Multiple competition reactions for RPA order the assembly of the DNA polymerase δ holoenzyme

Alexander Yuzhakov¹, Zvi Kelman²,
Jerard Hurwitz² and Mike O'Donnell^{1,3,4}

¹The Rockefeller University and ³Howard Hughes Medical Institute, 1230 York Avenue and ²Memorial Sloan-Kettering Cancer Institute, 1275 York Avenue, New York, NY 10021, USA

⁴Corresponding author
e-mail: odonnell@rockvax.rockefeller.edu

Processive extension of DNA in eukaryotes requires three factors to coordinate their actions. First, DNA polymerase α -primase synthesizes the primed site. Then replication factor C loads a proliferating cell nuclear antigen (PCNA) clamp onto the primer. Following this, DNA polymerase δ assembles with PCNA for processive extension. This report shows that these proteins each bind the primed site tightly and trade places in a highly coordinated fashion such that the primer terminus is never left free of protein. Replication protein A (RPA), the single-stranded DNA-binding protein, forms a common touchpoint for each of these proteins and they compete with one another for it. Thus these protein exchanges are driven by competition-based protein switches in which two proteins vie for contact with RPA.

Keywords: DNA polymerase δ /proliferating cell nuclear antigen/replication factor C/replication protein A

Introduction

Initiation of DNA replication requires multiple proteins to coordinate their actions on a small region of DNA (Kornberg and Baker, 1992). Replication is initiated by primase, a specialized RNA polymerase that synthesizes a short RNA primer of 8–12 nucleotides in both prokaryotes and eukaryotes. Following this, a ring-shaped sliding clamp protein is assembled around the primed site (Stukenberg *et al.*, 1991; Kong *et al.*, 1992). This assembly process is catalyzed by a multiprotein clamp loader that recognizes the primed template junction and couples ATP hydrolysis to open and close the protein ring around DNA (Kelman and O'Donnell, 1995). Finally, the replicative DNA polymerase assembles with the ring (Stukenberg *et al.*, 1991; Stillman, 1994; Naktinis *et al.*, 1996). As the DNA polymerase extends the primer, it drags the ring behind it. This continuous hold on DNA by the ring endows the polymerase with rapid speed and high processivity.

The coordinated action of these proteins in this multistep process has been examined previously in *Escherichia coli* (Yuzhakov *et al.*, 1999). The *E.coli* primase synthesizes the RNA primer and remains attached to it, protecting the primer from other proteins such as nucleases. Attachment of primase to DNA requires interaction with the single-stranded DNA-binding protein, SSB. Primase must be

displaced, however, in order for γ complex to perform its clamp loading function. A single subunit of γ complex is dedicated to this primase displacement task. The χ subunit of the γ complex binds SSB (Kelman *et al.*, 1998) and competes with primase for this protein–protein interaction, leading to dissociation of primase from its primed site. The clamp loader, having cleared primase from the primed site, is free to load the β subunit ring onto the DNA, after which it dissociates from β and DNA. DNA polymerase III core then couples with the β clamp for rapid and processive synthesis.

This report examines this initiation process in the human system. The eukaryotic primase, DNA polymerase α -primase complex (hereafter referred to as Pol α), is a four subunit assembly that combines its intrinsic RNA primase and DNA polymerase activities to synthesize an RNA–DNA hybrid primer of 35–50 residues (Murakami *et al.*, 1992). The five subunit clamp loader, replication factor C (RFC), couples hydrolysis of ATP to assembly of the ring-shaped sliding clamp, proliferating cell nuclear antigen (PCNA), onto a primed template (Tsurimoto and Stillman, 1990). The replicase, DNA polymerase δ (Pol δ), associates with the PCNA ring for efficient processive synthesis (Lee *et al.*, 1989, 1991; Tsurimoto and Stillman, 1990, 1991a; Tsurimoto *et al.*, 1990).

Previous work in this system has shown that Pol δ recruits the primed site from Pol α in an RFC/PCNA-dependent reaction termed 'polymerase switching' (Tsurimoto and Stillman, 1990, 1991a,b; Tsurimoto *et al.*, 1990; Waga and Stillman, 1994). Furthermore, there is evidence that the eukaryotic SSB, the heterotrimeric replication protein A (RPA) (Wold, 1997), may be involved in this process as it prevents Pol α action on the leading strand of the SV40 replicon (Tsurimoto and Stillman, 1991b).

The present study examines the human replication system for similarities and differences in how these proteins coordinate their action. In particular, we wished to understand the role of RPA in this switching process, as the *E.coli* study demonstrated a central role for SSB in coordinating transfer of the primer to the clamp loader. The findings reveal that the eukaryotic switching process is indeed coordinated by RPA, and that the overall process is a good deal more complicated than in *E.coli*. Similarly to the *E.coli* system, Pol α requires contact with RPA to remain stably attached to its primed site [that such contact exists was shown previously (Dornreiter *et al.*, 1992)]. Also like *E.coli*, RFC contacts RPA and disrupts the Pol α –RPA contact resulting in departure of Pol α from DNA. However, after this point, the behavior of the two systems diverges. Several subunits of RFC contact RPA and, after RFC has assembled PCNA onto a primed site, it does not dissociate but rather stays with the PCNA ring at the primed site through an RFC–RPA contact. The primed

site within this multiprotein complex is blocked from extension by a heterologous DNA polymerase, such as *E. coli* DNA polymerase I. However, Pol δ can access the primed site via contact with RPA. Pol δ competes with RFC for RPA, resulting in displacement of RFC from the 3' terminus, and replacement with Pol δ , which couples to the PCNA ring. Surprisingly, RFC is not displaced into solution but remains with the polymerase and sliding clamp in a holoenzyme structure.

