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Purification and characterization of replication protein A, a cellular protein required for *in vitro* replication of simian virus 40 DNA

(eukaryotic DNA replication/initiation of replication/single-stranded-DNA-binding proteins/origin-specific unwinding)

MARC S. WOLD AND THOMAS KELLY

Department of Molecular Biology and Genetics, The Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205

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ABSTRACT **The replication of simian virus 40 (SV40) DNA is largely dependent upon cellular replication proteins. To define these proteins we have made use of a cell-free system that is capable of replicating plasmid DNA molecules containing the SV40 origin of replication. Systematic fractionation–reconstitution experiments indicate that there are a minimum of six cellular proteins that are required for efficient viral DNA replication *in vitro*. We report here the purification of one of these proteins, replication protein A (RP-A), to homogeneity. RP-A is a multisubunit protein that contains four tightly associated polypeptides of 70, 53, 32, and 14 kDa. Partial proteolysis experiments indicate that the 53-kDa polypeptide is closely related to the 70-kDa polypeptide, suggesting that it may be a proteolytic fragment of the larger subunit. RP-A is absolutely required for reconstitution of SV40 DNA replication *in vitro*. The purified protein binds to single-stranded DNA and is required for the large tumor (T)-antigen-mediated unwinding of DNA molecules containing the SV40 origin of DNA replication. These properties are consistent with the possibility that RP-A plays a central role in the generation of a single-stranded region at the origin prior to initiation of DNA synthesis. The protein may also function to facilitate unwinding of the parental DNA strands during the elongation phase of SV40 DNA replication.**

The replication of chromosomal DNA in animal cells is not well understood. One approach to this problem is to analyze the replication of the relatively simple genomes of animal viruses (1–3). Simian virus 40 (SV40), a papovavirus, has been extensively studied because the replication of its genome is similar in many ways to the replication of chromosomal replicons (2–4). Only a single virus-encoded protein, the large tumor (T) antigen, is required for SV40 DNA replication. Thus, the replication process is largely dependent upon proteins encoded by the host cell.

The SV40 T antigen is a multifunctional protein that plays a central role in viral DNA replication (2, 3, 5). The initial step in DNA replication is the binding of T antigen to a specific nucleotide-sequence element within the viral origin of DNA replication. Once bound to the DNA, T antigen catalyzes local unwinding of the DNA in the origin region (6–8). This unwinding reaction is a function of the intrinsic helicase activity of T antigen (9) and requires ATP and at least one cellular protein (6). Unwinding at the origin is presumably a prerequisite for priming and subsequent chain elongation. In addition to its role in initiation of replication, the helicase activity of T antigen may function during chain elongation to unwind the parental strands in front of the advancing replication forks (10, 11).

As an approach to defining all of the cellular proteins involved in SV40 DNA replication we have made use of the *in vitro* SV40 DNA replication system originally described by Li and Kelly (12, 13). Analysis of the system indicates that there are a minimum of six cellular proteins in addition to T antigen that are required for the replication of SV40 DNA. In this report we describe the purification and characterization of a previously undescribed cellular replication protein, RP-A. RP-A is a multisubunit protein that binds to single-stranded DNA and is required for the T-antigen-mediated unwinding of DNA templates containing the SV40 origin of DNA replication. Thus, RP-A may act to facilitate the generation of a single-stranded region at the origin preparatory to initiation of DNA synthesis. It is also possible that RP-A facilitates unwinding of the parental strands during the elongation phase of the replication reaction.

MATERIALS AND METHODS

Reagents and Enzymes. The following were obtained from commercial suppliers: Affi-Gel Blue, Bio-Rad; DEAE-Sephacel, Pharmacia; potassium thiocyanate, Baker; *myo*-inositol and *n*-octyl glucoside, Sigma. DNA topoisomerase I was the generous gift of Leroy Liu (Department of Biological Chemistry, Johns Hopkins University, Baltimore), and single-stranded-DNA-binding proteins from *Escherichia coli* (SSB) and adenovirus were generously supplied by Roger McMacken (Department of Biochemistry, Johns Hopkins University, Baltimore) and Edward O'Neill (Department of Molecular Biology and Genetics, Johns Hopkins University, Baltimore), respectively.

