Functional Domains of the 70-Kilodalton Subunit of Human Replication Protein A[†]

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ABSTRACT: Human replication protein A (RPA) is a single-stranded DNA-binding protein that is composed of subunits of 70, 32, and 14 kDa. This heterotrimeric complex is required for multiple processes in DNA metabolism including DNA replication, DNA repair, and recombination. Previous studies have suggested that the 616 amino acid, 70-kDa subunit of RPA (RPA70) is composed of multiple structural/functional domains. We used a series of N-terminal deletions of RPA70 to define the boundaries of these domains and elucidate their functions. Mutant RPA complexes missing residues 1−168 of RPA70 bound ssDNA with high affinity and supported SV40 replication *in vitro*. In contrast, deletions extending beyond residue 168 showed a decreased affinity for ssDNA and were inactive in SV40 DNA replication. When residues 1−381 were deleted, the resulting truncated RPA70 was unable to bind ssDNA but still formed a stable complex with the 32- and 14-kDa subunits of RPA. Thus, the C-terminal domain of RPA70 is both necessary and sufficient for RPA complex formation. These data indicate that RPA70 is composed of three functional domains: an N-terminal domain that is not required for ssDNA binding or SV40 replication, a central DNA-binding domain, and a C-terminal domain that is essential for subunit interactions. For all mutant complexes examined, both phosphorylation of the 32-kDa subunit of RPA and the ability to support T antigen-dependent, origin-dependent DNA unwinding correlated with ssDNA binding activity.

Replication protein A (RPA)[1] is a single-stranded DNA-binding protein that has multiple roles in DNA metabolism (Wobbe *et al.,* 1987; Wold & Kelly, 1988; Fairman & Stillman, 1988). Human RPA (hRPA) is a stable heterotrimeric complex with subunits of 70, 32, and 14 kDa (Wold & Kelly, 1988; Fairman & Stillman, 1988). RPA homologues have been identified in all eukaryotes examined including several species of mammals, *S. cerevisiae*, *D. melanogaster*, *X. laevis*, and *C. fasiculata* (Brill & Stillman, 1989; Brown *et al.*, 1992; Mitsis *et al.*, 1993; Adachi & Laemmli, 1992; Atrazhev *et al.*, 1992; Georgaki *et al.*, 1992; Fang & Newport, 1993). All characterized homologues are highly conserved heterotrimers that possess single-stranded DNA-binding activity (Erdile *et al.,* 1990, 1991; Nakagawa *et al.,* 1991; Umbricht *et al.,* 1993; Adachi & Laemmli, 1992; Alani *et al.,* 1992; Heyer *et al.,* 1990; Brill & Stillman, 1991; Brown *et al.*, 1994). In spite of this high level of similarity, only some RPA homologues can substitute for hRPA in SV40 DNA replication, suggesting that species-specific interactions are required for RPA function (Brown *et al.,* 1993; Melendy & Stillman, 1993; Kamakaka *et al.*, 1994).

RPA has multiple functions during DNA replication and is required during both initiation and elongation stages of DNA replication (Wobbe *et al.,* 1987; Wold & Kelly, 1988; Fairman & Stillman, 1988; Melendy & Stillman, 1993; Murakami *et al.,* 1992; Collins & Kelly, 1991; Kenny *et al.,* 1990; Bjerke and Wold, unpublished observations). RPA is involved in both DNA repair and recombination (Coverley *et al.,* 1991, 1992; Heyer *et al.,* 1990; Moore *et al.,* 1991). It has also been suggested that RPA is involved in regulation of gene expression in *Saccharomyces cerevisiae* because of the finding that RPA can bind specifically to upstream repressing sequences that regulate the expression of genes involved in DNA repair and arginine metabolism (Singh & Samson, 1995; Luche *et al.,* 1993). The primary biochemical activity of RPA is high-affinity binding to ssDNA; the association constant of human RPA is $\sim 10^9$ M^{-1} (Kim *et al.,* 1992, 1994; Kim & Wold, 1995). Human RPA has a higher affinity for pyrimidines than for purines and binds oligonucleotides with low cooperativity (∼10−20) with a binding site size of ∼30 nucleotides (Kim *et al.,* 1992, 1994; Kim & Wold, 1995). Studies using chemical cross-linking have suggested that RPA has a second binding mode with a binding site size of 8−10 nucleotides (Blackwell & Borowiec, 1994). In addition to interacting with ssDNA, RPA interacts specifically with two proteins essential for initiation of SV40 DNA replication: large T antigen and DNA polymerase α−primase complex (Dornreiter *et al.,* 1992). These interactions are thought to be essential for the initiation of DNA replication (Murakami *et al.,* 1992; Melendy & Stillman, 1993; Collins & Kelly, 1991). RPA also interacts with proteins involved in DNA repair (XPA and XPG; Matsuda *et al.,* 1995; He *et al.,* 1995), transcriptional activators (GAL4 and VP16; He *et al.,* 1993; Li & Botchan, 1993), and the cellular regulator p53 (Li & Botchan, 1993; He *et al.,* 1993; Dutta *et al.,* 1993). The functional importance of these protein−protein interactions is not well understood. RPA has also been shown to stimulate the