Results

RFC displaces Pol α

Previous studies have shown that RFC and PCNA inhibit Pol α -catalyzed DNA synthesis (Tsurimoto and Stillman, 1991b; Eki *et al.*, 1992). Two mechanisms that may underlie inhibition include: RFC-PCNA displaces Pol α from DNA, or RFC-PCNA binds the 3' primer terminus thereby blocking chain extension by Pol α , but Pol α remains on the DNA. To distinguish these alternatives, we followed the location, on or off DNA, of these proteins. The first two panels of Figure 1A examine binding of Pol α to primed DNA. Pol α was pre-incubated with M13mp18 single-stranded DNA (ssDNA) primed with a DNA oligonucleotide in the presence of only two dNTPs, limiting extension to only a few nucleotides. To assay for stable association of Pol α with DNA, reactions were analyzed by gel filtration using BioGel A15m. This large pore resin excludes the large DNA (fractions 10–15), but includes proteins (fractions 15–30). Therefore, proteins bound to DNA resolve from proteins not bound to DNA. Pol α was detected in column fractions by its synthetic activity. In the first panel, RPA is absent; in the second panel, sufficient RPA is added to coat the ssDNA. The results show that Pol α does not bind primed DNA in the absence of RPA, but does remain attached to DNA in the presence of RPA. This experiment indicates that Pol α -RPA contact stabilizes interaction of Pol α with primed ssDNA, consistent with an earlier study documenting contact between Pol α and the 70 kDa subunit of RPA (Dornreiter *et al.*, 1992; Braun *et al.*, 1997). Pol α does not remain on unprimed RPA-coated ssDNA in the absence of a primer (triangles in the second panel of Figure 1A). Therefore, Pol α requires both a primed site and RPA for stable attachment to DNA. These experiments are performed in the presence of 70 mM NaCl. If no NaCl is added, Pol α co-migrates with RPA-coated ssDNA even in the absence of a primed site (data not shown).

To follow assembly of PCNA onto DNA, we used PCNA tagged with a kinase recognition motif allowing it to be labeled with ^{32}P . Addition of [^{32}P]PCNA in the absence of RFC has no effect on Pol α (third panel, Figure 1A). In the fourth panel, RFC was added. The analysis shows that RFC assembles [^{32}P]PCNA onto DNA and Pol α is displaced. This result indicates that inhibition of Pol α by RFC and PCNA is due to displacement of Pol α from DNA.

Displacement of Pol α from DNA may be a consequence of assembly of PCNA onto the primed site. Alternatively, RFC alone may displace Pol α from the primed site. This latter possibility is suggested by the ability of RFC alone, when supplied in excess, to inhibit Pol α (Eki *et al.*,

1992). Inhibition of Pol α by RFC is re-examined in Figure 1B using RPA-coated primed M13mp18 ssDNA.

Addition of either RFC or PCNA slightly inhibits Pol α , but the presence of both RFC and PCNA inhibits Pol α >80% (Figure 1B, left panel) (Tsurimoto and Stillman, 1991b). The second panel in Figure 1B demonstrates that a large amount of RFC inhibits Pol α in the absence of PCNA. The panel to the right, using a three subunit subassembly of RFC (RFC⁴⁰⁻⁷⁻³⁶) (Uhlmann *et al.*, 1997b), demonstrates that this three subunit form also inhibits Pol α . PCNA alone, even in large amounts, shows little effect on Pol α activity (second panel of Figure 1B). In experiments not presented here, we have determined that these large amounts of either RFC or RFC⁴⁰⁻³⁷⁻³⁶ displace Pol α from primed DNA. Therefore, Pol α displacement activity is inherent to RFC and does not require loading of PCNA onto DNA. However, PCNA increases the efficiency of RFC in displacing Pol α from DNA.

The previous experiments supplied a synthetic primed site for Pol α , as RPA inhibits *de novo* primer synthesis. However, SV40 large T antigen (T-Ag) supports *de novo* primer synthesis by Pol α on RPA-coated ssDNA (Collins and Kelly, 1991; Dornreiter *et al.*, 1992). In Figure 1C, Pol α was incubated with T-Ag on RPA-coated ssDNA in the presence of the four NTPs but in the absence of dNTPs. Under these conditions, T-Ag promotes formation of the RNA portion of the RNA-DNA hybrid primer synthesized by Pol α (in the range of 6–12 nucleotides; Collins and Kelly, 1991). Indeed, RNA synthesis is observed (middle panel of Figure 1C). Following the RNA synthesis phase, RFC and PCNA were added, and then the reaction was analyzed by gel filtration. The result, in Figure 1C, shows that RFC-PCNA is not capable of displacing Pol α from ssDNA primed using only NTPs (first panel, squares). However, RFC-PCNA displaced Pol α after incubation of Pol α and T-Ag with both NTPs and dNTPs (circles in the first panel of Figure 1C). Thus, the full RNA-DNA-primed product of Pol α is needed for RFC-PCNA to dislodge Pol α from RPA-coated DNA.

RFC binds RPA

How does RFC displace Pol α from DNA? The experiments in Figure 1A showed that Pol α requires RPA for stable attachment to DNA. One possible mechanism of RFC-mediated displacement of Pol α is an interaction of RFC with RPA that severs the connection between Pol α and RPA.

In Figure 2, we examined RFC and each of its subunits for interaction with RPA-coated ssDNA. To follow these proteins, the five genes encoding the subunits of RFC were translated *in vitro* using [^{35}S]methionine, and the resulting complex was separated from unassembled subunits by gel filtration. RFC prepared in this fashion is active in loading PCNA onto DNA, and in replication assays with Pol δ (data not shown). In addition, each ^{35}S -labeled RFC subunit was prepared individually and separated from free label by gel filtration. These preparations are shown in Figure 2A, as is an autoradiogram of an SDS-polyacrylamide gel of the pooled fractions from each preparation. In Figure 2B, these labeled proteins were examined for their ability to interact with ssDNA with and without RPA. Radiolabeled proteins were mixed with M13mp18 ssDNA either in the absence (circles) or

Fig. 2. RFC interacts with RPA. (A) Gel filtration profiles of [³⁵S]RFC and [³⁵S]subunits of RFC prepared by *in vitro* transcription/translation. Column fractions were pooled (hatched) and analyzed by SDS-PAGE followed by autoradiography (insert). Free [³⁵S]Met eluted later than fraction 50. (B) The interaction of [³⁵S]RFC and [³⁵S]-labeled subunits of RFC with ssDNA was analyzed by gel filtration. ○, absence of RPA; ■, presence of RPA. Controls in which no DNA was added are shown for RFC and p140 (△).

lacking RPA gave little or no signal. The earlier result, that RFC⁴⁰⁻³⁷⁻³⁶ displaces Pol α from DNA, combined with the finding that of these three subunits only p40 binds RPA, suggests that the p40-RPA contact is sufficient to displace Pol α from RPA. The need for a large excess of RFC to displace Pol α from DNA may be explained by the need to decorate all the RPA with RFC in order to also bind the RPA molecule that interacts with Pol α. PCNA, bound to RFC, may direct the clamp loader to the primed site, thus increasing the efficiency of RFC in displacing Pol α from DNA.

