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An Interaction between Replication Protein A and SV40 T Antigen Appears Essential for Primosome Assembly during SV40 DNA Replication*

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Replication protein A from human cells (hRPA) is a multisubunit single-stranded DNA-binding protein (ssb) and is essential for SV40 DNA replication *in vitro*. The related RPA from *Saccharomyces cerevisiae* (scRPA) is unable to substitute for hRPA in SV40 DNA replication. To understand this species specificity, we evaluated human and yeast RPA in enzymatic assays with SV40 T antigen (TAg) and human DNA polymerase α/primase, the factors essential for initiation of SV40 DNA replication. Both human and yeast RPA stimulated the polymerase and (at subsaturating levels of RPA) the primase activities of human DNA polymerase α/primase on homopolymer DNA templates. In contrast, both human and yeast RPA inhibited synthesis by DNA polymerase α/primase on natural single-stranded DNA (ssDNA) templates. T antigen reversed the inhibition of DNA polymerase α/primase activity on hRPA-coated natural ssDNA, as previously described, but was unable to reverse the inhibition on scRPA or *Escherichia coli* ssb-coated templates. Therefore, the ability of an ssb to reconstitute SV40 DNA replication correlated with its ability to allow the TAg stimulation of polymerase α/primase in this assay. Enzyme-linked immunoassays demonstrated that hRPA interacts with TAg, as previously described; however, scRPA does not bind to TAg in this assay. These and other recent results suggest that T antigen contains a function analogous to some prokaryotic DNA replication proteins that facilitate primosome assembly on ssb-coated template DNAs.

DNA replication is a highly regulated process and a key event during cell division. To study this process, the replication of the SV40 genome has proven to be an excellent model system. Only a single viral protein, the large T antigen (TAg),[1] is required for SV40 DNA replication, and all other functions are provided by the host cell. The development of an *in vitro* SV40 replication system has allowed biochemical investigations of the factors and mechanisms involved in SV40 DNA replication (for reviews, see Challberg and Kelly, 1989; Stillman, 1989; Hurwitz *et al.*, 1990; Melendy and Stillman, 1992). A biochemical approach by a number of laboratories has allowed the initiation and elongation stages of SV40 DNA replication to be reconstituted with purified factors (Tsurimoto *et al.*, 1990; Weinberg *et al.*, 1990; Eki *et al.*, 1992). Detailed mechanistic studies using these purified factors have told us much about how initiation and elongation are coupled during the replication process (Tsurimoto *et al.*, 1990; Weinberg *et al.*, 1990; Lee *et al.*, 1991; Tsurimoto and Stillman, 1991a, 1991b; Eki *et al.*, 1992).

Only three factors, the viral TAg, the three subunit single-stranded DNA-binding protein, replication protein A (RPA, also known as human ssb), and polymerase α/primase complex are essential for origin recognition, unwinding, and synthesis of the first nascent RNA/DNA strands (Wobbe *et al.*, 1987; Ishimi *et al.*, 1988; Borowiec *et al.*, 1990; Matsumoto *et al.*, 1990; Tsurimoto *et al.*, 1990; Weinberg *et al.*, 1990; Bullock *et al.*, 1991; Erdile *et al.*, 1991a; Murakami *et al.*, 1992). TAg binds to the SV40 origin and acts as a bidirectional helicase (for review see Borowiec *et al.*, 1990; Prives, 1990; SenGupta and Borowiec, 1992; Melendy and Stillman, 1992; Wessel *et al.*, 1992). The addition of RPA creates an unwound complex that, in the absence of nucleic acid synthesis, but in the presence of a topoisomerase, will continue to unwind the plasmid DNA to form a highly unwound plasmid called form U (Dean *et al.*, 1987; Wold and Kelly, 1988; Tsurimoto *et al.*, 1989). Since form U is the first labeled species in SV40 DNA replication reactions, polymerase α/primase presumably recognizes the unwound complex and begins nucleic acid synthesis (Bullock *et al.*, 1989). Synthesis with just these three factors (and topoisomerases to relieve torsional stress) can be quite extensive (Wobbe *et al.*, 1987; Ishimi *et al.*, 1988; Erdile *et al.*, 1991a; Tsurimoto and Stillman, 1991b; Eki *et al.*, 1992).

A number of critical protein-protein interactions commonly occur between the factors involved in the initiation process. In general, DNA polymerases often interact with and are stimulated by their cognate ssDNA-binding proteins (Kornberg and Baker, 1991; Wang, 1991). In the case of SV40 DNA replication, RPA has been shown to stimulate DNA polymerase α/primase activity (Kenny *et al.*, 1989; Tsurimoto and Stillman, 1989; Erdile *et al.*, 1991b), and recently Dornreiter *et al.* (1992) have reported a direct interaction between RPA and DNA primase. It has also been known for many years that there is an interaction between TAg and DNA polymerase α/primase that is essential for SV40 DNA replication (Smale and Tjian, 1986; Gannon and Lane, 1987; Dornreiter *et al.*, 1990; Gannon and Lane, 1990). It is, in fact, this

