Role of Protein−Protein Interactions in the Function of Replication Protein A (RPA): RPA Modulates the Activity of DNA Polymerase α by Multiple Mechanisms

Katherine A. Braun,[‡] Ye Lao,[‡] Zhigang He,[§] C. James Ingles,[§] and Marc S. Wold*,[‡]

Department of Biochemistry, University of Iowa College of Medicine, 51 Newton Road, Iowa City, Iowa 52242-1109, and Banting and Best Department of Medical Research and Department of Molecular and Medical Genetics, University of Toronto, Toronto, Ontario M5G 1L6, Canada

Received February 28, 1997; Revised Manuscript Received May 2, 1997[⊗]

ABSTRACT: Replication Protein A (RPA) from human cells is a stable complex of 70-, 32-, and 14-kDa subunits that is required for multiple processes in DNA metabolism. RPA binds with high affinity to single-stranded DNA and interacts with multiple proteins, including proteins required for the initiation of SV40 DNA replication, DNA polymerase α and SV40 large T antigen. We have used a series of mutant derivatives of RPA to map the regions of RPA required for specific protein−protein interactions and have examined the roles of these interactions in DNA replication. T antigen, DNA polymerase α and the activation domain of VP16 all have overlapping sites of interaction in the N-terminal half (residues 1−327) of the 70-kDa subunit of RPA. In addition, the interaction site for DNA polymerase α is composed of two functionally distinct regions, one (residues 1−∼170) which stimulates polymerase activity and a second (residues ∼170−327) which increases polymerase processivity. In the latter, both the direct protein−protein interaction and ssDNA-binding activities of RPA were needed for RPA to modulate polymerase processivity. We also found that SV40 T antigen inhibited the ability of RPA to increase processivity of DNA polymerase α, suggesting that this activity of RPA may be important for elongation but not during the initiation of DNA replication. DNA polymerase α, but not T antigen also interacted with the 32- and/or 14-kDa subunits of RPA, but these interactions did not seem to effect polymerase activity.

Human Replication Protein A (RPA)[1] is a ssDNA binding protein composed of three subunits of 70-, 32-, and 14-kDa (*1*, *2*; see also *3* for review of RPA). RPA is required for DNA replication, DNA repair, and recombination (*1*, *2*, *4*−*7*). Homologues of RPA have been identified in all eukaryotes examined (*8*−*14*); although, only human RPA and closely related homologues can support SV40 DNA replication (*15*−*17*). RPA is required for both the initiation and elongation phases of SV40 DNA replication (*1*, *2*, *4*, *16*, *18*−*20*). RPA stimulates the activity of many DNA helicases including the viral protein SV40 large T antigen (T antigen) (*1*, *21*−*24*). Also, RPA stimulates the activities of DNA polymerase α/primase complex (DNA polymerase α), DNA polymerase δ and DNA polymerase ϵ (*20*, *25*−*27*).

The initiation of SV40 DNA replication requires RPA, DNA polymerase α, and T antigen (*4*, *28*, *29*). T antigen recognizes and binds the SV40 origin of replication distorting the dsDNA (*30*−*33*). RPA stimulates the T antigen-mediated unwinding of the SV40 origin of replication (*21*, *34*). This reaction requires only the ssDNA-binding activity of RPA since other RPA homologues as well as *Escherichia coli* SSB can support this process (*1*, *21*, *35*). DNA polymerase α is recruited to the partially unwound SV40 origin of replication to begin the synthesis of the first RNA primer (*16*, *18*, *19*). Protein−protein interactions occur between the catalytic subunit of DNA polymerase α and T antigen, between the primase subunits of DNA polymerase α and RPA, and between RPA and T antigen (*36*−*38*). These interactions seem to be important in recruiting DNA polymerase α during initiation and can also affect the activity of DNA polymerase α (*16*, *19*, *21*, *39*). Both T antigen and RPA can modulate the polymerase and primase activities of DNA polymerase α (*16*, *25*, *40*). RPA stimulates DNA polymerase α in both polymerase assays as well as primer extension assays using a poly(dA) template and inhibits primer synthesis on ssM13 (*16*, *19*). This inhibition can be partially overcome by the addition of T antigen (*16*, *19*). T antigen stimulates both priming and polymerase activity of DNA polymerase α on ssM13 (*19*).

In addition to interacting with proteins involved in the initiation of DNA replication, RPA has been shown to interact with a number of other proteins including proteins involved in nucleotide excision repair (XPA, XPG, ERCC1/XPF) (*41*−*43*), recombination (RAD52) (*44*), the activation of transcription (and DNA replication) (GAL4, VP16, p53) (*45*−*48*), and organization of replication foci (FFA-1) (*49*). RPA interactions with DNA repair protein XPA appear to be important in damage recognition (*42*) and RPA−p53

[†] These studies were supported by grants from the National Institutes of Heath, GM44721 (K.A.B., Y.L., M.S.W.) and from the Medical Research Council of Canada (C.J.I.). Z.H. was the recipient of a Medical Research Council of Canada Studentship.

* To whom correspondence should be sent. Tel: (319) 335-6784. Fax: (319) 335-9570. E-mail: marc-wold@uiowa.edu.

[‡] University of Iowa College of Medicine.

[§] University of Toronto.

[⊗] Abstract published in *Advance ACS Abstracts,* June 15, 1997.

[1] Abbreviations: RPA, human replication protein A; scRPA, *S. cerevisiae* replication protein A; DNA polymerase α, DNA polymerase α/primase complex; ssDNA, single-stranded DNA; ELISA, enzyme-linked immunosorbent assay.

S0006-2960(97)00473-X CCC: $14.00

interactions may be involved in modulating the activity of p53 (*48*). The role of other specific RPA−protein interactions is not known, but it is likely that they are essential for various processes in DNA metabolism (*3*).

The precise role of the three subunits of RPA during DNA replication is not known. All three subunits are required to support SV40 DNA replication (*20*, *50*−*53*) and DNA excision repair (*54*−*59*). The 70-kDa subunit contains intrinsic ssDNA-binding activity as well as regions responsible for protein−protein interactions (*8*, *20*, *37*, *45*−*47*, *52*, *60*−*62*). Recently, it has been shown that the 70-kDa subunit is responsible for the stimulation of DNA polymerase α and DNA polymerase δ activity (*63*). The 70-kDa subunit can be divided into three functional domains: an N-terminal domain, a central ssDNA-binding domain, and a C-terminal subunit interaction domain (*35*, *52*, *63*−*67*). Only the N-terminal domain can be deleted without affecting the function of RPA in SV40 DNA replication (*35*, *63*). The 32-kDa subunit is phosphorylated in a cell cycle-dependent manner on multiple serines (*68*−*71*). The role of this phosphorylation is not known, but it may be involved in coordinating DNA metabolism with other cellular processes (*72*). The 32-kDa subunit also interacts with the DNA repair protein, XPA, and RAD52 (*42*, *44*, *62*) and has been reported to interact with T antigen (*53*). In addition, both the 32- and the 14-kDa subunits are essential for formation of a functional heterotrimeric RPA complex (*51*).

In these studies, we have mapped the regions of RPA required for interactions with DNA polymerase α, T antigen, and the activation domain of VP16 and have examined the role of specific domains of the 70-kDa subunit of RPA on the function of DNA polymerase α and T antigen. Using mutant forms of RPA in enzyme-linked immunosorbent assays (ELISA), we show that both the N-terminal region and a portion of the ssDNA-binding domain are primarily responsible for the interactions with T antigen, DNA polymerase α, and VP16. In addition, the interactions between RPA and DNA polymerase α are necessary for the stimulation of the polymerase activity on poly(dA):oligo(dT) templates. T antigen interfered with the ability of RPA to stimulate the activity of DNA polymerase α, suggesting that multiple sets of interactions may be required during DNA replication.

EXPERIMENTAL PROCEDURES

Materials. [α-^{32}P]dTTP and [α-^{32}P]dATP were obtained from Amersham. Poly(dA) was synthesized by Midland with a length of 1000−2000 nt. Oligo(dT)$_{30}$ was synthesized by Bio-Synthesis Inc.

DNA Polymerase α Purification. DNA polymerase α was purified using a SJK-237 Sepharose 4B affinity column (*60*, *73*). 21 mg of SJK-237, a non-neutralizing monoclonal antibody to DNA polymerase α (*74*), was coupled to 7 mL of CNBr-activated Sepharose 4B (Pharmacia) as described by the manufacturer. Unless noted, all manipulations were carried out at 4 °C. Cytoplasmic extracts (*60*) from 75.5 L (4.4 g of protein) of HeLa cells were passed over an Affi-Gel Blue column as described previously (*8*, *75*). 250 mL (∼1 g of protein) of Affi-Gel Blue flow-though was cleared by spinning at 15000*g* for 10 min before loading. The 7 mL affinity column was equilibrated with 10 column volumes of a buffer containing 50 mM KPO_4 (pH 7.5), 500 mM NaCl, 0.5% NP-40, 0.25% inositol, and 1 mM DTT (Wash Buffer 1). The cleared Affi-Gel blue flow-though was loaded at 1 column volume per hour onto the bottom of the affinity column. The affinity column was washed with Wash Buffer 1 until the protein concentration was near background as determined by a Bradford protein assay (using BSA as a standard) and then washed with 20 more column volumes of Wash Buffer 1. The KPO_4 was removed by washing the column with 3 column volumes of a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.25% inositol, and 1 mM DTT. The polymerase was eluted (top to bottom) using a buffer containing 50 mM Tris-HCl (pH 8.0), 3 M $MgCl_2$, and 1 mM DTT. The fractions containing protein as determined by a Bradford protein assay were pooled and diluted 2-fold with a buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1 mM DTT. The $MgCl_2$ was removed by dialysis in 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1 mM 2-mercaptoethanol, and 20% glycerol with three buffer changes. The EDTA was removed by dialysis in 50 mM Tris-HCl (pH 8.0), 1 mM 2-mercaptoethanol, and 20% glycerol. The protein is concentrated by dialysis in 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 1 mM 2-mercaptoethanol, and 50% glycerol.

