A novel assay for examining the molecular reactions at the eukaryotic replication fork: activities of replication protein A required during elongation

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ABSTRACT

Studies to elucidate the reactions that occur at the eukaryotic replication fork have been limited by the model systems available. We have established a method for isolating and characterizing Simian Virus 40 (SV40) replication complexes. SV40 rolling circle complexes are isolated using paramagnetic beads and then incubated under replication conditions to obtain continued elongation. In rolling circle replication, the normal mechanism for termination of SV40 replication does not occur and the elongation phase of replication is prolonged. Thus, using this assay system, elongation phase reactions can be examined in the absence of initiation or termination. We show that the protein requirements for elongation of SV40 rolling circles are equivalent to complete SV40 replication reactions. The DNA produced by SV40 rolling circles is double-stranded, unmethylated and with a much longer length than the template DNA. These properties are similar to those of physiological replication forks. We show that proteins associated with the isolated rolling circles, including SV40 T antigen, DNA polymerase α, replication protein A (RPA) and RF-C, are necessary for continued DNA synthesis. PCNA is also required but is not associated with the isolated complexes. We present evidence suggesting that synthesis of the leading and lagging strands are co-ordinated in SV40 rolling circle replication. We have used this system to show that both RPA–protein and RPA–DNA interactions are important for RPA's function in elongation.

INTRODUCTION

Eukaryotic DNA replication occurs by a semi-discontinuous mechanism, with the leading strand being synthesized continuously and the lagging strand being synthesized as a series of short Okazaki fragments. Biochemical analysis of replication proteins as well as functional studies *in vitro* have led to the proposal of a model of the eukaryotic elongation (reviewed in 1–3). This model is patterned after the mechanism of prokaryotic DNA replication (4). Most biochemical evidence indicates that the replicative DNA polymerase is DNA polymerase δ (reviewed in 5) although genetic studies have also implicated a second highly processive DNA polymerase ε in chromosomal DNA replication (6–9). Both polymerases require PCNA which acts as a sliding clamp to allow highly processive DNA synthesis (10). PCNA is loaded on the DNA by RF-C (11–18, reviewed in 2,19,20). On the leading strand, PCNA is thought to be loaded once to establish a processive polymerase complex which can synthesize long stretches of DNA without further modification. On the lagging strand, each Okazaki fragment is initiated after synthesis of an RNA primer by DNA polymerase α/primase complex. DNA polymerase α synthesizes a small segment of DNA, polymerase switching then occurs and DNA polymerase δ carries out the synthesis of the rest of the Okazaki fragment (15,21–23). Each Okazaki fragment is thought to require the loading of PCNA by RF-C. Okazaki fragments are subsequently processed by RNaseH, FEN1/RTH and DNA ligase to give a complete nascent DNA strand (23–25, reviewed in 1,2).

Although much is known about the biochemistry of replication, a number of questions remain about the reactions at a eukaryotic replication fork. Detailed analyses of the reactions at a replication fork have been limited by the difficulty in isolating functional replication fork complexes. In this manuscript we describe a system for examining elongation utilizing the *in vitro* replication of Simian Virus 40 (SV40) (26, reviewed in 27,28). SV40 encodes a single protein required for replication, the multifunctional initiator, SV40 large T antigen. All other proteins needed for replication are supplied by the host cell. SV40 has a double-stranded, circular genome which normally replicates *in vivo* via a bidirectional, circle-to-circle mechanism. Replication terminates when the two replication forks meet. *In vitro*, both circle-to circle and rolling circle mechanisms of replication have been observed (26,29). In rolling circle replication, a single replication fork moves around the circular genome and normal termination processes do not occur. We have developed a system for isolating SV40 rolling circles and characterizing DNA elongation *in vitro*. We also examined the role of replication protein A (RPA) in the elongation phase DNA replication.

RPA was initially identified as a protein absolutely required for SV40 DNA replication (30–32). Subsequently it was shown that RPA is a single-stranded DNA (ssDNA)-binding protein which is involved in multiple aspects of DNA metabolism in cells,

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including replication, repair and recombination (reviewed in 33). Human RPA is a stable complex of three subunits of 70, 32 and 14 kDa (33). RPA also interacts with multiple proteins (reviewed in 33) and can stimulate the activity of eukaryotic DNA polymerases and several known helicases (13,33–37). All three subunits of the RPA complex are required for RPA function (33). The 70 kDa subunit has high affinity ssDNA-binding activity and has been shown to specifically interact with several replication proteins including SV40 large T antigen and DNA polymerase α/primase (38–41). The 70 kDa subunit is composed of at least three domains, an N-terminal protein interaction domain, a central high-affinity ssDNA-binding domain and a C-terminal domain that participates in interactions with the other two subunits (42 and references therein). The 32 kDa subunit also contains an N-terminal regulatory domain and weakly binds to ssDNA (43–47). The 14 kDa subunit is required for the formation of the RPA complex (48). Several studies have indicated that RPA is probably required for both initiation and elongation phases of DNA replication (34,41,49–52); however, the role of RPA in the elongation process is not yet understood.

Using this novel assay, we examine the protein requirements for elongation and present evidence that leading and lagging strand synthesis is co-ordinated. We also show that both RPA–DNA interactions and RPA–protein interactions are necessary for the elongation phase of DNA replication. We also suggest that RPA may be associated with a replication complex, a 'replisome', that replicates both the leading and lagging strands.

MATERIALS AND METHODS

Materials

Redivue [α-^{32}P]dATP (3000 Ci/mmol) was obtained from Amersham. Dynabeads M-280 Streptavidin were purchased from Dynal. Biotin-14-dATP was obtained from Life Technologies. Restriction endonucleases *Sau*3AI and *Mbo*I were purchased from Stratagene; *Dpn*I was purchased from New England Biolabs and Life Technologies, Inc. Topoisomerase I was purchased from Life Technologies.