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[1] Abbreviations: RPA, replication protein A; hRPA, human replication protein A; rhRPA, recombinant human replication protein A; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; oligo-$(dT)_{30}$, oligodeoxythymidine 30 residues in length; RPA70, 70-kDa subunit of RPA; RPA32, 32-kDa subunit of RPA; RPA14, 14-kDa subunit of RPA; SSB, single-stranded DNA-binding protein; IPTG, isopropyl β-D-thiogalactopyranoside.

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activity of proteins involved in DNA metabolism including several DNA helicases (Seo *et al.,* 1991; Thömmes *et al.,* 1992; Georgaki *et al.,* 1994), SV40 large T antigen (Wold & Kelly, 1988; Kenny *et al.,* 1989), and DNA polymerases α, δ, and ϵ (Tsurimoto & Stillman, 1989; Kenny *et al.,* 1990; Lee *et al.,* 1991; Erdile *et al.,* 1991). In addition, RPA stimulates the priming activity of polymerase α-primase complex on physiological templates (Collins & Kelly, 1991; Murakami *et al.,* 1992; Melendy & Stillman, 1993).

Current evidence indicates that all three subunits of RPA are required for function. Isolated RPA subunits, a subcomplex of the 32- and 14-kDa subunits, and RPA deletion mutants do not substitute for RPA in a replication reaction (Kenny *et al.,* 1990; Erdile *et al.,* 1991; Henricksen *et al.,* 1994; Gomes & Wold, 1995; Lee & Kim, 1995). In addition, all three genes encoding subunits of RPA are essential for viability in *S. cerevisiae* (Heyer *et al.,* 1990; Brill & Stillman, 1991). Currently the precise role of the three subunits is not known. The 70-kDa subunit of RPA contains intrinsic DNA-binding activity (Wold *et al.,* 1989; Brill & Stillman, 1989; Kenny *et al.,* 1990; Erdile *et al.,* 1991; Gomes & Wold, 1995) and has been shown to participate in specific interactions with DNA polymerase α−primase complex, the DNA damage recognition protein XPA, tumor suppressor p53, and transcriptional activators like VP16 (Dornreiter *et al.,* 1992; Li *et al.,* 1995; Li & Botchan, 1993; He *et al.,* 1993; Dutta *et al.,* 1993). In addition, studies in yeast have demonstrated that mutations in the large subunit of RPA affect DNA replication, repair, and recombination *in vivo* (Longhese *et al.,* 1994; Firmenich *et al.,* 1995; Smith & Rothstein, 1995). The role of the 32- and 14-kDa subunits is not yet known. These two subunits can form a soluble subcomplex which may serve a structural role in the formation of a heterotrimeric complex (Henricksen *et al.,* 1994). In addition, RPA32 is phosphorylated in a cell cycle dependent manner (Din *et al.,* 1990; Dutta *et al.,* 1991; Fotedar & Roberts, 1992; Dutta & Stillman, 1992) and may participate in specific protein−protein interactions (Dornreiter *et al.,* 1992; Matsuda *et al.,* 1995; Lee & Kim, 1995; Li *et al.,* 1995). Thus, RPA32 may also be involved in regulating the function of RPA in the cell.