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[1] The abbreviations used are: TAg, SV40 large tumor antigen; RPA, replication protein A; hRPA, human RPA; scRPA, *S. cerevisiae* RPA; mRPA, murine RPA; pol α, DNA polymerase α/primase complex; ssb, single-stranded DNA-binding protein; ssDNA, single-stranded DNA; ELISA, enzyme-linked immunoassay.

interaction that appears to be responsible for the inability of mouse cell extracts to support SV40 DNA replication (Murakami *et al.*, 1986), although this has recently been questioned (Eki *et al.*, 1991). Furthermore, TAg has been shown to stimulate polymerase α/primase activity on an ssDNA template in the presence of an ssb (Collins and Kelly, 1991). These functional interactions have been supported by studies using enzyme-linked immunoassays (ELISAs) that demonstrate a direct interaction between TAg and the catalytic subunit of DNA polymerase α (Dornreiter *et al.*, 1992).

RPA purified from *Saccharomyces cerevisiae* (scRPA) has properties similar to human RPA (hRPA). Both RPAs contain three subunits. The largest subunit is an ssb; the second largest subunit is phosphorylated in a cell cycle-dependent manner; and RPA from both species will cooperate with TAg to generate form U DNA (Brill and Stillman, 1989; Wold *et al.*, 1989; Din *et al.*, 1990; Kenny *et al.*, 1990). Furthermore, all three RPA subunits are essential for growth in *S. cerevisiae* (Heyer *et al.*, 1990; Brill and Stillman, 1991). scRPA, however, cannot function in the reconstituted SV40 DNA replication system in the place of hRPA (Brill and Stillman, 1989). Conversely, murine RPA (mRPA) can replace hRPA in the replication reaction (Schneider *et al.*, 1992). We have compared hRPA, scRPA, mRPA, and *Escherichia coli* ssb in a number of assays to identify the basis for the species specificity for hRPA (or mRPA) in SV40 DNA replication with the expectation that the species specificity would provide insight into the mechanism of DNA replication. All the ssbs cooperated with TAg in unwinding, and surprisingly all the eukaryotic RPA proteins stimulated human polymerase α/primase. All the ssbs also inhibited *de novo* initiation by primase on ssDNA templates; however, only hRPA and mRPA (the two mammalian ssbs that support SV40 DNA replication) allowed TAg stimulation of polymerase α/primase synthesis on ssb-saturated ssDNA templates. ELISA assays corroborated that TAg interacts with hRPA but not scRPA. We suggest that TAg mediates the loading of DNA polymerase α/primase onto a DNA template via interactions with RPA bound to ssDNA. This function is analogous to that found in a number of prokaryotic replication proteins.

EXPERIMENTAL PROCEDURES

Materials—[α-^{32}P]dATP and dTTP (800 Ci/mmol) were obtained from Amersham Corp. and Du Pont-New England Nuclear, respectively. Poly(dA) (average length of 290 nucleotides), poly(dT) (average length of 167 nucleotides), and oligo(dT) (average length of 12–15 nucleotides) were purchased from Pharmacia LKB Biotechnology Inc. Klenow fragment was purchased from Boeringer Mannheim.

Replication Factors—SV40 TAg was purified from SF9 cells infected with a recombinant baculovirus expression vector using immunoaffinity chromatography (Simanis and Lane, 1985; Stillman and Gluzman, 1985; Lanford, 1988). Topoisomerases I and II were prepared from calf thymus according to published procedures with slight modifications (Liu and Miller, 1981; Schomburg and Grosse, 1986). RPA was purified to near homogeneity as previously reported (Brill and Stillman, 1989) from either FM3A cell cytosolic extract (mS100) (for mRPA), fraction I (Prelich *et al.*, 1987) (for hRPA), or the phosphocellulose flow-through from *S. cerevisiae* extracts (Fien and Stillman, 1992) (for scRPA). DNA polymerase α/primase (pol α) was purified from a 293-cell cytosolic extract (S100) by immunoaffinity chromatography with an anti-pol α monoclonal antibody (SJK 273-71)-Sepharose as described (Murakami *et al.*, 1986; Tsurimoto and Stillman, 1989). In some instances, the pol α complex was further purified by gradient elution from a 0.1-ml S-Sepharose column (30 mM potassium phosphate, pH 7.0, 1 mM Na_2EDTA, 10% (v/v) glycerol, 50–500 mM NaCl).

Assays—For all assays, incorporation of radiolabeled nucleotide was determined as the picomoles of dAMP or TMP that remained bound to DE-81 paper (Whatman) after five washes in 0.5 M Na_2HPO_4. The total volume of the reactions varied from 10 to 50 μl; incorporation levels from all experiments were normalized to reflect incorporation of a 50-μl assay.

DNA Replication—Reaction conditions for SV40 DNA replication were as previously described (Stillman and Gluzman, 1985) and were incubated for 60 min at 37 °C. The plasmid DNA substrate used was pSV011 that contains the SV40 origin region from *Hin*dIII (nucleotide 5171) to *Sph*I (nucleotide 128) in pUC18 (2.9 kilobases) (Prelich and Stillman, 1988). Each assay contained TAg and topoisomerase I and II at levels previously described (Melendy and Stillman, 1991) and immunoaffinity-purified polymerase α/primase complex at 50 μg/ml.