Reaction Conditions. The conditions for replication reactions were as described previously (14), with some modifications. Standard 25-μl reaction mixtures contained 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes, pH 7.5); 7 mM $MgCl_2$; 50 μM dCTP with 2.5 μCi (92.5 kBq) of [α-^{32}P]dCTP, 100 μM each dATP, dGTP, and dTTP; 200 μM each CTP, GTP, and UTP; 4 mM ATP; 40 mM creatine phosphate; 15 mM potassium phosphate (250 mM stock, pH 7.5 at 24°C); 50 ng of supercoiled pUC.HSO DNA template (6); 100 μg of creatine kinase; and protein fractions (shown in Fig. 1) as indicated in figure legends. Complementation assays for RP-A contained 5 μg of fraction CF IB, 20 μg of fraction CF II, 100 ng of topoisomerase I, and 0.8 μg of T antigen. [Fraction CF IC was not required under these conditions because fraction CF IB contains low levels of the active component(s) present in CF IC.] Replication reaction mixtures were incubated at 37°C for 2 hr. The reactions were terminated by the addition of 25 μl of 2% $NaDodSO_4$/50 mM EDTA/0.2% proteinase K followed by incubation at 37°C for 30 min. The products were collected by precipitation in 2 M ammonium acetate/50% isopropyl alcohol and analyzed by electrophoresis in 1% agarose gels.

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Abbreviations: SV40, simian virus 40; T antigen, large tumor antigen; RP-A, replication protein A; PCNA, proliferating-cell nuclear antigen; SSB, *Escherichia coli* single-stranded-DNA-binding protein.

Fractionation of HeLa Cell Cytoplasmic Extract and Purification of RP-A. The preparation of HeLa cytoplasmic extract and the generation of fractions CF I and CF II by phosphocellulose chromatography have been described (6, 12). Cytoplasmic extract (2.9 g of protein) from 103 liters of HeLa cells (logarithmically growing in suspension) was made 100 mM in KCl and passed over coupled phosphocellulose (500 ml) and hydroxylapatite (500 ml) columns. The flow-through was quantitatively depleted of protein. The columns were uncoupled and CF II was eluted from the phosphocellulose column (6). The hydroxylapatite column was washed with 500 ml of buffer F (30 mM Hepes, pH 7.5/100 mM KCl/1 mM dithiothreitol, 0.25% *myo*-inositol) and the active fraction was eluted with buffer F containing 70 mM potassium phosphate and 0.1% octylglucoside (11). The resulting fraction (CF I'; 900 mg) was dialyzed into buffer H (30 mM Hepes, pH 7.5/1 mM dithiothreitol/0.25 mM EDTA/0.25% *myo*-inositol/0.01% Nonidet P-40) containing 15 mM KCl and then loaded onto a 90-ml column of DEAE-Sephacel. After the column was washed sequentially with buffer H containing 15 mM (50 ml) and 100 mM (250 ml) KCl, bound material was eluted with an 800-ml linear gradient of KCl (100–300 mM in buffer H) followed by a 200 ml wash with buffer H containing 2 M KCl. Three peaks of replication activity (CF IA, CF IB, and CF IC) were identified by appropriate complementation assays, concentrated on small hydroxylapatite columns (as described above), and dialyzed into buffer H containing 15 mM KCl and 0.5% *myo*-inositol. The first peak (CF IA) contained 130 mg of protein and was eluted at 150 mM KCl. RP-A was purified from CF IA by chromatography over Affi-Gel Blue. CF IA (30 mg) was bound to a 5-ml column of Affi-Gel Blue equilibrated in buffer H with 15 mM KCl. Bound material was then eluted sequentially with buffer H containing either 1 M KCl, 0.5 M KSCN, 0.75 M KSCN, or 1.3 M KSCN (20 ml each). Highly purified RP-A (≈300 μg) was eluted in the 1.3 M KSCN wash. The resulting peak of activity was dialyzed into buffer H containing 15 mM KCl and 0.5% *myo*-inositol and was stored in aliquots at −80°C. RP-A was purified >100-fold on Affi-Gel Blue with a recovery of ≈80% of the activity in CF IA. RP-A is stable for at least 4 months at −80°C and through multiple freeze–thaw cycles. The purification of proliferating-cell nuclear antigen (PCNA; ref. 15) from CF IB, the fractionation of CF IC and CF II, and the purification of DNA polymerase α/primase complex will be described elsewhere.

