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# Purification of replication protein C, a cellular protein involved in the initial stages of simian virus 40 DNA replication *in vitro*

(eukaryotic DNA replication/simian virus 40 large tumor antigen/helicase/DNA unwinding/phosphoprotein phosphatase)

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ABSTRACT **The replication of simian virus 40 (SV40) DNA is dependent upon a single viral protein [tumor (T) antigen] and multiple cellular proteins. To define the required cellular proteins, we have made use of a cell-free system that supports the replication of plasmid DNA molecules containing the SV40 origin of replication. We report here the purification from HeLa cell extracts of replication protein C (RP-C), a previously undescribed protein that is required to reconstitute efficient DNA replication *in vitro*. Highly purified preparations of RP-C contain two closely related polypeptides of 32 and 34 kDa. Preincubation of purified RP-C with T antigen and the DNA template largely eliminates the delay normally observed before the onset of rapid DNA synthesis. In addition, RP-C stimulates the unwinding of duplex DNA molecules containing the SV40 replication origin in a reaction that requires T antigen and a single-stranded DNA binding protein. These observations suggest that RP-C is involved in the initial steps of SV40 DNA replication *in vitro*.**

The papovavirus simian virus 40 (SV40) has proven to be an excellent model system for studying the mechanisms of DNA replication in animal cells (for a recent review, see ref. 1). Since only a single virus-encoded protein, the SV40 tumor (T) antigen, is required for SV40 DNA replication, viral DNA replication is largely dependent on the cellular replication apparatus. The development (2) and subsequent analysis of an efficient cell-free system for SV40 DNA replication has resulted in the identification of several cellular proteins that are required for viral DNA replication. These include the DNA polymerase α–primase complex (3, 4), topoisomerases I and II (5), proliferating cell nuclear antigen (PCNA) (6), and replication protein A (RP-A) (7, 8). Reconstitution experiments *in vitro* indicate that additional cellular proteins are required as well.

The mechanism of initiation of SV40 DNA replication is not yet well understood, although there has been considerable recent progress on the problem. Kinetic studies have revealed that the replication reaction can be divided into two phases: a slow presynthetic phase followed by a phase of rapid DNA chain elongation (7, 9, 10). The slow phase can be eliminated by preincubation of the DNA template with T antigen, ATP, and cell extract, suggesting that the presynthetic reaction may involve the formation of a DNA–protein complex competent for elongation. One of the earliest molecular events in the replication reaction is the binding of T antigen to its recognition site in the viral origin of DNA replication. The binding reaction is facilitated by ATP (11–13) and appears to result in a significant perturbation of the DNA structure in the origin region (14). Once bound to the origin, the T antigen is capable of entering the duplex and catalyzing the local unwinding of the two DNA strands (10, 15, 16). The unwinding reaction, which is an expression of a helicase activity intrinsic to T antigen (17), also requires cellular proteins and ATP. Unwinding at the origin is presumably a prerequisite for priming and subsequent chain elongation.

This laboratory has previously reported the isolation and characterization of a protein fraction derived from HeLa cells (designated CF I) that contains all of the cellular factors required during the presynthetic phase of replication, including those necessary for the origin-dependent unwinding reaction (10). To identify the components of CF I that are active in these early steps of SV40 DNA replication, we have carried out systematic fractionation-reconstitution experiments (4). We and others have already reported the purification of RP-A, a multisubunit single-stranded DNA (ssDNA) binding protein that participates in the unwinding reaction (7, 8). In this report we describe the purification and characterization of another replication protein present in CF I that we have designated replication protein C (RP-C). Highly purified preparations of RP-C contain two closely related polypeptides of 32 and 34 kDa. Preincubation of purified RP-C with T antigen, ATP, and the template largely eliminates the slow presynthetic phase of the replication reaction. Moreover, the purified protein significantly stimulates unwinding of the viral origin of DNA replication in the presence of T antigen and RP-A. These data strongly suggest that RP-C plays an important role in the initial stages of *in vitro* SV40 DNA replication.