The subunit of RPA that interacts with RFC is identified in Figure 3B. Wells were coated with either the p70 subunit of RPA, or the p34-p14 complex, then [³⁵S]RFC (or [³⁵S]-labeled subunits) was added, followed by washing. The result shows that three RFC subunits (p140, p40 and p38) interact with the p70 subunit of RPA. Also shown in Figure 3B is a negative control using immobilized *E. coli* SSB (no appreciable interaction).

RFC and Pol α compete for RPA

Figures 2 and 3A demonstrate an interaction of RFC with RPA. Perhaps RFC displaces Pol α from DNA by severing the Pol α-RPA contact needed to hold Pol α to DNA. To

Fig. 3. RFC binds directly to RPA and competes with Pol α for RPA. (A) [³⁵S]RFC or [³⁵S]subunits of RFC were added to immobilized RPA. (B) Either p70 (top row), the p34-p14 complex (middle row) or *E. coli* SSB (bottom row) were immobilized to wells marked with a plus sign. Then [³⁵S]RFC or [³⁵S]-labeled RFC subunits were added, followed by washing and autoradiography. (C) Pol α and RFC compete for RPA. Lanes 1-7: [³⁵S]RFC was mixed with increasing amounts of Pol α then analyzed for interaction with immobilized RPA. Lane 8 is a specificity control in which RPA was omitted. Lane 9 is a specificity control showing that addition of a large excess of RPA to [³⁵S]RFC in solution lowers the signal of [³⁵S]RFC bound to immobilized RPA. Lane 10 tests for interaction between [³⁵S]RFC and immobilized Pol α.

test this, we designed an experiment to determine whether RFC and Pol α bind RPA in an exclusive fashion. In Figure 3C, RPA was immobilized to wells, and mixtures of [³⁵S]RFC with different amounts of Pol α were added, followed by washing and autoradiography. If both Pol α and RFC can bind RPA at the same time, the signal of bound [³⁵S]RFC will remain unchanged. On the other hand, if Pol α and RFC compete for RPA, the signal of [³⁵S]RFC will decrease as the concentration of Pol α is increased.

The result, in Figure 3C, demonstrates that as the concentration of Pol α is increased, the amount of [³⁵S]RFC retained on the immobilized RPA decreases. Hence, Pol α and RFC compete for RPA, consistent with a mechanism whereby RFC displaces Pol α from a primed site by competition for RPA. Whether such competition is via an allosteric mechanism or overlapping binding sites cannot be distinguished by these experiments. The large amount of Pol α needed to compete [³⁵S]RFC off RPA may reflect the biology of the interaction, as in the cell the competition is expected to be the reverse (i.e. RFC must compete Pol α from RPA and thus RFC probably

binds RPA more tightly than Pol α , see Discussion). The last well demonstrates that RFC does not bind immobilized Pol α , indicating that RFC does not displace Pol α by contacting it directly.

RFC remains with PCNA at the primed site

RFC is more efficient in loading PCNA onto *E. coli* SSB-coated primed ssDNA than onto RPA-coated primed ssDNA (N. Yao and M. O'Donnell, unpublished). A possible explanation for this observation is that RPA binds RFC, preventing it from acting in a catalytic fashion. Indeed, footprinting studies detect RFC in complex with PCNA at a primer terminus (Tsurimoto and Stillman, 1991a). Stoichiometric use of RFC predicts that only one PCNA clamp will be loaded onto a primed DNA when RPA is used. However, RFC does not interact with *E. coli* SSB, and thus may act catalytically to load multiple PCNA clamps onto *E. coli* SSB-coated primed DNA. These predictions are tested in Figure 4 using [32 P]PCNA, RFC and either RPA- or SSB-coated ssDNA primed with a 90mer DNA oligonucleotide. Several [32 P]PCNA clamps are assembled onto DNA coated with *E. coli* SSB, whereas approximately one [32 P]PCNA clamp is assembled onto DNA coated with RPA (Figure 4A). A similar experiment using a DNA 15mer primer is shown in Figure 4B. This length is only sufficient to accommodate one PCNA ring (PCNA is the thickness of one turn of B-form DNA) and therefore only one PCNA clamp should be loaded onto DNA whether it is coated with RPA or SSB. The result demonstrates that approximately one PCNA clamp is assembled onto this substrate whether SSB or RPA is used. These findings support the hypothesis that RPA holds RFC on the primed site with PCNA and prevents it from acting catalytically. This conclusion is substantiated further by experiments described below.

Stable interaction of RFC with PCNA at a primed site may protect the 3' terminus from reaction with other proteins. This is tested in Figure 4C by examining whether RFC and PCNA protect the 3' end from extension by *E. coli* Pol I Klenow fragment in an RPA-dependent fashion. The result shows that RFC prevents Pol I action on RPA-coated ssDNA (circles, right panel), but not on *E. coli* SSB-coated ssDNA (circles, left panel). Control experiments lacking RFC show similar Pol I extension rates on SSB- and RPA-coated DNA (squares in the left and right panels). These results indicate that RPA holds RFC on the primed site with PCNA and protects the 3' end from extension by Pol I. Further experiments that examine RFC directly for its presence on RPA- or SSB-coated ssDNA support this conclusion (shown later in Figure 6).

The effect of Pol δ on these reactions is presented in Figure 4D. The results show that Pol δ functions with RFC and PCNA to extend primed sites whether SSB or RPA is used. Hence, Pol δ is capable of recruiting the primer end to which RFC-PCNA is attached. How does Pol δ gain access to this primer terminus while Pol I cannot?