Monoclonal antibodies used: SJK-287 to DNA polymerase α/primase (53), α70C to RPA70 (35), mab71 to RPA32 (54), pab 414 to SV40 large T antigen (55), anti PCNA antibody (56) and 2-313 to RF-C (Bruce Stillman, personal communication). All are neutralizing antibodies.

Protein purification

All mutant forms of RPA and SV40 T antigen were purified as described previously (38,42,48,57, respectively). Fractionation of HeLa cytoplasmic extracts to yield cellular fraction IBC and II (CFIBC and CFII, respectively) was performed as described previously (48,53). Expression and purification of recombinant *Saccharomyces cerevisiae* RPA (scRPA) and (RPA70 F238A, W361A) have been described (47,58).

SV40 replication assay

SV40 replication reactions were carried out as described previously with minor modifications (48). Briefly, replication reactions were carried out in volumes of 25 or 35 µl with either 100 µg of HeLa

cytoplasmic extract or with partially purified replication proteins purified from HeLa cytoplasmic extracts. The 25 µl reactions had the following final concentrations of partially purified proteins: 300 ng of RPA, 9.4 µg of CFII (containing DNA polymerase α, DNA polymerase δ and RF-C) and 4.2 µg of CFIBC (containing PCNA and Protein Phosphatase 2A). Reactions were incubated at 37°C with the following components: 200 µM of CTP, GTP and UTP, 4 mM ATP, 50 µM dATP (with 0.5 µCi [α-^{32}P]dATP), 100 µM of dCTP, dGTP and dTTP, 30 mM HEPES (diluted from 1 M stock at pH 7.8), 7 mM MgCl$_2$, 50 ng pUC.HSO, 1 µg SV40 large T antigen, and 1 U of topoisomerase I (10 000 U/ml). Reactions were terminated by incubating with 2× Stop Solution (2% SDS, 50 mM EDTA, 1 mg/ml Proteinase K) for 15–30 min at 37°C or by phenol extraction (1 vol phenol saturated with TE) and chloroform:isoamyl alcohol (48:1 v/v) treatment. DNA replication products were precipitated with 3 vol ethanol, 6 M ammonium acetate and 1.2 mg/ml tRNA and vacuum dried. All synthesized DNA replication products were resolved on a 1% agarose gel in 1× TAE (40 mM Tris-acetate and 2 mM EDTA, pH 8.5), and analyzed by autoradiography. Total DNA synthesis was quantitated by TCA precipitation; specific species of replication products were quantitated after gel electrophoresis using a Packard Instant Imager.

The time course of SV40 DNA replication was carried out by incubating a large 250 µl reaction containing HeLa cytoplasmic extract at 37°C. At the indicated times, a 25 µl aliquot was removed and terminated with 1 vol of 2× Stop Solution. DNA products synthesized were resolved and analyzed as described above. For pulse labeling analysis, individual 25 µl HeLa cytoplasmic extract replication reactions were incubated in the absence of [α-^{32}P]dATP for the indicated times. 50 µM dATP with 0.5 µCi [α-^{32}P]dATP were then added to the reactions and incubated for 10 min. The reactions were then terminated and the synthesized DNA products were isolated and analyzed as described above.

Two stage SV40 elongation assay

Stage 1: a 700 µl SV40 replication reaction was assembled with HeLa cytoplasmic extract, 2 µg pUC.HSO (equivalent to 100 ng/1× reaction), 2 µM Biotin-14-dATP, and no [α-^{32}P]dATP. The reaction was incubated at 37°C for 2 h. Washing conditions: streptavidin Dynal beads (420 µg) washed three times with 1× SVRB buffer (200 µM of CTP, GTP, UTP, 4 mM ATP, 30 mM HEPES pH 7.8, 7 mM MgCl$_2$) were added to the 700 µl reaction and incubated with regular mixing at 37°C for 30 min. The incubated beads were then washed three times with 1× SVRB buffer and separated into 30 µg aliquots. Stage 2: the streptavidin beads with bound Biotin-14-dATP labeled DNA–protein elongation complexes were incubated for 1 h at 37°C with partially purified replication proteins in the presence of [α-^{32}P]dATP in standard SV40 replication conditions with the following modifications in dNTP concentrations: 57 µM dCTP, dTTP, dGTP and 28.5 µM dATP. Reactions were terminated by phenol extraction (1 vol of phenol equilibrated with TE). The aqueous phase was then extracted with 1 vol of chloroform:isoamyl alcohol [48:1 (v/v)] and precipitated with 3 vol ethanol, 6 M ammonium acetate and 1.2 mg/ml tRNA and vacuum dried. The DNA was then resuspended in 1× restriction endonuclease buffer and either treated with the indicated restriction endonuclease or left
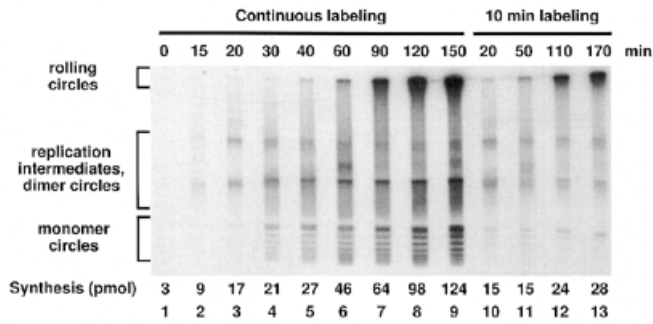
Figure 1. Time course of SV40 DNA replication. SV40 replication reactions containing HeLa cytosolic extract were carried out as described in Materials and Methods. Lanes 1–9 show a time course of a continuously labeled reaction. Length of incubation at 37°C is indicated. In lanes 10–13, replication reactions were pulse labeled for 10 min with [α-^{32}P]dATP beginning at the time indicated. Total DNA synthesis in pmol nucleotide is indicated.

untreated. DNA products were incubated at 37°C for 3 h and analyzed as described above.