Recent proteolytic studies of RPA suggest that RPA70 is composed of at least two structural domains (Gomes *et al.,* 1996). Studies of a series of C-terminal deletions of RPA70 also suggested that the DNA-binding domain for RPA is located in the N-terminal two-thirds of RPA70 and that the C-terminus of RPA70 was important for stable heterotrimeric complex formation (Gomes & Wold, 1995). In order to precisely define the functional domains of RPA70, we have generated and characterized a series of N-terminal deletions of RPA70. Using these mutations, we have shown that the N-terminal domain of RPA70 is not necessary for ssDNA-binding activity or SV40 replication *in vitro*. We mapped the boundaries of the DNA-binding domain and also show that the C-terminal domain of RPA70 is both necessary and sufficient for interactions with the 32- and 14-kDa subunits of RPA. Functional characterization of these mutants provided insights into the role of RPA70 in RPA phosphorylation and T antigen-dependent unwinding.

EXPERIMENTAL PROCEDURES

Materials. Restriction endonucleases, polynucleotide kinase, Vent DNA polymerase, and Klenow were purchased from New England BioLabs and Life Technologies, Inc. [γ-^{32}P]ATP (4500 Ci/mmol) and [α-^{32}P]dATP (3000 Ci/mmol) were obtained from Amersham. Oligonucleotides were purchased from Midland Certified Reagents or the University of Iowa DNA Core Facility. *E. coli* DH5α was from Life Technologies, Inc. *E. coli* expression strain BL21-(DE3) was from W. Studier (Studier *et al.,* 1990). Recombinant human RPA (rhRPA) was purified as described previously and has properties similar to those of native RPA (Henricksen *et al.,* 1994).

HI buffer contains 30 mM HEPES (diluted from 1 M stock at pH 7.8), 1 mM dithiothreitol, 0.25 mM EDTA, 0.5% (w/v) inositol, and 0.01% (v/v) Nonidet P-40. HI buffer was supplemented with different concentrations of salt as indicated in the text. 1× filter-binding buffer (FBB) contains 30 mM HEPES (pH 7.5), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 0.5% (w/v) inositol. 1× Tris−acetate/EDTA (TAE) gel buffer contained 40 mM Tris−acetate and 2 mM EDTA, pH 8.5 (Ausubel *et al.,* 1989). 1× Tris−borate/EDTA (TBE) buffer is 89 mM Tris-base, 89 mM borate, and 2 mM EDTA.

pET expression plasmids used were obtained from W. Studier and co-workers (Studier *et al.,* 1990). RPA expression plasmids (p11d-tRPA and p3d-RPA14/32) were described previously (Henricksen *et al.,* 1994).

DNA Manipulation. Restriction endonucleases and Klenow fragment were used according to manufacturer's recommendations. Oligonucleotides were radiolabeled with [γ-^{32}P]-ATP using polynucleotide kinase (Ausubel *et al.,* 1989). Polymerase chain reactions (PCR) were performed with Vent DNA (New England BioLabs) polymerase in a DNA Thermal Cycler (Perkin-Elmer). DNA amplification conditions were 20 cycles of 90 °C for 1 min, 50 °C for 1 min, and 72 °C for 3 min. PCR products and DNA fragments were isolated from 1% TAE−agarose gels using a Geneclean II kit (BIO 101, La Jolla, CA) according to manufacturer's specifications. Ligation reactions and transformations were as according to Ausubel and co-workers (Ausubel *et al.,* 1989). Recombinant plasmids were transformed into strain *E. coli* DH5α and isolated by the boiling lysis method (Ausubel *et al.,* 1989). DNA sequencing was performed using an Applied Biosystems 373A automatic DNA sequencer at the DNA core facility at the University of Iowa.

