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PCNA Is Required for Initiation of Recombination-Associated DNA Synthesis by DNA Polymerase δ

Xuan Li,[1,4] Carrie M. Stith,[3] Peter M. Burgers,[3] and Wolf-Dietrich Heyer[1,2,*]
[1]Department of Microbiology
[2]Department of Molecular and Cellular Biology
University of California, Davis, Davis, CA 95616-8665, USA
[3]Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, MO 63110, USA
[4]Present address: Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA
*Correspondence: wdheyer@ucdavis.edu
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SUMMARY

Genetic recombination ensures proper chromosome segregation during meiosis and is essential for genome stability and tumor suppression. DNA synthesis after Rad51-mediated DNA strand invasion is a crucial step during recombination. PCNA is known as the processivity clamp for DNA polymerases. Here, we report the surprising observation that PCNA is specifically required to initiate recombination-associated DNA synthesis in the extension of the 3′ end of the invading strand in a D loop. We show using a reconstituted system of yeast Rad51, Rad54, RPA, PCNA, RFC, and DNA polymerase δ that loading of PCNA by RFC targets DNA polymerase δ to the D loop formed by Rad51 protein, allowing efficient utilization of the invading 3′ end and processive DNA synthesis. We conclude that PCNA has a specific role in the initiation of recombination-associated DNA synthesis and that DNA polymerase δ promotes recombination-associated DNA synthesis.

INTRODUCTION

Homologous recombination is a key pathway to preserve genomic stability by repairing DNA double-stranded breaks, gaps, and interstrand cross-links, as well as by mediating recovery of stalled or broken replication forks (Li and Heyer, 2008). Recombination also facilitates the pairing and segregation of homologous chromosomes during meiosis (Hunter, 2007). The signature reactions of recombination, homology search, and DNA strand invasion are performed by the Rad51 protein, the eukaryotic homolog of the paradigmatic RecA protein (Krogh and Symington, 2004). Rad51 forms a right-handed helical filament on single-stranded DNA (ssDNA) in a gap or as a result of the processing of a double-stranded DNA (dsDNA) break. RPA, the eukaryotic ssDNA-binding protein, is involved in many DNA transactions and plays at least two distinct roles during recombination. First, RPA antagonizes secondary structure in the invading ssDNA to favor formation of the active Rad51-ssDNA filament (Sugiyama et al., 1997). Second, RPA binds to the displaced strand in the D loop to stabilize the invasion intermediate (Eggler et al., 2002). Binding of the displaced strand by RPA is likely occurring also during the DNA synthesis phase. Rad54 is a Snf2-family dsDNA motor protein with multiple functions during recombination in vitro. It stabilizes the Rad51 presynaptic filament and stimulates D loop formation (Mazin et al., 2003; Mazin et al., 2000; Van Komen et al., 2000). Owing to its specific interaction with the Rad51-dsDNA filament, Rad54 promotes the dissociation of Rad51 from the heteroduplex DNA product after DNA strand invasion (Kiianitsa et al., 2006; Li et al., 2007; Solinger et al., 2002). This activity is required to allow access of DNA polymerases to the invading 3′ end, because Rad51, unlike bacterial RecA protein, does not readily release dsDNA after ATP hydrolysis (Li and Heyer, 2009; Li et al., 2007; Zaitseva et al., 1999).

After DNA strand invasion, DNA synthesis is pivotal to restoring the integrity of the chromosome. The identity of the DNA polymerases involved in homologous recombination remains to be defined. A biochemical screen in human cells for activities that could catalyze DNA synthesis primed at a synthetic D loop identified DNA polymerase η (Pol η) and showed that DNA polymerase δ (Pol δ) was unable to do so (McIlwraith et al., 2005). However, genetic studies in budding yeast suggest that Pol δ is involved in homologous recombination (Fabre et al., 1991; Giot et al., 1997; Lydeard et al., 2007; Maloisel et al., 2004; Maloisel et al., 2008; Paques and Haber, 1997; Wang et al., 2004). Single mutants displayed a rather modest reduction in recombination, possibly owing to the use of conditional mutants necessitated by the essential function of Pol δ in DNA replication. In addition, the observed partial defects may indicate functional overlap with other DNA polymerases, such as Pol ε. Although Pol η is required for DSB-induced gene conversion in chicken DT40 cells, it does not appear to be involved in recombination in yeast (Kawamoto et al., 2005; McDonald et al., 1997).

PCNA is involved with a host of DNA polymerases during DNA replication and other DNA metabolic processes (Majka and Burgers, 2004; Moldovan et al., 2007). Like its bacterial homolog the β-clamp, PCNA endows DNA polymerases with high processivity. PCNA was shown to be required for mating-type switching in budding yeast, a model double-strand break repair event by homologous recombination (Wang et al., 2004). Modification of PCNA modulates its protein interactions, regulating access to the primer-template (Moldovan et al., 2007). PCNA

monoubiquitylation on K164 favors interaction with translesion DNA polymerases over replicative polymerases, whereas polyubiquitylation on the same residue may disengage polymerase interaction, favoring template switching by fork regression at stalled replication forks (Hoege et al., 2002; Stelter and Ulrich, 2003; Ulrich and Jentsch, 2000). In contrast, PCNA SUMOylation on K164 (and K127) favors an interaction with Srs2, a helicase that dissociates Rad51 from ssDNA exerting an antirecombinogenic effect (Papouli et al., 2005; Pfander et al., 2005). These results suggest that unmodified PCNA is the active form for recombination-associated DNA synthesis.