RESULTS

Temporal analysis of SV40 replication

Several different forms of DNA products are produced during the replication of SV40 origin containing DNA *in vitro*: monomer circles, dimer circles, replication intermediates (e.g. Cairns structures) and high molecular weight DNA that remains close to the origin of the gel (Fig. 1; 26,29,48,53). The Cairns structures and circular DNA are the products of circle-to-circle replication (4,26,29). In contrast, the high molecular weight DNA are long concatemers synthesized by a rolling circle mechanism (4,29). To examine the temporal appearance of these difference species, DNA was labeled continuously and the products analyzed at various times of incubation. As expected, replication intermediates were the first species of products observed (Fig. 1, lanes 2 and 3). These intermediates were then rapidly converted to monomer and dimer circles (Fig. 1, lane 4). The high molecular weight, rolling circle products were first observed after 40 min of incubation. However, we observed that the proportion of rolling circle products increased over time until at late times, rolling circle products predominate (Fig. 1, lanes 6–9). To confirm these observations, reactions were pulse labeled for 10 min at various times during a reaction. At late times (110 and 170 min) a majority of the radioactivity was incorporated into rolling circles (Fig. 1, lanes 10–13). We conclude that at late times during a reaction, the predominant mechanism of replication is rolling circle. This conclusion is not unexpected. Initiation events decrease over the course of a reaction and it takes only a few minutes for an SV40 containing plasmid to complete replication by the circle-to-circle mechanism (59,60; M.S.Wold, unpublished data). In contrast, rolling circles have only a single replication fork and no efficient mechanism of terminating replication. Thus, it is expected that rolling circles will accumulate throughout the reaction.

Isolation and characterization of rolling circle replication complexes

In rolling circle replication, the elongation phase of replication continues for an extended period of time without requiring
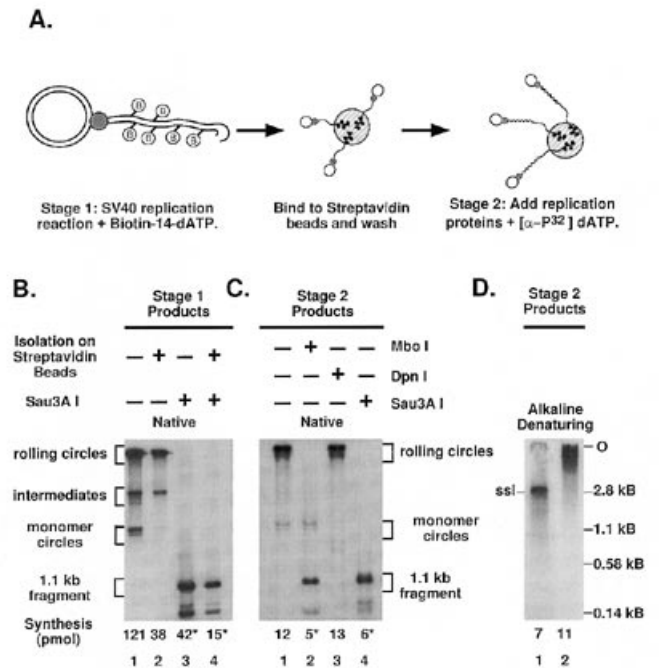

Figure 2. SV40 elongation. (**A**) Schematic of SV40 elongation assay. Stage 1: HeLa SV40 replication reactions are carried out in the presence of Biotin-14-dATP. Newly synthesized DNA is isolated on streptavidin paramagnetic beads and complexes are washed. Stage 2: isolated complexes are combined with partially purified replication proteins and incubated under replication conditions with [α-^{32}P]dATP. Lines, DNA; wavy lines, DNA synthesized in stage 2; gray circle, putative protein complex; Bs and black circles, biotin-14-dATP; hatched circle, paramagnetic beads. (**B**) Stage 1 reaction: replication reactions containing HeLa extracts, Biotin-14-dATP and [α-^{32}P]dATP were incubated at 37°C for 2 h. Where indicated, streptavidin beads were added and DNA isolated as described in Materials and Methods. DNA products were digested with *Sau*3AI where indicated. The positions of different DNA forms and the largest complete digestion product (1.1 kb) are shown. Approximately 31% of the labeled DNA products were isolated on the streptavidin beads. (**C**) Stage 2 reaction products were isolated as described in Materials and Methods. The DNA was then digested for 3 h with the indicated enzyme or left untreated. (**D**) Stage 2 reaction products were isolated as described previously and then digested for 3 h with *Hin*dIII (which has a unique site; lane 1) or left untreated (lane 2). Products were resolved on a 1% alkaline denaturing agarose gel. Total synthesis (pmol dNMP incorporated) or DNA present in the 1.1 kb fragment (*) is indicated for each reaction. Radioactivity present in the 1.1 kb fragment was quantitated using an Instant Imager (Packard). Note the 1.1 kb fragment is 40% the length of the pUC.HSO template.

re-initiation. We reasoned that if the molecules replicating by a rolling circle mechanism could be isolated and analyzed, they would be an excellent model for examining the reactions that occur at a eukaryotic DNA replication fork. We therefore developed a procedure to isolate the rolling circle replication complexes on paramagnetic streptavidin beads (shown schematically in Fig. 2A). In this procedure, SV40 replication reactions were carried out in the presence of low levels of biotin-14-dATP (stage 1). The newly synthesized DNA was isolated on Streptavidin paramagnetic beads, washed and incubated under replication conditions that allow continued DNA synthesis (stage 2).

Control stage 1 reactions were carried out in the presence of both [α-^{32}P]dATP and biotin-14-dATP. Biotin-14-dATP had no effect on DNA synthesis (data not shown). 30–50% of the newly

synthesized DNA could be isolated on the paramagnetic, streptavidin beads (e.g. Fig. 2B, lanes 1 and 2). There was a significant enrichment of high molecular weight products and replication intermediates during isolation (Fig. 2B, lane 2). Although the basis of this enrichment has not been determined, we hypothesize that these forms of DNA may have a more open shape and thus are more easily bound to the paramagnetic beads.