## MATERIALS AND METHODS

**Materials.** Reagents and enzymes were obtained as described (4, 8) with the following additions: *Escherichia coli* single-stranded DNA binding protein (SSB) was obtained from Pharmacia, and *Micrococcus luteus* DNA polymerase was from Midland Certified Reagent Company. Enzymes were used according to the manufacturer's instructions. Buffer HN is 30 mM Hepes diluted from 1 M stock, pH 7.8/1 mM dithiothreitol/0.5% *myo*-inositol/0.01% Nonidet P-40/0.5 mM EDTA/1 mM $NaN_3$. Buffer HN with 15 mM KCl is designated HN-15 and with 100 mM KCl is designated HN-100. EDTA was omitted from buffers used in hydroxylapatite chromatography. Protein assays were by the method of Bradford (18) with bovine serum albumin as a standard. $NaDodSO_4$/PAGE was performed with the buffers of Laemmli (19).

**Preincubation and Time-Course Studies.** Preincubation studies were performed as described (10). The initial reaction mixtures contained, in 54 μl, 200 ng of pUC.HSO, 3.2 μg of T antigen, 95 μg of concentrated CF I (CF I'), 800 ng of RP-A, and/or 400 ng of RP-C as indicated in Fig. 4. At the end of the 37°C preincubation, 52 μg of fraction CF II, 120 ng of PCNA, 100 ng of topoisomerase I, 20 μCi (1 μCi = 37 kBq) of

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Abbreviations: ssDNA, single-stranded DNA; PCNA, proliferating cell nuclear antigen; RP-A, replication protein A; RP-C, replication protein C; SSB, *Escherichia coli* ssDNA binding protein; SV40, simian virus 40; T antigen, tumor antigen; $R_S$, Stokes' radius.

[$\alpha$-$^{32}$P]dCTP, unlabeled nucleotides, and any components omitted from the preincubation were added in a volume of 54 $\mu$l. Aliquots (15 $\mu$l) were removed for quantitation at the intervals shown.

**Unwinding of Linear Duplex DNA.** pUC.HSO (10) was linearized with *Hin*dIII and labeled at the 3′ terminus by incubation with [$\alpha$-$^{32}$P]dATP, [$\alpha$-$^{32}$P]dCTP, and *M. luteus* DNA polymerase. Subsequent digestion with *Bam*HI yielded a 200-base-pair (bp) fragment containing the SV40 origin of replication (ori+ in Fig. 5), and a 2686-bp vector fragment (vector in Fig. 5) with no SV40 sequences. Control fragments of 171 and 1619 bp generated by digestion of pUC19 with *Hin*dIII followed by *Pvu* I were labeled in a similar fashion.

Unwinding reaction mixtures contained as indicated (see Fig. 5) 0.4 ng of labeled DNA, 200 ng of T antigen, 200 ng of RP-A or 300 ng of *E. coli* SSB, and 160 ng of RP-C in a final volume of 10 $\mu$l. All reaction mixtures contained 30 mM Hepes (pH 7.5), 15 mM potassium phosphate (pH 7.5), 7 mM $MgCl_2$, 4 mM ATP, 0.05% Nonidet P-40, 0.5 mM dithiothreitol, 40 mM creatine phosphate, and 100 $\mu$g of creatine kinase per ml and were assembled on ice. Reactions were allowed to proceed 30 min at 37°C and were terminated by the addition of 10 $\mu$l of 2% $NaDodSO_4$/2 mg of proteinase K per ml/50 mM EDTA. After an additional 30 min at 37°C and 5 min at 65°C, glycerol and bromphenol blue were added to final concentrations of 10% and 0.02%, respectively, and the samples were analyzed by PAGE on 8% gels in 89 mM Tris base/89 mM boric acid/2 mM EDTA. Gels were dried on DEAE-paper and subject to autoradiography on Kodak XAR film at −80°C. Exposure time was adjusted to compensate for differences in specific activity of the DNA so that film image densities were comparable.