Pol δ competes with RFC for RPA and for PCNA

One mechanism by which Pol δ may recruit the 3' end from RFC is that Pol δ binds RPA and competes with RFC for this contact, thereby releasing RFC from the

3' end. To test this hypothesis, we examined Pol δ for interaction with RPA. In Figure 5A, [35 S]-labeled Pol δ was produced by *in vitro* translation of both genes encoding the two subunit form of human Pol δ and then [35 S]Pol δ was isolated from free label and unassociated monomer units by gel filtration (Figure 5A). Also shown in Figure 5A is an autoradiogram of an SDS-polyacrylamide gel of the pooled fractions (shaded region) containing the [35 S]Pol δ .

[35 S]Pol δ is examined for interaction with RPA in Figure 5B. In lane 1, RPA was immobilized to the well and then [35 S]Pol δ was added, followed by washing. The result shows a strong signal of [35 S]Pol δ bound to immobilized RPA. In the absence of RPA (lane 2), no appreciable non-specific interaction of [35 S]Pol δ with the well was detected. Comparison of lanes 3 and 4 indicates that the [35 S]Pol δ -RPA interaction is mediated by the p70 subunit of RPA and not the p34-p14 subcomplex of RPA. Lanes 6 and 7 test for interaction between [35 S]Pol δ and either Pol α (lane 6) or RFC (lane 7). The results show no detectable interaction between Pol δ and Pol α , whereas they do indicate interaction between Pol δ and RFC, consistent with interaction between Pol δ and the p40 subunit of RFC (Pan *et al.*, 1993).

Next, we examined Pol δ and RFC to determine if they compete for RPA. In Figure 5B, [35 S]Pol δ was mixed with increasing amounts of RPA, and then incubated in wells containing immobilized RFC, followed by washing. The results demonstrate that as the concentration of RPA is increased, the amount of [35 S]Pol δ retained by immobilized RFC decreases. This result indicates that RFC and Pol δ compete for RPA, since if they could both bind RPA at the same time, the amount of [35 S]Pol δ bound to RFC should be unaffected by the presence of RPA.

The experiment of Figure 5D tests for competition between Pol δ and RFC for PCNA. Here, RFC was immobilized to wells and mixtures of [35 S]Pol δ with varying amounts of PCNA were added, followed by washing. The results indicate that as the concentration of PCNA is increased, the amount of [35 S]Pol δ that remains in the well decreases, suggesting that RFC and Pol δ compete for PCNA. This finding is consistent with results of other studies showing that RFC and Pol δ interact with the same side of the PCNA ring (Oku *et al.*, 1998).

Fate of RFC after Pol δ associates with PCNA

Competition between RFC and Pol δ for RPA (and PCNA) implies that when Pol δ recruits the 3' end from RFC, RFC is displaced from these proteins. One may presume that RFC dissociates into solution. However, the fact that RFC and Pol δ interact, combined with the observation that three different subunits of RFC contact RPA, raises the possibility that RFC may remain with Pol δ and PCNA through one or more of these additional contacts. Indeed, an earlier study observed that ATP γ S, an inhibitor of RFC, blocks ongoing DNA synthesis, suggesting that RFC travels with Pol δ and PCNA (Tsurimoto and Stillman, 1991b).

The experiments of Figure 6 were designed to examine the fate of RFC after Pol δ binds PCNA at a primed site. A 5' biotin end-labeled DNA 94mer that forms a hairpin-primed template (see the scheme in Figure 6) was coated with either RPA (left panels) or *E. coli* SSB (right panels),

Fig. 4. RPA holds RFC with PCNA on DNA. (A) RFC was used to load [32 P]PCNA onto 90mer-primed M13mp18 ssDNA coated with *E.coli* SSB (●) or RPA (○) followed by gel filtration. (B) Analysis of [32 P]PCNA loaded onto RPA- or SSB-coated DNA was performed as in (A) except that a DNA 15mer was used to prime the ssDNA. (C) Effect of RFC and PCNA on Pol I primer extension on M13mp18 ssDNA coated with either *E.coli* SSB (left) or RPA (right). ■, no RFC added; ○, RFC was present. (D) Pol δ extends a primer on M13mp18 ssDNA coated with either *E.coli* SSB (left panel), or RPA (right panel). Reactions contain PCNA (■) or PCNA and RFC (○).

then RFC was added. Following this, avidin-coated beads were added and then washed and analyzed for radiolabeled protein. Three sets of experiments were performed using either [35 S]RFC, [32 P]PCNA or [35 S]Pol δ (in each set, the other two proteins were unlabeled). Figure 6A and B presents results using [35 S]RFC with unlabeled PCNA (and Pol δ where indicated) on either RPA- or SSB-coated DNA. In both panels, lane 1 shows that [35 S]RFC is retained on the hairpin DNA regardless of whether RPA or SSB is used (left and right panels, respectively). Retention of [35 S]RFC on the *E.coli* SSB-coated hairpin template is probably mediated through the non-specific DNA-binding activity of the p140 subunit of RFC to the duplex portion of the DNA. In the case of the RPA-coated hairpin template, [35 S]RFC may bind RPA as well. Addition of a 20-fold molar excess of a non-biotinylated 94mer capture oligonucleotide (also coated with either

RPA or SSB) in a 2 min incubation preceding the avidin beads lowered [35 S]RFC binding to both RPA- and SSB-coated hairpin templates (lane 2). Presumably this incubation provided sufficient time for [35 S]RFC to dissociate from the biotinylated primer template whereupon it became trapped by the excess capture oligonucleotide. Hence RFC bound to DNA $\pm$ RPA is not sufficiently stable to resist challenge with capture oligonucleotide.

Lane 3 shows the effect of adding PCNA followed by addition of capture DNA. The presence of PCNA results in retention of [35 S]RFC on the RPA-coated hairpin template, even in the presence of excess RPA-coated capture oligonucleotide (compare lanes 2 and 3 in Figure 6A).

