BIOCHEMISTRY
including biophysical chemistry & molecular biology
Article

Biochemistry **2010,** *49,* 5919–5928 5919
DOI: 10.1021/bi100380n

Functions of Alternative Replication Protein A in Initiation and Elongation†

Aaron C. Mason,‡ Rupa Roy,§ Daniel T. Simmons,§ and Marc S. Wold*,‡

‡*Department of Biochemistry, Carver College of Medicine, University of Iowa, Iowa City, Iowa 52242, and* §*Department of Biological Sciences, University of Delaware, Newark, Delaware 19716*

Received March 11, 2010; Revised Manuscript Received April 30, 2010

ABSTRACT: Replication protein A (RPA) is a single-stranded DNA-binding complex that is essential for DNA replication, repair, and recombination in eukaryotic cells. In addition to this canonical complex, we have recently characterized an alternative replication protein A complex (aRPA) that is unique to primates. aRPA is composed of three subunits: RPA1 and RPA3, also present in canonical RPA, and a primate-specific subunit RPA4, homologous to canonical RPA2. aRPA has biochemical properties similar to those of the canonical RPA complex but does not support DNA replication. We describe studies that aimed to identify what properties of aRPA prevent it from functioning in DNA replication. We show aRPA has weakened interaction with DNA polymerase α (pol α) and that aRPA is not able to efficiently stimulate DNA synthesis by pol α on aRPA-coated DNA. Additionally, we show that aRPA is unable to support de novo priming by pol α. Because pol α activity is essential for both initiation and Okazaki strand synthesis, we conclude that the inability of aRPA to support pol α loading causes aRPA to be defective in DNA replication. We also show that aRPA stimulates synthesis by DNA polymerase α in the presence of PCNA and RFC. This indicates that aRPA can support extension of DNA strands by DNA polymerase ∂. This finding along with the previous observation that aRPA supports early steps of nucleotide excision repair and recombination indicates that aRPA can support DNA repair synthesis that requires polymerase δ, PCNA, and RFC and support a role for aRPA in DNA repair.

Replication protein A (RPA)[1] is a single-stranded DNA-binding protein that functions in many aspects of DNA metabolism (*1*−*3*). Since its discovery as an essential factor for simian virus 40 (SV40) DNA replication, RPA has been shown to play a crucial role in multiple processes of DNA metabolism. In addition to being essential in DNA replication, RPA is required for DNA recombination, DNA repair, and the response to DNA damage (*1*−*3*). RPA functions by binding and protecting exposed single-stranded DNA (ssDNA) and interacting with a multitude of proteins involved in DNA metabolism (*1*−*3*).

The canonical RPA is a heterotrimeric protein composed of 70 (RPA1), 32 (RPA2), and 14 kDa (RPA3) subunits that are conserved among eukaryotes (*1*, *3*, *4*). In addition to the three subunits that make up the canonical RPA complex, a fourth subunit termed RPA4 has been identified in primates (*5*, *6*). RPA4 is 63% homologous to RPA2 and on the basis of sequence comparison has a domain structure similar to that of RPA2: an N-terminal putative phosphorylation domain, a central DNA binding domain, DBD G, and a C-terminal winged helix domain (*5*, *6*). It has been shown that RPA4 can replace RPA2 in the trimeric complex creating a complex containing RPA1, RPA3, and RPA4, termed alternative RPA (aRPA) (*7*). This alternative complex has hydrodynamic properties indistinguishable from those of the canonical RPA complex but does not support SV40 or chromosomal DNA replication (*6*, *7*). It has been hypothesized that aRPA functions in DNA repair processes. Supporting this hypothesis, in vivo studies have shown that RPA4 localizes to sites of DNA damage when cells are challenged with inhibitors of either topoisomerase I or II (*6*). In vitro studies have shown that aRPA can support the dual incision−excision steps of nucleotide excision repair and stimulate Rad51-dependent strand invasion during the initial steps of recombination-mediated repair (*8*).

The role of RPA in DNA replication has been characterized in detail using the SV40 system. SV40 initiation requires the concerted action of four proteins, SV40 large T-antigen (Tag), polymerase α−primase (pol α), topoisomerase I (topo I), and RPA (*9*−*11*). Tag assembles at the origin of replication, bidirectionally unwinds the double-stranded DNA, and recruits other proteins to establish a replication fork (*12*). Topo I stimulates pol α by binding to Tag and releases torsional stress induced by unwinding of the parental strands (*13*, *14*). RPA is required to stabilize the emerging ssDNA and, along with Tag, recruits pol α (*15*, *16*). Pol α is a heterotetrameric complex of p180, p68, p58, and p48 subunits that synthesizes a short RNA primer on the leading strand and at the beginning of each Okazaki fragment on the lagging strand (*15*, *17*). After ∼10 ribonucleotides are incorporated, the complex transitions to DNA synthesis for ∼20 deoxynucleotides, creating the initial RNA−DNA primers used to start DNA replication and each Okazaki fragment (*18*). It has been shown that RPA acts as an auxiliary factor for pol α by stimulating synthesis and increasing processivity during the initiation of DNA replication (*19*). During initiation, RPA interacts with pol α to keep the polymerase

†This work was supported by National Institutes of Health Grant GM44721 to M.S.W. and National Cancer Institute Grant CA36118 to D.T.S.

*To whom correspondence should be addressed. E-mail: marc-wold@uiowa.edu. Phone: (319) 335-6784. Fax: (319) 384-4770.

[1]Abbreviations: RPA, human replication protein A; aRPA, alternative RPA; RPA1, 70 kDa subunit of RPA; RPA2, 32 kDa subunit of RPA; RPA3, 14 kDa subunit of RPA; RPA4, product of the *RPA4* gene; pol α, human DNA polymerase α−primase complex or human DNA polymerase α; PCNA, human proliferating cell nuclear antigen; RFC, human replication factor C; topo I, topoisomerase I; SV40, simian virus 40; Tag, SV40 large T-antigen; nt, nucleotide; ssDNA, single-stranded DNA; DBD, DNA binding domain; ssM13mp18, single-stranded M13mp18; BSA, bovine serum albumin; dNTPs, deoxynucleotides.

Published on Web 06/14/2010

at the primed site. To switch from initiation to elongation, RFC interacts with RPA, disrupting the pol α−RPA interaction and causing the release of pol α (*20*). RFC then loads PCNA and remains at the primed site by interacting with RPA. DNA polymerase δ (pol δ) can then access the primed site via contact with RPA. pol δ is one of the replicative polymerases in eukaryotes and is the major polymerase used for lagging strand synthesis (*21*). Pol δ competes with RFC for RPA, resulting in displacement of RFC from the 3′ terminus, and replacement with pol δ (*20*). RFC remains at the site by interacting with the PCNA ring. While in SV40 replication, pol δ can support synthesis of both leading and lagging strands (*22*), it is believed that generally once the elongation complex is established, pol δ extends the primers generated by pol α on the lagging strand while DNA polymerase ε continuously synthesizes DNA on the leading strand (*21*, *23*, *24*).

RPA has multiple roles in DNA replication. These include binding to exposed ssDNA being created by the helicase, helping recruit pol α, and coordinating the polymerase switch from polymerase α to polymerase δ/polymerase ε. Throughout the course of replication, RPA serves as a common interaction partner for many proteins and through a protein-mediated handoff mechanism coordinates the ordered assembly of the proteins (*3*). We have previously shown that aRPA does not support SV40 initiation or elongation. However, it is not known what activity prevents aRPA from functioning in DNA replication. This study examines the role of aRPA during the initiation and elongation reactions of DNA replication using purified recombinant proteins. In particular, we wished to understand how aRPA affects the activities of pol α and pol δ. We show that unlike RPA, aRPA has altered interactions with pol α and does not support efficient loading of or priming by pol α. The pattern of DNA synthesis by pol α in the presence of aRPA also suggests that aRPA cannot stabilize pol α on the DNA. In contrast, we find that aRPA does support pol δ synthesis in the presence of PCNA and RFC. These findings suggest that the defect of aRPA in replication is in promoting efficient priming by pol α but can function in processive DNA synthesis by pol δ.

EXPERIMENTAL PROCEDURES

Plasmids. pGBM-RFC1, pET-RFC4/2, and pCDFK-RFC5/3 were generous gifts from Y. Masuda (*25*). pET-hPold1 and pCOLA-hPold234 were generous gifts from Y. Matsumoto (*26*). p11d-tRPA and p11d-aRPA were described previously (*7*). pT7-hPCNA was a generous gift from B. Stillman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). This plasmid was used as a template for in vitro site-directed mutagenesis to insert an N-terminal six-histidine tag. The primers used to generate pT7-His-hPCNA were 5′-CCGTTTACTTTAAGAAGGAGATATACATATGCATCACCATCATCACCACGGATCCGCTATGTTCGAGGCGCGCCTGGTCCAGGGCTCC-3′ and 5′-GGAGCCCTGGACCAGGCGCGCCTCGAACATAGCGGATCCGTGGTGATGATGGTGATGCATATGTATATCTCCTTCTTAAAGTAAACG-3′. The mutations were confirmed by DNA sequencing.