Construction of RPA70 N-Terminal Deletion Mutants. A series of N-terminal deletion mutants were generated by amplifying specific regions of the RPA70 cDNA using PCR (Table 1). The N-terminal primers contained nucleotide base changes (underlined) to generate a *Bam*HI restriction site and a *Nco*I restriction site. The N-terminal PCR primers used to generate the various N-terminal deletion mutants were as follows: 5′ AACGGATCCATGGAGGAAGAACAA 3′ (RPA70Δ1−66); 5′ GGAGGATCCATGGGCAATCCAGTG 3′ (RPA70Δ1−112); 5′ TTTGGATCCATGGCAGGTCCCAGC 3′ (RPA70Δ1−168); 5′ ATCGGATCCATGGCTTTCAATGAG 3′ (RPA70Δ1−236); 5′ TTTGGATCCATGGGGATTGATGAC 3′ (RPA70Δ1−303); 5′ AAAGGATCCATGGTCTCTGATTTC 3′ (RPA70Δ1−382); 5′ TTTGGATCCATGGCCACAGTGGTG 3′ (RPA70Δ1−465). The C-terminal PCR primer (5′ ATGCTAGTTATTGCTCAGCGG 3′) was designed so that the PCR products would contain a *Bam*HI restriction site from p11d-RPA70 in the 3′ untranslated region of RPA70 cDNA. These primers were used to amplify specific regions of the RPA70

cDNA and the 3′ untranslated region. The individual PCR products were ligated to pUC18 which was digested with *Sma*I. The resulting plasmids were digested with *Bam*HI and *Nco*I, yielding a series of fragments containing truncated RPA70 coding sequences. Each fragment was isolated and ligated to the expression vector pET-11d digested with *Nco*I and *Bam*HI to give a series of expression vectors containing specific N-terminal deletions of the RPA70 gene (Table 1). Each plasmid contained a T7 promoter, Shine−Dalgarno ribosome-binding site, and the coding sequence of a RPA70 deletion mutant. DNA sequence analysis of each of the resulting vectors confirmed that the initiator codon ATG was maintained in each plasmid.

Construction of pSD-RPA14/32. p3d-RPA14/32 (Henricksen *et al.*, 1994) was digested with *Bam*HI, and the 5′ overhangs were filled in with DNA polymerase I (Klenow) fragment to generate blunt ends and then religated. This plasmid was digested with *Xba*I and *Pst*I to generate a 2.9 kb fragment containing the coding sequence for RPA14 and RPA32. This fragment was isolated and cloned into pUC18 which was also digested with *Xba*I and *Pst*I. The resulting plasmid contains the Shine−Dalgarno ribosome-binding site, the coding sequence of RPA14, and a second Shine−Dalgarno ribosome-binding site, the coding sequence of the RPA32. Restriction analysis confirmed that the *Bam*HI restriction site between RPA14 and RPA32 was eliminated.

Construction of Protein Expression Vectors Containing RPA32, RPA14, and Mutant RPA70 Genes. Vectors capable of expressing RPA32, RPA14, and individual N-terminal deletion mutants of RPA70 in *E. coli* were constructed using a procedure similar to that used to generate p11d-tRPA (Henricksen *et al.*, 1994). pSD-RPA14/32 was digested with *Bam*HI, *Aat*II, and *Alw*NI restriction enzymes and a 2.25 kb *Bam*HI−*Aat*II fragment containing the coding sequences of both the 14-kDa and 32-kDa subunits was isolated. This 2.25 kb fragment was ligated to each of the pET-11d vectors containing individual N-terminal deletion mutants of RPA70 digested with *Bam*HI and *Aat*II. For the construction of rhRPA·70Δ1−66 and rhRPA·70Δ1−465, an alternate cloning scheme was used. pSD-RPA14/32 was digested with *Bam*HI and *Pvu*I restriction enzymes, and a 2.8 kb fragment containing the RPA14 and RPA32 genes was isolated. pET-11d containing individual N-terminal deletion mutants of RPA70 was similarly digested with *Bam*HI and *Pvu*I, isolated, and ligated to the 2.8 kb fragment. The resulting expression vectors contain a single T7 RNA polymerase promoter followed by the coding sequence of the mutated 70-kDa and the wild-type 14- and 32-kDa subunits of RPA. Each coding sequence is preceded by a Shine−Dalgarno ribosome-binding site. This series of plasmids is summarized in Table 1.