Use of *E.coli* SSB (Figure 6B, lane 3) provided no detectable stability to the association of [35 S]RFC with PCNA on DNA. We examined the hairpin template for

Fig. 5. Pol δ competes with RFC for RPA and PCNA. (A) [³⁵S]Pol δ was produced by *in vitro* transcription/translation, gel filtered and the pool (hatched) was analyzed by SDS-PAGE/autoradiography. (B) [³⁵S]Pol δ interacts with immobilized RPA: lane 1, RPA; lane 2, no immobilized protein control; lane 3, p70 subunit of RPA; lane 4, RPA p34-p14 complex; lane 5, PCNA; lane 6, Pol α ; lane 8, RFC. (C) Pol δ competes with RFC for RPA. [³⁵S]Pol δ , with increasing amounts of RPA, was analyzed for interaction with immobilized RFC. (D) Pol δ and RFC compete for PCNA. [³⁵S]Pol δ , with increasing amounts of PCNA, was analyzed for interaction with immobilized RFC.

the presence of PCNA using [³²P]PCNA and unlabeled RFC (Figure 6C and D). The result shows that [³²P]PCNA is retained with RFC on the RPA-coated hairpin template (Figure 6C, lane 3), but neither PCNA nor RFC is retained on the SSB-coated hairpin template (Figure 6B and D, lane 3). It may be presumed that [³²P]PCNA, once loaded onto the template, requires RFC to prevent the PCNA ring from simply sliding off the duplex end of the hairpin template. These results imply that RFC-RPA contact is needed to stabilize RFC and PCNA on the hairpin template. This finding is consistent with a previous study using a similar experimental design that showed RFC has a half-life of ~5 min with PCNA on an RPA-coated primed template (Waga and Stillman, 1998). We have measured the half-life of [³⁵S]RFC with PCNA on the RPA-coated hairpin DNA by varying the time of incubation with the capture oligonucleotide prior to addition of avidin beads, and observe a $t_{1/2}$ of ~30 min (data not shown). There are several differences between the assays of the earlier report and this study, which may explain the greater half-life of RFC with PCNA and RPA. Among the most likely is that the capture oligonucleotide was coated with RPA in the

Fig. 6. Assembly of DNA polymerase δ holoenzyme at a primed site. A biotin end-labeled hairpin primer template was incubated with proteins, one of which is radiolabeled, and then removed from solution upon adding avidin-coated beads. Proteins that remain bound to DNA were quantitated by liquid scintillation. DNA was treated with either RPA (left panels) or *E. coli* SSB (right panels), then proteins were added and incubated for 3 min before adding avidin-coated beads. Addition of capture oligonucleotide was followed by a further 2 min incubation prior to adding the beads. (A) and (B) utilized [³⁵S]RFC, (C) and (D) utilized [³²P]PCNA, and (E) and (F) utilized [³⁵S]Pol δ .

current study. Hence, the biotinylated hairpin template should not be depleted for RPA during the experiment through transfer of RPA to the capture oligonucleotide. These results are also consistent with the experiments in Figure 4, indicating that RFC remains with PCNA on RPA-coated primed ssDNA, but not on SSB-coated primed ssDNA.

Next, the effect of adding Pol δ was examined (lane 4). Since Pol δ competes with RFC for RPA and PCNA (Figure 5), one may expect Pol δ to displace RFC from DNA. In Figure 6A, lane 4, Pol δ was added to the RPA-coated hairpin template along with PCNA and [³⁵S]RFC, followed by addition of capture oligonucleotide 2 min later. The results indicate that Pol δ does not displace RFC from PCNA, but that [³⁵S]RFC remains attached to DNA and possibly forms a Pol δ -RFC-PCNA holoenzyme. The comparable experiment using SSB in place of RPA shows that [³⁵S]RFC does not stably associate with PCNA and Pol δ (Figure 6B, lane 4). Thus, RPA is needed to form the putative Pol δ -RFC-PCNA holoenzyme. The presence of each protein in this putative holoenzyme was examined using [³²P]PCNA (Figure 6C, lane 4) and [³⁵S]Pol δ (Figure 6E, lane 4). The results show that both PCNA and RFC are indeed present with

Fig. 7. Assembly of Pol δ holoenzyme involves two competition switches in series. Pol α requires contact with RPA for stable attachment to its primed site (A). RFC binds RPA and competes Pol α from RPA, resulting in release of Pol α from DNA (B). RFC assembles PCNA onto DNA and remains with it via interaction with RPA (B). Pol δ competes with RFC for both RPA and PCNA, thereby recruiting PCNA and the 3' terminus for chain extension (C). RFC is retained in the resulting Pol δ holoenzyme.

Pol δ on DNA, supporting the presence of these proteins in a Pol δ –RFC–PCNA holoenzyme. The comparable experiments using *E. coli* SSB, shown in lane 4 of Figure 6B, D and F, indicate that Pol δ and PCNA form a complex on DNA, but that RFC is not present in this complex. Presumably Pol δ is associated with the PCNA clamp, thereby preventing it from sliding off the back of the hairpin template. The stability of these protein complexes (by varying the time of incubation with capture oligonucleotide) indicated the following half-times: Pol δ –RFC–PCNA–RPA ($t_{1/2} = 30$ min), Pol δ –PCNA (i.e. using *E. coli* SSB) ($t_{1/2} = 10$ min), indicating that RFC and RPA stabilize Pol δ with PCNA on DNA. A control reaction using Pol δ and RFC without PCNA shows that [35 S]Pol δ does not bind DNA stably in the absence of PCNA (Figure 6E and F, lane 5). As capture oligonucleotide was not added to this reaction, the [35 S]RFC remains bound to the hairpin template (Figure 6A and B, lane 5). In lane 6, dNTPs were supplied, allowing replication of the hairpin template. The results, in lane 6 of Figure 6A, C and E, show that [32 P]PCNA, [35 S]RFC and [35 S]Pol δ were all lost from the biotinylated DNA.