The activity of DNA polymerase α was assayed using activated salmon sperm DNA which was generated by incubating sheared salmon sperm DNA (Sigma) with DNase I for 20 min. The 25 μL reactions included 60 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 300 μg/mL of acetylated BSA, 1 mM DTT, 100 μM each of dCTP, dGTP, dTTP, 50 μM dATP with 2.5 μCi of [α-^{32}P]dATP, 200 μg/mL of activated salmon sperm DNA, and various amounts of DNA polymerase α. The reactions were incubated at 37 °C for 30 min and the amount of acid insoluble radioactivity was determined (1 unit is 1 nmol of dNTPs incorporated in 30 min at 37 °C).

SV40 T antigen was purified using affinity chromatography from Sf9 cells infected with a baculovirus vector containing the gene encoding T antigen as previously described (*60*, *76*).

Nomenclature for Mutant Forms of RPA. Mutant forms of RPA containing only the 70-kDa subunit will be referred to as RPA70ΔXX and heterotrimeric RPA complexes referred to as RPA•XX where XX indicates the subunit mutated and/or the residues changed. Deletions from the N- or C-terminus are indicted by an N or C, respectively, and a number indicating the first residue deleted. Thus, RPA70ΔC442 is a form of the 70-kDa subunit in which residues 442−616 are deleted. All forms of RPA used in these studies are shown schematically in Figure 1A.

Construction of Deletion Mutants of the 32-kDa Subunit of RPA. Three C-terminal deletion mutants of the 32-kDa subunit of RPA (RPA32Δ223−271, RPA32Δ238−271, and RPA32Δ256−271; shown schematically in Figure 1) were produced by amplifying specific regions of the coding sequence using PCR. p3a-RPA32 (*51*) was used as a template. The N-terminal PCR primer was: 5′ CCAGGATCCGTCCGGCGTAGAGGA 3′. This primer contains a unique restriction site for *Xba*I. The C-terminal PCR primers were: 5′ CTGAAAGTGGATCCCTTAAAGTCGGACA 3′ (for 32Δ223−271), 5′ TGAGGATCCTTACATGTGTTTCAG 3′ (for 32Δ238−271), and 5′ CACAGGATCCTAGATGTCCCC 3′ (for 32Δ256−271). In each primer, nucleotide changes (underlined) were made in the wild-type

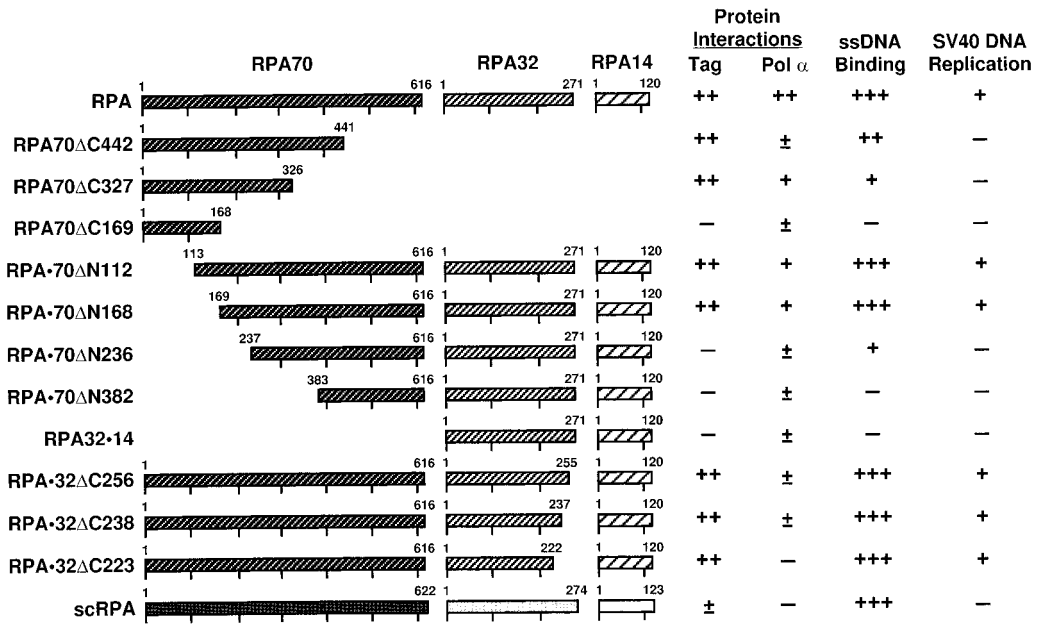

FIGURE 1: Schematic of RPA mutations. The left portion shows schematic diagrams of all RPA mutants used in this study. Beginning and ending amino acids of each mutant are indicated. The activities of each of the mutants in relation to wild-type RPA are shown to the right. The number of "+"s indicates the relative activity; −indicates no activity. Protein interactions with T antigen or DNA polymerase α were determined by ELISA in these studies. ssDNA-binding activity and ability to support SV40 DNA replication were determined previously (*35, 52*). The following RPA mutants were used in these studies (abbreviations in parentheses): RPA70ΔC442−616 (RPA70ΔC442), RPA70ΔC327−616 (RPA70ΔC327), RPA70ΔC169−616 (RPA70ΔC169), RPA·70ΔN1−112 (RPA·70ΔN112), RPA·70ΔN1−168 (RPA·70ΔN168), RPA·70ΔN1−236 (RPA·70ΔN236), RPA·70ΔN1−382 (RPA·70ΔN382), RPA32·14, RPA·32ΔC256−271 (RPA·32ΔC256), RPA·32ΔC238−271 (RPA·32ΔC238), RPA·32ΔC223−271 (RPA·32ΔC223).

sequence to generate a termination codon followed by a *Bam*HI restriction site. PCR were performed with Vent DNA polymerase (New England Biolabs) in a DNA Thermal Cycler (Perkin Elmer) using the following conditions for 25 cycles: 90 °C for 1 min, 50 °C for 1 min, and 72 °C for 3 min. The PCR products were isolated on a 1.2% agarose gel and purified from the gel using Wizard PCR Preps DNA purification system (Promega) according to the manufacturer's recommendations. The isolated PCR products and p3a-RPA32 were digested with *Xba*I and *Bam*HI (New England Biolabs), separated on a 1.2% agarose gel, and isolated using the Geneclean II kit (BIO 101, La Jolla, CA) according to manufacturer's recommendations. The DNA fragments containing mutated coding sequence of the 32-kDa subunit were individually ligated with the 4.9-kb *Xba*I−*Bam*HI fragment of p3a-RPA32 using T4 DNA ligase (New England Biolabs). Transformations were carried out as described (*77*). Recombinant plasmids were transformed into *E. coli* strain DH5α and isolated by the alkaline lysis method (*77*). The DNA sequence of each deletion mutant was confirmed by automated DNA sequencing at the DNA Core Facility at the University of Iowa. Plasmids expressing the mutated 32-kDa subunit with the other two subunits of RPA were made by digesting p3a-RPA32 derivatives with *Aat*II and *Bst*BI (New England Biolabs), isolating the 1.2−1.4 kb fragments containing the coding region of the C-terminal deletion mutants, and ligating each mutant coding region to the 8.3 kb *Aat*II and *Bst*BI fragment of p11d-tRPA (*51*) containing the coding regions for the 70- and 14-kDa subunits. The plasmids produced, ptRPA·32Δ223−271, ptRPA·32Δ238−271, and ptRPA·32Δ256−271, contained a T7 polymerase promoter, and cDNAs for the 70-, mutant 32-, and 14-kDa subunits, each proceeded by a Shine-Dalgarno ribosome binding site.

Induction and Purification of Mutant Forms of RPA. Recombinant human RPA (RPA), RPA32·14, and the yeast homologue of RPA (scRPA; Z. A. Sibenaller, unpublished data) were expressed in BL21(DE3) cells and purified as previously described (*51*). Deletion mutants of the 70-kDa subunit of RPA (RPA70ΔC442, RPA70ΔC327, RPA70ΔC169, RPA·70ΔN112, RPA·70ΔN168, RPA·70ΔN236, RPA·70ΔN382) were expressed and purified in a similar manner to RPA as previously described (*35*, *51*, *52*).

ptRPA·32Δ223−271, ptRPA·32Δ238−271, and ptRPA·32Δ256−271 plasmids were individually transformed into BL21 (DE3), grown and induced as described previously (*51*). Purification of the mutant forms of RPA was carried out as described previously with the modifications described below (*51*). Lysate from 2 L of induced culture was fractionated on Affi-Gel Blue and hydroxylapatite columns as described previously. Peak fractions from hydroxylapatite containing each mutant were applied to a Mono-Q column (HR 5/5, Pharmacia) equilibrated in HI buffer [30 mM HEPES (diluted from a 1 M stock at pH 7.8), 1 mM dithiothreitol (DTT), 0.25 mM EDTA, 0.5% (w/v) inositol, and 0.01% (v/v) Nonidet P-40] containing 50 mM KCl. The column was washed with 3 mL of HI-50 mM KCl buffer, followed by a 10 mL wash of HI buffer containing a linear salt gradient from 50−500 mM KCl. Both RPA·32ΔC238 and RPA·32ΔC256 were eluted between 300 and 400 mM KCl, which is consistent with wild-type RPA. RPA·32ΔC223 did not bind to the Mono-Q column. Therefore, the flow-through fractions containing RPA·32ΔC223 were applied directly on a 1 mL ssDNA cellulose column equilibrated in HI buffer with 200 mM NaSCN. The column was washed sequentially with 5 mL each of HI buffer containing 200 mM NaSCN, 600 mM NaSCN, 1 M NaSCN, and 1.5 M NaSCN. The protein was eluted at 600 mM

NaSCN. The purification of RPA mutants all resulted in yields of 0.5−1.5 mg of proteins per liter of culture. During purification, the mutant forms of RPA were monitored by 8%−14% gradient polyacrylamide gels followed by staining with silver or immunoblotting.