Protein Purification. Recombinant RPA and aRPA were expressed in BL21(DE3) cells and purified as previously described (*7*, *27*, *28*). Recombinant human pol α was expressed and purified as described previously (*29*). Recombinant human pol δ was expressed in BL21(DE3) cells harboring pRARE2 (Novagen) cotransformed with pET-hPold1 and pCOLA-hPold234 for 18 h at 16 °C after induction with 0.2 mM isopropyl β-D-1-thiogalactopyranoside and purified as described previously (*25*). The purity of pol δ (~85%) was confirmed by SDS−PAGE visualized with Coomassie Blue staining. Recombinant human RFC was expressed in BL21(DE3) cells harboring pRARE2 cotransformed with pGBM-RFC1, pET-RFC4/2, and pCDFK-RFC5/3 for 18 h at 16 °C after induction with 0.2 mM isopropyl β-D-1-thiogalactopyranoside and purified as described previously except the size exclusion column was omitted (*25*). The purity of RFC (~85%) was confirmed by SDS−PAGE visualized with Coomassie Blue staining. Recombinant human PCNA was expressed in BL21(DE3) cells transformed with pT7-His-hPCNA for 4 h at 37 °C. The cells were harvested and lysed in a manner similar to that for RPA and aRPA. The supernatant loaded onto a Ni-NTA Agarose (Qiagen) column equilibrated with buffer J [30 mM HEPES (pH 7.8), 0.25% (w/v) myoinositol, 1 mM tris(2-carboxyethyl)-phosphine, and 0.02% Tween 20 (v/v)] supplemented with 20 mM imidazole. Following a 3 column volume wash, PCNA was eluted with a 5 column volume linear gradient from 20 to 250 mM imidazole. The peak fractions were pooled and dialyzed for 16 h against buffer J supplemented with 150 mM KCl to remove the imidazole. The purity of PCNA (>95%) was confirmed by SDS−PAGE visualized with Coomassie Blue staining.

Enzyme-Linked Immunosorbent Assay (*ELISA*). An ELISA was used to examine interactions between purified proteins as described previously (*28*). Briefly, wells in microtiter plates were coated with 1 μg of RPA or aRPA for interactions with PCNA and pol α and 1 μg of RFC and pol δ in 50 μL of water and incubated for 1 h. Plates were washed with phosphate-buffered saline with 0.2% Tween 20 and blocked with 5% milk in phosphate-buffered saline. The indicated amount of pol α, PCNA, RPA, aRPA, or BSA was added to each well, incubated for 1 h, and washed. Primary antibodies in phosphate-buffered saline with 5% milk for pol α (1:100 for SJK237), RPA/aRPA (1:300 for 719A), and PCNA (1:50 for anti-human PCNA antibody, the generous gift of T. Kelly) were added to the plates, incubated for 30 min, and washed. Goat α-mouse IgG-HRP (1:1000) was added and incubated for 30 min. Plates were developed using 200 μL of 0.8 mg/mL *o*-phenylenediamine in 0.005 M phosphate citrate buffer with 0.03% sodium perborate. OD_{450} was quantified after 10−60 min using a microtiter plate reader. Background was determined by using BSA as the secondary protein, and all data shown have these values subtracted. In all assays, the background values were similar and close to zero.

Pol α Extension Assay. Pol α activity was assayed with a singly primed d24·d66-mer oligodeoxynucleotide (d24, 5′-CTCGGACAATTTGGTGTGCTAGGT-3′; d66, 5′-AGGATGTATGTCTAGTAGGTACATAACTATTCAGTAGTATAGACCTAGCACACCAAATTGTCCGAG-3′) as a template. The d24·d66-mer was prepared by labeling the 5′-end of the d24-mer primer with [γ-^{32}P]ATP and T4 polynucleotide kinase (NEB) according to the manufacturer's protocol. The d66-mer template oligonucleotide was then mixed with the complementary labeled d24-mer oligonucleotide in a 1:1 molar ratio in 20 mM Tris-HCl (pH 8.0) containing 20 mM KCl and 1 mM EDTA, heated for 5 min at 90 °C, and then incubated for 2 h at 65 °C and slowly cooled to room temperature. A final volume of 15 μL contained 50 mM Tris-HCl (pH 7.6), 0.25 mg/mL BSA, 1 mM dithiothreitol, 6 mM $MgCl_2$, 20 nM (3′-OH ends) 5′-^{32}P-labeled d24·d66-mer DNA template, 10 μM dNTPs, 1 nM pol α, and 50 nM RPA or aRPA as indicated. Reaction mixtures were assembled on ice; reactions were initiated by the addition of dNTPs, and mixtures were incubated

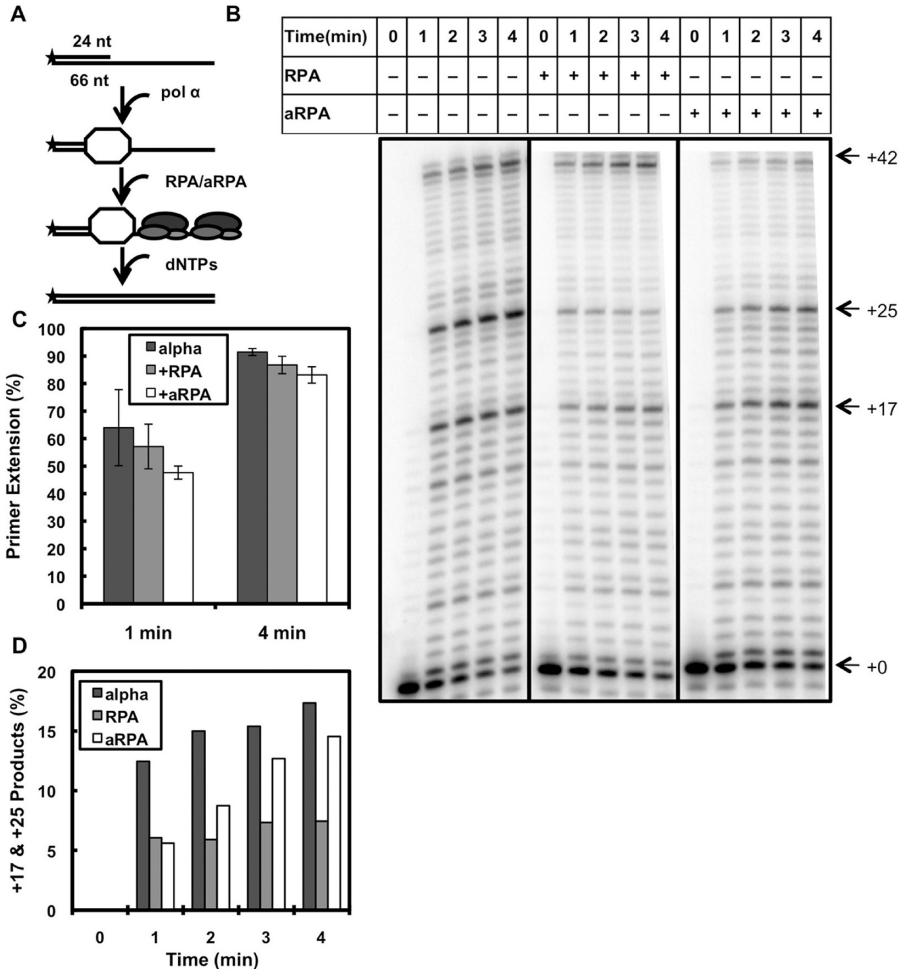

FIGURE 1: Effect of RPA and aRPA on pol α when polymerase is premixed with the DNA substrate. (A) Schematic illustrating the experimental setup and order of addition of proteins. The asterisk indicates the location of the ^{32}P label. (B) DNA pol α extension assays in which pol α (1 nM) has been preincubated with the DNA substrate (20 nM 3′-OH ends). Following preincubation, either RPA (50 nM) or aRPA (50 nM) was added and the reaction initiated by the addition of dNTPs (10 μM). Reaction products were separated by electrophoresis on a denaturing polyacrylamide sequencing gel and visualized on a Fuji FLA-7000 phosphorimager. (C) The results from three independent experiments were quantified and are presented. For each experiment, the amount of DNA in each band was determined. Percent primer extension was calculated by determining the ratio of all extended products (nucleotides 1−42) to total DNA [all products with an unextended primer (+0)]. Error bars indicate the standard deviation. (D) Amount of products at nucleotides 17 and 25 in the gel shown in panel B were quantified by dividing the sum of the amount of DNA at nucleotides 17 and 25 by total DNA.

for the indicated time at 37 °C. When the order of addition was varied, reaction mixtures were preincubated at 37 °C for 10 min and then reactions initiated by the addition of the indicated proteins and dNTPs. Reactions were quenched by the addition of formamide loading buffer [80% deionized formamide, 10 mM EDTA (pH 8.0), 1 mg/mL xylene cyanol, and 1 mg/mL bromophenol blue] and heated at 95 °C for 5 min, and products were separated in a 15% polyacrylamide sequencing gel containing 8 M urea. Products were visualized with a FLA-7000 phosphorimager (Fujifilm Global) and quantified using Multi Gauge (Fujifilm Global).