Discussion

Two serial competition switches remodel Pol α and Pol δ

The eukaryotic polymerase/primase, Pol α , is exchanged for Pol δ at a primed template junction in an RFC–PCNA-mediated reaction called polymerase switching (Tsurimoto *et al.*, 1990; Tsurimoto and Stillman, 1991b; Stillman, 1994; Waga and Stillman, 1994). This report reveals the mechanistic path by which these protein–DNA complexes are redefined in time as one protein is exchanged for another. Each protein exchange is driven by a competition-based protein switch in which two proteins vie for interaction with a third protein. For the Pol α to Pol δ switch, two competition reactions occur in series as illustrated in Figure 7. In Figure 7A, Pol α contacts RPA for firm attachment to its primed site. This report shows that RFC binds RPA and disrupts the Pol α –RPA interaction, leading to dissociation of Pol α from its primed site (Figure 7A→B). The primed site is then available for RFC to load the PCNA ring onto DNA, after which RFC remains with PCNA through contact with RPA. In the second remodeling step, Pol δ assembles with PCNA, which presumably necessitates movement of RFC from the 3' terminus for extension by Pol δ (Figure 7B→C). This second switch involves a dual competition in which Pol δ binds both PCNA and RPA, and it competes with

RFC for these same two proteins. RFC remains with the Pol δ –PCNA complex.

This report shows that RFC, attached to RPA at a primed site, prevents the primed site from extension by a heterologous DNA polymerase (i.e. Pol I). Contact between RPA and RFC is needed for RFC to remain with PCNA on primed DNA, as RFC does not remain if *E. coli* SSB is substituted for RPA. This conclusion is consistent with a study showing that RFC dissociates from DNA after assembly of PCNA onto *E. coli* SSB-coated DNA (Podust *et al.*, 1998), but remains with PCNA on RPA-coated DNA (Waga and Stillman, 1998). This finding is also consistent with RPA action as a specificity factor in enhancing formation of RFC–PCNA complex on primed DNA (Tsurimoto and Stillman, 1991a).

The *E. coli* primase to polymerase switch

The mechanism by which *E. coli* primase and Pol III trade places on DNA also involves a competition reaction, although it is simpler than in the human system (Yuzhakov *et al.*, 1999). The *E. coli* primase requires interaction with SSB for stable attachment to its primed site. One subunit of the γ complex clamp loader binds SSB and competes with primase for this contact, causing primase to dissociate from DNA. After assembling the β clamp onto DNA, γ complex dissociates from the primed site, making room for Pol III core to associate with the ring for processivity (Turner *et al.*, 1999). Pol III core and γ complex compete for the β ring, but the γ complex does not remain attached to β after transfer to DNA and thus would seem to make such competition unnecessary. However, the function of competition between core and γ complex for β is to prevent the unloading activity of γ complex from removing β from DNA while Pol III core is using β for processive extension (Naktinis *et al.*, 1996). Furthermore, in the Pol III holoenzyme, γ complex is attached to Pol III core through the τ subunit (Onrust *et al.*, 1995). This proximity may provide specificity for Pol III core, eliminating the need for γ complex to remain at the primed site in order to protect it from the action of other proteins (e.g. nucleases).

How are these competition reactions ordered?

Pol α , Pol δ , RFC, PCNA and RPA are present together in the cell, yet several interactions among them are competitive. How are these competition reactions ordered to bring about the smooth transition from primer synthesis to assembly of Pol δ holoenzyme on a primed site? It seems reasonable to expect that the product of one reaction leads to an increased affinity of the next protein component

for the product, thereby competing off the earlier protein and providing an ordered flow of events: from (i) primer formation to (ii) PCNA clamp assembly to (iii) association of Pol δ with PCNA. For example, RFC (along with PCNA) probably interacts more tightly with RPA at a primed template junction than with RPA on ssDNA. Thus, displacement of Pol α by RFC would be more efficient after Pol α has synthesized a primer. The results of this study indicate that this is indeed the case, as RFC does not displace Pol α at a stage prior to completion of the primer. Inability to displace Pol α prior to completion of the primer may involve a more complex association between the small RNA primer, RPA and Pol α . Previous studies reveal interaction between p34 of RPA and nascent RNA–DNA primers during early stages in lagging strand synthesis. This interaction is not detected with more advanced intermediates (Mass *et al.*, 1998).

Generalization of these principles to protein remodeling during origin activation

Studies in *E. coli*, phage λ and yeast indicate that protein remodeling occurs during origin activation. λ P protein binds *E. coli* DnaB helicase and assembles with it at the origin along with λ O protein (Alfano and McMacken, 1989). λ P inactivates DnaB helicase, preventing extensive DNA unwinding. Heat shock proteins, DnaJ and DnaK, release λ P from the origin thereby activating DnaB for DNA unwinding (Alfano and McMacken, 1989). It seems plausible that a competition-based switch underlies displacement of λ P from DnaB in which the DnaJ–DnaK complex and DnaB helicase compete for λ P. In this scenario, DnaJ–DnaK would win the competition in a reaction fueled by ATP, thereby freeing DnaB helicase from λ P.

In the *E. coli* system, DnaC protein (functionally analogous to λ P) binds DnaB and promotes its assembly at the *E. coli* origin, *oriC*, along with DnaA and other proteins that bind the origin (Kornberg and Baker, 1992). In the process of origin activation, DnaC is eliminated from the orisome, allowing DnaB to unwind DNA (Funnell *et al.*, 1987). Does a component of the orisome compete with DnaC for DnaB, causing DnaC to be ejected? It has been shown that DnaB interacts with DnaA (Marszalek and Kaguni, 1994). Thus, DnaA is a potential candidate as a member of a competition-based switch whereby DnaA competes with DnaC for DnaB, leading to release of DnaC from DNA.

In yeast, *cdc6* is required for assembly of the hexameric MCM complex at the origin. In conversion of the pre-replicative complex to the replication complex, *cdc6* is lost from the origin followed by movement of MCMs from the origin (Aparicio *et al.*, 1997; Waga and Stillman, 1998). It has been proposed that MCMs act as a helicase (Ishimi, 1997), and that *cdc6* loads MCMs onto DNA, analogously to λ P and *E. coli* DnaC (Perkins and Diffley, 1998). It seems possible that remodeling of *cdc6* may be rooted in competitive interactions among proteins at the origin of replication.

Competition-based switches in other DNA metabolic processes

Competition-based switches may operate in other areas of DNA enzymology in which multiple proteins are involved.