Enzyme-Linked Immunosorbent Assay (ELISA). ELISA was used to examine interactions between purified proteins (*36*). 96-well microtiter plates were coated with 10 pmol of RPA or a RPA mutant and incubated for 1 h at 25 °C. Plates were washed with phosphate-buffered saline (PBS) with 0.2% Tween-20 three times to remove unbound protein. Plates were blocked with 5% milk in PBS for 10 min at 25 °C and washed. Various amounts of either T antigen, DNA polymerase α, or BSA in PBS with 5% milk were added to each well, incubated for 1 h at 25 °C, and washed. Either anti-T antigen (419) (*78*) or anti-DNA polymerase α (SJK-237) (*74*) antibodies in PBS with 5% milk were added to the plates, incubated for 30 min at 25 °C, and washed. Anti-mouse IgG peroxidase conjugate (Sigma) in PBS with 5% milk was added to the plates, incubated for 30 min at 25 °C, and washed. Plates were developed using 0.8 mg/mL *o*-phenylenediamine in 0.05 M phosphate−citrate buffer with 0.03% sodium perborate. OD_{450} was then quantitated after 20−60 min at 25 °C using a microtiter plate reader. In each experiment, backgrounds were determined for wild-type and all mutant forms of RPA using BSA as the secondary protein. This allowed us to control for nonspecific interactions with the antibodies specific for either T antigen or DNA polymerase α. The data shown have these background values subtracted. In all assays the background values for all proteins were similar and close to zero.

Polymerase Assays. The polymerase activity of DNA polymerase α was examined in polymerase assays using a primed template in the absence of rNTPs (*16*, *21*). The template was generated by annealing oligo$(dT)_{30}$ primers to poly(dA) 1000−2000 nucleotides in length at a ratio of approximately one primer for every 600 nucleotides. The 10 μL reactions contained 30 mM HEPES (pH 7.8), 7 mM $MgCl_2$, 4 mM ATP, 50 μM dTTP with 2.5 μCi of [α-^{32}P]-dTTP, 40 μg/mL of acetylated BSA, 40 mM creatine phosphate, 100 μg/mL of creatine kinase, poly(dA):oligo-(dT) (20:1, 40 μM nucleotide), 75.6 ng of DNA polymerase α (0.70 units), and additional proteins as indicated. These reactions were incubated at 37 °C for 2 h, stopped by adding 10 μL of a solution containing 2% SDS, 50 mM EDTA, and 1 mg/mL of Proteinase K, and incubated at 37 °C for 30 min. The products were precipitated using 90 μL of 100% ethanol with the addition of 10 μL of a solution containing 6 M ammonium acetate and 0.6 mg/mL of tRNA. The precipitated products were resuspended in 10 μL of a solution containing 0.025% bromophenol blue, 0.025% xylene cyanol, 1.5% Ficoll Type 400, and 0.02% SDS. The products were denatured by incubating at 100 °C for 3 min and analyzed by electrophoresis on a denaturing 1% agarose gel (30 mM NaOH and 1 mM EDTA).

RESULTS

Interactions of RPA with T Antigen. Previously a number of mutant derivatives of RPA were made and characterized in our laboratory (*35*, *64*). Using these mutant forms, we have determined the regions of RPA involved in interactions with T antigen using ELISA. In these studies, 10 pmol of each RPA derivative were adsorbed to a microtiter plate. Increasing amounts of soluble T antigen were added, the plate was washed, and the bound T antigen was detected using antibodies specific to T antigen. The results from a typical assay are shown in Figure 2A. With wild-type RPA, addition of increasing amounts of T antigen resulted in an increased signal confirming the interaction between these two proteins. In contrast, when BSA was used in place of RPA, little change in the signal at 450 nm was observed as T antigen was added. This demonstrates that RPA-T antigen interactions are specific. When mutant forms of RPA were examined, three classes of interactions were observed: four derivatives interacted with an affinity close to wild-type RPA, four interacted very weakly giving signals only slightly higher than BSA, and scRPA interacted with an intermediate signal (Figure 2A).

The N-terminal 168 and the C-terminal 289 amino acids of the 70-kDa subunit of RPA were not required for interactions with T antigen, since RPA·70ΔN112, RPA·70ΔN168, RPA70ΔC442, and RPA70ΔC327 all gave signals similar to that of RPA. This indicates that residues between 168−327 of the 70-kDa subunit of RPA are essential for interactions with T antigen. In addition, both RPA·70ΔN236 and RPA·70ΔN382 lack portions of this region and did not interact with T antigen. The yeast homologue of RPA, scRPA, had a significantly reduced interaction with T antigen, suggesting that these interactions are primarily specific for human RPA. In contrast, RPA70ΔC169 did not interact with T antigen demonstrating that the first 168 amino acids of the 70-kDa subunit are not sufficient for interaction with T antigen. In these studies, the large subunit of RPA appears to be necessary and sufficient for interactions with T antigen, since the subcomplex containing the 32- and the 14-kDa subunits of RPA, RPA32·14, did not interact with T antigen and two mutants missing the two smaller subunits (RPA70ΔC442 and RPA70ΔC327) interacted well with T antigen.

Interactions of the 32-kDa Subunit of RPA with T Antigen. The data presented above indicate that the 70-kDa subunit of RPA is solely responsible for the interaction with T antigen. This differs from conclusions of a previous study which reported that a mutant form of RPA missing the C-terminal 30 residues of the 32-kDa subunit was unable to interact with T antigen (*53*). This particular mutant complex was also unable to support Tag-dependent unwinding or SV40 DNA replication, and these defects were postulated to be caused by the inability of this mutant derivative to properly interact with T antigen (*53*). In order to resolve this apparent contradiction, three mutant RPA complexes were made in which 14, 32, and 47 residues were deleted from the C-terminus of the 32-kDa subunit of RPA. These complexes were expressed in *E. coli*, purified to homogeneity. The ability of these mutant complexes to interact with SV40 T antigen was then examined. All three mutants, RPA·32ΔC223, RPA·32ΔC238, and RPA·32ΔC256, interacted with T antigen as well as wild-type RPA (Figure 2C). These data are consistent with those shown in Figure 2A and confirm that the C-terminus of the 32-kDa subunit RPA is not required for interactions with T antigen. We also determined the functional activities of these mutant complexes. The ssDNA-binding activity of all three mutant complexes was examined both in gel mobility shift assays (data not shown) and by monitoring quenching of intrinsic