**Purification of RP-C.** The peak fractions containing CF I subfraction CF IC activity from DEAE chromatography (4) were pooled (Table 1), phenylmethylsulfonyl fluoride was added to 200 $\mu$g/ml, and solid $NH_4SO_4$ was added to 25% saturation. The resulting precipitate was removed by centrifugation at 10,000 × *g* for 15 min. $NH_4SO_4$ was added to the supernatant to give 60% saturation. The precipitate was collected, resuspended in 10 ml of buffer HN-100, and applied to a 2.6 × 60 cm Sephacryl S-300 HR column in the same buffer. The column was eluted at 25 ml/hr, and 4-ml fractions were collected. Aliquots (200 $\mu$l) were dialyzed into buffer HN-15 and assayed for ability to stimulate SV40 DNA replication in a standard replication reaction (4) containing 50 ng of pUC.HSO, 0.8 $\mu$g of T antigen, 200 ng of RP-A, 30 ng of PCNA, and 15 $\mu$g of CF II. CF IC activity was eluted from the S-300 column as a broad peak between the elution volumes of ferritin and aldolase. Active fractions were pooled and stored at −80°C.

Table 1. Purification of RP-C

| Procedure | Protein, mg | Total activity,* units × $10^{-3}$ | Specific activity, units/$\mu$g | Yield,† % | Purification† |
|---|---|---|---|---|---|
| DEAE pool | 161 | | | | |
| S-300 load | 119 | 117 | 0.98 | 100 | 1 |
| HAP load | 47.6 | 147 | 3.1 | 126 | 4.2 |
| HAP Mg eluate | 2.0 | 62 | 30.9 | 53 | 31.5 |
| Superose 12 peak | 0.10 | 34 | 358 | 29 | 365 |

HAP, hydroxylapatite.
*One unit = 1 pmol of dNTP incorporated into trichloroacetic acid-precipitable material in 2 hr (background DNA synthesis subtracted).
†Yield and purification factor calculated from the S-300 load because of the variable presence of inhibitors in the DEAE pool.

**Hydroxylapatite Chromatography.** Pooled peak fractions from Sephacryl S-300 gel filtration were thawed and loaded directed onto a hydroxylapatite column (200 mg of matrix per mg of protein). The column was washed with 3 volumes of buffer HN-100, 6 volumes of buffer HN-100 containing 2 M $MgCl_2$, 2 volumes of buffer HN-100, and 4 volumes of buffer HN-100 containing 70 mM potassium phosphate (pH 7.5) (see Fig. 1). Approximately 60% of the RP-C activity and 5% of the protein were eluted with the 2 M $MgCl_2$ wash. Aliquots were dialyzed into buffer HN-15 and then assayed for protein and replication stimulation. Fractions indicated by the horizontal bar in Fig. 1 were pooled, dialyzed overnight against one change of HN-15, and concentrated by loading on a small hydroxylapatite column and eluting with HN-100 containing 70 mM potassium phosphate.

**Superose 12 Gel Filtration.** The 2 M $MgCl_2$ eluate from hydroxylapatite was further concentrated by precipitation with 60% $NH_4SO_4$, resuspended in a small volume of buffer HN-100 with 2 M $MgCl_2$, and subjected to gel filtration on a Superose 12 FPLC (fast protein liquid chromatography; Pharmacia) column in buffer HN-100 containing 2 M $MgCl_2$ at a flow rate of 0.25 ml/min. Fractions (400 $\mu$l) were dialyzed into HN-15 and analyzed for replication-complementing activity as above. The same fractions were analyzed by $NaDodSO_4$/PAGE on 5–15% gradient gels.

## RESULTS

**Purification of RP-C.** Li and Kelly have previously demonstrated that crude cytoplasmic extracts from HeLa cells contain all of the cellular proteins required for the *in vitro* replication of templates containing the SV40 origin of DNA replication (2, 20). Fractionation of such extracts has provided evidence that a minimum of seven cellular factors are involved in SV40 DNA replication (4). These include several proteins that have been purified to near homogeneity (DNA polymerase $\alpha$–primase complex, PCNA, RP-A, and DNA topoisomerases I and II) as well as two relatively crude fractions (CF IC and CF IIA). As described below, RP-C was purified from fraction CF IC.