Induction and Purification of the Deletion Mutants of RPA70. The N-terminal deletion mutants were expressed in BL21(DE3) as described previously (Henricksen *et al.*, 1994). Purification of rhRPA·70Δ1−112, rhRPA·70Δ1−168, and rhRPA·70Δ1−236 was carried out following the general procedure of Henricksen and co-workers (Henricksen *et al.*, 1994). All steps were carried out at 4 °C. During the purification, the N-terminal deletion mutants were monitored by Western blotting. Soluble lysate from 3 L of an induced culture was applied to a 50 mL Affi-Gel Blue (Bio-Rad) column equilibrated with HI buffer containing 80 mM KCl. The column was washed sequentially with 150 mL each of HI buffer containing 80 mM KCl, 0.8 M KCl, 0.5 M NaSCN, or 1.5 M NaSCN. Like wild-type RPA, rhRPA·70Δ1−112, rhRPA·70Δ1−168, and rhRPA·70Δ1−236 eluted in the 1.5 M NaSCN wash. The peak of protein from the 1.5 M NaSCN wash was applied directly to a 15 mL HAP column equilibrated with HI buffer. The column was eluted sequentially with 60 mL each of HI buffer containing 0, 80, or 500 mM potassium phosphate; rhRPA·70Δ1−112 eluted in HI buffer with 80 mM potassium phosphate and rhRPA·70Δ1−168 eluted in HI buffer with 0 mM potassium phosphate while rhRPA·70Δ1−236 eluted equally in the HI buffer with 0 and 80 mM potassium phosphate. The fractions containing the deletion mutant were applied to a Mono-Q (HR5/5) column (Pharmicia) equilibrated with HI buffer containing 14 mM KCl. In the case of rhRPA·70Δ1−236, the 0 mM and the 80 mM potassium phosphate fractions were pooled and applied to the Mono-Q column. The column was washed with 4 mL of HI buffer containing 50 mM KCl and then developed with a 10 mL linear salt gradient of 50−340 mM KCl; rhRPA·70Δ1−112, rhRPA·70Δ1−168, and rhRPA·70Δ1−236 eluted from the Mono-Q column at ∼300 mM KCl. The purification of the RPA70 mutant complexes is summarized in Table 2.

rhRPA·70Δ1−382 was purified using the same purification protocol as described above with a slight modification. One liter of an induced culture was applied to a 10 mL Affi-Gel Blue column equilibrated with HI buffer containing 80 mM KCl. The column was eluted with 30 mL of HI buffer containing 80 mM KCl followed by a 180 mL linear salt gradient of 80−800 mM KCl. The column was washed sequentially with 30 mL of HI buffer containing 800 mM KCl, 0.5 M NaSCN, or 1.5 M NaSCN. rhRPA·70Δ1−382 eluted in the 800 mM KCl wash. The peak of protein from the 800 mM KCl wash was applied directly to a 1.2 mL HAP column equilibrated with HI buffer. The column was eluted with 4 mL of HI buffer containing no added salts followed by a 10 mL linear salt gradient from 0 to 100 mM potassium phosphate. rhRPA·70Δ1−382 eluted equally in the HI buffer wash and at 40 mM potassium phosphate. The peak fractions containing the deletion mutant were pooled and applied to a Mono-Q (HR5/5) column (Pharmicia) equilibrated with HI buffer containing 14 mM KCl. The column was washed with 4 mL of HI buffer containing 50 mM KCl and then developed with a 10 mL linear salt gradient of 50−340 mM KCl; rhRPA·70Δ1−382 eluted from the Mono-Q column at 200 mM KCl. The purification of this mutant complex is summarized in Table 2. Protein concentrations were determined by Bradford assay using bovine serum albumin as a standard (Bradford, 1976).

Immunoblotting. Protein samples were separated on 8−14% SDS−polyacrylamide gels (Laemmli, 1970) and transferred to nitrocellulose (Bio-Rad) using a PolyBlot Electrotransfer System from Millipore as per manufacturer′s specifications. RPA70 deletion mutants were detected using monoclonal antibody RPA9 (B. Stillman, CSHL). Secondary antibodies (Sigma) used were sheep anti-mouse horseradish peroxidase conjugate. Secondary antibodies were detected using ECL chemiluminescense kit (Amersham) as recommended by the manufacturer.