DNA Polymerase Assays—The DNA polymerase assay contained poly(dA)/oligo(dT) (19:1, 0.04 mM nucleotide), 40 mM Tris-HCl, pH 6.9, 6 mM $MgCl_2$, 10% (v/v) glycerol, 40 μg/ml acetylated bovine serum albumin, 1 mM dithiothreitol, 0.04 mM dTTP with 1–2 μCi/ml [α-^{32}P]dTTP, and 15 μg/ml polymerase α/primase complex.

DNA Primase Assays—The DNA primase assay contained 50 mM Tris-HCl, pH 7.9, 9 mM $MgCl_2$, 4 mM dithiothreitol, 0.1 mg/ml acetylated bovine serum albumin, 5 mM ATP, 0.04 mM dATP with 1–2 μCi/ml [α-^{32}P]dATP, 0.1 mM poly(dT), 10 units/ml Klenow fragment, and 15 μg/ml polymerase α/primase complex.

DNA Polymerase/Primase Assays—The DNA polymerase/primase assay on unprimed ssM13 mp18 was performed under SV40 DNA replication conditions as described (Collins and Kelly, 1991). Reactions contained 42 μg/ml polymerase α/primase complex and 5–10 μCi/ml [α-^{32}P]dATP. To control for nonspecific priming, the template was assayed with 250 μg/ml polymerase α/primase in both the presence and absence of rNTPs. No synthesis was detected in the absence of rNTPs; hence, all synthesis using this template is dependent upon primase activity. In experiments where TAg concentration was held constant, it was present at 35 μg/ml. When the RPA or ssb concentrations were held constant to saturate the template DNA, RPA was used at 25 μg/ml and *E. coli* ssb at 45 μg/ml. The levels of RPA and *E. coli* ssb selected had resulted in >95% inhibition of incorporation by polymerase α/primase in the absence of TAg (at 210 μg/ml of polymerase α/primase, data not shown). We designated this level of inhibition to be "biochemically saturated" for this assay.

Enzyme-linked Immunoassays—ELISAs were performed as described (Dornreiter *et al.*, 1992). Different levels of primary antisera (Din *et al.*, 1990) were used in ELISA analyses against varying amounts of immobilized hRPA and scRPA to ascertain the appropriate dilution of primary antisera so that similar levels of RPA gave similar levels of absorbance in the assay. The rabbit antiserum raised against hRPA was used at a 1:1000 dilution, whereas the rabbit antiserum raised against scRPA was used at a 1:4000 dilution. The incubations using hRPA and scRPA were performed in buffer with 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 30 units/ml micrococcal nuclease. The chromogenic substrate used was 3,3′-5,5′-tetramethylbenzidine. After 10 min, the reactions were stopped with 2 M sulfuric acid, and the absorbance was measured at 450 nm.

RESULTS

scRPA is unable to substitute for hRPA in the SV40 DNA replication system (Brill and Stillman, 1989). To discover the basis for this, we compared the functions of hRPA and scRPA by a variety of less demanding assays. The ssDNA-binding properties of RPA from yeast and human cells (both maximal levels of ssDNA bound and off-rate analyses) indicated that the ssDNA-binding properties of yeast and human RPA are very similar (data not shown; Brill and Stillman, 1989; Kim *et al.*, 1992; Alani *et al.*, 1992). Thus, it is unlikely that RPA-DNA interactions are responsible for the inability of scRPA to support SV40 DNA replication.

These findings suggested that some protein-protein contact may be responsible for the inability of scRPA to reconstitute SV40 DNA replication. The fact that *E. coli* ssb and scRPA can replace hRPA for DNA synthesis by the leading strand DNA replication complex (consisting of replication factor C (RFC), proliferating cell nuclear antigen (PCNA), DNA polymerase δ, and an ssb), indicates that the failure of scRPA to substitute for hRPA is not due to an inability to synthesize leading strands at a replication fork (Kenny *et al.*, 1989; Fien and Stillman, 1992). To determine whether the RPA specificity could be ascribed to the factors involved in initiation of

DNA replication and subsequent lagging strand synthesis, we investigated whether scRPA could replace hRPA in the SV40 origin-dependent DNA synthesis system first described by Wobbe *et al.* (1987). This system requires only TAg, RPA, polymerase α/primase, and a DNA topoisomerase and, depending on the conditions, only synthesizes the lagging strand or can synthesize both leading and lagging strands (Ishimi *et al.*, 1988; Tsurimoto and Stillman, 1991b). In this assay, TAg binds to the SV40 origin sequence and, in the presence of RPA and a topoisomerase, unwinds the plasmid. Polymerase α/primase then synthesizes RNA-primed DNA strands using the unwound DNA as a template. We titrated human, murine, and *S. cerevisiae* RPA into reactions containing DNA, TAg, topoisomerases I and II, and DNA polymerase α/primase. Either hRPA or mRPA fully supported DNA synthesis, but as previously published, scRPA did not (Fig. 1) (Erdile *et al.*, 1991a). This result suggested that the protein-protein interaction responsible for the species specificity of RPA in SV40 DNA replication is between RPA and either polymerase α/primase, TAg, or a combination of both.