The crude cytoplasmic extract was initially resolved into two fractions, CF I and CF II, by phosphocellulose chromatography. CF I was found to contain the factors required during the presynthetic phase of SV40 DNA replication, including those necessary for the origin-dependent unwinding reaction mediated by T antigen (10). CF II contained all of the DNA polymerase $\alpha$–primase activity and additional unidentified replication factors. CF I was concentrated on hydroxylapatite, yielding fraction CF I′. CF I′ was then separated into three subfractions, CF IA, CF IB, and CF IC, by chromatography on DEAE-Sephacel (4). RP-A (7, 8) was purified from CF IA, and PCNA (6) was purified from CF IB (4). RP-A was shown to be required for the origin-dependent unwinding reaction (8), while PCNA does not appear to be involved in the early steps of DNA replication.

Our efforts to purify the active component in fraction CF IC were initially frustrated by the finding that the replication activity was eluted as a rather broad peak from a variety of chromatographic matrices (see below). However, the following three-step protocol reproducibly gave good yields of the pure protein. Fraction CF IC was first subjected to gel filtration chromatography on Sephacryl S-300. The fractions containing replication activity, which were eluted in a broad zone between ferritin and aldolase [Stokes' radius ($R_S$) ≈ 50 Å; ref 21], were then loaded onto a hydroxylapatite column. Approximately 60% of the replication activity was eluted from hydroxylapatite with 2 M $MgCl_2$ in a fraction that contained only about 5% of the total protein (Fig. 1). The use

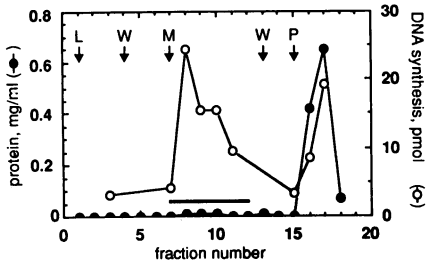


FIG. 1. Hydroxylapatite chromatography of partially purified RP-C. The Sephacryl S-300 fraction was loaded onto a hydroxylapatite column (arrow L) and washed with buffer containing 100 mM NaCl (arrows W). Approximately 60% of the replication activity and 5% of the protein were eluted with a buffer containing 2 M $MgCl_2$ (arrow M). The active fractions were pooled for further purification by gel filtration (see Fig. 2). The residual replication activity and the bulk of the protein were eluted in a buffer containing 70 mM phosphate (arrow P). This material was not studied further. ○, Replication stimulation; ●, protein.

of 2 M $MgCl_2$ was suggested by the work of Gorbunoff and Timasheff (22), who demonstrated that a class of proteins with isoelectric points between pH 5 and 7 are selectively eluted from hydroxylapatite under these conditions. In the final step of the purification, the hydroxylapatite eluate was concentrated and fractionated by gel filtration chromatography on a Superose 12 FPLC column in the presence of 2 M $MgCl_2$. After dialysis to remove the $MgCl_2$, the fractions were assayed for replication activity in reaction mixtures containing cellular fraction II and the purified proteins RP-A, PCNA, topoisomerase I, and T antigen. The replication activity was eluted from the Superose 12 column in a single peak corresponding to an estimated $R_S$ of 25 Å (Fig. 2). Analysis of the active fractions by $NaDodSO_4$/PAGE revealed the presence of two closely migrating polypeptides of 32 and 34 kDa, which together accounted for more than 90% of the protein recovered (Fig. 2). The two-dimensional chymotryptic peptide maps (23) of the 32- and 34-kDa polypeptides were virtually identical, indicating that the two species are closely related in structure (data not shown). The observed difference in electrophoretic mobility may be due to proteolysis or to an unidentified posttranslational modification. The three-step purification procedure described above resulted in a 300- to 400-fold purification of RP-C from the DEAE eluate fraction CF IC (Table 1). Approximately 40 μg of the purified protein were obtained per 100 liters of HeLa cell suspension culture.