Primer RNA−DNA Synthesis Assay. These assays were conducted as described by Khopde et al. (*14*). Briefly, reaction mixtures (40 μL) contained 400 ng of CsCl-purified pSKori, 245 nM T-antigen (monomer), 7 nM pol α, and 200 nM RPA or aRPA with or without 55 nM topo I in replication buffer [30 mM HEPES-KOH (pH 8.0), 7 mM $MgCl_2$, 40 mM creatine phosphate, 25 μg/mL creatine phosphokinase, 0.5 mM dithiothreitol, 50 μg/mL BSA, 4 mM ATP, CTP, GTP, and UTP (0.2 mM each), and 10 units of RNasin (Sigma)]. After 1 h at 37 °C, newly synthesized RNA−DNA primers were pulse labeled for 1 min with 10 μCi of [α-^{32}P]dCTP, in the presence of 100 μM dATP, dTTP, and dGTP. Purified DNA was incubated with 15 μL of 95% formamide, 10 mM EDTA, 0.01% bromophenol blue, and 0.01% xylene cyanol at 90 °C for 3 min and subjected to electrophoresis on 12% polyacrylamide−urea sequencing gels. Products were visualized by being exposed to phosphorimager screens.

Pol δ Extension via a Singly Primed ssM13mp18 Assay. Pol δ activity was assayed on singly primed single-stranded M13mp18. The standard reaction mixture (10 μL) contained 20 mM HEPES-NaOH (pH 7.5), 0.2 mg/mL BSA, 1 mM dithiothreitol, 1 mM ATP, 1 mM EDTA, 50 fmol (364 pmol for nucleotides) of singly primed ssM13mp18 (5′-^{32}P-labeled 36-mer primer, CAGGGTTTTCCCAGTCACGACGTTGTAAAACGACGG, complementary to nucleotides 6330−6295), 555 nM RPA or aRPA, 50 nM PCNA, 50 nM RFC, and 20 nM pol δ. Reactions were initiated by the addition of dNTPs and $MgCl_2$ to final concentrations of 150 μM and 10 mM, respectively. After incubation at 37 °C for the indicated time, the reactions were quenched by the addition of formamide loading buffer [80% deionized formamide, 10 mM EDTA (pH 8.0), 1 mg/mL xylene cyanol, and 1 mg/mL bromophenol blue] and heated at 95 °C for 5 min,

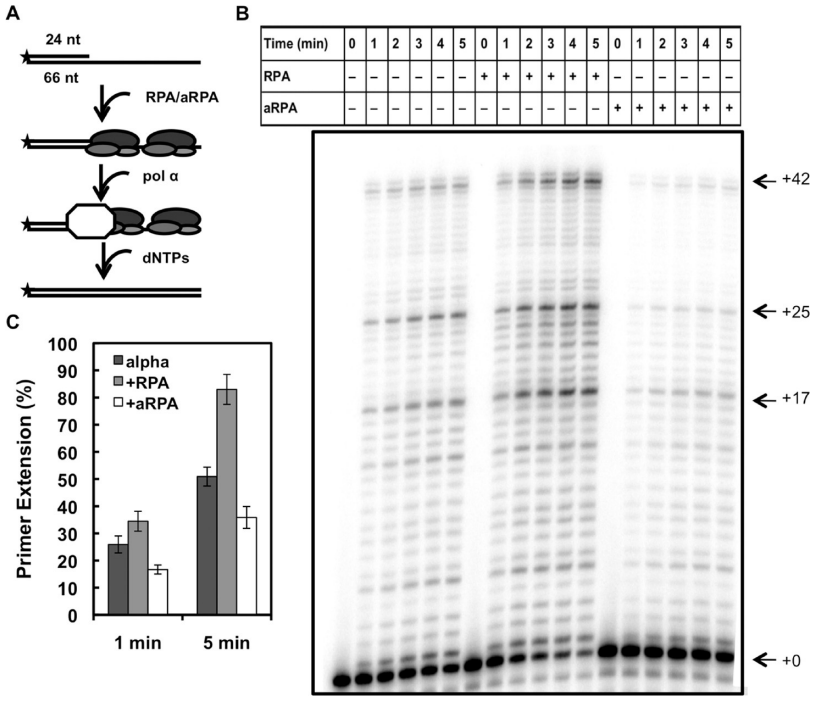

FIGURE 2: Effect on pol α when either RPA or aRPA is prebound to the DNA substrate. (A) Schematic illustrating the experimental setup and order of addition of proteins. The asterisk indicates the location of the ^{32}P label. (B) DNA pol α extension assays in which either RPA (50 nM) or aRPA (50 nM) was preincubated with the DNA substrate (20 nM 3′-OH ends). Following preincubation, pol α (1 nM) was added and the reaction initiated by the addition of dNTPs (10 μM). Reaction products were separated and visualized as described in the legend of Figure 1. (C) The results from three independent experiments were quantified and are presented. Percent primer extension was determined as described in the legend of Figure 1. Error bars indicate the standard deviation.

and products were separated in a 15% polyacrylamide sequencing gel containing 8 M urea. Products were visualized with a FLA-7000 phosphorimager (Fujifilm Global) and quantified using Multi Gauge (Fujifilm Global).

RESULTS

Effect of aRPA on Pol α. Initiation of SV40 DNA replication requires the concerted action of SV40 large T-antigen (Tag), topoisomerase I, pol α, and RPA, which form an "initiation complex" (*10*). Previously, we have shown that aRPA interacts with Tag at levels similar to that of RPA (*7*). This suggests that the inability of aRPA to support DNA replication is a result of aRPA affecting one of the other proteins in initiation or at the replication fork. We initially examined the effect of aRPA on the function of pol α using a 66-mer oligonucleotide primed with a 24-mer oligonucleotide. Pol α and either RPA or aRPA were incubated with the primed template in the presence of deoxynucleotides (dNTPs), and products of the reaction were separated on a 15% denaturing polyacrylamide gel, which allowed for separation of single-nucleotide incorporation events creating a laddering of products from nucleotide 1 to nucleotide 42. By allowing some components to prebind to the DNA, we were able to examine the effect of aRPA on the polymerization of a prebound pol α (Figure 1A) or on the loading and subsequent polymerization of pol α (Figure 2A). When pol α was allowed to bind to the template before the addition of dNTPs, it efficiently synthesized DNA, with the full-length product being observed in <1 min (Figure 1B, lanes 1−5). Intermediate-length products, notably two major pause sites at positions 17 and 25, were observed. The major pause sites are both two purines in a row (AA and GG, respectively), and the degree of pausing is consistent with the low processivity of pol α (*19*, *30*). Similar experiments in which pol α was allowed to bind to the primer-template junction and then either RPA or aRPA was added with the initiating dNTPs were conducted. The amount of RPA or aRPA used was enough to saturate the ssDNA region of the substrate with two RPA molecules bound per DNA substrate. In these reactions, addition of either RPA or aRPA resulted in levels of synthesis similar to that observed with pol α alone (Figure 1B,C). Addition of RPA caused a decrease in the level of accumulation of products at the two major pause sites by an average of 56% over the time course (Figure 1D). In contrast, there was only a slight change in the level of pausing (16%) when aRPA was added. Together, these findings suggest that aRPA does not affect the polymerization of pol α that is associated with the primer-template junction.

We next examined the ability of aRPA to facilitate the loading of pol α. This was done using the template described above, but the order of addition was changed: RPA or aRPA was allowed to prebind to exposed ssDNA on the template strand, and reactions were initiated by the addition of pol α (Figure 2A). The level of total synthesis with pol α was reduced under these conditions (Figure 2B). This is consistent with association of the polymerase being the rate-limiting step with this type of template. When RPA is prebound, total DNA synthesis by pol α is similar to that under conditions in which pol α is prebound to the template (compare Figure 1B with Figure 2B). This suggests RPA promotes loading of pol α on primer-template junctions. In contrast, when aRPA was prebound to the ssDNA of the template, there was a 64% decrease in the total level of DNA synthesis [compared to prebinding RPA and a decrease of 42% compared to pol α alone (Figure 2C)]. This suggests, that unlike RPA, aRPA does not support efficient loading of pol α and actually inhibits its association with the primer-template junction.