RPA from either human or yeast cells did not affect the DNA helicase or ATPase activities of TAg (data not shown). Because of these observations and the fact that both RPAs could support SV40 origin-dependent unwinding of the DNA (Brill and Stillman, 1989), we tested for differential effects on DNA polymerase α/primase activities.

Initially, a relatively simple template DNA (oligo(dT) hybridized to poly(dA)) was employed. Increasing amounts of either hRPA, scRPA, or *E. coli* ssb were incubated with the template DNA for 5 min prior to the addition of the polymerase α/primase complex. The results in Fig. 2*A* clearly indicated that both scRPA and hRPA were capable of stimulating polymerase α/primase in this assay, although to slightly different degrees. In contrast, *E. coli* ssb did not stimulate the DNA polymerase activity of polymerase α/primase. The various ssbs were also tested for their effect on primase activity. In this assay (which uses excess poly(dT) template), both hRPA and scRPA stimulated the primase activity of polymerase α/primase, whereas *E. coli* ssb did not (Fig. 2*B*). Therefore, on synthetic homopolymer template DNAs, both hRPA and scRPA stimulated DNA polymerase and DNA primase activities. These results suggest that the inability of scRPA to support SV40 DNA replication was not due to an inability to stimulate polymerase α or primase activity on a simple DNA template.

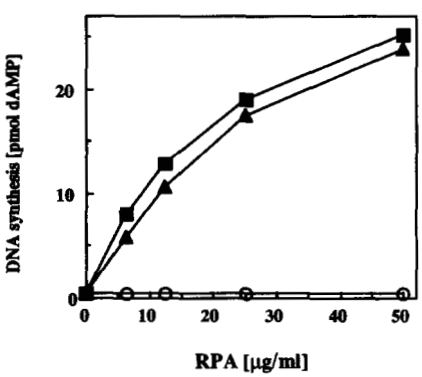

FIG. 1. **Yeast RPA cannot replace mammalian RPA for DNA synthesis from the SV40 origin.** Increasing amounts of hRPA (*filled squares*), mRPA (*filled triangles*), or scRPA (*empty circles*) were titrated into identical SV40 DNA synthesis reactions containing optimized levels of TAg, topoisomerases I and II, polymerase α/primase, and plasmid containing the SV40 origin. DNA synthesis is expressed as picomoles of dAMP incorporated in 60 min in a 0.05-ml reaction.

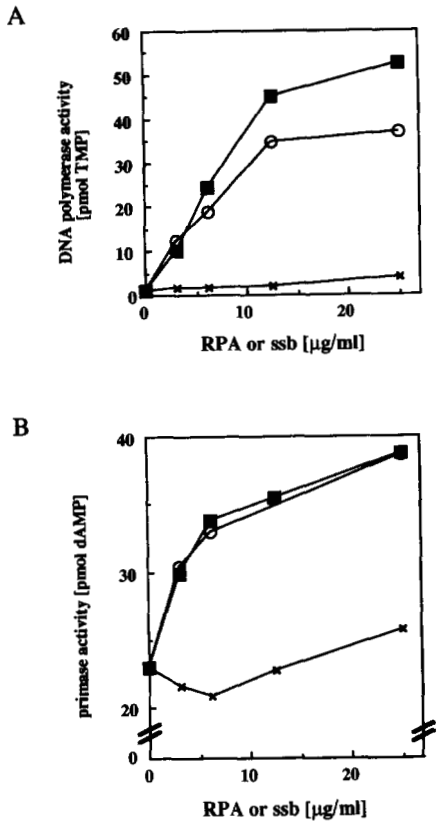

FIG. 2. **Yeast RPA can stimulate both the polymerase and primase activities of human polymerase α/primase.** Increasing amounts of hRPA (*filled squares*), scRPA (*empty circles*), or *E. coli* ssb (*crosses*). *A*, polymerase assay. Proteins were preincubated for 5 min at 37 °C with the poly(dA)/oligo(dT) template, and polymerase α/primase was then added and incubated for 15 min at 37 °C. Polymerase activity is expressed as picomoles of TMP incorporated in 15 min in a 0.05-ml reaction. *B*, primase assay. Proteins were preincubated with poly(dT) template for 5 min at 37 °C, and polymerase α/primase was then added and incubated for 20 min at 37 °C in the presence of an excess of *E. coli* DNA polymerase I (Klenow) fragment. Primase activity is expressed as picomoles of dAMP incorporated by the Klenow fragment polymerase in 25 min in a 0.05-ml reaction. Incorporated nucleotides were quantitated as described under "Experimental Procedures."