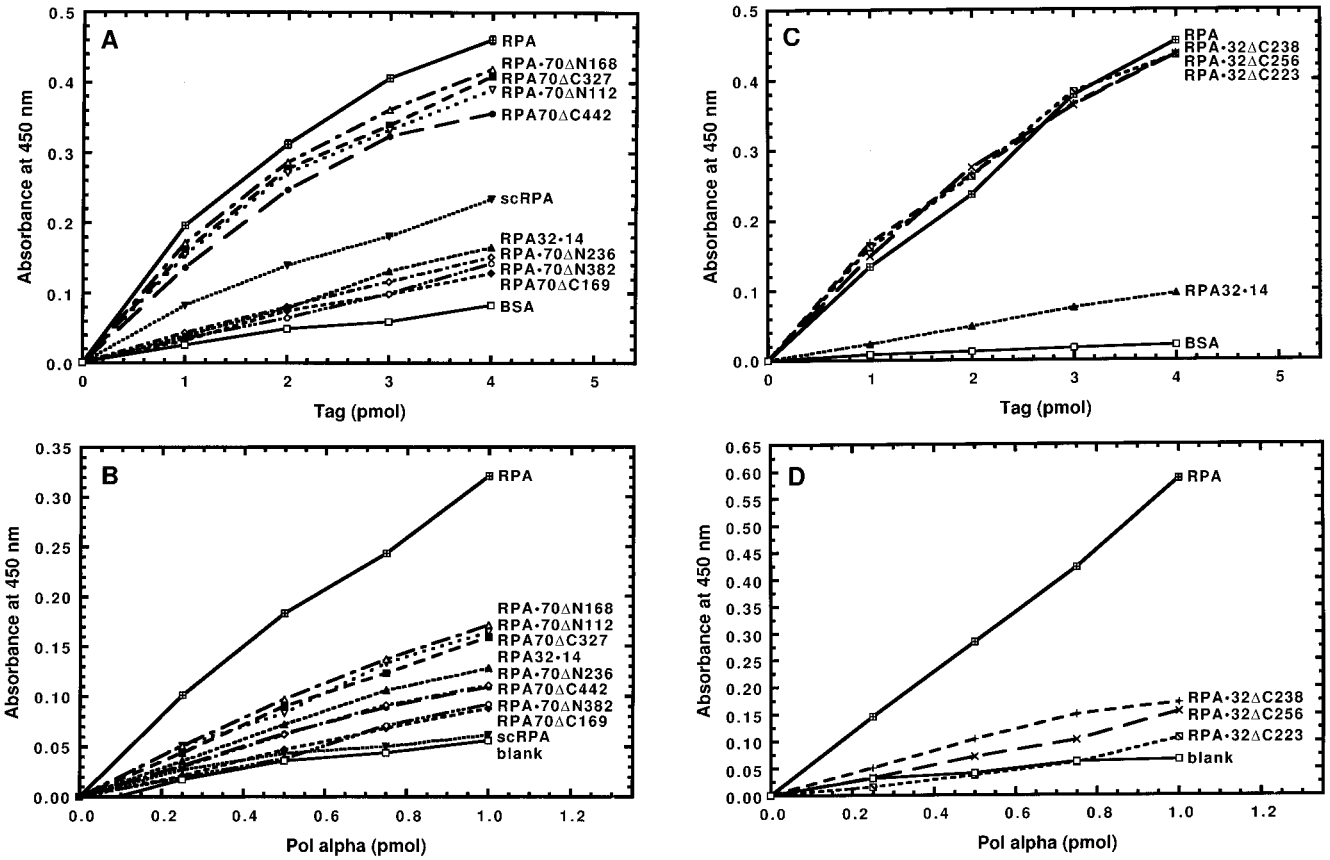

FIGURE 2: RPA−T antigen and RPA−DNA polymerase α interactions. (A, C) Wild-type or mutant RPA was immobilized on microtiter plates. Increasing amounts of T antigen (0, 1, 2, 3, or 4 pmol) were added to the plates and washed, and the bound protein was detected by an antibody specific to T antigen (419) followed by a peroxidase-coupled anti-mouse IgG antibody. Interactions were monitored at 450 nm after the addition of the substrate and plotted against the amount of T antigen. (B, D) Wild-type or mutant RPA was immobilized on microtiter plates. Increasing amounts of DNA polymerase α (0, 0.25, 0.5, 0.75, or 1 pmol) were added to the plates and washed, and the bound protein was detected by an antibody specific to DNA polymerase α (SJK-237) followed by a peroxidase-coupled anti-mouse IgG antibody. Interactions were monitored at 450 nm after the addition of the substrate and plotted against the amount of DNA polymerase α.

Table 1[a]

	protein			
	RPA	RPA·32ΔC223	RPA·32ΔC238	RPA·32ΔC256
K_A	2.6×10^7	3.2×10^7	2.9×10^7	3.2×10^7
error	4.4×10^6	3.7×10^6	2.7×10^6	5.7×10^6

[a] Binding constants were determined by monitoring the quenching of intrinsic fluorescence that occurs upon binding of oligo$(dT)_{30}$ as described previously (*35*, *84*). 50 nmol of wild-type or the indicated mutant form of RPA was titrated with oligo $(dT)_{30}$ in FB buffer [30 mM HEPES (diluted from 1 M stock at pH 7.8), 100 mM NaCl, 5 mM $MgCl_2$, l mM DTT, and 0.5% (w/v) inositol] or FB buffer with 1.5 M NaCl to obtain the RPA activities and ssDNA-binding constants, respectively. The quenching of intrinsic RPA fluorescence by titration was monitored using an SLM 4800 fluorometer. Binding isotherms were then fit to the Langmuir equation as described previously to obtain apparent binding constants (K_A) (*75*).

fluorescence upon binding to ssDNA (Table 1). These experiments showed that all three mutant complexes bound oligonucleotides with the same affinity as wild-type RPA. These results demonstrate that the C-terminus of 32-kDa subunit is not involved in ssDNA binding. We also determined the activity of the three mutant RPA complexes in T antigen-dependent unwinding and in SV40 DNA replication. In contrast to the previous study which used a mutant derivative similar to RPA·32ΔC238 (*53*), we found that all three mutant complexes had activity in both assays. When a 226 base pair origin-containing fragment of DNA was incubated with either T antigen or RPA alone, no unwinding was observed (Figure 3A). In the presence of both T antigen and wild-type or mutant forms of RPA, unwinding occurred and the levels of unwinding observed were similar with each of the four proteins (Figure 3A). When the activity of the three mutant forms was examined in SV40 replication reactions, RPA·32ΔC223, RPA·32ΔC238, and RPA·32ΔC256 all supported replication (Figure 3B). The activity of these three mutant complexes varied between individual assays, ranging between 30% and 80% that of wild-type RPA (data not shown). To examine whether deletions in the 32 kDa subunit caused significant changes in the structure of the RPA complex, proteolytic mapping was carried out on all three deleted forms of RPA. We found that protease sensitivity of both the 70- and 32-kDa subunits in the mutant complexes differed from that of wild-type RPA. When the mutant complexes were treated with proteases, the kinetics of digestion were slightly altered and at least one new proteolytic cleavage site in the 70-kDa subunit was observed [data not shown; see also (*64*)]. Therefore, deletion of the C-terminus of the 32-kDa subunit of RPA seems to have an effect on the overall structure of the RPA complex. We conclude that the C-terminus of RPA has a structural role in the RPA complex but is not needed directly for interactions with ssDNA or T antigen or for activity in DNA replication.

Interactions of RPA with DNA Polymerase α. The interactions between DNA polymerase α and individual RPA mutant complexes were also quantitated using ELISA (Figure

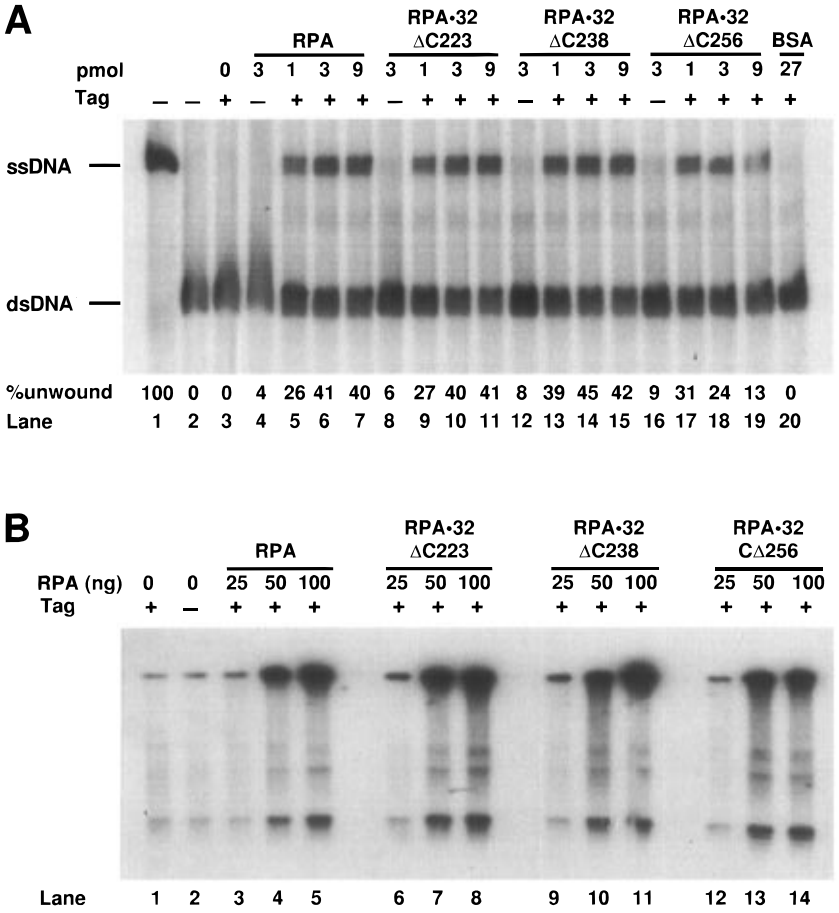

FIGURE 3: Activities of RPA complexes with C-terminal deletions in the 32-kDa subunit. (A) Stimulation of T antigen-dependent unwinding. The unwinding assays were performed as described (*35*): a labeled origin-containing fragment from pUC-HSO, T antigen, and increasing amounts (1, 3, or 9 pmol) of wild-type or mutant forms of RPA were incubated at 37 °C for 30 min. The stimulation of unwinding was examined by an 8% polyacrylamide gel in 1× TBE buffer followed by autoradiography. The percentage of the unwound DNA was determined after quantitation on a Packard Instant Imager. The positions of single- and double-stranded DNA are indicated. (B) Stimulation of SV40 replication. SV40 replication assays were carried out as described (*51*): T antigen (500 ng where indicated), pUC-HSO (50 μg), fraction CFII (20 μg; containing DNA polymerase α and δ and RF-C), fraction CF IBC (5.6 μg; containing proliferating nuclear cell antigen and protein phosphatase 2A), topoisomerase I (1 unit) and the indicated amounts of wild-type or mutant RPA were incubated at 37 °C for 2 h. The replication products were then isolated by isopropyl alcohol precipitation and examined by electrophoresis on a 1% agarose gel in 1x TAE followed by autoradiography.