Significant DNA synthesis was observed when, products from a stage 1 reaction were isolated and incubated in a stage 2 reaction containing all required replication proteins and $[\alpha\text{-}^{32}P]\text{dATP}$ (Fig. 2C, lane 1). The products of this stage 2 reaction were predominantly high molecular weight DNA (Fig. 2C, lane 1). This demonstrates that the high molecular weight products isolated from stage 1 were capable of directing further DNA synthesis. We routinely observed 10–20 pmol of synthesis in a 60 min stage 2 incubation (Fig. 2C, lane 1). Overall, this is similar to the amount of DNA isolated on beads from a stage 1 reaction (compare 15.2 pmol/60 min at 37°C in Fig. 2B, lane 2 with 12 pmol/60 min at 37°C in Fig. 2C, lane 1). Thus, the level of synthesis in stage 2 is consistent with an efficient replication reaction since we expect some dissociation of replication complexes during isolation. This suggests that rate of DNA synthesis in stage 2 is similar to that in stage 1 reactions. The distribution of high molecular weight products from stage 2 reactions incubated for short times (5 or 15 min) were identical to those observed in Fig. 2C, lane 1 (data not shown). Since initiation of SV40 DNA replication takes 15–20 min (Fig. 1), these data argue that the synthesis occurring in stage 2 is not due to new initiation events but rather is due to continued synthesis on previously initiated molecules.

The number of times a DNA molecule is replicated determines its methylation state. The template DNA used in these studies was purified from *Escherichia coli* and is fully methylated. One round of replication produces hemimethylated DNA and subsequent rounds of replication produce unmethylated DNA products. To determine the methylation state of the products of stage 2 reactions, the DNA was treated with the isoschizomers *Mbo*I [which only cleaves unmethylated double-stranded DNA (dsDNA)], *Dpn*I (which only cleaves methylated dsDNA) or *Sau*3AI (which cleaves dsDNA regardless of the methylation state). Replication products from both stage 1 and stage 2 reactions were completely digested by *Sau*3AI, as indicated by the appearance of a 1.1 kb fragment and other appropriately sized fragments [Fig. 2B (lanes 3 and 4) and C (lane 4)]. Thus, the DNA produced in both stage 1 and stage 2 reactions was predominantly double-stranded. The high molecular weight products from a stage 2 reaction were also fully digested by *Mbo*I but insensitive to *Dpn*I (Fig. 2C, lanes 2 and 3). This indicates that the DNA synthesized during stage 2 synthesis is fully unmethylated and that both strands are composed of nascent DNA. In addition, the stage 2 replication products were not affected by treatment with topoisomerase II, demonstrating that they are not catenated (linked) circles (data not shown). Analysis of the products on denaturing gels showed that they were composed of strands that were much longer than unit length (Fig. 1D). No nascent DNA was observed in the 100–200 nt size range suggesting that processing of lagging strand synthesis was occurring in stage 2. We conclude that the high molecular weight products from stage 2 are long concatemers of unmethylated dsDNA.

A small amount of monomer circular products was also observed in stage 2 reactions. These products were not susceptible
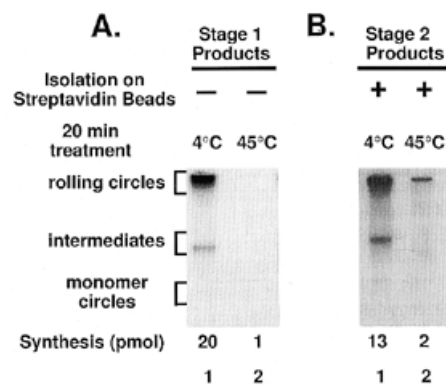

Figure 3. Heat treatment of protein–DNA complexes. (**A**) Products of an SV40 replication reaction in which the complete reaction was treated for 20 min at either 4 or 45°C and then incubated at 37°C for 1 h. (**B**) DNA–protein complexes were isolated on streptavidin beads from Stage 1 reactions. DNA–protein complexes bound on streptavidin beads were incubated at 4 or 45°C for 20 min, washed and added to a Stage 2 reaction. Total synthesis (pmol dNMP incorporated) is shown for each reaction.

to digestion by *Mbo*I indicating partial methylation (Fig. 2C, lane 2). These data are consistent with this DNA being synthesized by one round of circle-to-circle replication or by repair synthesis. These products are probably the result of completion of replication intermediates that were isolated on the streptavidin beads.

Protein requirements for elongation

The DNA synthesis observed in stage 2 could either be caused by continued synthesis of pre-existing replication complexes or by the assembly of new complexes on the isolated rolling circles (or a combination of both processes). To determine whether the isolated rolling circles contained proteins necessary for stage 2 synthesis, we examined the heat sensitivity of the isolated complexes. Human replication proteins are irreversibly inactivated when incubated at 45°C for 20 min (Fig. 3A, lane 2). When rolling circles were isolated on streptavidin beads, incubated at 45°C for 20 min and then added to a complete stage 2 reaction, DNA synthesis was almost totally inhibited (Fig. 3B, lane 2). [Heat treatment neither disrupted the DNA nor the interactions between the DNA and the beads (data not shown).] We conclude that protein components associated with the isolated complexes are necessary for efficient synthesis in stage 2.