For example, during eukaryotic excision repair, several proteins function in a localized area to recognize a lesion, cut and remove the incised DNA, then fill in the ssDNA gap (Sancar, 1996). RPA, PCNA and RFC are involved in this reaction and may participate in competitive switching reactions during this repair process.

Mismatch repair also utilizes the clamp, clamp loader and SSB (Modrich, 1997). In this process, several proteins utilize the 3' terminus, which is sequestered away from DNA polymerase, excised past the mismatch by a nuclease, and then the clamp and clamp loading system of Pol III holoenzyme regains the 3' end to refill the gap. Perhaps these successive transfers of the 3' terminus from one protein to the next are ordered through competition-based protein switches such as described in this report.

Materials and methods

Materials

Labeled nucleotides were from New England Nuclear; unlabeled nucleotides from Pharmacia-LKB; [35 S]Met (1000 Ci/mmol) and [3 H]Leu (184 Ci/mmol), Amersham; Klenow fragment, New England BioLabs; and T7 RNA polymerase and RNasin, Promega. M13mp18 ssDNA was prepared as described (Turner and O'Donnell, 1995), and primed with a DNA 30mer (Stukenberg *et al.*, 1991). Proteins were purified as described: Pol α (Eki *et al.*, 1992), RPA (Lee *et al.*, 1989), p70 and p34–p14 complex of RPA (Zhang *et al.*, 1998), RFC (Yao *et al.*, 1996), Pol δ (Lee *et al.*, 1991), *E. coli* SSB (Studwell and O'Donnell, 1990), RFC^{40,37,36} (Cai *et al.*, 1997). Buffers were: TPBS: 0.1 M NaH₂PO₄ pH 7.2, 0.15 M NaCl and 0.1% Tween-20. Replication buffer: 20 mM Tris–HCl pH 7.5, 8 mM MgCl₂, 0.7 mM ATP, 200 μ M each of CTP, GTP and UTP, 0.1 mM EDTA, 5 mM dithiothreitol (DTT), 4% glycerol, 40 μ g/ml bovine serum albumin (BSA) and 50 mM NaCl. Column buffer: 20 mM Tris–HCl pH 7.5, 8 mM MgCl₂, 5 mM DTT, 5% glycerol, 50 μ g/ml BSA. Incubation buffer: 20 mM Tris–HCl pH 7.5, 1 mM EDTA, 2 mM DTT, 5 mM MgCl₂, 150 mM NaCl, 20% glycerol, 2 mM CaCl₂, 1% gelatin, 10 μ g/ml DNase.

Radiolabeled proteins

Human PCNA^{PK} contains an N-terminal 32 residue tag (Kelman *et al.*, 1995) having a kinase recognition motif for labeling using [γ - 32 P]ATP (Stukenberg *et al.*, 1994). Human RFC, RFC subunits, two subunit human Pol δ and human RPA p70 and p34–p14 complex were radiolabeled with 35 S or 3 H by *in vitro* transcription/translation (Promega). Labeling was performed using [35 S]Met or [3 H]Leu. Reactions (200 μ l) were incubated at 30°C for 1.5 h, then NaCl was added to 1.66 M and reactions were applied to Superose 12 (individual subunits) or Superose 6 (RFC complex and Pol δ) equilibrated with column buffer + 500 mM NaCl at 4°C. Fractions of 200 μ l were collected and 5 μ l aliquots were analyzed by scintillation counting and by 8% SDS–PAGE/autoradiography.

Specific activities were determined from the known amounts of Met and Leu in each protein. Concentrations of Met (9.15 μ M) and Leu (5.12 μ M) in the reticulocyte lysate were determined by amino acid analysis (Rockefeller Protein/DNA Technology Center). Specific activities were (in c.p.m./fmol): [35 S]RFC, 13 000; [35 S]p140, 5600; [35 S]p40, 2400; [35 S]p38, 2400; [35 S]p37, 800; [35 S]p36, 1800; [3 H]RFC, 217 115; [35 S]Pol δ , 12 000; [3 H]RPA p70, 37 195; [3 H]RPA (p34–p14), 21 625.

Interaction of RFC with RPA

Assays were performed in 96-well vinyl plates (Costar). A 100 μ l aliquot of RPA (0.12 μ g/ml), or 0.1 μ g/ml p70 or p34–p14 was incubated in each well for 12 h at 4°C in incubation buffer. Solutions were removed, wells were washed four times using 100 μ l of phosphate-buffered saline (PBS) containing 0.1% Tween-20 (TPBS) and then blocked with 200 μ l of 2% rabbit reticulocyte lysate in TPBS for 3 h at 22°C. Wells were washed three times using 100 μ l of TPBS and then [35 S]protein in incubation buffer was added to each well. The same amount of total radioactivity was added to all the wells of a row. The first row contained 30 000 c.p.m., the second 15 000 c.p.m. and the third 3000 c.p.m. The plate was incubated for 1 h at 22°C, then washed three times with TPBS, dried for 2 h at 22°C and analyzed using a phosphorimager.