2B). All of the mutant forms of RPA tested had a reduced ability to interact with DNA polymerase α, suggesting that each was missing a region important for its interaction with DNA polymerase α. Mutant derivatives in which the N-terminus of the 70-kDa subunit was deleted, RPA·70ΔN112 and RPA·70ΔN168, have signals half that of RPA, indicating that the N-terminal 112 amino acids are involved in interactions with DNA polymerase α. To test this conclusion, the interaction of DNA polymerase α with RPA70ΔC169 which contains only the first 168 residues of the 70-kDa subunit was examined. When RPA70ΔC169 was assayed, the signal was slightly above background (Figure 2B); however, when higher concentrations were assayed, an increased signal was observed (data not shown). This increased interaction at high protein concentration was not observed with other weak binding mutants, suggesting that RPA70ΔC169 is weakly but specifically interacting with DNA polymerase α. Large deletions from the N-terminus, RPA·70ΔN236 and RPA·70ΔN382, decreased interactions with DNA polymerase α further, suggesting that residues between 112 and 327 are also involved. These conclusions are supported by the finding that a mutant derivative containing only residues 1−326 (RPA70ΔC327) also interacted with DNA polymerase α, though at a level approximately half that of RPA. The interaction of RPA70ΔC442 with DNA polymerase α is much lower than RPA70ΔC327, which is unexpected since RPA70ΔC442 contains more of the 70-kDa subunit than RPA70ΔC327. This may be due to differences in folding between these two mutant derivatives. Significant interactions were also observed with RPA32·14. This suggests that the 32- and the 14-kDa subunits are also capable of interacting with DNA polymerase α. The three complexes containing C-terminal deletions in the 32-kDa subunit, RPA·32ΔC223, RPA·32ΔC238, and RPA·32ΔC256, were unable to interact with DNA polymerase α (Figure 2D). However, because these mutations affect the structure of the RPA complex, interpretation of these results is difficult. Therefore, we conclude that two regions of RPA are necessary for full interactions with DNA polymerase α: amino acids between 1−327 of the 70-kDa subunit of RPA and a region(s) on the 32- and/or 14-kDa subunits. The interactions between RPA and DNA polymerase α appear to be very specific because scRPA did not interact with DNA polymerase α (Figure 2B).

Interactions of RPA with VP16. In order to determine whether the regions of RPA essential for interactions with T antigen and DNA polymerase α overlap sites for interaction with regulators of the initiation of DNA replication, we

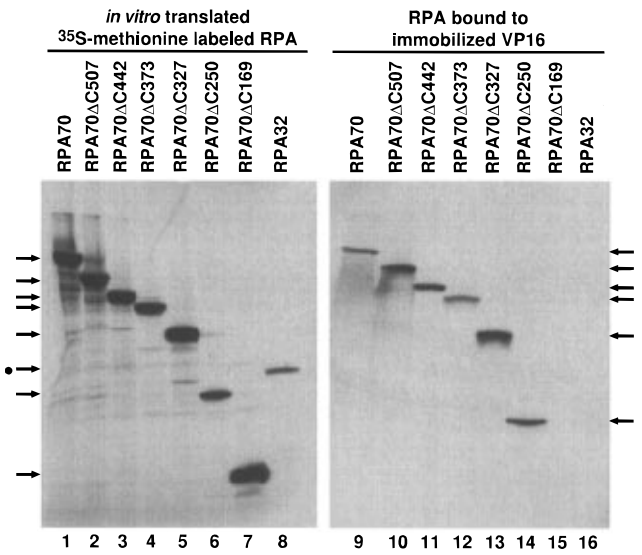

FIGURE 4: Mapping the interaction between RPA and the transactivation domain of VP16. The general procedure used for these studies was described previously (46). [^{35}S]Methionine-labeled wild-type or C-terminal deletions of the 70-kDa subunit of RPA were synthesized *in vitro* in a coupled transcription/translation rabbit reticulocyte lysate system and then were either analyzed directly by SDS−PAGE and autoradiography (A), or were incubated with glutathione-Sepharose beads containing immobilized GST-VP16 and then the bound proteins were analyzed by SDS−PAGE and autoradiography (B). Binding of the full-length 70-kDa subunit (RPA70), the 32-kDa subunit (RPA32), or of individual C-terminal deletions of the 70-kDa subunit of RPA to GST-VP16 was assessed. The mutant forms of RPA used are RPA70ΔC507−616 (RPA70ΔC507), RPA70ΔC442−616 (RPA70ΔC442), RPA70ΔC373−616 (RPA70ΔC373), RPA70ΔC327−616 (RPA70ΔC327), RPA70ΔC250−616 (RPA70ΔC250), and RPA70ΔC169−616 (RPA70ΔC169). Arrows indicate the position of full-length deriviatives of the 70-kDa subunit of RPA; the arrow with "•" indicates the position of the full-length 32-kDa subunit of RPA.

also mapped the interactions between RPA and the activation domain of the herpes simplex virion protein, VP16. A chimeric activator which contains the binding domain of GAL4 and the activation domain of VP16, GAL4-VP16, had been shown earlier to be capable of activating replication when bound to DNA sequences flanking the core elements of the origins of replication of both polyoma (*79*) and SV40 viral DNAs (*80*, *81*). The activation domains of VP16 and other transactivators bind directly to the 70-kDa subunit of RPA (*46*). We have now defined the region within this polypeptide required for this interaction. Individual C-terminal deletions of the 70-kDa subunit were transcribed and translated *in vitro* and assessed for their abilities to bind to GST-VP16 immobilized on glutathione beads. Figure 4 shows the results obtained with this series of C-terminal deletions. The smallest polypeptide derived from the 70-kDa subunit of RPA that still interacts strongly with VP16 is RPA70ΔC250 (Figure 4). The 32-kDa subunit of RPA did not interact with VP16 (Figure 4) [see also (*46*)]. This suggests that the first 249 amino acids of the 70-kDa subunit of RPA are important for interactions with VP16. In defining this N-terminal interaction site in more detail, we also examined the effect of several N-terminal deletions within the 70-kDa polypeptide. Any deletions which removed more than the first 65 amino acids abrogated binding to VP16. We conclude that the region of the 70-kDa subunit of RPA that interacts with this transactivation domain of VP16 overlaps but is not identical to the regions that interact with T antigen and DNA polymerase α.

RPA-Mediated Stimulation of DNA Polymerase α. It has been postulated that the specific interaction between RPA and DNA polymerase α are important for the initiation of DNA replication (*16*). In order to try to define the role of these interactions, we determined the effects of RPA and various RPA derivatives on DNA polymerase α activity using poly(dA):oligo(dT) as a template. These assays contained no ribonucleotides so that DNA polymerase activity could be examined independent of priming activity. When DNA polymerase α was assayed alone, a low level of synthesis of small molecular weight products was observed (lane 2, Figures 5A and 5B). As increasing levels of RPA were added, the amount of DNA synthesized increased greatly (Figure 5A, lanes 3−7) and the length of the products also increased. These results are consistent with previous studies (*16*, *19*, *21*, *25*, *40*) and show that RPA stimulates both the activity and the processivity of DNA polymerase α. In contrast to human RPA, addition of scRPA caused very little stimulation of DNA polymerase α activity (Figure 5B, lanes 18−22). scRPA has ssDNA-binding activity similar to that of RPA but is unable to interact with DNA polymerase α (see above). We conclude that the stimulation of DNA polymerase α on poly(dA):oligo(dT) templates by RPA requires specific protein−protein interactions.

When RPA32·14 was used in place of the heterotrimeric RPA complex, no stimulation of DNA synthesis was observed (Figure 5A, lanes 18−22). This indicated that the 70-kDa subunit of RPA is required for the stimulation of DNA polymerase α activity and that any interactions between the 32- and 14-kDa subunits and DNA polymerase α are not sufficient to affect polymerase activity. When a mutant form containing only residues 1−326 of the 70-kDa subunit (RPA70ΔC327) was used, both the level of synthesis and processivity of DNA polymerase α was increased; however, approximately 2-fold more protein was needed for maximal stimulation (Figure 4A, lanes 8−12). This shows that residues 1−326 of the 70-kDa subunit are sufficient for the stimulation of both properties of DNA polymerase α. Complexes in which portions of the N-terminal half of the interaction region had been deleted stimulated DNA polymerase α activity modestly but did cause high molecular weight products to be synthesized (see RPA·70ΔN112 and RPA·70ΔN168, Figure 5B, lanes 3−12). In contrast, when a deleted form of RPA containing only residues 1−168 (RPA70ΔC169) was used, the level of synthesis was stimulated weakly and no high molecular weight products were observed (Figure 5A, lanes 13−17). When all of the DNA polymerase α interaction domain of the 70-kDa subunit was deleted as in RPA·70ΔN382, no change in the level of DNA synthesis was observed (Figure 5B, lanes 13−17). We conclude that the stimulation of DNA polymerase α by RPA requires residues 1−327 of the 70-kDa subunit and that this domain affects DNA polymerase α by both stimulating polymerase activity and by increasing the processivity of DNA polymerase α. Furthermore the interaction region appears to be divided into two subdomains: residues 1−168 being required to stimulate polymerase activity and residues 169−327 being required both to increase processivity and stimulate activity.