Using an in vitro system reconstituted with yeast proteins coupling Rad51-mediated DNA strand invasion in the presence of RPA and Rad54 with DNA synthesis, we show that DNA polymerase δ is efficiently targeted to the D loop by PCNA, resulting in efficient usage of the invading 3′ end and processive DNA synthesis. This function of PCNA to specifically recruit DNA polymerases to a D loop significantly extends the earlier biochemical work with human Pol δ in recombination (McIlwraith et al., 2005), resolving the apparent discrepancy between biochemical and genetic evidence suggesting a role for Pol δ in recombination.

RESULTS

DNA Polymerase δ Requires PCNA for Recombination-Associated DNA Synthesis

To study the DNA synthesis step during homologous recombination, we reconstituted recombination in vitro using the D loop reaction, in which a 95-mer ssDNA invades a homologous supercoiled duplex DNA by the combined action of yeast Rad51, Rad54, and RPA (Figure 1A). Rad54 is a dsDNA motor protein that enhances D loop formation and dissociates Rad51 from the product heteroduplex DNA after DNA strand exchange. We have previously shown that this activity allows access to the invading 3′ end by DNA polymerase (Li and Heyer, 2009).

We compared recombination-associated DNA synthesis by yeast Pol δ with that by yeast Pol η by measuring the incorporation of α-^{32}P-dCTP (Figure 1B). Consistent with previous results using D loops that were preassembled from oligonucleotides (McIlwraith et al., 2005), Pol η but not Pol δ could extend the 3′ end in a D loop, albeit very inefficiently (Figure 1B, lanes 10–17). Both polymerases were equally efficient in extending standard primed templates under these reaction conditions (see Figure S1 available online). Both Pol δ and Pol η require PCNA for processive DNA synthesis (Haracska et al., 2001; Prelich et al., 1987). Addition of the processivity clamp PCNA and its clamp loader RFC resulted in strong stimulation of DNA synthesis by Pol η (Figure 1B, lanes 2–5). Strikingly, recombination-associated DNA synthesis by Pol δ was completely dependent on PCNA. These results were confirmed for Pol δ in experiments with an end-labeled 95-mer, which allows for quantitation of both the D loop (no DNA synthesis) and the D loop with an extended 3′ end (extended D loop) (Figures 1C–1F). Extension of the invading 95-mer by Pol δ depended on the combined presence of both PCNA and RFC, consistent with the model that PCNA requires appropriate loading by its clamp loader RFC (Figures 1C and 1D; Figure S2). The presence of PCNA not only allowed DNA synthesis by Pol δ, but also led to increased total product formation (D loops plus extended D loops) (Figure S3). This finding suggests that PCNA not only acts as a processivity factor, its known function in DNA replication (Majka and Burgers, 2004), but also recruits Pol δ to the 3′ end of the D loop.

RPA is the eukaryotic, heterotrimeric ssDNA-binding protein that enhances D loop formation by binding to the displaced strand (Eggler et al., 2002). As expected, RPA stimulated D loop formation (Figure S4**)**, leading to a corresponding increase in extension products (Figure 1D).

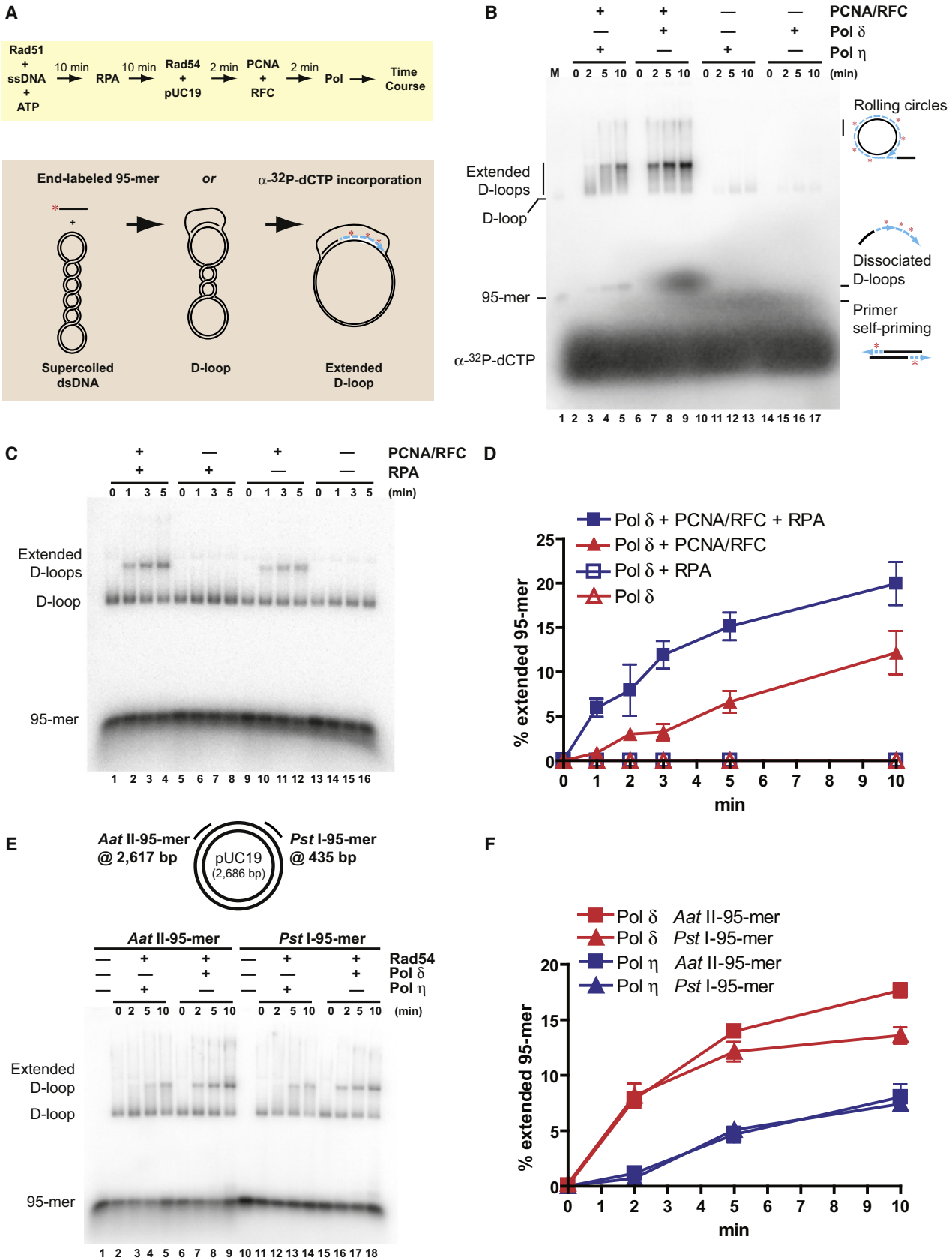
PCNA-stimulated DNA synthesis by Pol δ appeared more robust than that by Pol η (Figure 1B, lanes 2–5 vs. lanes 6–9). This inference was confirmed in experiments using 5′ end-labeled 95-mers (Figures 1E and 1F; Figure S5). We tested two different homology regions in the duplex DNA target, designated *Aat*II-95-mer and *Pst*I-95-mer, with virtually identical results, indicating that the apparent preference for extension by Pol δ is not dependent on the sequence context.