The chromatographic properties of RP-C suggest that the protein may form high molecular weight complexes, either by self-association or by interactions with other cellular proteins. The success of the purification procedure may be due to the ability of 2 M $MgCl_2$ to dissociate such complexes. For example, when the hydroxylapatite eluate was subjected to gel filtration in the absence of 2 M $MgCl_2$, we observed two peaks of DNA replication activity. One peak was eluted at the same position as observed previously ($R_S \approx 25$ Å), while the other peak was eluted with an apparent $R_S \approx 50$Å. The 32/34-kDa doublet was present in fractions from both peaks as shown by $NaDodSO_4$/PAGE. We have also observed two peaks of RP-C activity following sucrose density gradient sedimentation and chromatography on red Sepharose (data not shown).

**Biological Properties of RP-C.** Fig. 3 shows an analysis of the products of replication reactions reconstituted with purified RP-C, RP-A, PCNA, topoisomerase I, cellular fraction II, and DNA. The distribution of reaction products was essentially identical to that observed with crude extracts. DNA synthesis was highly dependent upon RP-C, and at saturating levels of the purified protein, the observed extent of replication was 8-fold above background. Low levels of DNA synthesis were observed in the absence of RP-C. This

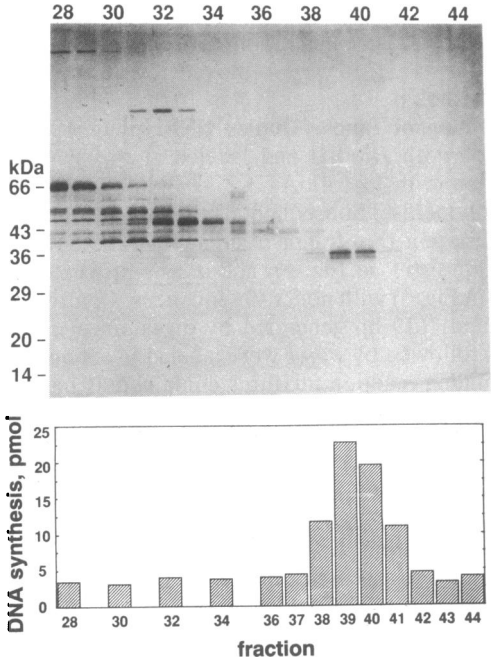


FIG. 2. Gel filtration of RP-C in 2 M $MgCl_2$. The concentrated hydroxylapatite Mg eluate was loaded onto a Superose 12 FPLC column and eluted with a buffer containing 2 M $MgCl_2$. (*Upper*) $NaDodSO_4$/PAGE. Aliquots of the indicated fractions were analyzed by electrophoresis on a 5–15% gradient gel and visualized by silver staining. (*Lower*) Replication activity. Samples of the indicated fractions were assayed for replication activity in reaction mixtures containing T antigen, RP-A, PCNA, cellular fraction II, and topoisomerase I.

residual DNA synthesis may be due to the presence of trace amounts of RP-C in other protein fractions; alternatively, RP-C may not be absolutely required for DNA replication. DNA replication in the reconstituted reaction was completely dependent upon T antigen and upon the presence of the wild-type SV40 origin of DNA replication in the template.

Previous kinetic analysis of SV40 DNA replication *in vitro* showed that CF I, the parent fraction of RP-A, RP-C, and PCNA, contained activities required for early steps in the replication reaction (10). In particular, the 15- to 30-min lag that normally occurs prior to the onset of rapid DNA synthesis could be eliminated by the 37°C preincubation of CF I (or CF I′) with T antigen, ATP, and the DNA template (Fig. 4 *Right*). It has been shown previously that PCNA is not required during this presynthetic phase of DNA replication (ref. 7; M. S. Wold, J. J. Li, and T.J.K, unpublished results). To assess the role of the other proteins derived from CF I in the presynthetic reaction, preincubation experiments were performed with purified RP-A and RP-C. In these experiments the two proteins were preincubated for 20 min at 37°C with T antigen, ATP, and a template containing the SV40 origin of DNA replication. After the preincubation, dNTPs and the remaining