We next examined the concentration dependence of RPA and aRPA on pol α synthesis. The concentrations of RPA and aRPA were varied from 0 to 100 nM with a fixed amount of DNA

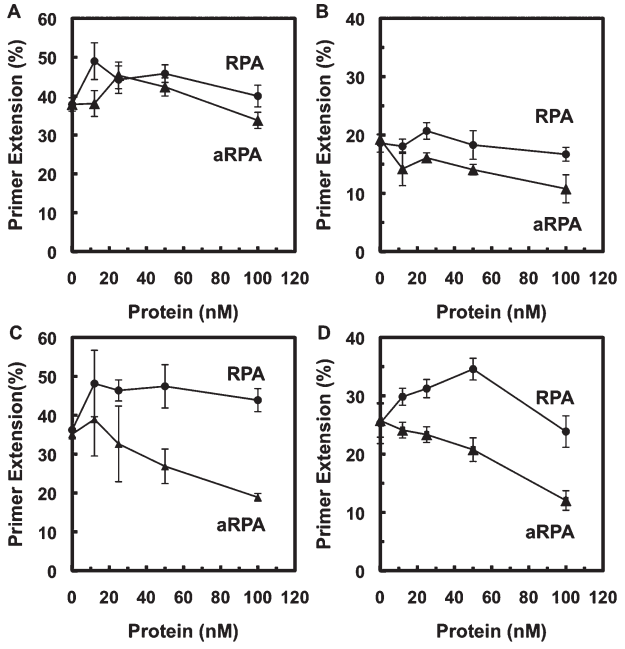

FIGURE 3: Titration of RPA and aRPA in the pol α extension assay. (A and B) Pol α was preincubated with the DNA substrate (20 nM 3′-OH ends) as described in the legend of Figure 1. Following preincubation, RPA (●) or aRPA (▲) was added and the reaction initiated by the addition of (A) dNTPs (10 μM) or (B) only dCTP (10 μM). (C and D) RPA (●) or aRPA (▲) was preincubated with the DNA substrate (20 nM 3′-OH ends) as described in the legend of Figure 2. Following preincubation, pol α (1 nM) was added and the reaction initiated by the addition of (C) dNTPs (10 μM) or (D) dCTP (10 μM). Primer extension was quantified as described in the legend of Figure 1. Averages of two independent experiments are shown with error bars to indicate the range of the data.

substrate (20 nM). RPA and aRPA had a minimal effect on pol α when the polymerase was allowed to prebind the DNA substrate (Figure 3A,B). When either RPA or aRPA was allowed to prebind the DNA substrate, there was a minimal change in DNA synthesis at low concentrations but the level of DNA synthesis quickly decreased when aRPA concentrations went above 30 nM (Figure 3C). However, the level of DNA synthesis with RPA increased to 30 nM and then remained constant for multiple incorporation events (Figure 3C). We also examined incorporation of the first nucleotide to determine whether the form of RPA affected the initial polymerization reaction. Single-nucleotide incorporation was examined by conducting the reactions in the presence of only the next nucleotide in the sequence (dCTP). RPA had a minimal effect up to 50 nM but inhibited synthesis at higher concentrations (Figure 3D). In contrast, aRPA decreased the amount of single-nucleotide incorporation at all concentrations examined (Figure 3D). The results suggest that over this concentration range, aRPA is inhibitory while RPA had a minimal effect on synthesis by pol α.

Mechanism of Pol α Inhibition by aRPA. We next determined whether there were altered interactions between aRPA and pol α. Enzyme-linked immunosorbant assays were conducted with purified proteins. Compared to that of RPA, the extent of interaction between aRPA and pol α was decreased by ~75% (Figure 4A). Both RPA1 and RPA2 interact with the pol α complex so either the pol α interaction with RPA2 is most important for the interactions monitored in these assays or the presence of RPA4 in the aRPA complex causes altered interactions of pol α with RPA1. These findings suggest that aRPA has a reduced

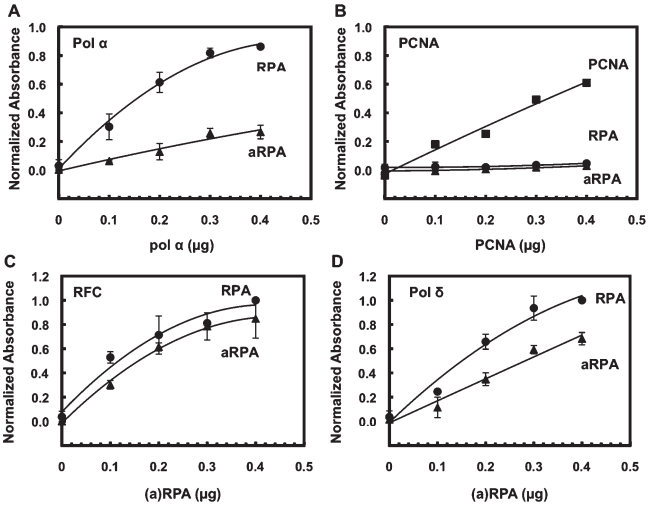

FIGURE 4: Enzyme-linked immunosorbant assay in which interactions were assessed between different forms of RPA and either (A) pol α, (B) PCNA, (C) RFC, or (D) pol δ. Forms of RPA used: RPA (●) and aRPA (▲). The data from each experiment were normalized to the highest absorbance in each experiment, averaged, and plotted. Error bars indicate the average of two or more independent replicates. BSA was used to determine nonspecific background (< 0.1) in each assay and subtracted. (B) PCNA (■) was also placed directly on the plate as a positive control.

level of interaction with pol α and that this prevents efficient loading when aRPA is bound to the DNA. However, they do not rule out aRPA allosterically modulating the activity of pol α. To test this possibility, a series of mixing experiments were conducted. Either RPA or aRPA was prebound to the DNA substrate, while at the same time, DNA pol α was preincubated with the other form of RPA. DNA synthesis was then initiated by a combination of the two mixtures and primer extension monitored (Figure 5A). When RPA was prebound and the reaction initiated by the addition of the aRPA−pol α complex, there was a slight decrease in the level of DNA synthesis compared to that for pol α alone (Figure 5B). In contrast, when aRPA was prebound to ssDNA and the reaction initiated by the addition of the RPA−pol α complex, there was a larger decrease in the level of DNA synthesis (Figure 5B). Together, these findings suggest that the majority of the inhibition of pol α by aRPA is a result of weakened protein interactions between aRPA and pol α preventing either pol α from associating on aRPA-coated primer-template junctions or pol α from displacing aRPA from the DNA template.

Effect of aRPA on the Synthesis on RNA−DNA Primers. To examine the effect these altered interactions have on initiation, we examined the ability of aRPA to support pol α-dependent priming using a SV40-based monopolymerase assay. We identified newly synthesized RNA−DNA primers by labeling them in the presence of [^{32}P]dCTP for 1 min following a 1 h incubation in the presence of rNTPs. RNA−DNA primers of approximately 36 nucleotides were synthesized and readily detected in the presence of RPA (Figure 6, lanes 1 and 2) or RPA and topoisomerase I (Figure 6, lanes 3 and 4). However, no synthesis was detected in the presence of aRPA (Figure 6, lanes 5−8). This demonstrates that aRPA is unable to support efficient initiation of DNA replication from the SV40 origin by preventing priming by pol α.

Effect of aRPA on Pol δ DNA Synthesis. Thus far, we have shown that aRPA does not support the efficient loading of pol α onto the primer-template junction. This agrees with our earlier findings that aRPA does not support the initiation steps of

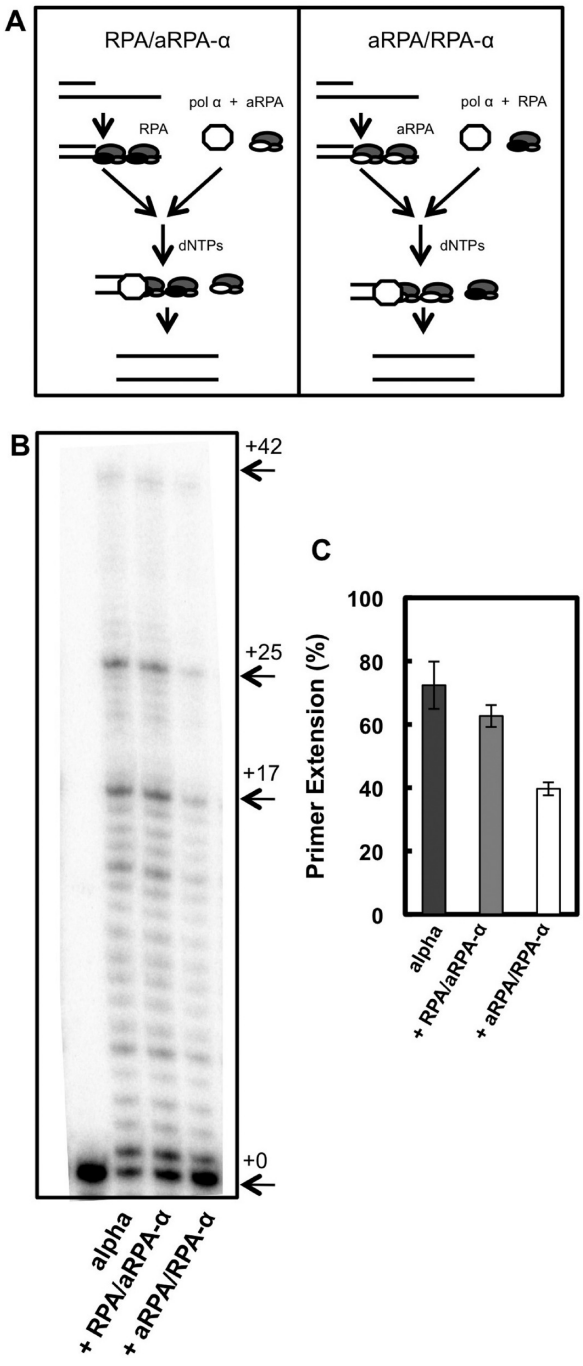

FIGURE 5: Mechanism of pol α inhibition. (A) Schematic illustrating the experimental setup, order of addition of proteins, and preincubation of proteins and DNA substrate. (B) DNA pol α extension assays in which either RPA (50 nM) or aRPA (50 nM) was preincubated with the DNA substrate (20 nM 3′-OH ends) and the other form of RPA was preincubated with pol α (1 nM). The preincubated samples were mixed, and the reaction was initiated by the addition of dNTPs (10 μM). Reaction products were separated by electrophoresis on a denaturing polyacrylamide sequencing gel and visualized by phosphorimaging. (C) The results from two independent experiments were quantified and are presented. Percent primer extension was determined as described in the legend of Figure 1. Error bars indicate the range of data.