Control experiments omitting Rad51 demonstrated that the observed DNA synthesis depended on recombination (Figure 2B, lanes 10–13). Also the Rad54 protein (lanes 6–9) was essential for recombination-associated DNA synthesis. Substituting with the Rad54-K341R mutant protein, which eliminates Rad54 ATPase and motor activities (Amitani et al., 2006), abrogated recombination-associated DNA synthesis consistent with previous studies (Li and Heyer, 2009). In the reactions with Pol δ, we observed low, but reproducible, incorporation of α-^{32}P-dCTP in the absence of Rad51 or Rad54 (Figure 2B, lanes 9, 13, and 17) or with Rad54-K341R protein (Figure 2C, lane 9). This signal is due to Pol δ priming after spontaneous annealing of the 95-mer to the homologous sequence in the negatively supercoiled DNA. Accordingly, in reactions using a heterologous 95-mer, this low-level incorporation was essentially lost (Figure 2C, lane 13).

D Loop Extension Is Limited by Topological Constraint

To determine the length of DNA synthesis in individual reaction products, the extended D loops were analyzed by native/denaturing 2-dimensional gel electrophoresis. In both assays (α-^{32}P-dCTP incorporation, 5′ end-labeled 95-mer), the products of both polymerases center around a length of 500 nt with evidence of some full-length (2.7 kb) products that have replicated around the entire plasmid (Figure 3; Figure S6). In accordance with the data from the incorporation assay (Figure 1B), the absence of PCNA prevented detectable extension of the 5′ end-labeled 95-mer by Pol δ (Figure 3B, left panel). In titrating the ssDNA-binding protein RPA, we showed that the efficiency of extension was stimulated by the presence of RPA but not limited by the RPA concentration used (Figure S7).

In the D loop assay, extension of the 3′ end can lead to topological constraints, because with each 10.5 nt synthesized, one positive supercoil is induced in the closed circular template DNA. Experiments with the bacterial RecA protein showed that D loop extension by *Escherichia coli* Pol III holoenzyme to full-length products required gyrase to relieve this topological stress (Xu and Marians, 2002). In contrast, studies with the phage T4 recombination system have shown that DNA synthesis was unobstructed by topological problems because the D loop migrated along with DNA synthesis. Such D loop migration (bubble migration) relieves the topological stress by dissociating


A
Rad51 + ssDNA + ATP
10 min
RPA
10 min
Rad54 + pUC19
2 min
PCNA + RFC
2 min
Pol
Time Course
End-labeled 95-mer
or
α-32P-dCTP incorporation
Supercoiled dsDNA
D-loop
Extended D-loop
B
PCNA/RFC
Pol δ
Pol η
M 0 2 5 10 0 2 5 10 0 2 5 10 0 2 5 10 (min)
Extended D-loops
D-loop
95-mer
α-32P-dCTP
Rolling circles
Dissociated D-loops
Primer self-priming
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
C
PCNA/RFC
RPA
0 1 3 5 0 1 3 5 0 1 3 5 0 1 3 5 (min)
Extended D-loops
D-loop
95-mer
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
D
Pol δ + PCNA/RFC + RPA
Pol δ + PCNA/RFC
Pol δ + RPA
Pol δ
% extended 95-mer
min
E
Aat II-95-mer @ 2,617 bp
pUC19 (2,686 bp)
Pst I-95-mer @ 435 bp
Aat II-95-mer
Pst I-95-mer
Rad54
Pol δ
Pol η
0 2 5 10 0 2 5 10 0 2 5 10 0 2 5 10 (min)
Extended D-loop
D-loop
95-mer
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
F
Pol δ Aat II-95-mer
Pol δ Pst I-95-mer
Pol η Aat II-95-mer
Pol η Pst I-95-mer
% extended 95-mer
min