Protein Analysis. Analysis of polypeptides was performed by electrophoresis in $NaDodSO_4$/polyacrylamide (8–14% linear gradient) gels according to the method of Laemmli (16). Polypeptides were visualized by silver staining (17). Protein concentrations were determined by the method of Bradford (18) with bovine serum albumin as a standard.

RESULTS

Identification and Purification of RP-A. Previous work demonstrated that crude cytoplasmic extracts derived from HeLa cells contain all of the cellular proteins required for the *in vitro* replication of DNA templates containing the SV40 origin of DNA replication (12, 13). To identify and characterize these cellular replication proteins, we fractionated the crude extract by a variety of methods and determined which fractions are required to reconstitute DNA replication *in vitro*. This approach resulted in the identification of five distinct protein fractions, each containing at least one component that is absolutely required for replication (Fig. 1). RP-A was purified from the fraction designated cellular fraction IA (CF IA).

The crude cytoplasmic extract was initially separated into two fractions, CF I and CF II, by chromatography on phosphocellulose (19, 20). In previous studies we demonstrated that CF I contains factors required during the early steps of SV40 DNA replication, including the origin-dependent unwinding of the template catalyzed by T antigen (6, 19). For this reason we focused our attention on the further resolution of this fraction. CF I was purified by hydroxylapatite chromatography and then separated into three subfractions, CF IA, CF IB, and CF IC, by chromatography on DEAE-Sephacel. All three subfractions contained factors required for reconstitution of SV40 DNA replication *in vitro*, but only CF IA appeared to be required

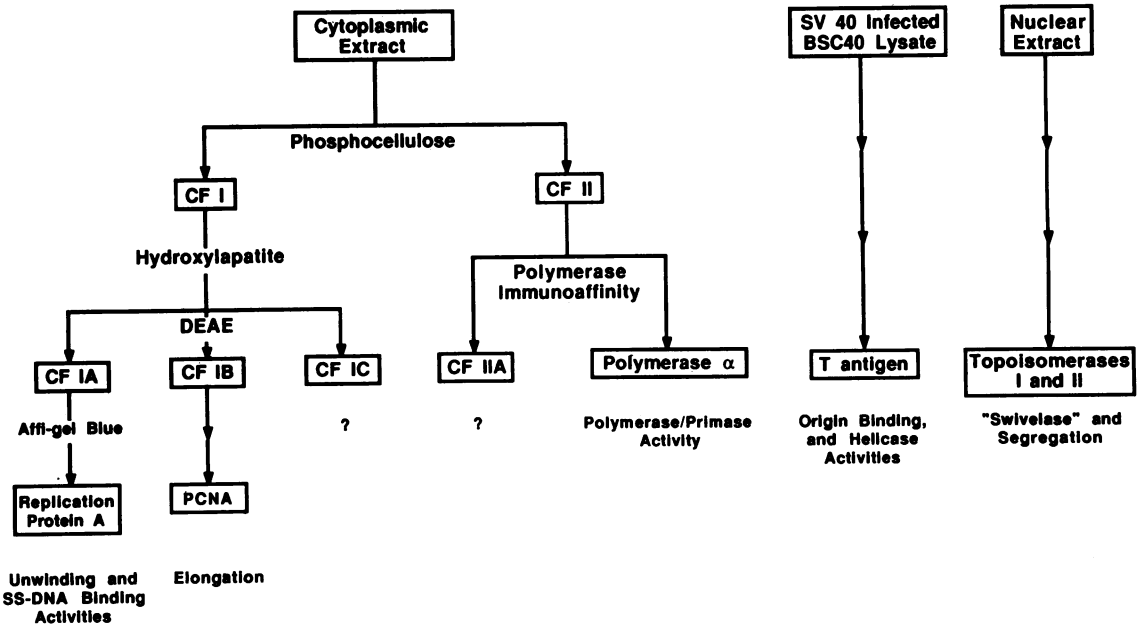

FIG. 1. Proteins required for SV40 DNA replication *in vitro*. T antigen was isolated from SV40-infected BSC40 cells (a monkey kidney cell line). Cytoplasmic extract derived from HeLa cells was fractionated by the indicated chromatographic procedures (see *Materials and Methods*). Efficient replication of SV40 DNA *in vitro* required RP-A, PCNA, DNA polymerase α/primase, topoisomerase I, fraction CF IC, and fraction CF II. Relevant biochemical activities of the fractions (where known) are indicated in the lower portion of the figure. SS-DNA, single-stranded DNA.