Interaction of Pol δ with RPA, RFC and PCNA

Interaction of RFC with RPA bound to ssDNA

Displacement of Pol α from primed ssDNA coated with RPA

Inhibition of Pol α by RFC and PCNA

Polymerase extension assays in the presence of RFC

Assembly of PCNA onto primed DNA

Assembly of RFC, Pol δ and PCNA on a small hairpin template

Acknowledgements

References

- 6198

- the simian virus 40 origin of replication by the combined action of DNA polymerases α and δ . *Proc. Natl Acad. Sci. USA*, **86**, 7361–7365.
- Lee, S.-H., Kwong, A.D., Pan, Z.Q. and Hurwitz, J. (1991) Studies on the activator 1 protein complex, an accessory factor for proliferating cell nuclear antigen-dependent DNA polymerase δ . *J. Biol. Chem.*, **266**, 594–602.
- Marszalek, J. and Kaguni, J.M. (1994) DnaA protein directs the binding of DnaB protein in initiation of DNA replication in *Escherichia coli*. *J. Biol. Chem.*, **269**, 4883–4890.
- Mass, G., Nethanel, T. and Kaufmann, G. (1998) The middle subunit of replication protein A contacts growing RNA–DNA primers in replicating simian virus 40 chromosomes. *Mol. Cell. Biol.*, **18**, 6399–6407.
- Modrich, P. (1997) Strand-specific mismatch repair in mammalian cells. *J. Biol. Chem.*, **272**, 24727–24730.
- Murakami, Y., Eki, T. and Hurwitz, J. (1992) Studies on the initiation of simian virus 40 replication *in vitro*: RNA primer synthesis and its elongation. *Proc. Natl Acad. Sci. USA*, **89**, 952–956.
- Naktinis, V., Turner, J. and O'Donnell, M. (1996) A molecular switch in a replication machine defined by an internal competition for protein rings. *Cell*, **84**, 137–145.
- Oku, T., Ikeda, S., Sasaki, H., Fukuda, K., Morioka, H., Ohtsuka, E., Yoshikawa, H. and Tsurimoto, T. (1998) Functional sites of human PCNA which interact with p21 (Cip1/Waf1), DNA polymerase δ and replication factor C. *Genes Cells*, **3**, 357–369.
- Onrust, R., Finkelstein, J., Turner, J., Naktinis, V. and O'Donnell, M. (1995) Assembly of a chromosomal replication machine: two DNA polymerases, a clamp loader and sliding clamps in one holoenzyme particle. *J. Biol. Chem.*, **270**, 13366–13377.
- Pan, Z.Q., Chen, M. and Hurwitz, J. (1993) The subunits of activator 1 (replication factor C) carry out multiple functions essential for proliferating-cell nuclear antigen-dependent DNA synthesis. *Proc. Natl Acad. Sci. USA*, **90**, 6–10.
- Perkins, G. and Diffley, J.F.X. (1998) Nucleotide-dependent prereplicative complex assembly by Cdc6p, a homolog of eukaryotic and prokaryotic clamp-loaders. *Mol. Cell*, **2**, 23–32.
- Podust, V.N., Tiwari, N., Stephan, S. and Fanning, E. (1998) Replication factor C disengages from proliferating cell nuclear antigen (PCNA) upon sliding clamp formation, and PCNA itself tethers DNA polymerase δ to DNA. *J. Biol. Chem.*, **273**, 31992–31999.
- Sancar, A. (1996) DNA excision repair. *Annu. Rev. Biochem.*, **65**, 43–81.
- Stillman, B. (1994) Smart machines at the DNA replication fork. *Cell*, **78**, 725–758.
- Studwell, P.S. and O'Donnell, M. (1990) Processive replication is contingent on the exonuclease subunit of DNA polymerase III holoenzyme. *J. Biol. Chem.*, **265**, 1171–1178.
- Stukenberg, P.T., Studwell-Vaughan, P.S. and O'Donnell, M. (1991) Mechanism of the sliding β -clamp of DNA polymerase III holoenzyme. *J. Biol. Chem.*, **266**, 11328–11334.
- Stukenberg, P.T., Turner, J. and O'Donnell, M. (1994) An explanation for lagging strand replication: polymerase hopping among DNA sliding clamps. *Cell*, **78**, 877–887.
- Tsurimoto, T. and Stillman, B. (1990) Functions of the replication factor C and proliferating cell nuclear antigen: functional similarity of DNA polymerase accessory proteins from human cells and bacteriophage T4. *Proc. Natl Acad. Sci. USA*, **87**, 1023–1027.
- Tsurimoto, T. and Stillman, B. (1991a) Replication factors required for SV40 DNA replication *in vitro*. I. DNA structure-specific recognition of a primer–template junction by eukaryotic DNA polymerases and their accessory proteins. *J. Biol. Chem.*, **266**, 1950–1960.
- Tsurimoto, T. and Stillman, B. (1991b) Replication factors required for SV40 DNA replication *in vitro*. II. Switching of DNA polymerase α and δ during initiation of leading and lagging strand synthesis. *J. Biol. Chem.*, **266**, 1961–1968.
- Tsurimoto, T., Melendy, T. and Stillman, B. (1990) Sequential initiation of lagging and leading strand synthesis by two different polymerase complexes at SV40 DNA replication origin. *Nature*, **346**, 534–539.
- Turner, J. and O'Donnell, M. (1995) Cycling of *Escherichia coli* DNA polymerase III from one sliding clamp to another: model for lagging strand. *Methods Enzymol.*, **262**, 442–449.
- Turner, J., Hingorani, M.M., Kelman, Z. and O'Donnell, M. (1999) The internal workings of a DNA polymerase clamp-loading machine. *EMBO J.*, **18**, 771–783.
- Uhlmann, F., Cai, J., Gibbs, E., O'Donnell, M. and Hurwitz, J. (1997a) Deletion analysis of the large subunit p140 in human replication factor C reveals regions required for complex formation and replication activities. *J. Biol. Chem.*, **272**, 10058–10064.
- Uhlmann, F., Gibbs, E., Cai, J., O'Donnell, M. and Hurwitz, J. (1997b) Identification of regions within the four small subunits of human replication factor C required for complex formation and DNA replication. *J. Biol. Chem.*, **272**, 10065–10071.
- 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.
- Waga, S. and Stillman, B. (1998) Cyclin-dependent kinase inhibitor p21 modulates the DNA primer–template recognition complex. *Mol. Cell. Biol.*, **18**, 4177–4187.
- 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.
- Yao, N., Turner, J., Kelman, Z., Stukenberg, P.T., Dean, F., Chechter, D., Pan, Z.-Q., Hurwitz, J. and O'Donnell, M. (1996) Clamp loading, unloading and intrinsic stability of the PCNA, β and gp45 sliding clamps of human, *E. coli* and T4 replicases. *Genes Cells*, **1**, 101–113.
- Yuzhakov, A., Kelman, Z. and O'Donnell, M. (1999) Trading places on DNA—a three-point switch underlies primer handoff from primase to the replicative DNA polymerase. *Cell*, **96**, 153–163.
- Zhang, D., Frappier, L., Gibbs, E., Hurwitz, J. and O'Donnell, M. (1998) Human RPA (hSSB) interacts with EBNA1, the latent origin binding protein of Epstein–Barr virus. *Nucleic Acids Res.*, **26**, 631–637.

Received June 28, 1999; revised and accepted September 13, 1999