the D loop at one end as it is extended at the other end, thereby keeping it at a constant size (Formosa and Alberts, 1986). To distinguish between these possibilities, we supplemented the reconstituted recombination reactions with eukaryotic topoisomerase I (Topo I), which relieves both positive and negative supercoils. Topo I was added 2.5 min after DNA synthesis was initiated, when D loops have formed and synthesis is ongoing. The addition of Topo I stimulated the extent of DNA synthesis by Pol δ. In the absence of Topo I, ~50% of the extension products (11.8% of total label; Figure 3D left) had an average length of 500 nt. After Topo I addition, this entire population shifted to a size of about 1500 nt; Figure 3D, right). As expected, the addition of Topo I led to the disappearance of D loops (Figure 3C; Figure S8), because D loop stability depends on the negative superhelicity of the duplex DNA (Van Komen et al., 2000). The addition of saturating amounts of RPA (1 μM, enough to cover a full-length displaced strand) led to the accumulation of a new, slower migrating species (Figure 3C arrow). This band represents full-length extension of the 95-mer (Figure 3D arrow) and accumulated to 2% of the input DNA, corresponding to more than 10% of the extended D loops. These results suggest that DNA synthesis initiated at the D loop is limited by topological constraints.

Loading of PCNA at the D Loop Targets DNA Polymerase δ

In DNA replication, RFC loads PCNA at the primer-template to act as a processivity clamp for the DNA polymerase. Control experiments showed that RFC was required for recombination-associated DNA synthesis by Pol δ and PCNA (Figure S2). To corroborate the sequence of events (D loop formation → PCNA loading → polymerase recruitment), we performed order-of-addition experiments, where we changed the relative order of the addition of PCNA, RFC, and Pol δ. As schematically diagrammed in Figure 4A, dsDNA, Rad54, PCNA, RFC, and Pol δ were either added at the same time (order #1), in sequence (dsDNA + Rad54 → PCNA/RFC → Pol δ = order #2), or in a scrambled sequence (dsDNA + Rad54 → Pol δ → PCNA/RFC after 5 min [order #3] or 10 min [order #4]). The results show that recombination-associated DNA synthesis has faster kinetics and higher yield when PCNA/RFC is supplied after D loop formation and Pol δ is added last (Figure 4B, lanes 5–7; order #2). This order is consistent with a model that loading of PCNA by RFC at the D loop recruits Pol δ to the 3′ end of the invading strand for processive DNA synthesis.

DISCUSSION

DNA synthesis plays a key role in the restoration of the genetic information during recombinational DNA repair. The identities of the DNA polymerases involved in homologous recombination remained rather uncertain, in particular the involvement of DNA polymerase δ, because of conflicting genetic and biochemical results. Genetic experiments in *Saccharomyces cerevisiae* suggested a role of Pol δ and demonstrated a requirement for PCNA (and by implication RFC) (Fabre et al., 1991; Giot et al., 1997; Lydeard et al., 2007; Maloisel et al., 2004; Maloisel et al., 2008; Paques and Haber, 1997; Wang et al., 2004). Break-induced replication, a recombination-mediated pathway, depends on the Pol δ holoenzyme (the complex that includes the nonessential Pol32 subunit) (Jain et al., 2009; Lydeard et al., 2007). Moreover, a small C-terminal truncation of the Pol δ catalytic subunit affects conversion tract length in mitotic and meiotic recombination (Maloisel et al., 2004; Maloisel et al., 2008). Finally, the proofreading exonuclease activity of Pol δ was shown to process terminal heterology of the invading 3′ end, suggesting that Pol δ has access to the nascent (unextended) D loop (Figure 4C) (Paques and Haber, 1997). These genetic data are consistent with a central role for Pol δ in homologous recombination, and our data now provide biochemical evidence for this role. The previous biochemical experiments with human Pol δ omitted PCNA/RFC (McIlwraith et al., 2005) and found that Pol δ alone could not access the 3′ end of the invading strand, consistent with our data.

Using an in vitro reconstituted recombination reaction, we coupled DNA strand invasion and D loop formation by Rad51, Rad54, and RPA with the ensuing DNA synthesis catalyzed by Pol δ and its accessory factors PCNA and RFC. This system allowed us to identify a role for PCNA in the initiation of DNA synthesis at a recombination-mediated D loop. This surprising finding consolidates the genetic evidence in yeast that

Figure 1. PCNA/RFC Stimulate Pol η in Recombination-Associated DNA Synthesis and Are Necessary for Pol δ-Dependent DNA Synthesis during D Loop Extension

(A) Schematic representation of the reconstituted recombination reaction (top) and the assays (bottom) using either a 5′ end-labeled invading 95-mer or α-^{32}P-dCTP incorporation to identify DNA synthesis products.

(B) α-^{32}P-dCTP incorporation assay. Reactions were either complete (Pol η, lanes 2–5; Pol δ, lanes 6–9) or lacked PCNA/RFC (Pol η, lanes 10–13; Pol δ, lanes 14–17) using the *Aat*II-95-mer. Lane 1 contains a D loop reaction with 5′ end-labeled *Aat*II-95-mer as a marker (M), and the positions of the 95-mer and D loop are indicated. The signal above the extended D loops in lanes 3–5 and 7–9 represents products of rolling circle replication with greater than full-length synthesis products (see Figure 3D) that also depend on DNA strand invasion (Figure 2B). The signal running just above the 95-mer marker in lanes 3–5 and 7–9 represents dissociated D loops, which form during sample processing and electrophoresis (see Figure S6). The signal comigrating with the 95-mer in lanes 10–17 is due to self-priming of the 95-mer, and was particularly evident with Pol η (see also Figures 2B and 2C).

(C) D loop extension by Pol δ with 5′ end-labeled *Aat*II-95-mer. Reactions were either complete (lanes 1–4), lacking PCNA/RFC (lanes 5–8), lacking RPA (lanes 9–12), or lacking PCNA/RFC and RPA (lanes 13–16).