We next tested DNA synthesis by polymerase α/primase on a natural, unprimed single-stranded DNA template, and for this purpose we used single-stranded M13 mp18 phage DNA. Collins and Kelly (1991) reported that TAg and various ssbs have opposing effects on polymerase α/primase activity with this template DNA. TAg stimulated, whereas hRPA and *E. coli* ssb both inhibited polymerase α/primase activity. Interestingly, TAg overcame the inhibition of DNA synthesis by both hRPA or ssb (Collins and Kelly, 1991), although this may depend upon the conditions of the reaction (see below).

Various levels of either hRPA, mRPA, scRPA or *E. coli* ssb were added to reactions containing unprimed ssM13 DNA and DNA polymerase α/primase, both in the presence and absence of TAg (Fig. 3*A*). In the absence of TAg, none of the ssbs had a large effect on the low level of synthesis by polymerase α/primase (Fig. 3*A*, *dashed lines*). When 5-fold higher levels of polymerase α/primase were used, all three ssbs inhibited synthesis (data not shown). As observed previously (Collins and Kelly, 1991), TAg stimulated DNA synthesis by polymerase α/primase about 10-fold in the absence of other factors. Both scRPA and *E. coli* ssb completely inhibited the stimulated level of DNA synthesis, whereas

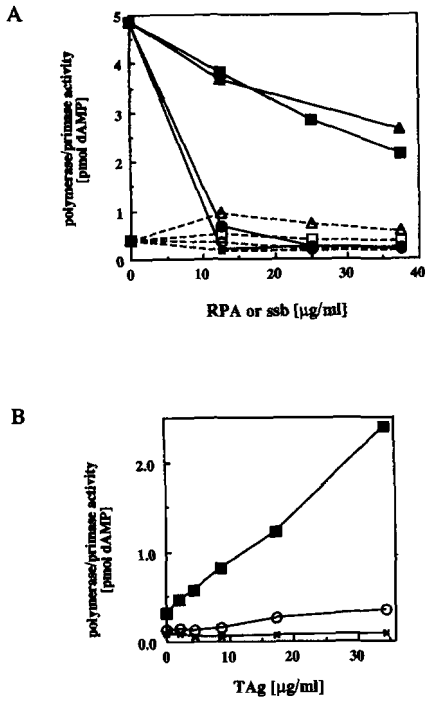

FIG. 3. **Yeast RPA cannot cooperate with TAg and polymerase α/primase in primosome assembly.** *A*, increasing amounts of hRPA (*squares*), mRPA (*triangles*), scRPA (*circles*), or *E. coli* ssb (*crosses*) were titrated into polymerase/primase reactions containing M13 mp18 ssDNA template and a fixed amount of polymerase α/primase either in the absence (*empty symbols, dashed lines*) or the presence (*filled symbols, solid lines*) of a fixed level of TAg. *B*, increasing amounts of TAg were titrated into polymerase/primase reactions containing M13 mp18 ssDNA template, a fixed amount of polymerase α/primase, and fixed levels of either hRPA (*filled squares*), scRPA (*empty circles*), or *E. coli* ssb (*crosses*). In both assays, polymerase/primase activity is expressed as picomoles of dAMP incorporated in 30 min in a 0.05-ml reaction.

hRPA and mRPA only partially attenuated the TAg stimulation (Fig. 3*A*, *solid lines*). In the converse experiment, increasing amounts of TAg were added to reactions containing ssM13 DNA template, polymerase α/primase, and a fixed level of each RPA or *E. coli* ssb. Under these conditions, TAg could not stimulate polymerase α/primase activity in the presence of *E. coli* ssb or scRPA, but was able to stimulate polymerase α/primase activity in the presence of hRPA (Fig. 3*B*).

These results are summarized in Table I. The only differences in activities between human and yeast RPA were the inability of scRPA to allow TAg-stimulated DNA synthesis by polymerase α/primase on an ssDNA template and the inability of scRPA to support SV40 origin-dependent DNA replication. In support of this correlation, murine RPA, which can replace hRPA in SV40 DNA replication reactions (Schneider *et al.*, 1992), behaves identically with hRPA in the origin-dependent DNA synthesis assay and in the TAg-stimulated polymerase α/primase assay on unprimed ssDNA template. Furthermore, *Crithidia fasciculata* RPA, which cannot reconstitute SV40 DNA replication (Brown *et al.*, 1992), does not allow TAg stimulation of polymerase α/primase on an ssb-coated ssDNA template (data not shown).

The differing effect of RPAs from human and yeast cells on DNA synthesis by human polymerase α/primase only occurred in the presence of TAg. This suggested that a TAg-RPA interaction might be mediating the effect on polymerase α/primase. To test the hypothesis that there is a direct physical interaction between hRPA and TAg and not between

TABLE I

Function of RPA from divergent species in enzymatic assays

Assay	hRPA	mRPA	Sc RPA	*E. coli* ssb
SV40 origin unwinding (+ TAg)[a]	+	+	+	+
Polymerase α stimulation[b]	+	+	+	−
Primase stimulation[c]	+	+	+	−
TAg stimulation of polymerase α/primase[d]	+	+	−	−
ori-dependent DNA synthesis[e]	+	+	−	−
SV40 DNA replication[f]	+	+	−	−

[a] Origin-unwinding assay used a plasmid containing the SV40 origin and purified TAg and topoisomerase I (Brill and Stillman, 1989).