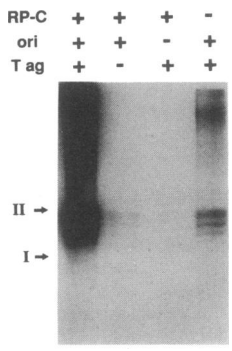


FIG. 3. RP-C is required for efficient SV40 DNA replication. The complete reaction mixture contained plasmid pUC.HSO (ori+), T antigen (T ag), RP-A, PCNA, CF II, and topoisomerase I. In the lane marked ori−, the plasmid pUC.8-4, which contains a 4-bp deletion in the SV40 replication origin (10), was substituted for pUC.HSO.

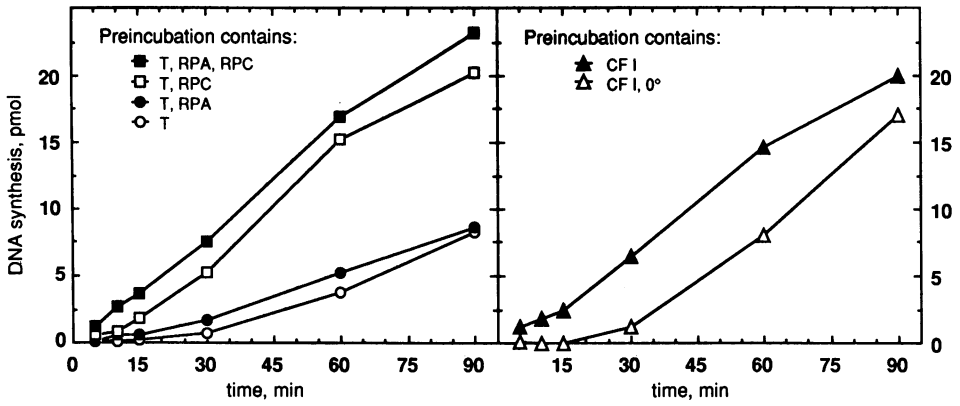


FIG. 4. Time course of DNA synthesis after preincubation of the template with various proteins. Preincubation reactions were carried out as described (10). All preincubation mixtures contained T antigen (T), plasmid pUC.HSO (ori+), ATP, and the proteins or fractions indicated below. After preincubation for 20 min at 37°C, the remaining components required for DNA replication (PCNA, CF II, topoisomerase I, the four dNTPs, and the omitted protein fractions) were added, and aliquots were removed at intervals for quantitation of DNA synthesis. (*Left*) Preincubation reactions contained purified RP-A and/or RP-C in addition to T antigen and the template. (*Right*) Preincubation reactions contained fraction CF I′ in addition to T antigen and the template and were preincubated at 37°C or on ice.

proteins required for SV40 DNA replication were added, and the time course of DNA synthesis was determined. The preincubation with purified RP-A, RP-C, and T antigen largely eliminated the initial lag in DNA synthesis (Fig. 4). Indeed, the time course of DNA synthesis was essentially identical to that observed after preincubation with the crude fraction CF I′. Omission of RP-A from the preincubation reaction had little effect upon the kinetics of DNA synthesis as has been reported (7). Thus, it appears that the major protein components required during the rate-limiting phase of the presynthesis reaction are T antigen and RP-C.