(D) Quantitation of the results in (C) and additional experiments. Shown are means from at least three independent experiments and one standard deviation, except for the reactions containing Pol δ only, which were repeated twice.

(E) Efficiency of D loop extension is independent of sequence context. The relative position of the homologies on the duplex DNA target for the *Aat*II- and PstI-95-mers is schematically indicated. Reactions were complete with either Pol η (lanes 2–5 and 11–14) or Pol δ (lanes 6–9 and 15–18) with 5′ end-labeled invading ssDNA, either the *Aat*II-95-mer (lanes 1–9) or the PstI-95-mer (lanes 10–18). Lanes 1 and 10 were controls with complete reactions lacking only Rad54 and polymerase.

(F) Quantitation of the results in (E) and additional experiments. Shown are means from at least three independent experiments and one standard deviation.

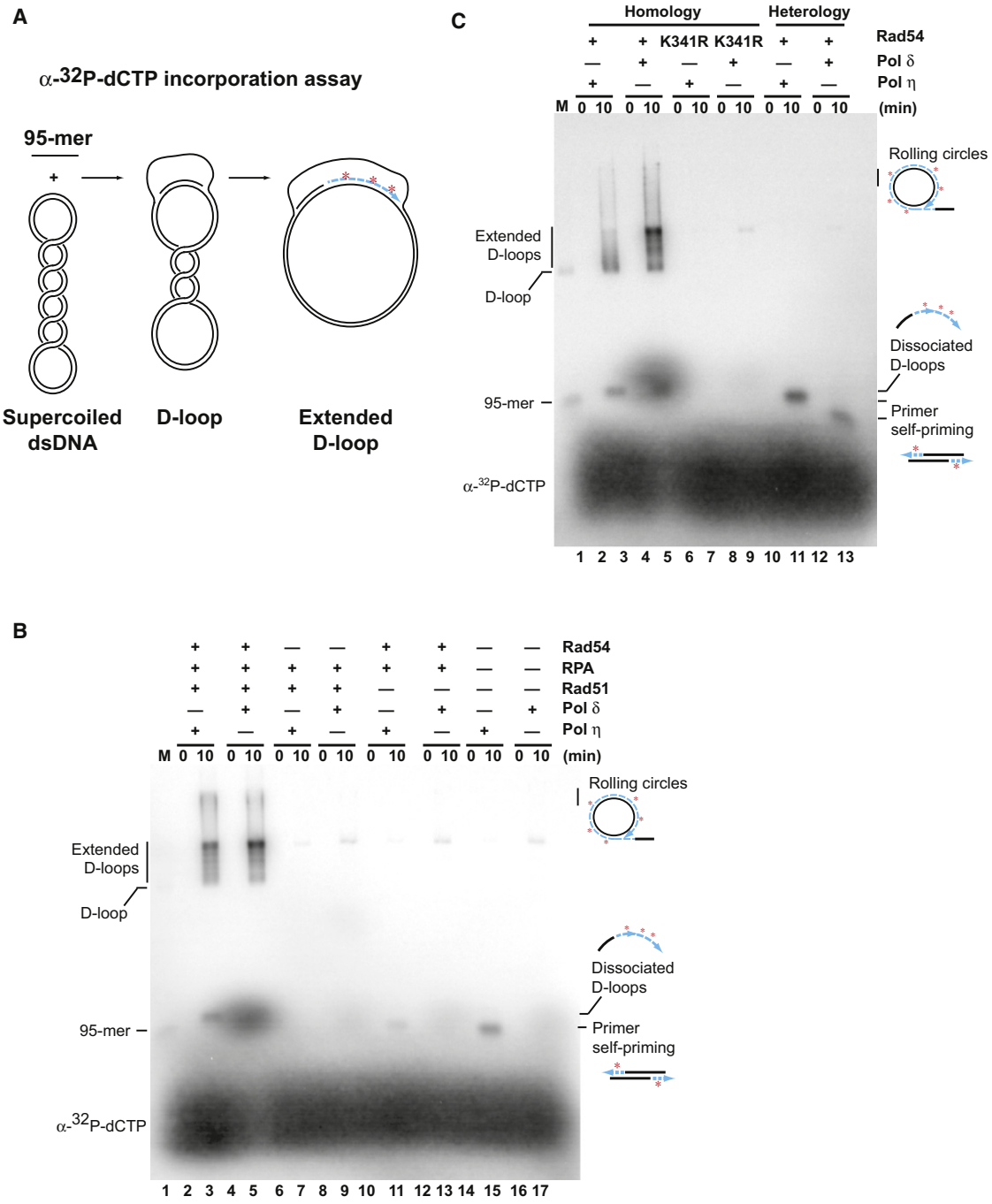


Figure 2. Recombination-Associated DNA Synthesis Depends on Rad51, Rad54, and Homology

(A) Scheme of the α-^{32}P-dCTP incorporation assay.

(B) D loop extension requires Rad51 and Rad54. α-^{32}P-dCTP incorporation assays were complete with either Pol η or Pol δ (lanes 2–5), omitted Rad54 (lanes 6–9), Rad51 (lanes 10–13), or RPA, Rad51, and Rad54 (lanes 14–17). Lane 1 contains a D loop reaction with 5′ end-labeled *Aat*II-95-mer as a marker (M), and the positions of the 95-mer and D loop are indicated.

(C) D loop extension requires the Rad54 ATPase activity and homology. Reactions were complete with either Pol η or Pol δ and wild-type Rad54 protein (lanes 2–5) or the ATPase-deficient Rad54-K341R protein (lanes 6–9). In lanes 10–13, reactions using a heterologous 95-mer (olWDH640) were analyzed. Lane 1 contains size markers as described in (B). The signals representing rolling-circles, dissociated D loops and primer self-priming were described in the legend to Figure 1B.