[b] Polymerase α stimulation was evaluated by dTMP incorporation using poly(dA)/oligo(dT) as a template.

[c] Primase assay used a large excess of poly(dT) template; priming was quantitated by the incorporation of dAMP on the RNA primers by excess DNA polymerase I (Klenow fragment).

[d] Polymerase α/primase activity was assayed through dAMP incorporation on unprimed ssDNA (M13 mp18).

[e] ori-dependent DNA synthesis was measured by dAMP incorporation using a dsDNA plasmid containing the SV40 origin and purified TAg, topoisomerases I and II, and polymerase α/primase.

[f] SV40 DNA replication was measured by dAMP incorporation in an assay containing purified TAg, PCNA, RFC, topoisomerases I and II, and the DNA polymerase containing fraction IIA (Tsurimoto *et al.*, 1989).

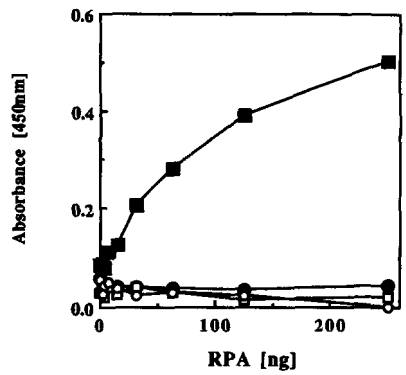

FIG. 4. **Yeast RPA does not bind to TAg.** Either TAg (*filled symbols*) or bovine serum albumin (*empty symbols*) was bound in ELISA plates (1 μg/well). After blocking and washing, increasing amounts of scRPA (*circles*) and hRPA (*squares*) were incubated in both the TAg- and BSA-coated wells for 30 min. After washing, the bound scRPA and hRPA were detected with the appropriate polyclonal rabbit antiserum and subsequent incubations with peroxidase-conjugated swine anti-rabbit antibody and a chromogenic substrate.

scRPA and TAg, we utilized an ELISA similar to that described by Dornreiter *et al.* (1992). The solid phase was either TAg or bovine serum albumin. These were incubated with varying amounts of human or yeast RPA. The presence or absence of RPA bound to TAg was then determined by addition of rabbit sera raised against the appropriate RPA molecule, followed by antibody detection with swine anti-rabbit immunoglobulin linked to horseradish peroxidase. The results, shown in Fig. 4, indicated that TAg bound to hRPA (as had been shown previously by Dornreiter *et al.* (1992)) but not to scRPA. To eliminate DNA-mediated interactions in the ELISA analyses, the RPA was preincubated with micrococcal nuclease and the nuclease was present throughout the incubation with TAg.

DISCUSSION

scRPA cannot replace hRPA in the SV40 DNA replication reaction *in vitro* (Brill and Stillman, 1989). This led us to investigate what biochemical function of hRPA the scRPA

could not perform. We reasoned that if scRPA mirrored hRPA in all functional aspects but one, the difference must indicate a function of RPA essential for DNA replication.

Both hRPA and scRPA support unwinding from the SV40 origin in conjunction with TAg, as has been shown for several other ssbs (Brill and Stillman, 1989; Kenny *et al.*, 1989). We and others have investigated the ssDNA-binding properties of the two RPAs and found them similar (Brill and Stillman, 1989; Alani *et al.*, 1992, Kim *et al.*, 1992).[2] In addition, neither RPA had any detectable effect on the helicase or ATPase activities of TAg.[2]

We suspected that the difference between human and yeast RPA in replication might be due to the reported difference in their ability to stimulate polymerase α/primase on a multiply primed homopolymer DNA template (Erdile *et al.*, 1991b). Our results, however, conflicted with the results in the literature since both hRPA and scRPA stimulated polymerase α activity. We confirmed our results by purifying scRPA from *S. cerevisiae* grown and harvested during logarithmic growth, as well as from yeast grown to stationary phase. These RPA preparations differ in the state of phosphorylation of the RPA middle-sized subunit. All preparations stimulated human polymerase α activity to a similar extent.[2] There is a great deal of variability in the extent of RPA stimulation in this assay (we have seen from 2-fold to greater than 60-fold stimulation), depending on parameters such as the time and temperature of preincubation of RPA with the DNA template and the levels of polymerase α/primase used in the assay. This variability cannot, however, explain the discrepancy between our findings and those of Erdile *et al.* (1991b), since both RPAs vary similarly in their ability to stimulate the polymerase activity of polymerase α/primase. Although there is a small difference between human and yeast RPA in their relative ability to stimulate polymerase α, this difference is negligible and does not appear to be of the magnitude that could account for the complete lack of SV40 DNA replication and DNA synthesis when scRPA is used in place of hRPA.