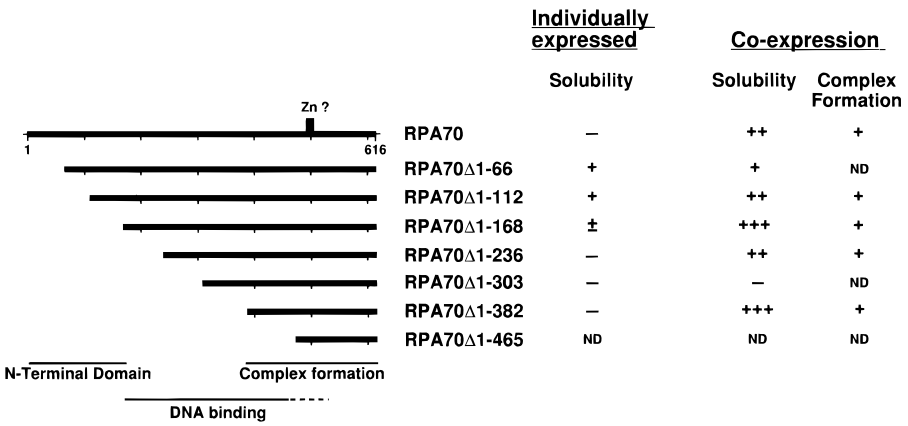

FIGURE 1: Schematic of individual N-terminal deletion mutants. The left-hand side of the figure shows a schematic of wild-type RPA70 and the seven deletion mutants (RPA70Δ1−66, RPA70Δ1−112, RPA70Δ1−168, RPA70Δ1−236, RPA70Δ1−303, RPA70Δ1−382 and RPA70Δ1−465). Each mutant was named for the amino acid deleted (e.g., RPA70Δ1−66 has amino acids 1 through 66 deleted). Thick bars indicate residues contained in each polypeptide. Ticks are positioned every 100 amino acids; the position of a conserved putative zinc finger motif is indicated by the solid box (Zn?) (Erdile *et al.,* 1991). Lines under the schematic indicate the location of putative functional domains. (The dashed line indicates the C-terminal region of the ssDNA-binding domain which is probably not needed for DNA binding but whose boundary has not been precisely defined.) The right-hand side summarizes the properties of the deletion mutants when expressed alone or when coexpressed in the presence of RPA32 and RPA14: solubility (− denotes insoluble; + 20% soluble; ++ 30−50% soluble; +++ >50% soluble) and complex formation (ND denotes not determined).

Table 1: Summary of RPA70 Deletion Mutants and Plasmids Constructed

subunits[a]	predicted mass (kDa)	expression plasmid[b]	coexpression plasmid[c]	complex name[d]	abbreviation
RPA70	68	p11d-RPA70[e]	p11d-tRPA[e]	rhRPA	rhRPA
RPA70Δ1−66	61	p11d-RPA70Δ1−66	ptRPA·70Δ1−66	rhRPA·70Δ1−66	ΔN66
RPA70Δ1−112	56	p11d-RPA70Δ1−112	ptRPA·70Δ1−112	rhRPA·70Δ1−112	ΔN112
RPA70Δ1−168	50	p11d-RPA70Δ1−168	ptRPA·70Δ1−168	rhRPA·70Δ1−168	ΔN168
RPA70Δ1−236	43	p11d-RPA70Δ1−236	ptRPA·70Δ1−236	rhRPA·70Δ1−236	ΔN236
RPA70Δ1−303	35	p11d-RPA70Δ1−303	ptRPA·70Δ1−303	rhRPA·70Δ1−303	ΔN303
RPA70Δ1−382	27	p11d-RPA70Δ1−382	ptRPA·70Δ1−382	rhRPA·70Δ1−382	ΔN382
RPA70Δ1−465	18	p11d-RPA70Δ1−465	ptRPA·70Δ1−465	rhRPA·70Δ1−465	ΔN465

[a] Protein designations indicate amino acid residues deleted (e.g., Δ1−66 has residues 1−66 deleted). [b] Plasmid derived from pET-11d capable of directing the expression of individual RPA mutants in *E. coli*. Construction of plasmids is described under Experimental Procedures. [c] Plasmid derived from pET-11d capable of directing the simultaneous expression of individual RPA mutants and the 32- and 14-kDa subunits of RPA. Construction of plasmids is described under Experimental Procedures. [d] Each mutant RPA complex contains RPA70XX, RPA32, and RPA14 subunits and will be referred to by the amino acids deleted (e.g., rhRPA·70Δ1−112 is a recombinant complex of RPA70Δ1−112, RPA32, and RPA14). [e] Henricksen *et al.,* 1994.