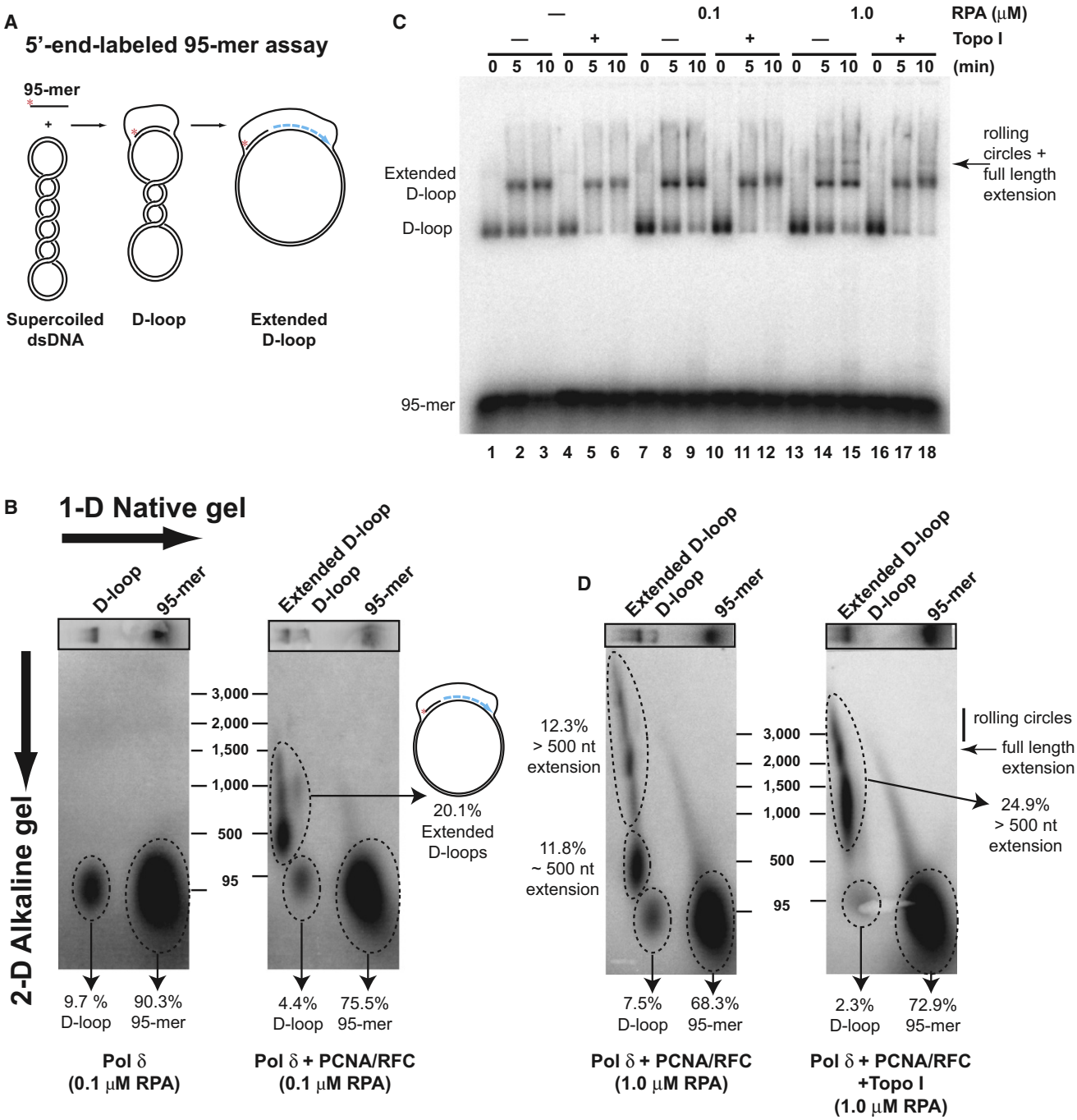


Figure 3. Topological Constraint Limits DNA Synthesis

(A) Scheme of the assay with the 5′ end-labeled 95-mer.

(B) Extension of the invading 3′ end in a D loop by Pol δ requires PCNA. Two-dimensional product analysis assays with 5′ end-labeled invading ssDNA (*Aat*II-95-mer). D loop extension products in complete reactions (10 min time point) with Pol δ in the presence (right) or absence (left) of PCNA/RFC were separated in the first dimension by native electrophoresis and in the second dimension under denaturing, alkaline conditions. Quantitation of the substrate and product species is provided in percentage of total.

(C) Product length is limited by topological constraint. Complete reactions with Pol δ were performed with a titration of RPA (0–1 μM) using end-labeled *Aat*II-95-mer. Eukaryotic topoisomerase I (lanes 4–6, 10–12, and 16–18) or buffer control (lanes 1–3, 7–9, and 13–15) was added at 2.5 min after addition of Pol δ, and products were analyzed on native gels.

(D) Two-dimensional product analysis. Products of the 10 min time points of reactions containing 1 μM RPA (see lanes 15 and 18 in C) supplemented (right) or not (left) with topoisomerase I were analyzed by 2-dimensional gel electrophoresis as in (B). Quantitation of the different substrate and product species is provided as a percentage of the total. The positions of full-length extension products (2.7 kb) and rolling circle products (>2.7 kb) are indicated.