When a homopolymer template (poly(dT)) was used, both human and yeast RPA stimulated primase activity to a similar degree. In contrast, it has been published that hRPA inhibits primase activity on ssDNA M13 DNA templates (Collins and Kelly, 1991), and we have confirmed their results (data not shown). The difference between the two observations may be explained by a difference in the levels of template DNA in each assay (33 μg/ml poly(dT) *versus* 1 μg/ml M13 DNA). This difference, as well as the differing reaction conditions, could explain why RPA stimulates primase in one assay but not in the other. Stimulation of priming could be a result of TAg targeting polymerase α/primase to the DNA template, as was previously suggested to account for the ability of TAg to stimulate the polymerase activity of polymerase α/primase (Collins and Kelly, 1991).

It was in looking at the effect of the RPAs on TAg stimulation of polymerase α/primase synthesis where we detected a major difference between human and yeast RPA. Although all RPAs and *E. coli* ssb inhibited polymerase α/primase activity on unprimed DNA templates, in the presence of TAg hRPA (and mRPA) was much less effective than either scRPA or *E. coli* ssb in this inhibition. In other words, T antigen could stimulate polymerase α/primase activity when the template DNA was coated with hRPA (or mRPA), but not when the template DNA was coated with scRPA or *E. coli* ssb. Thus, the only obvious biochemical difference between human and yeast RPA that correlated with their respective abilities to reconstitute SV40 DNA replication was the ability to biochemically interact with T antigen and allow polymerase α/primase to function on coated ssDNA templates. The fact that this correlation extended to murine RPA further strengthens our conclusion. It is this activity that likely explains why hRPA supports synthesis on artificial fork templates whereas *E. coli* ssb does not (Erdile *et al.*, 1991a). Such templates have no potential primers and little ssDNA (therefore easily saturated by any ssb or RPA present). All synthesis depends upon priming by polymerase α/primase that, as noted above, can be stimulated by T antigen to prime on hRPA-coated ssDNA templates but not on *E. coli* ssb-coated templates.

It was noted by Collins and Kelly (1991) that TAg stimulated the synthesis of polymerase α/primase on ssM13 templates in the presence of either hRPA or *E. coli* ssb. At first glance, our findings with *E. coli* ssb were not consistent with their results. The difference, however, in the Collins and Kelly result and our own seems to depend upon the relative levels of ssb and DNA template. In their experiment, both the RPA and ssb were used at lower concentrations (12 μg/ml of hRPA and 4 μg/ml of *E. coli* ssb at maximal levels), as compared with our experiments (25–50 μg/ml). The DNA template was present at 1.0 μg/ml in both experiments. The ssDNA-binding characteristics of human and yeast RPA indicate that the template DNA is saturated (completely coated) at levels of RPA in the range between 12.5 and 25 μg/ml (Kim *et al.*, 1992).[2] At subsaturating levels of protein (1.0 μg/ml ssDNA with $<$12.5 μg/ml RPA), TAg was still able to stimulate synthesis by polymerase α/primase with any RPA or with *E. coli* ssb.[2]

Our results suggested that the difference between human and yeast RPA in their ability to support synthesis by polymerase α/primase was TAg-dependent. This implied that there was an interaction between TAg and hRPA, but not between TAg and scRPA. This hypothesis was directly confirmed by ELISA analyses.

As mentioned in the introduction, direct physical interactions occur between polymerase α and TAg and primase and hRPA (Smale and Tjian, 1986; Gannon and Lane, 1987, 1990; Dornreiter *et al.*, 1990, 1992). Moreover, TAg and hRPA also directly interact (Dornreiter *et al.*, 1992). These results are entirely consistent with, and help explain, the functional interactions reported herein. Together, the data suggest the following model (Fig. 5). During initiation of DNA replication at the SV40 origin and during lagging strand DNA synthesis at a replication fork, TAg (via an RPA interaction) actively promotes primer formation and DNA synthesis by polymerase α/primase by loading the polymerase/primase complex onto the template DNA. It is likely that these interactions occur when TAg is active as a hexameric DNA helicase, since T antigen hexamers appear to be functional at the replication fork (SenGupta and Borowiec, 1992; Wessel *et al.*, 1992).

Other DNA replication systems also require specific proteins to allow priming on ssb-coated templates; however, these systems often require two proteins to accomplish the two roles played by TAg. One role is the stimulation of priming on naked ssDNA by the cognate helicase. Just as polymerase α/primase is stimulated by TAg on ssDNA templates, prokaryotic primases are stimulated by their cognate helicases (Nossal, 1980; Arai and Kornberg, 1981; Liu and Alberts, 1981). The second role TAg plays is in negating the inhibition of priming by hRPA (accomplished via a TAg/RPA interaction). *E. coli* primase (dnaG) is also ineffective in priming on ssDNA templates coated by *E. coli* ssb. Bacteriophage λ O and P proteins can cooperate to stimulate priming on such a tem-

[2] T. Melendy and B. Stillman, unpublished observations.