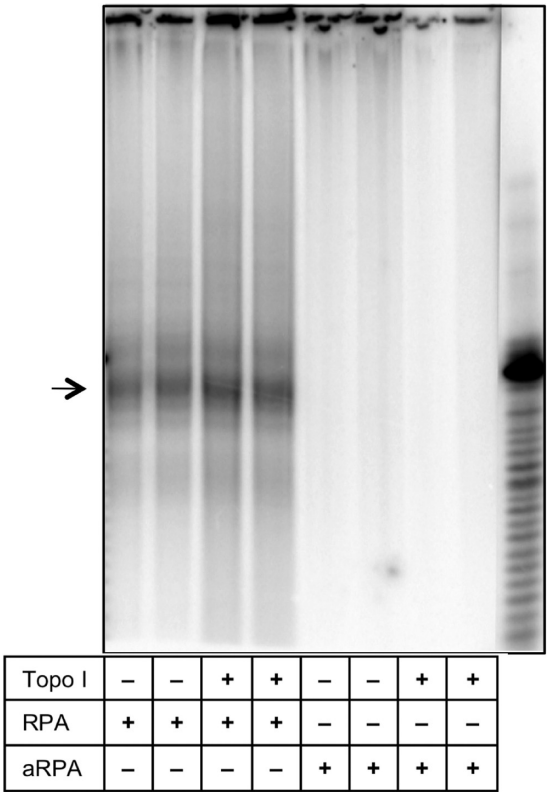

FIGURE 6: Primer RNA−DNA synthesis in the presence of RPA or aRPA. Primer RNA−DNA synthesis was assessed by incorporation of [^{32}P]dCTP in a 1 min labeling reaction mixture that contained pSKori DNA (400 ng), Tag (800 ng), pol α (100 ng), Topo I (200 ng), RPA (900 ng), and aRPA (900 ng) as indicated. After deproteination, the DNA was denatured with formamide and subjected to electrophoresis on a 12% acrylamide sequencing gel followed by detection of the labeled DNA with a phosphorimager. The major band in the right lane represents a 42 nt single-stranded DNA marker. The arrow indicates predominant length primers.

SV40 DNA replication. However, we have shown that aRPA also does not support the elongation phase of SV40 DNA replication (*7*). One possibility is that like pol α inhibition, aRPA could also inhibit DNA synthesis by pol δ. Another possibility is that the inhibition of pol α, which is required for Okazaki fragment synthesis, is enough to uncouple leading and lagging strand synthesis, thus halting DNA synthesis.

To discriminate between these two possibilities, pol δ, RFC, and PCNA were purified and the effect of aRPA on pol δ DNA synthesis was examined on a singly primed ssM13mp18 plasmid. As shown in lanes 2 and 3 of Figure 7A, DNA synthesis by pol δ by itself is limited to a small number of nucleotides incorporated, which is consistent with its low processivity in the absence of the accessory factors PCNA and RFC (*20*). When RFC and PCNA were added to the reaction mixture (Figure 7A, lanes 11 and 12), there was an increase in the length of products formed, indicating that RFC actively loaded PCNA onto the single-strand plasmid and PCNA formed a complex with pol δ. The addition of RPA to the RFC, PCNA, and pol δ reaction mixture showed a dramatic increase in the length of the products formed (Figure 7A, lanes 5 and 6). Interestingly, addition of aRPA to RFC, PCNA, and pol δ resulted in products that are identical to those synthesized in the presence of RPA (in Figure 7A, compare lanes 8 and 9 to lanes 5 and 6, and quantitation of the products in Figure 7B). A time course of these reactions indicated that while there was a slight lag with aRPA, overall the rate of synthesis is similar with either RPA or aRPA (Figure 7C). We conclude that aRPA does support processive DNA synthesis by pol δ in the presence of RFC and PCNA.

We also examined whether there were altered interactions between aRPA and RFC, PCNA, and pol δ. Both RFC and pol δ

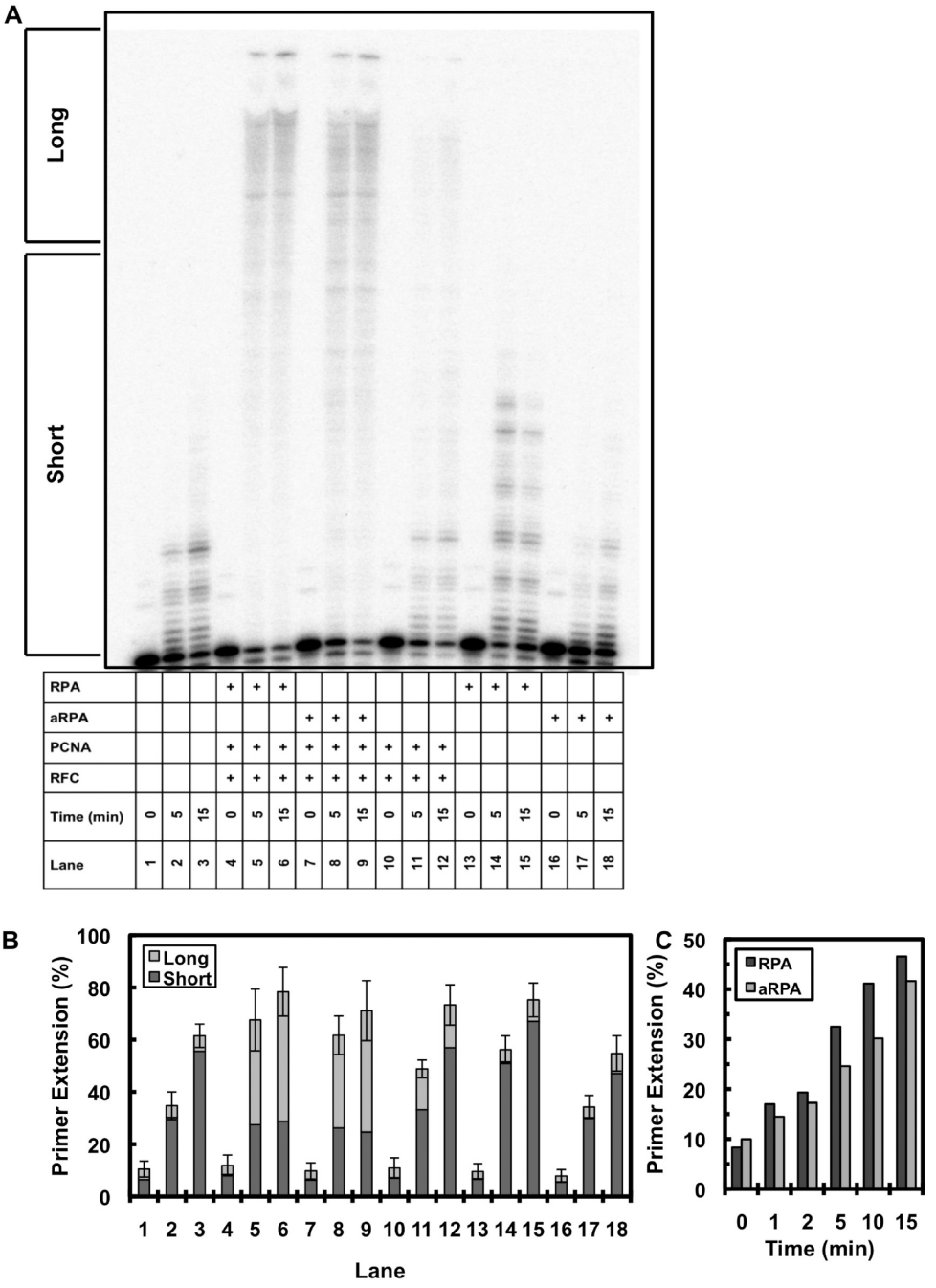

FIGURE 7: Synthesis of pol δ on singly primed single-stranded M13mp18 template. (A) Pol δ activity was assayed on singly primed single-stranded M13mp18 (50 fmol) in a reaction initiated by the addition of dNTPs (150 μM). Addition of individual components is indicated by a plus sign: pol δ (20 nM), RPA (555 nM), aRPA (555 nM), PCNA (50 nM), and RFC (50 nM). The time of the reaction is indicated (0, 5, or 15 min). Reaction products were separated by electrophoresis on a denaturing polyacrylamide sequencing gel and visualized by phosphorimaging. (B) The results from three independent experiments were quantified and are presented. Primer extension was quantified by dividing either short products ($\leq$40 nt, dark gray) or long products ($\geq$41 nt, light gray) by total DNA. Error bars indicate the standard deviation of the data for the long products. The standard deviation for the short products is shown in supplemental Figure 1 of the Supporting Information. (C) Expanded time course of complete reactions [pol δ (20 nM), PCNA (50 nM), and RFC (50 nM)] with the addition of either RPA (555 nM, dark gray) or aRPA (555 nM, light gray).