for the origin-dependent unwinding reaction (6). Further fractionation of CF IB yielded homogeneous PCNA/cyclin, a protein that was recently shown to be required for efficient DNA chain elongation but does not appear to be involved in the initiation of replication (refs. 15 and 20; unpublished data). The replication activity in CF IA was purified by chromatography on a column of Affi-Gel Blue. After binding CF IA, the column was washed with a buffer containing 1 M KCl and bound proteins were eluted stepwise with the same buffer containing increasing concentrations of potassium thiocyanate. After dialysis to remove the thiocyanate, the fractions were assayed for replication activity in reaction mixtures containing the complementing fractions CF IB and CF II, as well as T antigen, topoisomerase I, and a plasmid template (pUC.HSO) containing the wild-type SV40 origin of DNA replication. Replication activity was eluted at 1.3 M KSCN in a fraction that contained <1% of the total protein. The overall extent of purification on the Affi-Gel Blue column was at least 100-fold and the overall purification from crude extract was ≈1600-fold.

Analysis of the active fractions from the Affi-Gel Blue column by $NaDodSO_4$/polyacrylamide gel electrophoresis revealed the presence of four polypeptides of 70, 53, 32, and 14 kDa (Fig. 2). To determine whether these polypeptides were associated, samples of the purified material were analyzed by sedimentation in glycerol gradients under several different conditions. Fig. 3 shows the results of such a sedimentation analysis carried out at relatively low ionic strength (50 mM KCl). It is evident that under these conditions the four polypeptides cosedimented precisely. As expected, the fraction containing the four polypeptides also contained replication activity as determined by the complementation assay. Under these low-salt conditions this complex has a sedimentation coefficient of 6.7 S. The four polypeptides also cosedimented in glycerol gradients containing 0.5 M KCl or 0.5 M KCl with 1.7 M urea. We conclude from these results that the four polypeptides are tightly associated in a single protein, which we have designated RP-A. Futhermore preliminary data indicated that RP-A also behaved as a complex on a Superose-12 column and had a

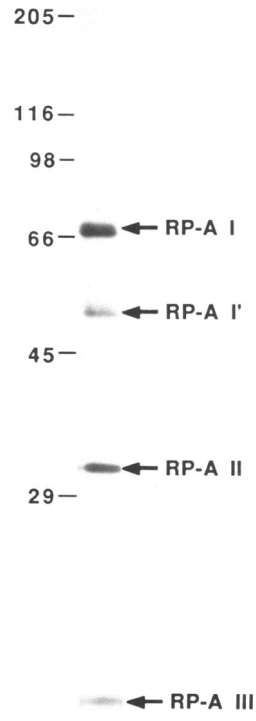

FIG. 2. $NaDodSO_4$/polyacrylamide gel analysis of purified RP-A. Positions of RP-A subunits are indicated by arrows. Standards are (in order of decreasing size, kDa) myosin heavy chain, β-galactosidase, phosphorylase *b*, bovine serum albumin, ovalbumin, and carbonic anhydrase.