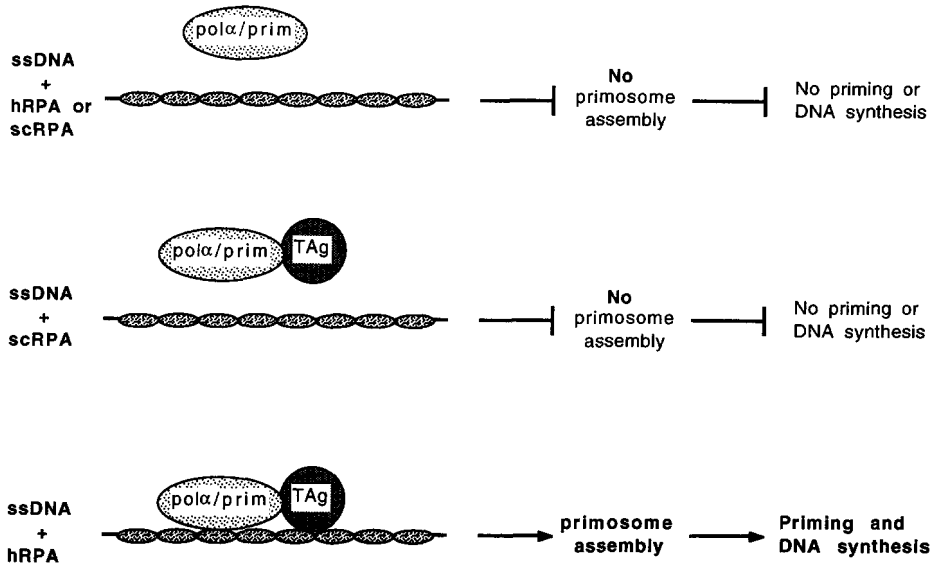

FIG. 5. **Primosome assembly in SV40 DNA replication.** When any RPA (or *E. coli* ssb) coats a ssDNA template, the polymerase α/primase complex is blocked from priming on that template. TAg, which stimulates priming by polymerase α/primase strongly on naked ssDNA, cannot stimulate priming when the ssDNA template is coated by a noncognate ssb (such as scRPA or *E. coli* ssb). In contrast, when the ssDNA is coated by hRPA (or mRPA), TAg is capable of stimulating priming by polymerase α/primase. Hence, TAg, hRPA, and polymerase α/primase act cooperatively during primosome assembly.

TABLE II

Conserved functions of DNA replication initiation proteins

Human/SV40	Phage T4	*E. coli*	Function
RPA	Gene 32p	ssb	Single-stranded DNA binding; stimulates DNA polymerase
pol α/primase[a]	Gene 61p	dnaG	Priming
T antigen	Gene 41p	dnaB	Helicase; stimulation of priming on ssDNA
T antigen	Gene 59p	dnaC	Allows stimulation of priming on ssb-coated template DNA (primosome assembly)

[a] In eukaryotes, primase is tightly associated with DNA polymerase α. In prokaryotes, however, the primase (either gene 61p or dnaG) is not tightly associated with a DNA polymerase.

plate in the presence of dnaB protein, the cognate helicase. λ O protein appears to bind to the DNA, whereas λ P binds both λ O and dnaB (LeBowitz *et al.*, 1985). In replication of the *E. coli* chromosome from *ori* C, the dnaC protein may be playing this role by effecting the binding of dnaB protein to ssb-coated templates. It is unknown whether this is an active process of dnaC bringing dnaB to the template or simply the result of dnaC stimulating the ssDNA binding properties of dnaB through allosteric mechanisms (Wahle *et al.*, 1989a, 1989b). Once the dnaB helicase is bound to the ssb-coated DNA, the dnaG primase can recognize the template and make primers for DNA synthesis. The primosome assembly proteins (PriA (n′), PriB (n), PriC (n″), dnaB, dnaC, and dnaT (i)) also cooperate to load primase onto single-stranded ϕX174 DNA that is coated with *E. coli* ssb. The PriA protein, like dnaB and T antigen, is a DNA helicase (for a review, see Kornberg and Baker (1991).

An analogous situation exists in the bacteriophage T4 system. The gene 59 protein binds tightly to DNA and to the gene 32 protein (ssb). In addition, it assists in fork movement with the gene 41 protein (helicase) and stimulates priming by gene 61 protein (primase) on gene 32 protein-coated templates in the presence of gene 41 protein (Hacker *et al.*, 1989).[3] It therefore appears that proteins such as the *E. coli* dnaC protein, the λ P protein, and the phage T4 gene 59-encoded protein interact with a DNA helicase and the ssb-coated template DNA to facilitate DNA primase activity. We propose that SV40 TAg also functions like dnaC, λ P protein, and the T4 phage gp59 protein to facilitate primase recognition of RPA-coated DNA. TAg appears to be playing a dual role in primosome assembly: that of the DNA helicase, which stimulates primase function (like dnaB and gene 41 protein), and the bridging protein, which, with its cognate helicase, can allow priming on ssb-coated templates (like dnaC, λ P, and T4 gp59 proteins) (see Table II). Taking these analogies further, we speculate that eukaryotic cells contain proteins that might function like TAg, either as a DNA helicase or as a dnaC-, λ P-, or T4 gp59-like activity, or both. Such a protein from human cells might stimulate the activity of polymerase α/primase on hRPA-coated template DNAs. We are currently searching for such proteins.

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[3] B. Alberts, personal communication.

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