Efficient synthesis required the same fractions as needed for complete SV40 DNA replication (Fig. 4A). In this experiment, duplicate reactions were carried out. The upper panel shows complete reaction products and the lower panel shows the 1.1 kb fragment obtained after digestion with *Mbo*I. This enzyme does not digest circular products from a stage 2 reaction (Fig. 2) and thus, *Mbo*I digestion allows rolling circle synthesis to be visualized as a discrete species without contributions by circular products. When no exogenous RPA was added to a stage 2 reaction, the amount of DNA synthesized decreased to ~25% of that of the complete reaction (Fig. 4A, compare lanes 1 and 2). Addition of a neutralizing antibody to RPA caused a larger decrease in elongation synthesis, indicating that there is RPA associated with the isolated DNA–protein complexes (Fig. 4A, lane 3). [This inhibition was specific for RPA because it could be
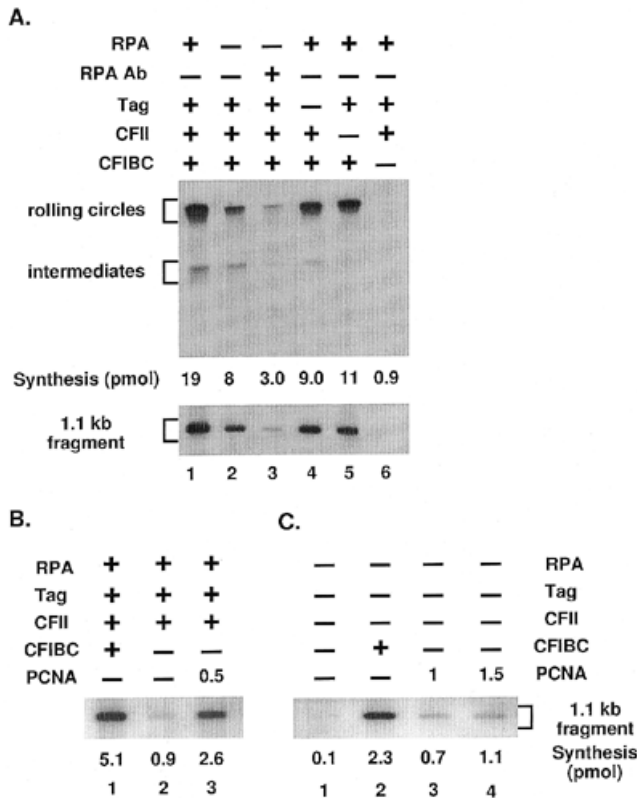
Figure 5. Inhibition of elongation with monoclonal antibodies. Complete Stage 2 reactions were carried out with all protein components in the presence or absence of neutralizing antibody. Antibody additions consisted of dialyzed antibody (Ab) or antibody sample depleted of antibody with Protein-A sepharose prior to addition (Ab buffer). Pol α Ab, monoclonal antibody to DNA polymerase α (SJK 287); PCNA Ab, monoclonal antibody to PCNA; Tag Ab, monoclonal antibody to SV40 Large T antigen (pab 414); RF-C Ab, monoclonal antibody to RF-C (2–313). The amount of nascent DNA (pmol dNMP incorporated) in 1.1 kb *Mbo*I digestion product shown was quantitated as described in Figure 2.

Figure 4. Protein requirements for elongation. (**A**) DNA–protein complexes from Stage 1 reactions were isolated on streptavidin beads, combined with the indicated protein fractions and then incubated in the presence of [α-^{32}P]dATP for 60 min at 37°C. Duplicate reactions were carried out in each case with one reaction analyzed without digestion (top panel) and the second reaction digested for 3 h with *Mbo*I prior to separation on an agarose gel. Only the 1.1 kb (*Mbo*I 1.1 kb) complete digestion fragment is shown (bottom panel). Total synthesis (pmol dNMP incorporated) is shown for each reaction. Protein fractions used: RPA, human RPA; RPA antibody, α70C: neutralizing monoclonal antibody to RPA; T antigen (Tag); fraction CFII, contains polymerases α and δ and RF-C; CFIBC, contains PCNA and protein phosphatase 2A. (**B** and **C**) Stage 2 reactions were carried out and digested with *Mbo*I as described in (A). In (C) the only exogenous proteins added were either CFIBC or human PCNA [purified from HeLa cytosolic extracts (53), amounts in μg]. The amount of nascent DNA (pmol dNMP incorporated) in 1.1 kb *Mbo*I digestion product shown was quantitated as described in Figure 2.

reversed by the addition of RPA (data not shown, see also Fig. 7).] We conclude that RPA is required for elongation phase synthesis (see also below). When either SV40 large T antigen or fraction CFII (which contains DNA polymerase α, DNA polymerase δ and RF-C) were omitted, synthesis was reduced to ~50% that of the complete reaction. This suggested that the isolated complexes contained sufficient T antigen, DNA polymerases α and δ and RF-C to support significant though not optimal DNA synthesis (Fig. 4A, lanes 4 and 5). When fraction CFIBC (which contains PCNA and Protein Phosphatase 2A) was left out, no elongation was seen (Fig. 4A, lane 6). The addition of 0.5 μg of purified PCNA restores synthesis to a level ~50% that of CFIBC (Fig. 4B). We conclude that PCNA is necessary for elongation. We also conclude that under these conditions, CFIBC contains additional factors that stimulate DNA synthesis.
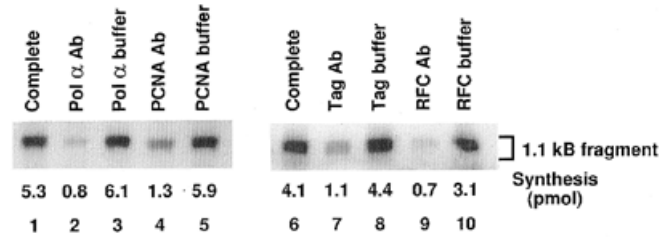
To examine the requirement for PCNA in more detail, a stage 2 reaction was carried out in which the only exogenous protein fraction added was CFIBC. In this reaction, low but significant levels of DNA synthesis were observed (synthesis was 20 times background, Fig. 4C, compare lanes 1 and 2). Similar results were observed when only PCNA was added (Fig. 4C, lanes 3–4). (Again synthesis with PCNA alone was ~50% that of CFIBC, suggesting that under these conditions, additional components are needed for optimal synthesis.) This indicated that at least some complexes isolated on streptavidin beads contain all proteins necessary for DNA synthesis except PCNA. This suggested that T antigen, DNA polymerase α, DNA polymerase δ, RPA and RF-C were associated with the isolated complexes and that these complexes were capable of synthesizing double-stranded nascent DNA when exogenous PCNA is added. The increased synthesis observed in the presence of all replication proteins suggested that the isolated protein complexes were not completely stable during purification and can re-form under replication conditions. However, the heat inactivation studies shown in Figure 3 indicate that at least some proteins must be associated with the rolling circles to obtain efficient DNA synthesis.