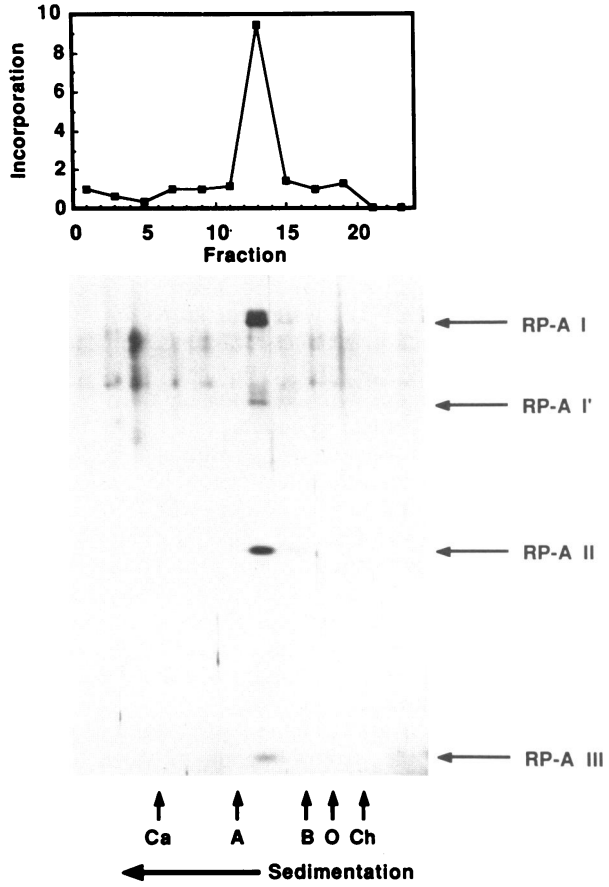

FIG. 3. Glycerol gradient analysis of RP-A. Pure RP-A (8 μg) was sedimented on a 4.8-ml 15–35% (vol/vol) glycerol gradient in buffer H containing 50 mM KCl. After centrifugation at 48,000 rpm for 20 hr at 4°C in a Beckman SW50.1 rotor, the gradient was fractionated and an aliquot of every other fraction was analyzed by $NaDodSO_4$/8–14% polyacrylamide gel electrophoresis. The sedimentation position of protein standards (catalase, Ca; aldolase, A; bovine serum albumin, B; ovalbumin, O; chymotrypsinogen, Ch) from a parallel gradient are shown on the bottom along with the direction of sedimentation (long arrow). Positions of RP-A subunits are indicated at right. The bands visible between the positions of RP-A I and RP-A I' in every lane of the gel are an artifact of staining. Aliquots of each fraction (10 μl dialyzed into buffer H with 15 mM KCl) were also assayed for replication activity in reaction mixtures containing 0.8 μg of T antigen, 5μg of CF IB, 20 μg of CF II, 100 ng of topoisomerase I, and 50 ng of a plasmid (pUC.HSO) containing the wild-type origin of SV40 DNA replication; results are shown as total incorporation (pmol) of deoxynucleotides.

Stokes radius of ≈54 Å (data not shown). Thus RP-A has a molecular mass of ≈160 kDa; however, determination of the precise molecular size and subunit stoichiometry of RP-A must await more detailed hydrodynamic studies.

We have consistently observed that the 53-kDa polypeptide is present in significantly smaller amounts than the other polypeptides. Moreover, partial proteolysis experiments with *Staphylococcus aureus* V8 protease (21) indicated that the 53-kDa polypeptide is closely related in structure to the 70-kDa polypeptide. All but one of the peptide fragments released from the 53-kDa polypeptide by protease digestion were identical to fragments of the 70-kDa polypeptide (data not shown). Thus, the 53-kDa polypeptide may well be a proteolytic breakdown product of the 70-kDa polypeptide. The 32-kDa polypeptide appears to be unrelated to the 70-kDa or 53-kDa polypeptide, since no common peptide fragments were observed. We cannot make a definitive statement about the possible structural relationship of the

smallest (14-kDa) polypeptide to the others, since no proteolytic fragments of this polypeptide were observed.

Properties of RP-A. RP-A was identified and purified on the basis of its ability to complement other cellular replication proteins in the reconstitution of SV40 DNA replication *in vitro*. Fig. 4 shows an analysis of the products of such a reconstitution reaction by agarose gel electrophoresis. The reaction mixture contained purified RP-A, CF IB, CF II, topoisomerase I, and T antigen, as well as a template containing the SV40 origin of DNA replication. (Similar results were obtained when the purified polymerase α/primase complex and CF IIA were substituted for CFII.) The distribution of reaction products was quite similar to that observed previously with crude cytoplasmic extracts (12, 13, 19) or in reconstitutions with CF IA instead of RP-A (Fig. 4). Both monomeric circular products and higher molecular weight forms were observed. The synthesis of both types of products was completely dependent upon the presence of the SV40 T antigen. In the absence of RP-A no DNA synthesis above background was observed, indicating that SV40 DNA replication *in vitro* is completely dependent upon this cellular protein. However, the maximal extent of DNA synthesis in the presence of saturating levels of RP-A was somewhat lower than that observed with saturating quantities of CF IA, suggesting that CF IA may contain additional factors that stimulate DNA synthesis.