These two subdomains within the 70-kDa subunit correlate to two previously identified structural domains of RPA: the

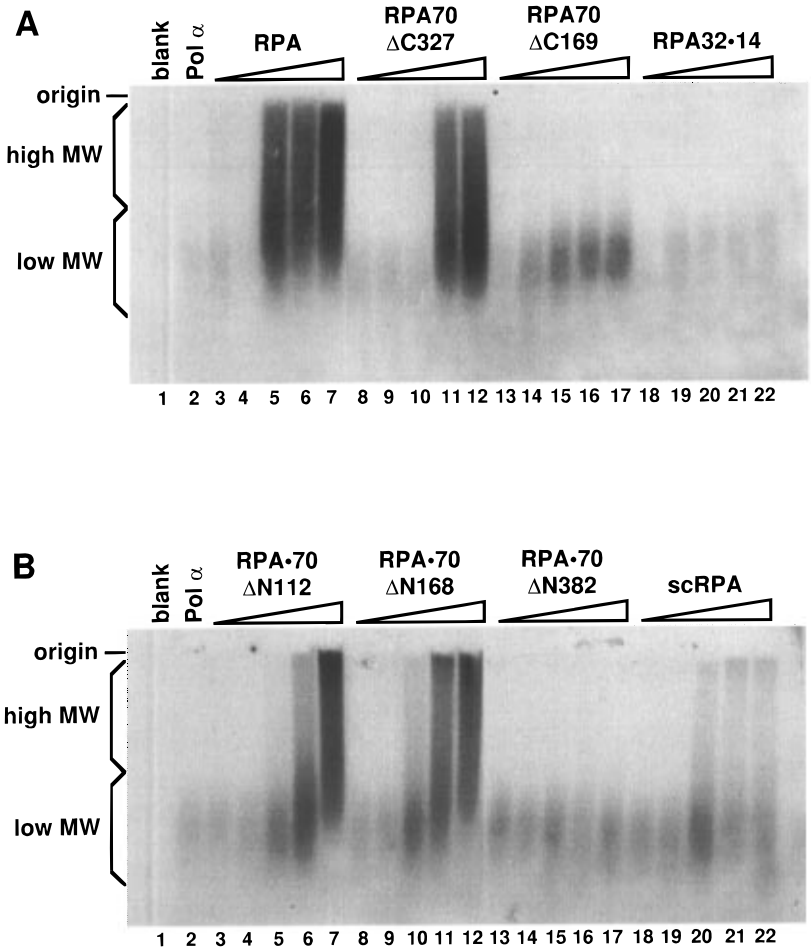

FIGURE 5: DNA polymerase α stimulation assay. (A) Reaction mixtures (10 μL) contained 400 μmol of poly(dA):oligo(dT)$_{30}$ (nt), 75.6 ng (0.7 units) of DNA polymerase α, and increasing amounts of either RPA, RPA70ΔC327, RPA70ΔC169, or RPA32•14 (0.2, 0.4, 0.8, 1.6, and 3.2 pmol) as indicated. (B) Reactions were performed as in part A using increasing amounts of RPA•70ΔN112, RPA•70ΔN168, RPA•70ΔN382, and scRPA. Lane labeled "blank" contains no DNA polymerase α. Positions of origin and high and low molecular weight products (high MW and low MW, respectively) are indicated.

N-terminal domain (residues 1−∼170) and the central DNA-binding domain (residues ∼170−450) (*35*, *52*, *64*, *67*). This raises the question of whether ssDNA-binding activity is playing a role in the stimulation and increased processivity caused by residues 169−327. In order to examine this question, competition experiments were carried out in which RPA was incubated with saturating amounts of oligo(dT)$_{30}$, and this mixture was added to a DNA polymerase assay. Previous studies have shown that oligo(dT)$_{30}$ fully occupies the DNA binding site of RPA and is bound with 50-fold higher affinity than oligo(dA) (*75*, *82*). As shown in Figure 6, when the ssDNA binding site on RPA was saturated with oligo(dT)$_{30}$, this complex was able to stimulate the synthesis of low molecular weight products but it did not increase the amount of high molecular weight products. Thus, the RPA·-oligo(dT)$_{30}$ complex behaves like RPA70ΔC169. These data show that ssDNA-binding activity is important for the RPA-mediated stimulation of DNA polymerase α processivity.

T Antigen Inhibition of RPA-Mediated Stimulation of DNA Polymerase α. In order to determine the effect of T antigen on DNA polymerase α and the ability of RPA to stimulate DNA polymerase α, a series of assays were carried out using all three proteins. T antigen has no effect on the synthesis by DNA polymerase α using poly(dA):oligo(dT) as a template (Figure 7, lanes 3−7). In the presence of 1 pmol of RPA, DNA polymerase α activity and processivity were stimulated as seen in previous figures. When increasing amounts of T antigen were added to RPA containing reactions, the amount of DNA synthesized and the size of the DNA products both decreased dramatically. We conclude that T antigen can inhibit the ability of RPA to stimulate DNA polymerase α activity on poly(dA):oligo-(dT) templates.

DISCUSSION

In these studies we have used a series of deleted forms of RPA to map sites of protein interactions and to examine the functional role of different regions of RPA on DNA polymerase α activity. Deletions can affect protein function either by removing amino acid residues essential for a particular function or perturbing folding to cause more global effects. In these studies, we believe that the former mechanism is responsible for our results. With the exception of the C-terminal deletions of the 32-kDa subunit of RPA, none of the deletions appears to causes major changes in the protease sensitive sites, indicating that the gross structure of the deleted complexes is close to normal [(*64*) and data not shown]. In addition, the major conclusions from these studies are generally based upon multiple polypeptides, reducing the possibility that they are based on an aberrantly folded RPA.

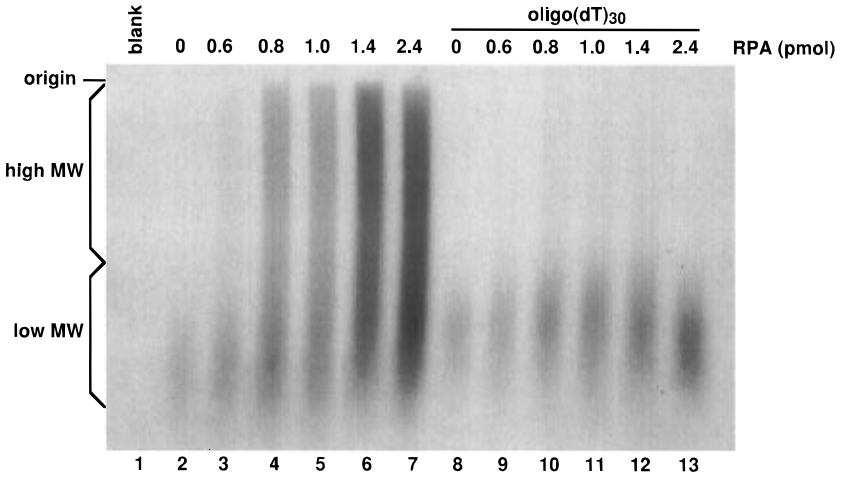

FIGURE 6: Role of ssDNA-binding domain of RPA in the stimulation of DNA polymerase α activity. The indicated amounts of RPA were incubated with 10 pmol of oligo(dT)$_{30}$ for 20 min at 37 °C. Increasing amounts of RPA alone or the RPA−oligo(dT)$_{30}$ complexes were added to a polymerase assay containing 400 μmol of poly(dA):oligo(dT)$_{30}$ (nt) and 75.6 ng (0.7 units) of DNA polymerase α. Lane labeled "blank" contains no DNA polymerase α. Positions of origin and high and low molecular weight products (high MW and low MW, respectively) are indicated.

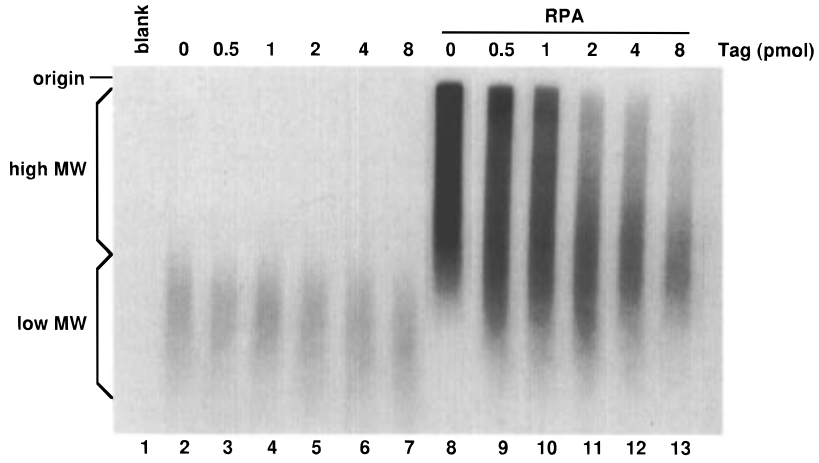

FIGURE 7: Effect of T antigen on the ability of RPA to stimulate DNA polymerase α. Increasing amounts of T antigen (0, 0.5, 1, 2, 4, or 8 pmol) in the absence or presence of 1 pmol of RPA as indicated were added to a polymerase assay containing 400 μmol of poly(dA):oligo(dT)$_{30}$ (nt) and 75.6 ng (0.7 units) of DNA polymerase α. Lane labeled "blank" contains no DNA polymerase α. Positions of origin and high and low molecular weight products (high MW and low MW, respectively) are indicated.

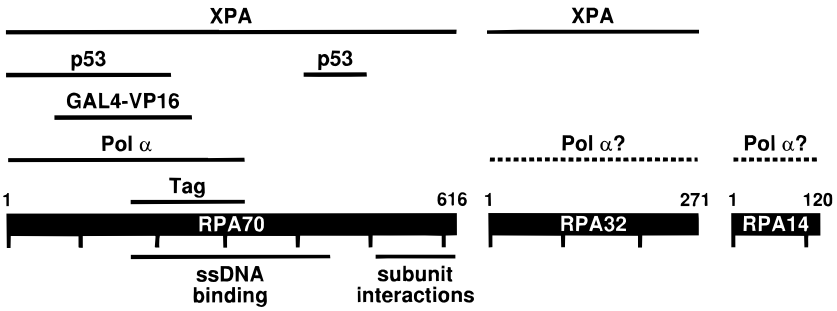

FIGURE 8: This diagram summarizes schematically the regions of RPA that interact with T antigen (Tag), DNA polymerase α (Pol α), the activation domain of VP16, p53 (*65*) and XPA (*42*, *44*, *62*). Horizontal lines above the schematic indicate sites of interaction, and dashed lines indicate possible sites of interaction; horizontal lines below schematic indicate previously identified functional domains.