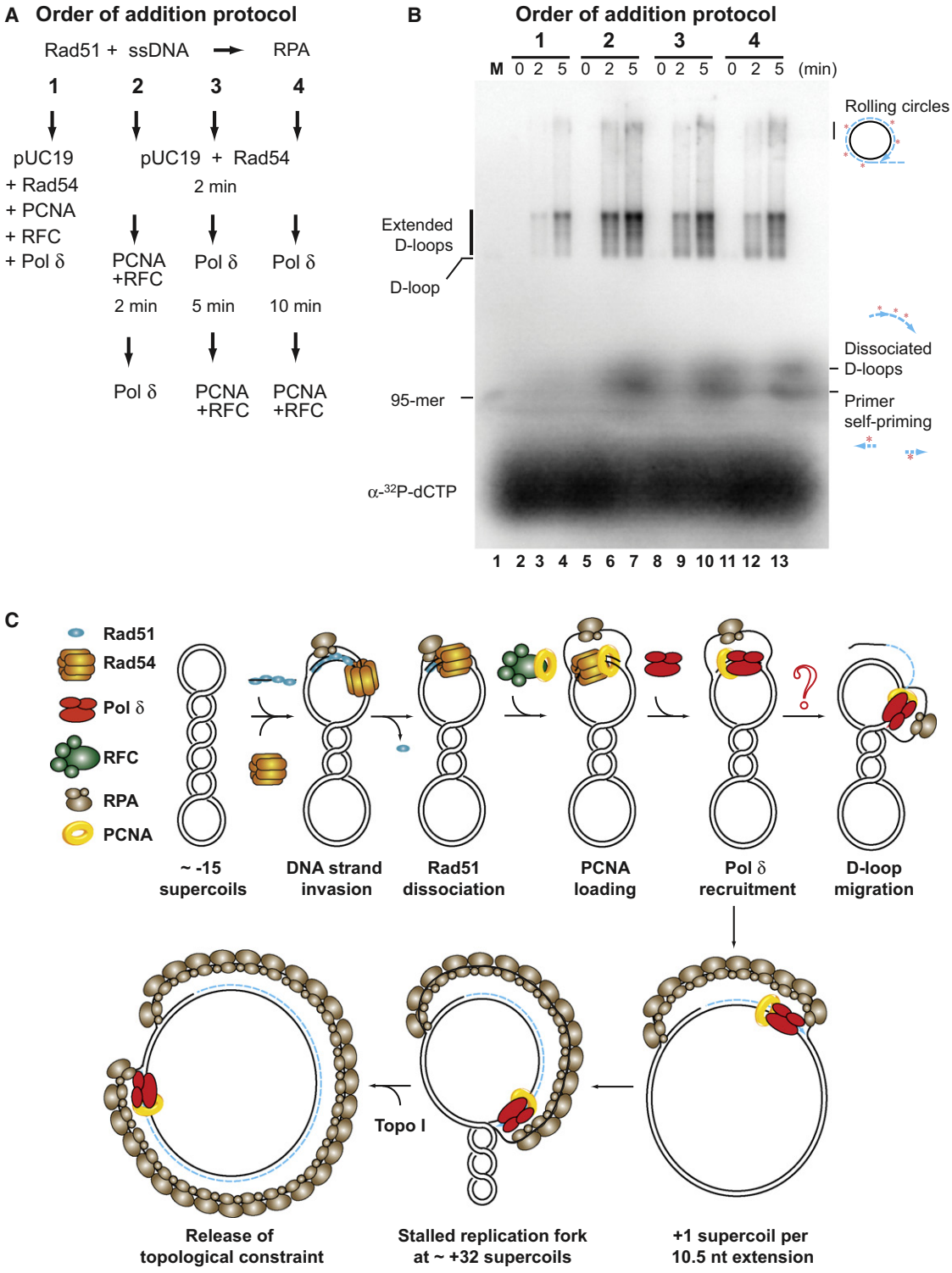


Figure 4. Order-of-Addition Experiments

(A) Scheme of the order-of-addition experiments.

(B) Complete α-^{32}P-dCTP incorporation assays with the *Aat*II-95-mer containing Pol δ were staged by the different protocols schematically indicated on the left hand side. Lane 1 contains a D loop reaction with end-labeled *Aat*II-95-mer as size markers (M), and the positions of the 95-mer and D loop are indicated. The signals representing rolling-circles, dissociated D loops, and primer self-priming were described in the legend to Figure 1B.

suggested a role for Pol δ in homologous recombination with biochemical data. Taken together, our studies suggest the following model (Figure 4C). First, the Rad51-ssDNA filament performs homology search and DNA strand invasion. Next, D loops are stabilized by RPA binding to the displaced strand. Rad54 dissociates Rad51 from the duplex DNA after DNA strand exchange to allow access to the 3′ end of the invading strand (Li and Heyer, 2009). This is in agreement with the need for *E. coli* RecA protein to hydrolyze ATP and dissociate from the heteroduplex DNA before Pol III holoenzyme can access the invading 3′ end (Xu and Marians, 2002). Finally, loading of PCNA by RFC targets DNA Pol δ to the D loop, leading to efficient and processive DNA synthesis from the invading 3′ end.

The unmodified form of PCNA used here is likely the relevant form for recombination, because mono- and polyubiquitylation of PCNA favor alternative pathways, and SUMOylation antagonizes recombination (Moldovan et al., 2007). The in vitro system identified a preference of Pol δ over Pol η in D loop extension, consistent with unmodified PCNA favoring binding to Pol δ over the translesion polymerase η (Garg and Burgers, 2005). It is unclear how DNA polymerases are selected for access at the D loop beyond the inherent preference identified here. This may involve posttranslational modification of the polymerases themselves or other mechanisms. Although the involvement of Pol α-primase had been excluded in genetic experiments (Wang et al., 2004), clearly further studies are needed to address the potential for functional overlap between the other five nuclear polymerases (δ, ε, η, ζ, and λ) in recombination.