Previous kinetic analysis of the replication reaction indicated that CF I, the parent fraction of RP-A, contains activities that are required for the early events in SV40 DNA replication (6). For example, the 20- to 30-min lag that normally occurs prior to the onset of rapid DNA synthesis can be eliminated by preincubation of T antigen, CF I, ATP, and the DNA template. If topoisomerase I is present during this presynthetic reaction, it is possible to detect extensive unwinding of the template, dependent upon the presence of the SV40 origin of replication (6). The unwinding reaction is catalyzed by the helicase activity of T antigen but requires CFI and ATP as cofactors. To examine the ability of RP-A to facilitate the origin-dependent unwinding reaction, the 30-min preincubation was carried out in the presence of topoisomerase I, and the template DNA was immediately analyzed by agarose gel electrophoresis (Fig. 5). A rapidly migrating, underwound form (arrow) was observed when the preincubation reaction mixture contained both RP-A and T antigen, but no such product was observed if either protein was absent. The underwound nature of the product was verified by demonstrating that it could be relaxed by *E. coli* ω protein, a type I topoisomerase that acts specifically on negatively supercoiled DNA (data not shown). The efficiency of the unwinding reaction mediated by T antigen and RP-A was somewhat lower than that observed with T antigen and CF I, suggesting the existence of a stimulatory factor(s) in the less purified fraction. We recently observed that fraction CF IC stimulates unwinding significantly (data not shown). However, unwinding in the presence of CF IC is still completely dependent upon RP-A.

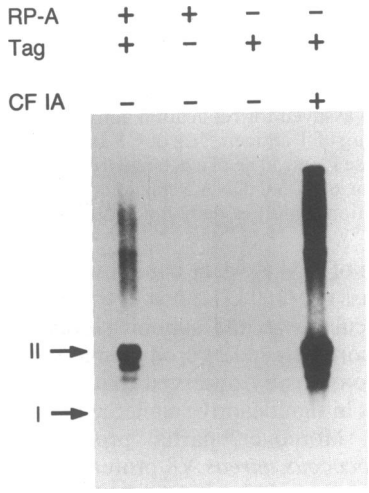

FIG. 4. RP-A is required for SV40 DNA replication. Reaction mixtures contained 5 μg of CF IB. 21 μg of CF II, 100 ng of topoisomerase I, 50 ng of plasmid pUC.HSO, and, where indicated, 90 ng of RP-A, 0.8 μg of T antigen (Tag); and 15 μg of CF IA. The reaction products were analyzed by agarose gel electrophoresis and visualized by autoradiography. Note: the lack of requirement for CF IC in the reaction is due to the fact that low levels of the replication factor in CF IC are present in fraction CF IB. The dependence of replication of CF IC becomes apparent when PCNA is substituted for CF IC. Positions of pUC.HSO forms I and II are indicated by arrows.

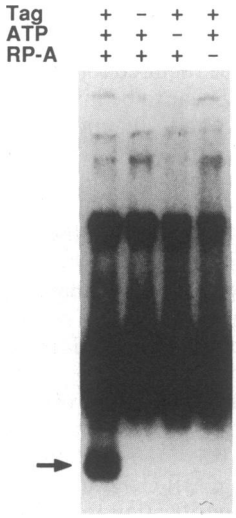

FIG. 5. RP-A is required for T-antigen-dependent unwinding of the template. Unwinding was assayed as described (6). Reaction mixtures contained 2.2 μg of T antigen (Tag), 4 mM ATP, 250 ng of RP-A (where indicated), and 100 ng of plasmid pUC.HSO. The products of the unwinding reaction were analyzed by agarose gel electrophoresis and visualized by the Southern blot hybridization procedure (6).

It has been shown (6, 7) that the origin-dependent unwinding reaction catalyzed by T antigen can be facilitated by *E. coli* single-stranded-DNA-binding protein (SSB). For this reason, we examined the ability of the purified RP-A to bind to single-stranded DNA. Using a nitrocellulose filter binding assay, it was found that RP-A could bind single-stranded DNA (comparable in this qualitative assay to *E. coli* SSB and adenovirus DNA-binding protein) and may also have some affinity for duplex DNA (Table 1). It should be noted that although *E. coli* SSB will function in the unwinding assay, it cannot replace RP-A in the replication reaction (data not shown). This suggests that the role of RP-A in the early steps of SV40 DNA replication may be more complex than simply binding to single-stranded DNA.

