neither Pol δ nor Pol η bypassed the abasic site (Fig. 4*B*, lanes 2 and 7). Coating of the single-stranded template by RPA did not significantly alter the distribution of replication pause sites (data not shown). This result was expected because of the lack of significant secondary structure in the mostly poly(dT) template.

In the presence of PCNA and RFC, but without streptavidin or RPA, we again detected no bypass of the abasic site by Pol δ nor by Pol η (lanes 6 and 11, respectively). However, either the addition of streptavidin or the addition of RPA resulted in PCNA-dependent bypass synthesis. RPA, which avidly binds the two poly(dT) tracts upstream and downstream of the primer, locks PCNA onto the DNA thereby allowing TLS to take place (lanes 5 and 10) (33, 34). As replication proceeds, the downstream RPA molecule is displaced and replication up to the end of the template takes place, particularly with Pol δ (lane 5). The presence of just the streptavidin moieties, without RPA, promoted efficient PCNA-dependent translesion synthesis (lanes 4 and 9). However, streptavidin sterically blocked Pol δ from replicating up to the end of the template (compare lane 5 with lane 4). No further significant improvement in bypass synthesis by Pol δ or Pol η was observed when, in addition to the streptavidin blocks, the DNA was also coated with RPA (lanes 3 and 8).

In contrast to Pol δ and Pol η , Pol ζ catalyzed bypass past the abasic site even in the absence of PCNA (lane 12). However, PCNA strongly stimulated bypass provided either RPA or streptavidin or both were present to retain PCNA on the DNA. As with Pol δ , the biotin-streptavidin block prevented Pol ζ from replicating the final few template residues (compare lanes 13 and 14 with lanes 15 and 16). An analysis of the rate and processivity of DNA synthesis by Pol ζ on undamaged DNA showed that PCNA increased both primer recognition by Pol ζ , measured as percentage of primers elongated, and the processivity of the enzyme (Fig. 5*A*). When the template contained the abasic site, PCNA decreased pausing by Pol ζ at the -1 position

and at the abasic site and promoted bypass synthesis (Fig. 5B).

Does Pol ζ Have a Novel PCNA-binding Domain?—Pol ζ lacks an identifiable consensus PCNA-binding domain (Qxx-LxxFF) in either the Rev3 or Rev7 subunit. All Y-class DNA polymerases use this domain to interact with PCNA during TLS (reviewed in Refs. 1 and 13). However, Pol δ has multiple domains for interaction with PCNA. One of these is a consensus domain in the third subunit which has an auxiliary function during DNA replication (8, 35). A precise knowledge of the PCNA interaction domains in the catalytic and/or second subunit is still lacking although certain regions in these subunits have been implicated by peptide mapping studies (36, 37). A sequence comparison analysis of six evolutionarily diverse Rev3 orthologs revealed several conserved motifs outside of the core polymerase domains; however, none of these motifs were present in a similar alignment of Pol δ catalytic subunit sequences. Therefore, it appears that not even one of these putative interaction domains determined by peptide binding studies is present in Pol ζ .

Conclusions—This study not only underscores the importance of studying TLS under proper conditions with the necessary accessory factors present but also highlights the importance of the experimental approach that needs to be used to study PCNA-dependent TLS on model linear oligonucleotide substrates. In particular, appropriate measures need to be taken to stabilize the PCNA-polymerase complex on the linear DNA and to prevent it from sliding off. In our study, RPA served this function with some efficiency, mainly because of the presence of strong RPA-binding sites both upstream and downstream of the primer terminus. However, biotin-streptavidin blocks on both sides provide a superior stability (Fig. 4). Under those conditions, we were able to measure complete bypass synthesis of an abasic site by Pol δ or by Pol η , although only the latter enzyme was able to bypass UV-damage. In contrast, Pol ζ alone could bypass both types of damage, but both the rate and efficiency of bypass were strongly enhanced by PCNA. The inference that the PCNA-Pol ζ interaction involves a novel motif raises the possibility that even more proteins involved in DNA metabolism than currently known may physically and functionally interact with PCNA.

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