To confirm the identity of the proteins required for elongation synthesis in a stage 2 reaction, antibody inhibition studies were carried out. Stage 2 reactions were incubated with monoclonal antibodies to individual proteins known to be needed for SV40 DNA replication. Monoclonal antibodies to DNA polymerase α, PCNA, T antigen and RF-C all strongly inhibit DNA synthesis (Fig. 5). This inhibition was specific for the antibodies because no inhibition was seen with the same fractions after depletion of the antibodies using protein A beads (Fig. 5). The requirement for both PCNA and RF-C strongly suggests that DNA polymerase δ is also required in this system.

DNA polymerase α is known to be required for synthesis of the lagging strand; its primase activity is needed for initiation of each Okazaki fragment. However, after initiation, DNA polymerase α is not thought to be required for leading strand synthesis (1,2). We observed strong inhibition of DNA synthesis by a monoclonal DNA polymerase α antibody. This indicated that inhibition of lagging strand synthesis also blocked leading strand synthesis. An alternated explanation of these results is that in the absence of DNA polymerase activity, leading strand synthesis occurred and
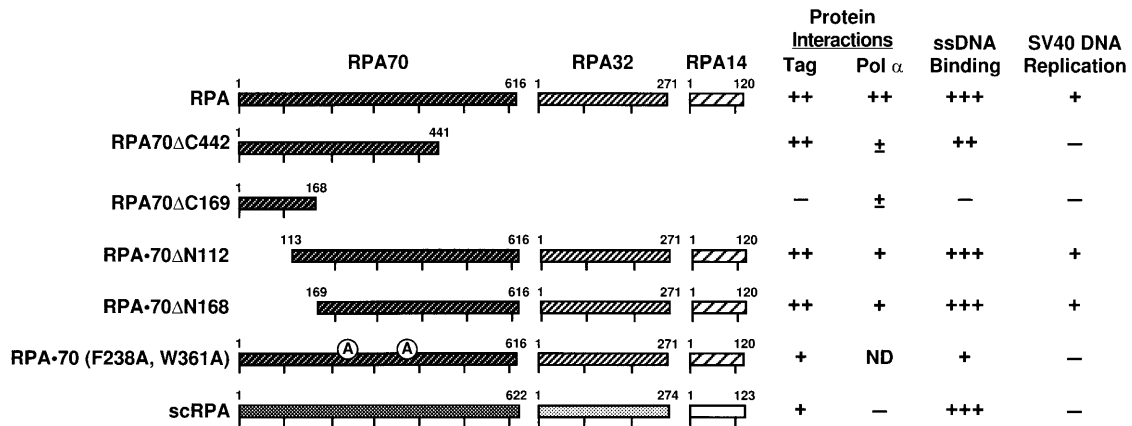
						Protein Interactions		ssDNA Binding	SV40 DNA Replication
						Tag	Pol α		
RPA						++	++	+++	+
RPA70ΔC442						++	±	++	–
RPA70ΔC169						–	±	–	–
RPA·70ΔN112						++	+	+++	+
RPA·70ΔN168						++	+	+++	+
RPA·70 (F238A, W361A)						+	ND	+	–
scRPA						+	–	+++	–

Figure 6. Schematic of RPA mutants and homologues used in elongation assays. The left portion shows schematic diagrams of all RPA mutants used in this study. Beginning and ending amino acids of each mutant are indicated. The activities of each of the mutants in relation to wild-type RPA are shown to the right. Number of +s indicates relative activity; ± indicates minimal activity; – indicates no activity. Protein interactions with T antigen or DNA polymerase α were determined by ELISA (40,58). ssDNA-binding activity and ability to support SV40 DNA replication were determined previously (38,42,58). The following forms of RPA were used in these studies (abbreviations in parentheses): RPA70ΔC442–616 (RPA70ΔC442), RPA70ΔC169–616 (RPA70ΔC169), RPA•70ΔN1–112 (RPA•70ΔN112), RPA•70ΔN1–168 (RPA•70ΔN168), RPA•70(F238A,W361A), homologue of RPA from scRPA.

then the resulting ssDNA was rapidly degraded. To test this possibility, we incubated radiolabeled, linear ssDNA under stage 2 reaction conditions (including all protein fractions) for 60 min. No significant degradation was observed (data not shown). This argues that leading- and lagging-strand synthesis in this system are co-ordinated.

Activities of RPA needed for elongation

RPA is absolutely required for SV40 DNA replication and is required for both initiation of DNA synthesis and elongation. To test the utility of isolated rolling circles for probing the mechanism of elongation we examined the activities of RPA necessary for elongation using mutant forms of RPA and other ssDNA binding proteins (shown schematically in Fig. 6). Initially we examined the requirement for ssDNA-binding activity. RPA•70(F238A,W361A) is a heterotrimeric RPA complex in which two aromatic residues in the high affinity DNA binding domain of RPA70 (F238 and W361) have been changed to alanine. This mutant form of RPA has an affinity for ssDNA which is 1000 times lower than wild-type RPA (58). RPA•70(F238A,W361A) was unable to support elongation; only background synthesis was observed when this mutant form was added to a stage 2 reaction (Fig. 7A, lanes 2 and 3). This indicates that high affinity ssDNA-binding activity is necessary for elongation synthesis.