T antigen is capable of catalyzing the unwinding of duplex DNA molecules containing the wild-type SV40 origin of DNA replication (10, 16). Unwinding is also dependent upon ATP and a ssDNA binding protein. Purified RP-A, which binds with high affinity to ssDNA, is the only cellular protein required for this reaction (8). Other ssDNA binding proteins, including *E. coli* SSB, will also support unwinding (10, 16) but cannot replace RP-A in the complete DNA replication reaction. In our earlier studies (4, 8), the extent of unwinding observed with purified RP-A and T antigen was consistently less than that seen with the crude fraction CF I and T antigen, suggesting the existence of an additional stimulatory factor. This stimulatory activity was initially localized to CF IC and copurified with RP-C. Fig. 5 shows the effects of purified RP-C on the unwinding of small linear duplex DNA fragments containing the SV40 origin. Unwinding required T antigen and RP-A. The addition of purified RP-C stimulated the reaction 3-fold. Similar results were obtained when *E. coli* SSB was substituted for RP-A (Fig. 5, lanes 7 and 8). Both reactions were dependent upon the presence of the wild-type SV40 origin in the template (Fig. 5, lanes 9–12). RP-C did not appear to have significant ssDNA binding activity (data not shown), and, indeed, RP-C did not stimulate T antigen-mediated DNA unwinding in the absence of RP-A or *E. coli* SSB (Fig. 5). These results indicate that RP-C stimulates unwinding by a different mechanism than RP-A.

## DISCUSSION

In this paper we describe the purification of RP-C, a previously undescribed cellular protein that is required for efficient SV40 DNA replication *in vitro*. Two lines of evidence strongly suggest that RP-C plays an important role in the initial stages of the replication reaction. First, kinetic experiments indicate that RP-C is required, together with T antigen, for a slow step(s) in the reaction that occurs prior to the initiation of DNA synthesis. Second, purified RP-C stimulates the origin-dependent unwinding of duplex DNA molecules in a reaction mediated by T antigen and RP-A. Unwinding of the two DNA strands is presumably a prerequisite for initiation. Thus, the availability of purified RP-C, as well as RP-A and T antigen, should now make possible a more detailed analysis of the earliest molecular events in SV40 DNA replication.

Preparations of purified RP-C contain two major polypeptides with apparent molecular masses by $NaDodSO_4$/PAGE of 32 and 34 kDa. While peptide mapping experiments indicate that the two polypeptides are closely related in amino acid sequence, their precise relationship is not yet clear. The 32-kDa species may simply be derived from the 34-kDa species by proteolysis during purification, but it is

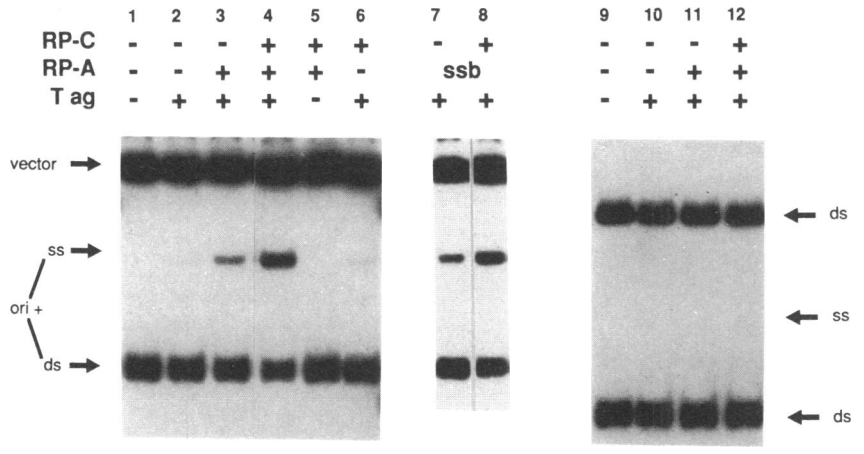


FIG. 5. RP-C stimulates the T antigen (T ag)-dependent unwinding of origin-containing duplex DNA. The substrate for the unwinding reactions in lanes 1–8 was a $^{32}P$-labeled 200-bp duplex DNA fragment containing the SV40 origin of replication. For the reactions in lanes 9–12, the substrate was a similar-size fragment lacking SV40 sequences. Reaction mixtures also contained, as indicated: T antigen, RP-A (lanes 1–6; 9–12) or *E. coli* SSB (lanes 7 and 8), and purified RP-C. Arrows indicate the positions of the duplex substrate (ds) and single-stranded unwound product (ss).