Proteolytic mapping and deletion analysis indicate that the 70-kDa subunit of RPA consists of two structural domains: an 18-kDa N-terminal domain (residues 1−∼170) and a 52-kDa C-terminal domain (residues ∼170−616), the latter being composed of an ssDNA-binding domain (residues 168−450) and a subunit interaction domain (residues 507−616) (*35*, *52*, *64*). In addition, recent genetic and structural studies have shown that the DNA-binding domain is composed of two subdomains with highly conserved structures (*67*, *83*). In our studies, we have shown that T antigen, DNA polymerase α, and the activation domain of VP16 all interact with overlapping regions of the 70-kDa subunit of RPA (Figure 8). All three proteins interact with the N-terminal half of the DNA-binding domain (residues between ∼170 and 327) and DNA polymerase α and VP16 also required additional N-terminal residues of this subunit for their interactions. In addition, previous studies have mapped interactions with p53 to residues 1−221 and 411−

492 of the 70-kDa subunit of RPA (*65*; see also Figure 8). Thus, it appears that the N-terminal half of the 70-kDa subunit of RPA may be a general site for protein interactions.

We observed no interactions between T antigen and either the 32-kDa or the 14-kDa subunits of RPA. This is in contrast to one previous study which suggested that there were interactions between the C-terminus of the 32-kDa subunit of RPA and T antigen and that these interactions were essential for the function of RPA in DNA replication (*53*). In order to resolve these contradictory results, we made and characterized a series of C-terminal deletions of the 32-kDa subunit of RPA. Multiple preparations of all three deleted complexes interacted normally with T antigen and had identical activity in DNA-binding and T antigen-dependent unwinding assays. These mutants also functioned in SV40 DNA replication, although their activity was reduced from that of wild-type. Proteolytic mapping experiments suggested that the decrease in activity was probably due to the fact that the structure of these mutant complexes was different from that of wild-type RPA. Thus, the 32-kDa subunit of RPA does not appear to interact with T antigen but it may play a role in maintaining the proper structure of the RPA complex. This conclusion is consistent with our previous studies on the formation of the RPA complex (*51*).

Although the 32-kDa subunit of RPA does not interact directly with T antigen, it may contact DNA polymerase α. A subcomplex of the 32- and 14-kDa subunits was able to interact significantly with DNA polymerase α and all forms of RPA in which the 32- and 14-kDa subunits were missing had reduced interactions with DNA polymerase α. We also found that mutant RPA complexes in which the C-terminus of the 32-kDa subunit has been deleted were unable to interact with DNA polymerase α; however, because these deletions also affected the structure of the RPA complex, these data must be interpreted with caution.

Interactions between RPA and DNA polymerase α are essential for RPA stimulation of DNA polymerase α on poly(dA):oligo(dT) templates. scRPA binds ssDNA similarly to RPA but is unable to interact with DNA-polymerase α or to stimulate polymerase efficiently (see Figures 2B and 5). Deletion mutants of the 70-kDa subunit of RPA which contain a portion or all of the DNA polymerase α interaction domain were able to stimulate DNA synthesis to varying degrees, while mutants lacking this domain were unable to stimulate DNA synthesis. In addition, RPA32·14 was unable to stimulate DNA synthesis even though it interacts with DNA polymerase α. Thus, it appears that the interactions with the N-terminus of the 70-kDa subunit of RPA are important in modulating the activity of DNA polymerase α. This conclusion is consistent with previous genetic studies of scRPA (*83*), which showed that yeast cells containing an RPA in which the C-terminus of the 32-kDa subunit of RPA was deleted, were viable. In contrast, these same studies showed that the N-terminus of the yeast 70-kDa subunit was essential for viability, consistent with this region participating in critical protein−protein interactions.

Our studies indicate that ssDNA-binding activity is necessary for stimulation of the processivity of DNA polymerase α. When RPA was prebound to ssDNA, it had properties similar to the mutant RPA70ΔC169; it stimulated polymerase activity but was unable to stimulate processivity. However, several lines of evidence indicate that ssDNA-binding activity alone is not sufficient for stimulation of processivity. RPA70ΔC327 and RPA70ΔC442 both stimulated processivity, but the level of stimulation did not directly correlate with the ssDNA-binding activity of these polypeptides; RPA70ΔC442 binds ssDNA with 40-fold higher affinity than does RPA70ΔC327 (*35*) but stimulates DNA polymerase α more weakly. Also, scRPA, which binds ssDNA with high affinity, does not stimulate DNA polymerase α efficiently even when mixed with the N-terminal portion of the 70-kDa subunit of RPA (RPA70ΔC169, data not shown). We conclude that RPA modulates DNA polymerase α by at least two different mechanisms: the N-terminal domain of the 70-kDa subunit (residues 1−168) stimulates the activity of DNA polymerase α through direct protein−protein interactions, and the ssDNA-binding domain of the 70-kDa subunit contains a region (residues 168−327) which both stimulates polymerase α and increases its processivity. Both ssDNA-binding activity and protein−protein interactions seem to be necessary for the latter stimulation. There are several general mechanisms by which RPA could be stimulating DNA polymerase α: (i) interactions with RPA could modulate DNA polymerase α enzymatic activity, (ii) RPA interactions with ssDNA could make it a better substrate for DNA polymerase α (and/or reduce nonproductive binding by polymerase), or (iii) RPA could help recruit DNA polymerase α to the template though a combination of direct RPA−polymerase and RPA−ssDNA interactions. These studies do not distinguish between these models but are most consistent with RPA acting through at least two or possibly all three of these mechanisms.

T antigen has been shown to stimulate DNA polymerase α-mediated DNA synthesis on a primed M13 template (*19*). When a poly(dA):oligo(dT) template was used, T antigen had no effect on DNA synthesis (Figure 7). Thus the effect of T antigen appears to be template-dependent. The reason for this dependence is not known. T antigen inhibited the stimulatory effect of RPA on DNA polymerase α activity on a poly(dA):oligo(dT) template (see Figure 7). RPA interacts with the primase subunits of DNA polymerase α whereas T antigen interacts with the catalytic subunit (*36*−*38*), making it possible that both T antigen and RPA could interact with DNA polymerase α simultaneously. In contrast, T antigen and DNA polymerase α have overlapping binding sites on RPA. Thus, our observed inhibition of RPA stimulation of DNA polymerase α by T antigen may result from competition between T antigen and DNA polymerase α for RPA. Previously it has been shown that T antigen stimulates the synthesis of primers on RPA coated ssDNA by DNA polymerase α (*16*). It seems likely that multiple complexes and different interactions may be occurring between these three proteins during the initiation of SV40 DNA replication. These data and previous data suggest a hypothetical model in which interactions with RPA stimulate DNA synthesis by DNA polymerase α. In this case, interactions with (or competition by) T antigen may modulate the effect of RPA and promote priming. The competition between T antigen and DNA polymerase α for RPA could play a role in switching the activity of DNA polymerase α from priming to DNA synthesis. Alternatively, T antigen could modulate the enzymatic properties of DNA polymerase α. Additional studies should define the roles of these multiple sets of interactions during the initiation and elongation phases of SV40 DNA replication.

ACKNOWLEDGMENT

We thank L. A. Henricksen and Z. A. Sibenaller for supplying purified recombinant hRPA and scRPA, respectively. We thank C-G. Lee, Z. A. Sibenaller, L. A. Henricksen, and B. F. Paulus for SV40 T antigen and HeLa cellular fractions. We thank the members of the Wold laboratory for scientific discussions and critical reading of this manuscript. We thank the University of Iowa DNA Core Facility for oligonucleotide synthesis and DNA sequencing.