interact with RPA, while PCNA does not directly interact with RPA (*3*, *20*). As shown in Figure 4C, the interaction between aRPA and RFC was the same as that between RPA and RFC. RPA and aRPA did not directly interact with PCNA (Figure 4B). aRPA also interacted with pol δ but at a slightly reduced level relative to RPA [60% (Figure 4D)]. In the absence of the accessory proteins RFC and PCNA, RPA but not aRPA caused a modest stimulation of pol δ DNA synthesis (in Figure 7A, compare lane 3 to lanes 15 and 18). This suggests that the altered interactions between aRPA and pol δ might reduce the level of direct stimulation of pol δ by aRPA. However, even if there is a miscommunication between aRPA and pol δ, it is readily overcome by the accessory proteins (Figure 7A).

While optimizing pol δ DNA synthesis on the ssM13mp18 plasmid, we examined the salt dependence of the reaction. Pol δ is sensitive to ionic strength, and most previous analyses with RFC and PCNA have been conducted under low-ionic strength conditions (*25*, *31*, *32*). We find that the addition of RPA or aRPA can overcome the salt inhibition of pol δ DNA synthesis (Figure 8). Synthesis was monitored, and both short ($\leq$40 nt) and long ($\geq$41 nt)

products were quantified. Under all salt conditions examined, minimal long products were observed with pol δ or with pol δ, PCNA, and RFC in the absence of either form of RPA [Figure 8B (○ and ◇)]. At low salt concentrations, short products were observed but the amount of synthesis decreased as salt concentration increased (Figure 8A). In contrast, when RPA or aRPA was added to RFC, PCNA, and pol δ, high levels of synthesis and full-length (long) products were observed from low to near-physiological ionic strength {0−125 mM NaCl [Figure 8B (filled symbols)]}. There is inhibition of synthesis at 250 mM NaCl in the presence of RPA or aRPA; however, even at this high ionic strength, synthesis of short products was observed while there was virtually complete inhibition of pol δ in the absence of RPA. These data clearly show that aRPA can stimulate pol δ under a variety of conditions and that both RPA and aRPA stimulate pol δ activity at physiological ionic strength.

DISCUSSION

RPA has a central role in DNA replication, playing an essential function in both initiation and elongation (*33*, *34*). We have previously shown that aRPA does not support DNA synthesis during the initiation and elongation phases of SV40 DNA replication or S-phase progression in human cells (*6*, *7*). The studies presented here provide a molecular explanation for this difference in activity. We have shown that aRPA bound to ssDNA prevents the synthesis of RNA−DNA primers by pol α by preventing efficient loading of the polymerase onto the ssDNA. This effect is probably caused by altered interactions between pol α and aRPA. However, aRPA has minimal effects on the polymerization reaction by pol α once it has started synthesizing DNA. These findings indicate that aRPA is unlikely to support the association of pol α on ssDNA, leading to priming and the initiation of DNA replication. This mechanism is also supported by the finding that aRPA is unable to support primer synthesis in an SV40 origin-dependent initiation reaction. This defect would also be expected to prevent priming of Okazaki fragments needed for lagging strand synthesis, which would presumably stall replication forks during elongation synthesis. Walther et al. showed that inhibition of pol α during the elongation phase of SV40 replication completely halts DNA synthesis, consistent with coupled synthesis of leading and lagging strands (*34*). This indicates that the loss of pol α loading on the lagging strand in the presence of aRPA would be expected to cause a defect in elongation. We also show that aRPA supports pol δ DNA synthesis in the presence of RFC and PCNA to the same extent as canonical RPA and that both aRPA and RPA increase the processivity of the polymerase more than PCNA and RFC alone. This suggests that aRPA can support processive DNA synthesis on primed DNA templates. This activity would have little consequence during DNA replication in the absence of priming by pol α but would further support a role for aRPA in genome maintenance. It has been shown that aRPA can function in multiple aspects of DNA repair from localization to sites of damage to supporting the dual incision−excision steps of nucleotide excision repair to supporting Rad51-dependent strand invasion (*8*). Our findings here suggest that aRPA can complete the nucleotide excision repair process by filling the gap left when the damaged DNA is removed. This gap filling reaction is conducted by PCNA, RFC, and either pol δ or pol ε, in a continuous manner using the free 3′-OH left by the removal of the damaged DNA (*35*). Similar gap filling reactions

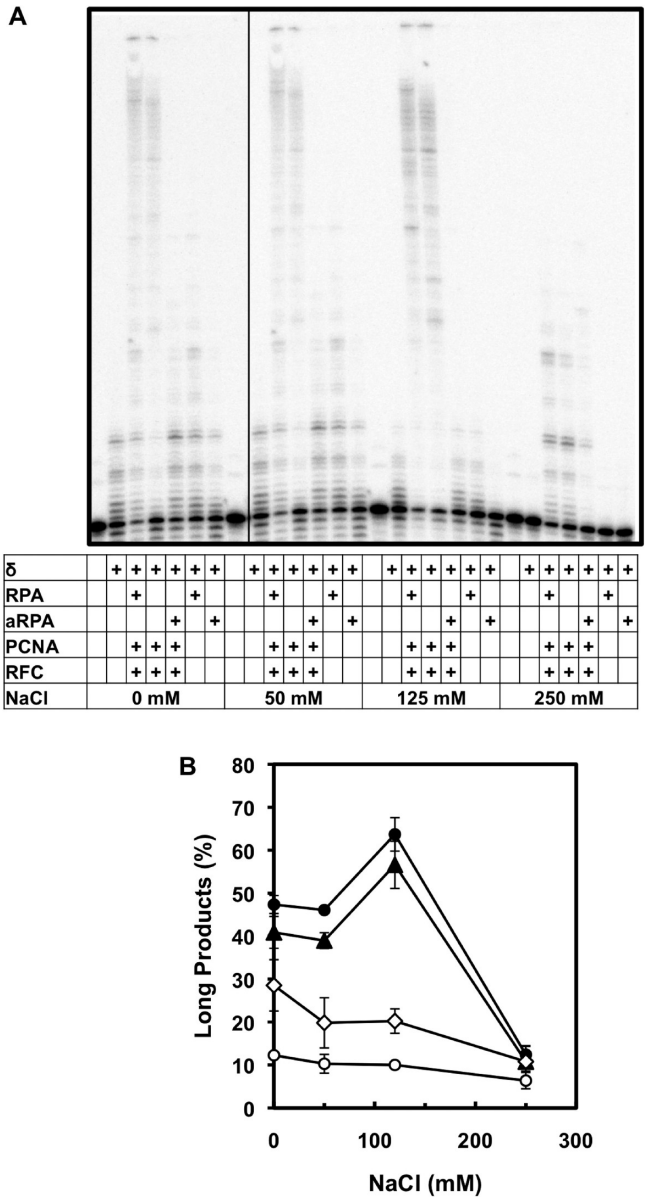

FIGURE 8: Salt dependence of pol δ activity. Pol δ activity was assayed on singly primed single-stranded M13mp18 (50 fmol) in varying concentrations of NaCl initiated by the addition of dNTPs (150 μM). Reaction components: pol δ (20 nM) (○); pol δ (20 nM), RPA (555 nM), PCNA (50 nM), and RFC (50 nM) (●); pol δ (20 nM), aRPA (555 nM), PCNA (50 nM), and RFC (50 nM) (▲); and pol δ (20 nM), PCNA (50 nM), and RFC (50 nM) (◇). (A) Reaction products were separated by electrophoresis on a denaturing polyacrylamide sequencing gel and visualized by phosphorimaging. (B) The results from two independent experiments were quantified and are presented. Percent long products (≥41 nt) were calculated as described in the legend of Figure 7. Error bars indicate the range of the data.

by a high-fidelity polymerase, such as pol δ and DNA polymerase ε, are common to most other forms of DNA repair (*36*). We suggest that aRPA, like canonical RPA, is capable of supporting gap synthesis in repair and thus helps the cell maintain genome stability. Of the 14 identified human polymerases, RPA has been shown to interact with at least pol α, δ, ε, λ, and κ (*32*, *37*−*39*). Interestingly, only pol α is able to initiate strand synthesis in DNA replication. All other human DNA polymerases extend previously initiated DNA strands.