The in vitro system demonstrates that Pol δ is capable of efficient displacement synthesis in the context of a D loop, where the displaced strand is contiguous, unlike in lagging strand replication, where the displaced strand is discontiguous (Jin et al., 2003). The D loop appears to grow in size during recombination-associated DNA synthesis, to about 500 nt, before stalling as a result of topological constraints estimated to be about 32 positive supercoils (Figure 4C). The topological stalling can be relieved by the addition of eukaryotic Topo I, but it is unclear whether this is a physiological role of type I topoisomerases. It is unlikely that topological constraint limits the length of conversion tracts to 500 nt in vivo, because mitotic gene conversion tracts are significantly longer (Lee et al., 2009). However, it is possible that topological constraint leads to polymerase stalling and D loop dissociation followed by reinvasion, which might explain the observed template-switching during break-induced replication (Smith et al., 2007). In this scenario, recombination events would involve multiple rounds of invasion and DNA synthesis. Alternatively, our reconstituted system might be lacking an activity that is required for D loop migration (see Figure 4C). It is possible that Sgs1/BLM (Bachrati et al., 2006; Gangloff et al., 2000; Lo et al., 2006; van Brabant et al., 2000), members of the RecQ 3′-5′ DNA helicase family, or other helicases/motor proteins (Srs2?' Dupaigne et al., 2008) are involved. Biochemical experiments have also suggested a potential role of the Rad54 motor protein in D loop migration (Bugreev et al., 2007); however, this function of Rad54 appears to be limited in our reconstituted system.

The identification of Pol δ as a DNA polymerase involved in D loop extension is a critical step in developing a complete mechanistic understanding of homologous recombination and will help to unravel the mechanisms that govern the differences between various recombination modes (synthesis-dependent strand annealing, gap repair, double Holliday junction formation, and break-induced replication) that are distinguished by their DNA synthesis reactions (Jain et al., 2009).

EXPERIMENTAL PROCEDURES

Proteins and DNA Substrates

Proteins were purified as described elsewhere (Fortune et al., 2006; Garg et al., 2005; Gomes et al., 2000; Solinger et al., 2002). Oligonucleotide sequences are available upon request. pUC19 DNA (2686 bp) was prepared by Triton/SDS lysis and purified by isopycnic density centrifugation on CsCl/Ethidium bromide gradients. T4 polynucleotide kinase and DNA polymerase I Klenow fragment were from New England Biolabs; wheat germ topoisomerase I was from Promega.

D Loop Extension Assay

The reaction was performed at 30°C in SEB buffer (30 mM Tris-acetate [pH 7.5], 1 mM DTT, 50 μg/ml BSA, 4 mM ATP, 5 mM Mg^{2+}-acetate, 20 mM phosphocreatine, and 20 U/ml creatine kinase) supplemented with three dNTPs (dATP, dGTP, and dTTP at 11.1 μM each) and α-^{32}P-dCTP (0.22 μM; 6000 μCi/mmol, Perkin Elmer); 95-mer (2 μM nt) was incubated with Rad51 (0.67 μM) for 10 min to assemble nucleoprotein filaments. RPA (0.1 μM) was then added to the reaction for an additional 10 min. The formation of D loops was catalyzed by addition of Rad54 (72 nM) and pUC19 plasmid (56.6 μM bp, 20 nM molecule) to the reaction for 2 min. PCNA (20 nM) and RFC-1D complex (20 nM) were added to the D loop reaction for an additional 2 min. The initiation of D loop extension was started by addition of either Pol δ (20 nM) or Pol η (20 nM). At each time point, aliquots were mixed with stop buffer (1% SDS, 0.1 M EDTA, and 4 mg/ml Proteinase K) and were incubated for 10 min at 30°C. The DNA was separated on 1% agarose gels at 6 V/cm for 150 min. The gels were dried and analyzed by a phosphoImager. For the assays with 5′ end labeled 95-mers, the experimental procedure was as described except that all four unlabeled dNTPs were present at 100 μM each. To release the topological constraint introduced by DNA synthesis, at 2.5 min after the addition of DNA polymerase, wheat germ topoisomerase I (0.42 U/μl) was added to the reaction, and the reactions were incubated another 7.5 min before analysis on gels.

Two-Dimensional Gel Electrophoresis

For analysis of the extension products, the D loop extension assays were performed with either Pol δ or Pol η, and the reactions were stopped at 10 min. The sample of each reaction was split into two halves and separated in two lanes on a 0.8% native agarose gel at 5.5 V/cm for 70 min (α-^{32}P-dCTP incorporation assay) or for 90 min (assays with 5′ end-labeled 95-mer). After electrophoresis, both lanes were excised, and one lane was dried and served as one-dimensional reference. The other lane was soaked into the alkaline gel running buffer (50 mM NaOH and 1 mM EDTA) for 40 min, and then was inserted into a 1% alkaline agarose gel. Electrophoresis was performed at 2 V/cm for 11 hr. The gel was dried and analyzed by a phosphoImager.

(C) PCNA targets DNA polymerase δ to the 3′-OH end of the invading strand in a recombination-mediated D loop. Working model depicting the sequence of DNA strand invasion and D loop extension by Pol δ in the presence of PCNA/RFC. The topological constraint limiting the extent of DNA synthesis can be relieved by the addition of Topo I. The question mark indicates a possible pathway leading to D loop migration, which would require additional activities. For more discussion, see text.

SUPPLEMENTAL DATA

Supplemental Data include eight supplemental figures and can be found with this article online at http://www.cell.com/molecular-cell/supplemental/S1097-2765(09)00696-0.

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