To determine whether ssDNA binding activity was sufficient for elongation, the RPA homologue from scRPA and *E.coli* ssDNA-binding protein (ecSSB) were tested for activity. Both proteins have high affinity for ssDNA yet neither protein supported efficient DNA synthesis when added to a stage 2 reaction (data not shown). These experiments were repeated with neutralizing antibody to the 32 kDa subunit of RPA added to the stage 2 reactions. This antibody strongly inhibited DNA synthesis, thereby reducing the background in the assay (Fig. 7B, lanes 2 and 3). This inhibition was specific because it could be largely reversed by the addition of human RPA (Fig. 7B, lane 4). In the presence of RPA neutralizing antibody, neither scRPA nor ecSSB was able to support elongation (Fig. 7B, compare lanes 5 and 6

with lane 4). We conclude that ssDNA-binding activity is not sufficient for elongation of SV40 rolling circles. The DNA binding parameters for scRPA and ecSSB differ from those of human RPA (47,61). Therefore, either specific interactions between RPA and DNA or RPA–protein interactions (or both) are necessary for elongation.

To examine the role of RPA–protein interactions in elongation, we assayed the activity of several additional mutant forms of RPA (Fig. 6). RPA70ΔC442 lacks residues 442–661 and is unable to form a complex with the 32 and 14 kDa subunits of RPA. This mutant binds ssDNA with high affinity (Ka is 10% that of RPA) and interacts with T antigen and DNA polymerase α (40,42). (This mutant is unable to support SV40 DNA replication but it was not known whether this is a defect in initiation, elongation or both.) RPA70ΔC442 was unable to support elongation of isolated rolling circle molecules (Fig. 7B, lane 7). Elongation synthesis was also not observed when RPA70ΔC442 was present at 10-fold higher concentrations (Fig. 7B, lane 8). This suggests that the either C-terminal domain of RPA70 and/or the 32 and 14 kDa subunits are important for RPA function in elongation.

N-terminal deletion mutants RPA•70ΔN112 and RPA•70ΔN168 (heterotrimeric RPA complexes missing residues 1–112 and 1–168 of RPA70, respectively; Fig. 6) were both able to support elongation synthesis at a level ~50% less than wild-type RPA (Fig. 7C, lanes 4 and 5). These mutants have ssDNA binding activity similar to wild-type RPA, interact normally with T antigen but show decreased interactions with DNA polymerase α (40,42). The combination of RPA70ΔC169 (RPA70 residues 1–168) and RPA70ΔN168 which together represent all of the residues of RPA, was unable to reconstitute wild-type activity (Fig. 7C, lane 6). This suggests that although RPA–protein interactions and ssDNA-binding are in overlapping regions, they must be linked for wild-type function of RPA. Taken together, these studies show that high affinity ssDNA binding activity is necessary but not sufficient for elongation. RPA–protein interactions also appear to be important but not sufficient for optimal elongation.
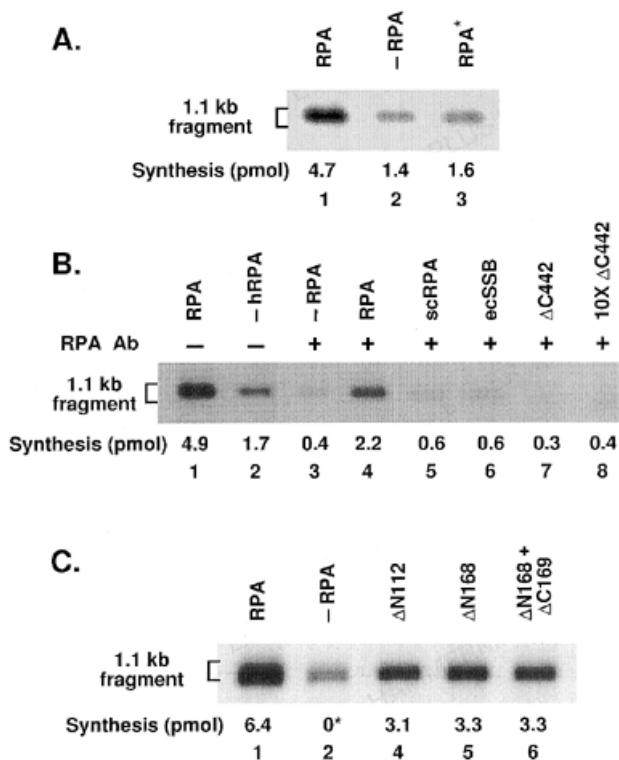
Figure 7. Activities of RPA required in elongation. Stage 2 reactions containing 3 pmol of wild-type or individual mutant forms of RPA were indicated. Reaction products were digested for 3 h with *Mbo*I and separated on an agarose gel. The amount of nascent DNA (pmol dNMP incorporated) in 1.1 kb *Mbo*I digestion product shown was quantitated as described in Figure 2. (**A**) Activity of wild-type RPA (RPA) and RPA•70(F238A,W361A) (RPA*) in elongation. (**B**) Activity of RPA and other ssDNA-binding proteins in elongation. Wild-type RPA (RPA), yeast RPA (scRPA), *E.coli* SSB (ecSSB) or RPA70ΔC442–616 (ΔC442) were added where indicated. Lane 4 contains an additional 3 pmol RPA. 10× ΔC442 contains 30 pmol of ΔC442. Neutralizing monoclonal antibody to the 32 kDa subunit of RPA (mab71; RPA antibody) was added where indicated. This antibody does not interact with scRPA, ecSSB or ΔC442. (**C**) Activity of mutant forms of RPA in elongation. Reactions contain 3 pmol of RPA, RPA70ΔC169–616 (ΔC169), RPA•70ΔN1–112 (ΔN112), and RPA•70ΔN1–168 (ΔN168) where indicated. *The minus RPA lane had 1.2 pmol of DNA synthesis and was subtracted from all other lanes.