The number of and the role of RPA-like complexes in different processes in eukaryotic cells are diverse. Up until the past decade,

it was thought that eukaryotic cells had primarily one nuclear single-stranded DNA-binding protein involved in DNA metabolism, canonical RPA. However, it is now clear that there are a number of RPA-like proteins in cells. Several domains of the tumor suppressor BRCA2 are structurally and functionally similar to the DNA-binding domains of RPA (*40*, *41*). Other examples include the recently identified pol α accessory proteins that are homologous to RPA and function in DNA metabolism (*42*) and the RPA-related complex, consisting of Cdc13, Stn1, and Ten1, that is involved in telomere maintenance (*43*−*45*). Mammals also have non-RPA-related single-stranded DNA binding proteins that function in DNA repair (*46*, *47*). Furthermore, a number of eukaryotes have multiple RPA complexes. *Cryptosporidium parvum* has two forms of RPA1 (*48*). Plants such as *Oryza sativa* and *Arabidopsis thaliana* have multiple copies of RPA genes that form multiple different heterotrimeric RPA complexes (*49*). These plant RPA complexes have nonredundant functions with respect to each other. For example, in rice the B type RPA plays a role in DNA damage repair while the C type RPA is required for DNA replication (*50*, *51*). RPA4 and RPA2 could be functioning similarly in human cells with aRPA and canonical RPA in humans playing the same roles as B type and C type RPA in rice, respectively.

RPA4 appears to have emerged relatively recently in evolution. RPA4-like sequences have been identified only in mammals, and intact *RPA4* genes have been maintained only in horses and primates (*6*). It has been estimated that approximately 3% of human genes are restricted to primates, and these genes are termed orphan genes (*52*). However, very few orphan genes have been well characterized experimentally. One characterized gene, dermcidin, encodes a peptide with antimicrobial activity that is secreted in sweat glands and has been reported to be involved in neural survival and cancer (*53*). Another example of a primate-specific orphan gene with described function is the SPHAR, which is involved in the regulation of DNA synthesis (*54*). Two more genes that function in DNA metabolism pathways are FAM9B and FAM9C, expressed solely in the testis, and they have been suggested to play a role in mediating recombination during meiosis (*55*). There has also been speculation that primate-specific genes are preferentially expressed in the reproductive system (*56*). *RPA4* is expressed in reproductive tissues (placenta, ovary, prostate, testis, and oocytes) but is also expressed in non-reproductive tissues (e.g., lung, esophagus, and bladder) (*8*, *57*). Therefore, while the cellular function of aRPA is not understood, it appears that the role(s) of RPA4 in the cell is not limited to reproduction.

The findings presented here reveal the mechanism that prevents aRPA from functioning in DNA replication. They also show that aRPA can support DNA repair synthesis that depends on pol δ with its accessory proteins, RFC and PCNA. These and other recent findings on aRPA suggest that it functions in repair processes to maintain the genomic stability in nondividing cells.

ACKNOWLEDGMENT

We thank Cathy Hass and Troy Humpreys for useful discussion and critical reading of the manuscript. We also thank Dr. Bruce Stillman, Dr. Yuji Masuda, and Dr. Yoshihiro Matsumoto for kindly providing expression plasmids for replication proteins. We thank Dr. Thomas Kelly for the antiserum to human PCNA.

SUPPORTING INFORMATION AVAILABLE

Results from three independent experiments described in Figure 7 quantified with error bars for the standard deviation of the short products. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Wold, M. S. (1997) Replication Protein A: A heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. *Annu. Rev. Biochem. 66*, 61–92.
2. Iftode, C., Daniely, Y., and Borowiec, J. A. (1999) Replication Protein A (RPA): The eukaryotic SSB. *CRC Crit. Rev. Biochem. 34*, 141–180.
3. Fanning, E., Klimovich, V., and Nager, A. R. (2006) A dynamic model for replication protein A (RPA) function in DNA processing pathways. *Nucleic Acids Res. 34*, 4126–4137.
4. Rider, S. D., Jr., Cai, X., Sullivan, W. J., Jr., Smith, A. T., Radke, J., White, M., and Zhu, G. (2005) The protozoan parasite *Cryptosporidium parvum* possesses two functionally and evolutionarily divergent replication protein A large subunits. *J. Biol. Chem. 280*, 31460–31469.
5. Keshav, K. F., Chen, C., and Dutta, A. (1995) Rpa4, a homolog of the 34-kilodalton subunit of the replication protein A complex. *Mol. Cell. Biol. 15*, 3119–3128.
6. Haring, S. J., Humphreys, T. D., and Wold, M. S. (2010) A naturally occurring human RPA subunit homolog does not support DNA replication or cell-cycle progression. *Nucleic Acids Res. 38*, 846–858.
7. Mason, A. C., Haring, S. J., Pryor, J. M., Staloch, C. A., Gan, T. F., and Wold, M. S. (2009) An alternative form of replication protein A prevents viral replication in vitro. *J. Biol. Chem. 284*, 5324–5331.
8. Kemp, M. G., Mason, A. C., Carreira, A., Reardon, J. T., Haring, S. J., Borgstahl, G. E., Kowalczykowski, S. C., Sancar, A., and Wold, M. S. (2010) An alternative form of replication protein a expressed in normal human tissues supports DNA repair. *J. Biol. Chem. 285*, 4788–4797.
9. Bullock, P. A. (1997) The initiation of Simian Virus 40 DNA replication in vitro. *Crit. Rev. Biochem. Mol. Biol. 32*, 503–568.
10. Taneja, P., Nasheuer, H. P., Hartmann, H., Grosse, F., Fanning, E., and Weisshart, K. (2007) Timed interactions between viral and cellular replication factors during the initiation of SV40 in vitro DNA replication. *Biochem. J. 407*, 313–320.
11. Fanning, E., and Zhao, K. (2009) SV40 DNA replication: From the A gene to a nanomachine. *Virology 384*, 352–359.
12. Simmons, D. T. (2000) SV40 large T antigen functions in DNA replication and transformation. *Adv. Virus Res. 55*, 75–134.
13. Khopde, S., and Simmons, D. T. (2008) Simian virus 40 DNA replication is dependent on an interaction between topoisomerase I and the C-terminal end of T antigen. *J. Virol. 82*, 1136–1145.
14. Khopde, S., Roy, R., and Simmons, D. T. (2008) The binding of topoisomerase I to T antigen enhances the synthesis of RNA-DNA primers during simian virus 40 DNA replication. *Biochemistry 47*, 9653–9660.
15. Melendy, T., and Stillman, B. (1993) An interaction between replication protein A and SV40 T antigen appears essential for primosome assembly during SV40 DNA replication. *J. Biol. Chem. 268*, 3389–3395.
16. Weisshart, K., Taneja, P., and Fanning, E. (1998) The replication protein A binding site in simian virus 40 (SV40) T antigen and its role in the initial steps of SV40 DNA replication. *J. Virol. 72*, 9771–9781.
17. Huang, S. G., Weisshart, K., Gilbert, I., and Fanning, E. (1998) Stoichiometry and mechanism of assembly of SV40 T antigen complexes with the viral origin of DNA replication and DNA polymerase α-primase. *Biochemistry 37*, 15345–15352.
18. Waga, S., and Stillman, B. (1994) Anatomy of a DNA replication fork revealed by reconstitution of SV40 DNA replication in vitro. *Nature 369*, 207–212.
19. Maga, G., Frouin, I., Spadari, S., and Hubscher, U. (2001) Replication protein a as a "fidelity clamp" for DNA polymerase α. *J. Biol. Chem. 276*, 18235–18242.
20. Yuzhakov, A., Kelman, Z., Hurwitz, J., and O'Donnell, M. (1999) Multiple competition reactions for RPA order the assembly of the DNA polymerase δ holoenzyme. *EMBO J. 18*, 6189–6199.
21. Nick McElhinny, S. A., Gordenin, D. A., Stith, C. M., Burgers, P. M., and Kunkel, T. A. (2008) Division of labor at the eukaryotic replication fork. *Mol. Cell 30*, 137–144.
22. Zlotkin, T., Kaufmann, G., Jiang, Y. Q., Lee, M. Y. W. T., Uitto, L., Syväoja, J., Dornreiter, I., Fanning, E., and Nethanel, T. (1996) DNA polymerase ε may be dispensable for SV40- but not cellular-DNA replication. *EMBO J. 15*, 2298–2305.