REFERENCES

1. Wold, M. S., and Kelly, T. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2523−2527.
2. Fairman, M. P., and Stillman, B. (1988) *EMBO J. 7*, 1211−1218.
3. Wold, M. S. (1997) *Annu. Rev. Biochem. 66*, 61−91.
4. Wobbe, C. R., Weissbach, L., Borowiec, J. A., Dean, F. B., Murakami, Y., Bullock, P., and Hurwitz, J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1834−1838.
5. Coverley, D., Kenny, M. K., Munn, M., Rupp, W. D., Lane, D. P., and Wood, R. D. (1991) *Nature 349*, 538−541.
6. Heyer, W.-D., Rao, M. R. S., Erdile, L. F., Kelly, T. J., and Kolodner, R. D. (1990) *EMBO J. 9*, 2321−2329.
7. Moore, S. P., Erdile, L., Kelly, T., and Fishel, R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9067−9071.
8. Brill, S. J., and Stillman, B. (1989) *Nature 342*, 92−95.
9. Brown, G. W., Melendy, T. E., and Ray, D. S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10227−10231.
10. Mitsis, P. G., Kowalczykowski, S. C., and Lehman, I. R. (1993) *Biochemistry 32*, 5257−5266.
11. Adachi, Y., and Laemmli, U. K. (1992) *J. Cell Biol. 119*, 1−15.
12. Atrazhev, A., Zhang, S., and Grosse, F. (1992) *Eur. J. Biochem. 210*, 855−865.
13. Georgaki, A., Strack, B., Podust, V., and Hübscher, U. (1992) *FEBS Lett. 308*, 240−244.
14. Fang, F., and Newport, J. W. (1993) *J. Cell Sci. 106*, 983−994.
15. Brown, G. W., Melendy, T., and Ray, D. S. (1993) *Mol. Biochem. Parasitol. 59*, 323−326.
16. Melendy, T., and Stillman, B. (1993) *J. Biol. Chem. 268*, 3389−3395.
17. Kamakaka, R. T., Kaufman, P. D., Stillman, B., Mitsis, P. G., and Kadonaga, J. T. (1994) *Mol. Cell. Biol. 14*, 5114−5122.
18. Murakami, Y., Eki, T., and Hurwitz, J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 952−956.
19. Collins, K. L., and Kelly, T. J. (1991) *Mol. Cell. Biol. 11*, 2108−2115.
20. Kenny, M. K., Schlegel, U., Furneaux, H., and Hurwitz, J. (1990) *J. Biol. Chem. 265*, 7693−7700.
21. Kenny, M. K., Lee, S.-H., and Hurwitz, J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9757−9761.
22. Seo, Y.-S., Lee, S.-H., and Hurwitz, J. (1991) *J. Biol. Chem. 266*, 13161−13170.
23. Thömmes, P., Ferrari, E., Jessberger, R., and Hübscher, U. (1992) *J. Biol. Chem. 267*, 6063−6073.
24. Georgaki, A., Tuteja, N., Sturzenegger, B., and Hübscher, U. (1994) *Nucleic Acids Res. 22*, 1128−1134.
25. Tsurimoto, T., and Stillman, B. (1989) *EMBO J. 8*, 3883−3889.
26. Lee, S.-H., Kwong, A. D., Pan, Z.-Q., and Hurwitz, J. (1991) *J. Biol. Chem. 266*, 594−602.
27. Erdile, L. F., Heyer, W.-D., Kolodner, R., and Kelly, T. J. (1991) *J. Biol. Chem. 266*, 12090−12098.
28. Tsurimoto, T., Melendy, T., and Stillman, B. (1990) *Nature 346*, 534−539.
29. Weinberg, D. H., Collins, K. L., Simancek, P., Russo, A., Wold, M. S., Virshup, D. M., and Kelly, T. J. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8692−8696.
30. Dodson, M., Dean, F. B., Bullock, P., Echols, H., and Hurwitz, J. (1987) *Science 238*, 964−967.
31. Borowiec, J. A., and Hurwitz, J. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 64−68.
32. Borowiec, J. A., Dean, F. B., and Hurwitz, J. (1991) *J. Virol. 65*, 1228−1235.
33. SenGupta, D. J., and Borowiec, J. A. (1994) *EMBO J. 13*, 982−992.
34. Wold, M. S., Li, J. J., and Kelly, T. J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3643−3647.
35. Gomes, X. V., and Wold, M. S. (1996) *Biochemistry 35*, 10558−10568.
36. Dornreiter, I., Höss, A., Arthur, A. K., and Fanning, E. (1990) *EMBO J. 9*, 3329−3336.
37. Dornreiter, I., Erdile, L. F., Gilbert, I. U., von Winkler, D., Kelly, T. J., and Fanning, E. (1992) *EMBO J. 11*, 769−776.
38. Dornreiter, I., Copeland, W. C., and Wang, T. S.-F. (1993) *Mol. Cell. Biol. 13*, 809−820.
39. Murakami, Y., and Hurwitz, J. (1993) *J. Biol. Chem. 268*, 11008−11017.
40. Tsurimoto, T., and Stillman, B. (1991) *J. Biol. Chem. 266*, 1961−1968.
41. Matsuda, T., Saijo, M., Kuraoka, I., Kobayashi, T., Nakatsu, Y., Nagai, A., Enjoji, T., Masutani, C., Sugasawa, K., Hanaoka, F., Yasui, A., and Tanaka, K. (1995) *J. Biol. Chem. 270*, 4152−4157.
42. He, Z., Henricksen, L. A., Wold, M. S., and Ingles, C. J. (1995) *Nature 374*, 566−569.
43. Matsunaga, T., Park, C. H., Bessho, T., Mu, D., and Sancar, A. (1996) *J. Biol. Chem. 271*, 11047−11050.
44. Park, M. S., Judwig, D. L., Stigger, E., and Lee, S.-H. (1996) *J. Biol. Chem. 271*, 18996−19000.
45. Li, R., and Botchan, M. R. (1993) *Cell 73*, 1207−1221.
46. He, Z., Brinton, B. T., Greenblatt, J., Hassell, J. A., and Ingles, C. J. (1993) *Cell 73*, 1223−1232.
47. Dutta, A., Ruppert, J. M., Aster, J. C., and Winchester, E. (1993) *Nature 365*, 79−82.
48. Miller, S. D., Moses, K., Jayaraman, L., and Prives, C. (1997) *Mol. Cell. Biol. 17*, 2194−2201.
49. Yan, H., and Newport, J. (1995) *Science 269*, 1883−1885.
50. Erdile, L. F., Collins, K. L., Russo, A., Simancek, P., Small, D., Umbricht, C., Virshup, D., Cheng, L., Randall, S., Weinberg, D., Moarefi, I., Fanning, E., and Kelly, T. (1991) *Cold Spring Harbor Symp. Quant. Biol. 56*, 303−313.
51. Henricksen, L. A., Umbricht, C. B., and Wold, M. S. (1994) *J. Biol. Chem. 269*, 11121−11132.
52. Gomes, X. V., and Wold, M. S. (1995) *J. Biol. Chem. 270*, 4534−4543.
53. Lee, S.-H., and Kim, D. K. (1995) *J. Biol. Chem. 270*, 12801−12807.
54. Longhese, M. P., Plevani, P., and Lucchini, G. (1994) *Mol. Cell. Biol. 14*, 7884−7890.
55. Firmenich, A. A., Elias-Arnanz, M., and Berg, P. (1995) *Mol. Cell. Biol. 15*, 1620−1631.
56. Smith, J., and Rothstein, R. (1995) *Mol. Cell. Biol. 15*, 1632−1641.
57. Santocanale, C., Neecke, H., Longhese, M. P., Lucchini, G., and Plevani, P. (1995) *J. Mol. Biol. 254*, 595−607.
58. Longhese, M. P., Neecke, H., Paciotti, V., Lucchini, G., and Plevani, P. (1996) *Nucleic Acids Res. 24*, 3533−3537.
59. He, Z., Wong, J. M. S., Maniar, H. S., Brill, S. J., and Ingles, C. J. (1996) *J. Biol. Chem. 271*, 28243−28249.
60. Wold, M. S., Weinberg, D. H., Virshup, D. M., Li, J. J., and Kelly, T. J. (1989) *J. Biol. Chem. 264*, 2801−2809.
61. Erdile, L. F., Wold, M. S., and Kelly, T. J. (1990) *J. Biol. Chem. 265*, 3177−3182.
62. Li, L., Lu, X. Y., Peterson, C. A., and Legerski, R. J. (1995) *Mol. Cell. Biol. 15*, 5396−5402.
63. Kim, D. K., Stigger, E., and Lee, S. H. (1996) *J. Biol. Chem. 271*, 15124−15129.
64. Gomes, X. V., Henricksen, L. A., and Wold, M. S. (1996) *Biochemistry 35*, 5586−5595.
65. Lin, Y. L., Chen, C., Keshav, K. F., Winchester, E., and Dutta, A. (1996) *J. Biol. Chem. 271*, 17190−17198.

66. Pfuetzner, R. A., Bochkarev, A., Frappier, L., and Edwards, A. M. (1997) *J. Biol. Chem. 272*, 430−434.
67. Bochkarev, A., Pfuetzner, R. A., Edwards, A. M., and Frappier, L. (1997) *Nature 385*, 176−181.
68. Din, S.-U., Brill, S. J., Fairman, M. P., and Stillman, B. (1990) *Genes Dev. 4*, 968−977.
69. Dutta, A., Din, S., Brill, S. J., and Stillman, B. (1991) *Cold Spring Harbor Symp. Quant. Biol. 56*, 315−324.
70. Fotedar, R., and Roberts, J. M. (1992) *EMBO J. 11*, 2177−2187.
71. Dutta, A., and Stillman, B. (1992) *EMBO J. 11*, 2189−2199.
72. Henricksen, L. A., Carter, T., Dutta, A., and Wold, M. S. (1996) *Nucleic Acids Res. 24*, 3107−3112.
73. Wang, T. S. F., Copeland, W. C., Rogge, L., and Dong, Q. (1995) *Methods Enzymol. 262*, 77−84.
74. Tanaka, S., Hu, S. Z., Wang, T. S., and Korn, D. (1982) *J. Biol. Chem. 257*, 8386−8390.
75. Kim, C., Paulus, B. F., and Wold, M. S. (1994) *Biochemistry 33*, 14197−14206.
76. O'Reilly, D. R., and Miller, L. K. (1988) *J. Virol. 62*, 3109−3119.
77. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1989) in *Current Protocols in Molecular Biology*, John Wiley and Sons, New York.
78. Harlow, E., Crawford, L. V., Pim, D. C., and Williamson, N. M. (1981) *J. Virol. 39*, 861−869.
79. Bennett-Cook, E. R., and Hassell, J. A. (1991) *EMBO J. 10*, 959−969.
80. Cheng, L., Workman, J. L., Kingston, R. E., and Kelly, T. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 589−593.
81. Guo, Z.-S., and DePamphilis, M. L. (1992) *Mol. Cell. Biol. 12*, 2514−2524.
82. Kim, C., Snyder, R. O., and Wold, M. S. (1992) *Mol. Cell. Biol. 12*, 3050−3059.
83. Philipova, D., Mullen, J. R., Maniar, H. S., Gu, C., and Brill, S. J. (1996) *Genes Dev. 10*, 2222−2233.
84. Kim, C., and Wold, M. S. (1995) *Biochemistry 34*, 2058−2064.

BI970473R