DISCUSSION

In this manuscript we describe a system in which protein–DNA complexes from SV40 replication reactions are isolated on streptavidin beads and placed under conditions that allow continued synthesis. We show that continued elongation of these isolated complexes has the same protein requirements as a full SV40 replication reactions and produces long, double-stranded concatemers as expected for the rolling circle mechanism of replication. There does not appear to be significant initiation in the second stage reactions with isolated replication complexes, allowing analysis of elongation in the absence of initiation.

In vivo, SV40 replicates predominantly by a circle-to-circle mechanism (62). In contrast, there is significant replication by a rolling circle mechanism *in vitro*. Rolling circle replication has the same protein and cofactor requirements as does circle-to-circle replication and seems to be an intrinsic property of *in vitro* reactions (26,29,63). The level of rolling circle replication can be influenced by the concentrations of certain proteins and ionic strength. For example, rolling circle replication occurs at elevated levels when topoisomerase I is limiting (64). At the same time, the level of rolling circle replication seems to be negligible in optimized reactions using purified proteins (65,66). It appears most likely that rolling circles arise when one of the two forks in circle-to-circle replication stalls and breaks down leaving a single replication complex on the template. If one of the two template strands then becomes nicked through nuclease action, a rolling circle is established. Thus, we believe that the complexes present on SV40 rolling circles represent bona fide SV40 replication forks and are a good model system for examining reactions at the replication fork.

There is substantial evidence that *in vivo* a protein complex, a 'replisome', exists at the replication fork. In addition, a number of protein complexes have been isolated that contain multiple replication proteins. These range from multi-functional forms of DNA polymerase α (67–70) to a large 17S complex that is active in SV40 DNA replication (71,72). However, in spite of these advances, details of the putative replisome have remained elusive. In our studies we present evidence supporting the presence of a replisome-like complex associated with SV40 rolling circles. We find that isolated SV40 replication complexes have associated proteins that are required for efficient DNA replication. These complexes appear to contain SV40 large T antigen, RPA, DNA polymerase α, DNA polymerase δ and RF-C. All of these proteins interact with one or more of the other proteins and with DNA so there are multiple specific interactions that stabilize this putative replication complex (19,39,40,73). Furthermore, these complexes are dynamic because after isolation, supplemental amounts of replication proteins must be added for optimal DNA synthesis. The only protein fraction that was absolutely required for DNA synthesis with isolated elongation complexes is the fraction containing PCNA which is needed for processive DNA synthesis by DNA polymerase δ. This is consistent with a mechanism in which a new PCNA complex must be loaded for the synthesis of each Okazaki fragment. Our results are consistent with recent studies by Maga and Hübscher, who isolated a complex containing DNA polymerase α, DNA polymerase δ and RF-C from calf thymus (70). This complex could carry out efficient DNA synthesis on singly-primed M13 in the presence of ATP and PCNA (70). These results are also consistent with previous studies indicating that SV40 large T antigen is present at the SV40 replication fork (60,74). Our data also indicate that leading and lagging strand synthesis are co-ordinated in this system. DNA polymerase α is thought to be only required for synthesis of the lagging strand of DNA, yet antibodies to DNA polymerase α inhibited synthesis of both strands (Fig. 5).

We have used this system to examine the role of RPA in elongation of SV40 rolling circles. We demonstrate that RPA is required for elongation. Furthermore, both ssDNA binding activity and RPA–protein interactions are required for elongation synthesis. We found that other ssDNA-binding proteins and partially active forms of RPA could not substitute for human RPA in this system. These results differ from studies of leading strand synthesis *in vitro* in both prokaryotic (75,76) and eukaryotic systems (11,70) which found either that no ssDNA-binding proteins were required for efficient DNA synthesis or that any non-specific ssDNA-binding protein would promote DNA synthesis. However, our studies are consistent with genetic studies which show that multiple functions of RPA are needed for chromosomal DNA

replication (77–80). The difference between our data and that obtained with model templates probably indicates that additional interactions are required for co-ordinated synthesis of leading and lagging strands. It seems likely that RPA–protein interactions are important for productive lagging-stand synthesis. This hypothesis is consistent with several recent studies of prokaryotic ssDNA binding proteins. A recent study examining interactions of *E.coli* replication proteins indicated that specific interactions between ecSSB and the χ subunit of DNA polymerase III are important for loading the PCNA homologue β and probably for increasing the processivity (81). Also protein interactions by T7 ssDNA-binding protein appear to be essential for the co-ordinated synthesis of leading and lagging strands in a model system established with T7 replication proteins (82).

These studies do not allow us to determine whether RPA is a component of the replication fork complex or is associated with ssDNA at the fork. Other ssDNA-binding proteins cannot substitute for RPA which strongly suggests that RPA–protein interactions are important and that RPA is at least interacting transiently with other proteins at the replication fork. We expect additional studies with the system described in this manuscript will allow us to elucidate the role of RPA during elongation.

Over the past several years there has been extensive analysis of the mechanisms of eukaryotic DNA replication. Studies to understand the molecular mechanisms of the elongation phase of DNA replication have principally involved biochemical analysis of replication proteins in defined systems or analysis of replication in model replication systems like SV40. Both types of assays have contributed significantly to our understanding of this process. Studies of model systems have determined the proteins necessary for replication and identified the basic properties of the replication apparatus. Defined studies with model templates have identified processes such as polymerase switching, the role of RF-C in loading PCNA, mechanism by which PCNA increases the processivity of DNA polymerase δ and the roles of RNaseH and FEN1/RTH in processing Okazaki fragments. In spite of these advances, both types of studies have limitations. In model systems it is difficult to separate the processes required for initiation from those of elongation. In defined biochemical assays, there is always a concern that there may be additional reactions present at a replication fork. In this manuscript we describe a method which allows replication complexes to be easily isolated and manipulated. These complexes have properties similar to those expected for physiological replication forks. Future studies using highly purified proteins will permit analysis of protein interactions in a defined system. We believe that this assay is likely to be a valuable tool for defining the molecular mechanisms at the replication fork.

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