Table 1. RP-A binds to single-stranded DNA

Protein	DNA bound, %	
	ss DNA	ds DNA
None	1	1
RP-A	79	11
E. coli SSB	30	3
Adeno DBP	91	ND
BSA	1	0.5

Nitrocellulose filter binding assays were carried out as described (21). The substance for binding was a ^{32}P-labeled 120-base-pair oligonucleotide that was used in its native, double-stranded form (ds DNA) or after thermal denaturation (single-stranded (ss) DNA]. Data are presented as percent of DNA retained on the filter. Binding reaction mixtures contained 80 pg of DNA and either 100 ng of RP-A, 600 ng of *E. coli* SSB, 400 ng of adenovirus DNA-binding protein (Adeno DBP), or 2 μg of bovine serum albumin (BSA). ND, not determined.

DISCUSSION

Systematic fractionation of HeLa cell cytoplasmic extracts resulted in the identification of five protein fractions that are absolutely required for SV40 DNA replication *in vitro*. We purified active proteins to near homogeneity from three cytoplasmic fractions: polymerase α/primase complex from fraction CF II, PCNA from fraction CF IB, and RP-A from fraction CF I. DNA polymerase α/primase complex and PCNA have been clearly implicated in the elongation phase of the replication reaction (12, 15, 20, 22). RP-A is potentially involved in both the initiation and the elongation phases (see below). In addition to the five replication proteins derived from cytoplasmic extracts, previous work demonstrated that topoisomerase activity is required for both the elongation of nascent chains (topoisomerase I or II) and the segregation of daughter molecules (topoisomerase II) (19, 23). As indicated in Fig. 1, the topoisomerases, though present in cytoplasmic extracts, are more easily obtained from nuclear extracts. Thus, at this writing the evidence indicates that at least six cellular proteins are required in addition to the SV40 T antigen for efficient SV40 DNA replication *in vitro*.

RP-A is the most recent addition to the panel of proteins required for SV40 DNA replication. RP-A contains four tightly associated polypeptides of 70, 53, 32, and 14kDa. The 53-kDa polypeptide is present in smaller amounts than the other polypeptides and was shown to be structurally related to the 70-kDa subunit. We strongly suspect, therefore, that the 53-kDa polypeptide represents a product of proteolysis. Our data indicate that RP-A binds to single-stranded DNA and that this property is intrinsic to both the 70-kDa and the 53-kDa subunits (data not shown). There has been a report of a single-stranded-DNA-binding protein that stimulates SV40 DNA replication *in vitro* (24). This protein migrates as a doublet of about 70-kDa in $NaDodSO_4$/polyacrylamide gels and could potentially be related to the 70-kDa subunit of RP-A. However, at present there is no direct evidence on this point.

RP-A has no intrinsic helicase or ATPase activity (data not shown) but is required for the efficient unwinding of duplex DNA by T antigen. The unwinding reaction requires a DNA substrate that contains the SV40 origin of DNA replication and is also dependent upon ATP hydrolysis. Although T antigen and RP-A are the only proteins that are required for origin-dependent DNA unwinding, we have identified an additional cellular factor that significantly stimulates the reaction.

Given the origin-dependence of the unwinding reaction, it is likely that one role of RP-A is to facilitate the generation of a single-stranded region in the vicinity of the origin preparatory to initiation of DNA synthesis. Such a single-stranded region is presumably a prerequisite for synthesis of the first primer(s) by polymerase α/primase and perhaps other proteins. A second possible role for RP-A may be to facilitate the unwinding of the parental strands at the replication fork during the elongation phase of SV40 DNA replication. Fork movement clearly requires the action of a helicase, and recent inhibitor studies are consistent with the hypothesis that T antigen may provide this function (11). However, it is also possible that one or more cellular helicases play this role. In either case RP-A could act to increase the efficiency of unwinding and, hence, enhance fork movement.

The mechanism by which RP-A facilitates the T-antigen-mediated unwinding of the template is not completely understood. The finding that RP-A binds to single-stranded DNA suggests that one function of the protein may be to stabilize the single-stranded region generated by the helicase activity of T antigen. Indeed, previous work has demonstrated that *E. coli* SSB can partially substitute for RP-A in the unwinding reaction (6, 7). However, given that the subunit structure of RP-A is considerably more complex than any of the previously described prokaryotic and eukaryotic single-stranded-DNA-binding proteins, we suspect that RP-A may play additional roles in SV40 DNA replication. In support of this view, we find that *E. coli* SSB is unable to substitute for RP-A in the complete reconstituted replication reaction.

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