23. Kunkel, T. A., and Burgers, P. M. (2008) Dividing the workload at a eukaryotic replication fork. *Trends Cell Biol. 18*, 521–527.
24. Burgers, P. M. (2009) Polymerase dynamics at the eukaryotic DNA replication fork. *J. Biol. Chem. 284*, 4041–4045.
25. Masuda, Y., Suzuki, M., Piao, J., Gu, Y., Tsurimoto, T., and Kamiya, K. (2007) Dynamics of human replication factors in the elongation phase of DNA replication. *Nucleic Acids Res. 35*, 6904–6916.
26. Fazlieva, R., Spittle, C. S., Morrissey, D., Hayashi, H., Yan, H., and Matsumoto, Y. (2009) Proofreading exonuclease activity of human DNA polymerase δ and its effects on lesion-bypass DNA synthesis. *Nucleic Acids Res. 37*, 2854–2866.
27. Henricksen, L. A., Umbricht, C. B., and Wold, M. S. (1994) Recombinant replication protein A: Expression, complex formation, and functional characterization. *J. Biol. Chem. 269*, 11121–11132.
28. Binz, S. K., Dickson, A. M., Haring, S. J., and Wold, M. S. (2006) Functional assays for replication protein A (RPA). *Methods Enzymol. 409*, 11–38.
29. Simmons, D. T., Gai, D., Parsons, R., Debes, A., and Roy, R. (2004) Assembly of the replication initiation complex on SV40 origin DNA. *Nucleic Acids Res. 32*, 1103–1112.
30. Hohn, K. T., and Grosse, F. (1987) Processivity of the DNA polymerase α-primase complex from calf thymus. *Biochemistry 26*, 2870–2878.
31. Podust, V. N., Chang, L. S., Ott, R., Dianov, G. L., and Fanning, E. (2002) Reconstitution of human DNA polymerase δ using recombinant baculoviruses: The p12 subunit potentiates DNA polymerizing activity of the four-subunit enzyme. *J. Biol. Chem. 277*, 3894–3901.
32. Chilkova, O., Stenlund, P., Isoz, I., Stith, C. M., Grabowski, P., Lundstrom, E. B., Burgers, P. M., and Johansson, E. (2007) The eukaryotic leading and lagging strand DNA polymerases are loaded onto primer-ends via separate mechanisms but have comparable processivity in the presence of PCNA. *Nucleic Acids Res. 35*, 6588–6597.
33. Wold, M. S., and Kelly, T. (1988) Purification and characterization of replication protein A, a cellular protein required for in vitro replication of simian virus 40 DNA. *Proc. Natl. Acad. Sci. U.S.A. 85*, 2523–2527.
34. Walther, A. P., Bjerke, M. P., and Wold, M. S. (1999) A novel assay for examining the molecular reactions at the eukaryotic replication fork: Activities of replication protein A required during elongation. *Nucleic Acids Res. 27*, 656–664.
35. Shivji, M. K., Podust, V. N., Hubscher, U., and Wood, R. D. (1995) Nucleotide excision repair DNA synthesis by DNA polymerase ε in the presence of PCNA, RFC, and RPA. *Biochemistry 34*, 5011–5017.
36. Sancar, A., Lindsey-Boltz, L. A., Unsal-Kacmaz, K., and Linn, S. (2004) Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annu. Rev. Biochem. 73*, 39–85.
37. Dornreiter, I., Erdile, L. F., Gilbert, I. U., von Winkler, D., Kelly, T. J., and Fanning, E. (1992) Interaction of DNA polymerase α-primase with cellular replication protein A and SV40 T antigen. *EMBO J. 11*, 769–776.
38. Maga, G., Ramadan, K., Locatelli, G. A., Shevelev, I., Spadari, S., and Hubscher, U. (2005) DNA elongation by the human DNA polymerase λ polymerase and terminal transferase activities are differentially coordinated by proliferating cell nuclear antigen and replication protein A. *J. Biol. Chem. 280*, 1971–1981.
39. Haracska, L., Unk, I., Johnson, R. E., Phillips, B. B., Hurwitz, J., Prakash, L., and Prakash, S. (2002) Stimulation of DNA synthesis activity of human DNA polymerase κ by PCNA. *Mol. Cell. Biol. 22*, 784–791.
40. Saeki, H., Siaud, N., Christ, N., Wiegant, W. W., van Buul, P. P., Han, M., Zdzienicka, M. Z., Stark, J. M., and Jasin, M. (2006) Suppression of the DNA repair defects of BRCA2-deficient cells with heterologous protein fusions. *Proc. Natl. Acad. Sci. U.S.A. 103*, 8768–8773.
41. Bochkarev, A., and Bochkareva, E. (2004) From RPA to BRCA2: Lessons from single-stranded DNA binding by the OB-fold. *Curr. Opin. Struct. Biol. 14*, 36–42.
42. Casteel, D. E., Zhuang, S., Zeng, Y., Perrino, F. W., Boss, G. R., Goulian, M., and Pilz, R. B. (2009) A DNA polymerase-α·primase cofactor with homology to replication protein A-32 regulates DNA replication in mammalian cells. *J. Biol. Chem. 284*, 5807–5818.
43. Miyake, Y., Nakamura, M., Nabetani, A., Shimamura, S., Tamura, M., Yonehara, S., Saito, M., and Ishikawa, F. (2009) RPA-like mammalian Ctc1-Stn1-Ten1 complex binds to single-stranded DNA and protects telomeres independently of the Pot1 pathway. *Mol. Cell 36*, 193–206.
44. Sun, J., Yu, E. Y., Yang, Y., Confer, L. A., Sun, S. H., Wan, K., Lue, N. F., and Lei, M. (2009) Stn1-Ten1 is an Rpa2-Rpa3-like complex at telomeres. *Genes Dev. 23*, 2900–2914.
45. Surovtseva, Y. V., Churikov, D., Boltz, K. A., Song, X., Lamb, J. C., Warrington, R., Leehy, K., Heacock, M., Price, C. M., and Shippen, D. E. (2009) Conserved telomere maintenance component 1 interacts with STN1 and maintains chromosome ends in higher eukaryotes. *Mol. Cell 36*, 207–218.
46. Richard, D. J., Bolderson, E., Cubeddu, L., Wadsworth, R. I., Savage, K., Sharma, G. G., Nicolette, M. L., Tsvetanov, S., McIlwraith, M. J., Pandita, R. K., Takeda, S., Hay, R. T., Gautier, J., West, S. C., Paull, T. T., Pandita, T. K., White, M. F., and Khanna, K. K. (2008) Single-stranded DNA-binding protein hSSB1 is critical for genomic stability. *Nature 453*, 677–681.
47. Li, Y., Bolderson, E., Kumar, R., Muniandy, P. A., Xue, Y., Richard, D. J., Seidman, M., Pandita, T. K., Khanna, K. K., and Wang, W. (2009) HSSB1 and hSSB2 form similar multiprotein complexes that participate in DNA damage response. *J. Biol. Chem. 284*, 23525–23531.
48. Millership, J. J., and Zhu, G. (2002) Heterogeneous expression and functional analysis of two distinct replication protein A large subunits from *Cryptosporidium parvum*. *Int. J. Parasitol. 32*, 1477–1485.
49. Sakaguchi, K., Ishibashi, T., Uchiyama, Y., and Iwabata, K. (2009) The multi-replication protein A (RPA) system: A new perspective. *FEBS J. 276*, 943–963.
50. Ishibashi, T., Kimura, S., and Sakaguchi, K. (2006) A Higher Plant Has Three Different Types of RPA Heterotrimeric Complex. *J. Biochem. 139*, 99–104.
51. Ishibashi, T., Kimura, S., Furukawa, T., Hatanaka, M., Hashimoto, J., and Sakaguchi, K. (2001) Two types of replication protein A 70 kDa subunit in rice, *Oryza sativa*: Molecular cloning, characterization, and cellular and tissue distribution. *Gene 272*, 335–343.
52. Toll-Riera, M., Bosch, N., Bellora, N., Castelo, R., Armengol, L., Estivill, X., and Alba, M. M. (2009) Origin of primate orphan genes: A comparative genomics approach. *Mol. Biol. Evol. 26*, 603–612.
53. Schittek, B., Hipfel, R., Sauer, B., Bauer, J., Kalbacher, H., Stevanovic, S., Schirle, M., Schroeder, K., Blin, N., Meier, F., Rassner, G., and Garbe, C. (2001) Dermcidin: A novel human antibiotic peptide secreted by sweat glands. *Nat. Immunol. 2*, 1133–1137.
54. Digweed, M., Gunthert, U., Schneider, R., Seyschab, H., Friedl, R., and Sperling, K. (1995) Irreversible repression of DNA synthesis in Fanconi anemia cells is alleviated by the product of a novel cyclin-related gene. *Mol. Cell. Biol. 15*, 305–314.
55. Martinez-Garay, I., Jablonka, S., Sutajova, M., Steuernagel, P., Gal, A., and Kutsche, K. (2002) A new gene family (FAM9) of low-copy repeats in Xp22.3 expressed exclusively in testis: Implications for recombinations in this region. *Genomics 80*, 259–267.
56. Tay, S. K., Blythe, J., and Lipovich, L. (2009) Global discovery of primate-specific genes in the human genome. *Proc. Natl. Acad. Sci. U.S.A. 106*, 12019–12024.
57. Menezo, Y., Jr., Russo, G., Tosti, E., El Mouatassim, S., and Benkhalifa, M. (2007) Expression profile of genes coding for DNA repair in human oocytes using pangenomic microarrays, with a special focus on ROS linked decays. *J. Assisted Reprod. Genet. 24*, 513–520.