also possible that both polypeptides are present in the cell and that the observed difference in mobility reflects some functionally significant difference in structure. In buffers containing 2 M $MgCl_2$, RP-C appears to have a $R_S \approx 25$ Å, consistent with a monomeric protein with a molecular weight $\approx 30$ kDa. However, in more physiological buffers, active species with $R_S \approx 50$ Å are also observed. These species presumably reflect the formation of larger complexes, either by self-association of the 32/34-kDa polypeptides or by association with other cellular proteins. The latter possibility is of considerable interest, as interactions between RP-C and other proteins could potentially have functional significance for cellular DNA replication.

This study has shown that preincubation of RP-C with T antigen, ATP, and the DNA template largely eliminates the 15- to 30-min delay that normally occurs prior to rapid DNA synthesis. While this result indicates that RP-C participates in a slow presynthetic step(s) in replication, the nature of the molecular events that occur during this step(s) is not yet clear. One possibility is that RP-C alters the structure of the template to facilitate opening of the duplex for initiation and priming. We do not currently favor this model because we have not detected strong DNA binding activity in highly purified preparations of RP-C. A second possibility consistent with our data is that the presynthesis reaction involves the interaction of RP-C, T antigen, and the DNA template to form a nucleoprotein complex that is competent for initiation of DNA synthesis. Such a complex could serve to induce local alterations in DNA structure and/or to nucleate the assembly of proteins that participate in subsequent steps of DNA replication (24). Finally, it is possible that the presynthesis reaction involves modification of the structure or association state of T antigen so that it is more active in initiation. In this model the putative modification reaction would be mediated by RP-C. Each of these models makes strong predictions that can be tested experimentally.

The finding that purified RP-C stimulates the T antigen-mediated unwinding of DNA molecules containing the SV40 origin of DNA replication provides additional support for an interaction between RP-C and T antigen. The unwinding reaction requires T antigen, ATP, and a ssDNA binding protein to stabilize the exposed ssDNA. While it is possible that the stimulatory effect of RP-C on unwinding is due to interactions with the ssDNA binding protein, the fact that stimulation is observed with either HeLa RP-A or *E. coli* SSB renders this possibility unlikely.

Fairman and Stillman (7) have reported that a fraction from extracts of human 293 cells, designated SSI, can reduce the early lag in DNA synthesis when preincubated with T antigen and the DNA template. We suggest that the active component present in fraction SSI is RP-C. The reported chromatographic properties of the SSI factor are consistent with this view. Like RP-C, the SSI factor does not bind to phosphocellulose or ssDNA cellulose at low ionic strengths.

Recently, Roberts and D'Urso (25) have found that extracts from cells in the $G_1$ phase of the cell cycle are deficient (relative to extracts from S-phase cells) in an activity required for efficient SV40 DNA replication. Such extracts are also deficient in an activity that stimulates the T antigen-mediated unwinding of plasmids containing the SV40 origin. Although the relevant factor(s) has not yet been purified, it is evident that these observations could be explained by a deficiency of RP-C in the $G_1$ extracts. Thus, it is possible that the amount or the activity of RP-C varies during the cell cycle. This interesting possibility can now be tested directly.

It seems reasonable to expect that RP-C plays a role in cellular DNA replication, although there is no direct evidence on this point. By analogy with its role in SV40 DNA replication, RP-C may interact with cellular initiation proteins, the putative counterparts of T antigen, to facilitate initiation of DNA replication at chromosomal origins.

**Note Added in Proof:** We have recently obtained amino acid sequences from several tryptic fragments of the 32/34-kDa protein. The sequences are identical to the sequence of the catalytic subunit of human protein phosphatase 2A ($PP2A_c$) (26), an intracellular protein of 37.3 kDa often found associated with regulatory subunits of 55 and 60 kDa (27). This finding suggests that RP-C/$PP2A_c$ may stimulate SV40 replication *in vitro* by dephosphorylation of T antigen and/or other replication proteins.

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