RESULTS

Construction and Expression of RPA70 N-Terminal Deletion Mutants. To map the location of the structural/functional domains of RPA70 and to elucidate their roles in DNA replication, we have generated and characterized a series of N-terminal deletion mutants of RPA70 (shown schematically in Figure 1). The nomenclature used for these mutants is summarized in Table 1. Each mutant was expressed in *E. coli* either alone or with RPA32 and RPA14. All the single-subunit expression plasmids, except p11d-RPA70Δ1−465, generated polypeptides of the appropriate size (data not shown). Sequence analysis confirmed that the RPA70Δ1−465 gene was in-frame with an initiator methionine codon at position 465. This suggested that the inability to detect RPA70Δ1−465 was caused by instability of this polypeptide, perhaps because of improper folding.

When these N-terminal deletions were expressed in *E. coli* and their solubility properties examined (Henricksen *et al.,* 1994; Gomes & Wold, 1995), they had properties similar to wild-type RPA70 (Henricksen *et al.,* 1994). They were predominantly insoluble when expressed individually but became substantially more soluble when expressed with RPA32 and RPA14 (data summarized in Figure 1). This suggested that, like full-length RPA70, these mutant polypeptides were interacting with RPA32 and RPA14 to form soluble, heterotrimeric complexes. Two mutants had properties different from this general pattern. RPA70Δ1−66 did not increase in solubility when expressed with the two small subunits of RPA (Figure 1). However, this mutant was consistently expressed at low levels, making it difficult to characterize in detail (data not shown). RPA70Δ1−303 was predominantly in the pellet fraction even when expressed with the two small subunits of RPA (Figure 1 and data not shown). We believe that this deletion disrupted the proper folding of this mutant. Four soluble mutant RPA complexes, rhRPA·70Δ1−112, rhRPA·70Δ1−168, rhRPA·70Δ1−236, and rhRPA·70Δ1−382, were purified and characterized in detail.

Purification and Hydrodynamic Characterization of Mutant RPA Complexes. Mutant RPA complexes were purified to near-homogeneity and characterized on SDS−polyacrylamide gels (Figure 2). An immunologically related ∼40-kDa polypeptide was observed in the purified rhRPA·70Δ1−236 (Figure 2). This polypeptide is probably a proteolytic breakdown product of RPA70Δ1−236. The 72-kDa polypeptide observed in the highly purified rhRPA·70Δ1−236 fraction was an *E. coli* protein that coeluted with rhRPA·70Δ1−236 in the final purification step.

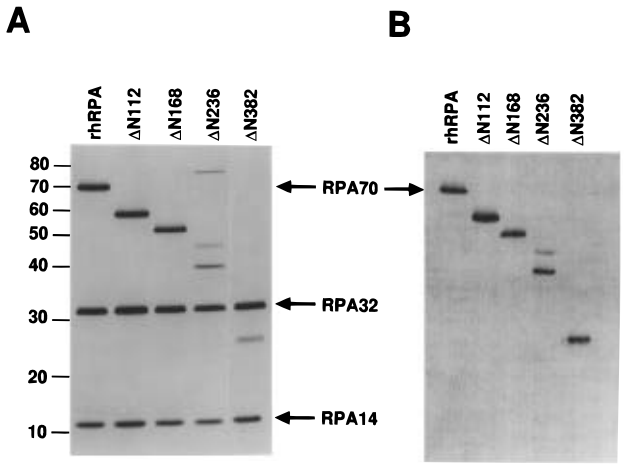

FIGURE 2: Purified RPA mutant complexes. One microgram of purified rhRPA or deletion mutant complexes (ΔN112, ΔN168, ΔN236, and ΔN382) was separated on an 8−14% SDS−polyacrylamide gels and visualized by staining with silver (A) or transferred to nitrocellulose detected by immunoblotting (B). Arrows indicate the positions of the full-length RPA70, RPA32, and RPA14. The positions of the molecular mass markers are indicated at the left.

To determine whether these deletions of RPA70 caused significant changes in the shape or stoichiometry of the RPA complex, the hydrodynamic properties of the mutant complexes were examined. The sedimentation constants and Stokes radii of the mutant complexes were determined by glycerol gradient sedimentation and gel permeation chromatography, respectively (Table 3). These values were then used to determine the molecular mass and frictional coefficient of each of the recombinant mutant complexes (Table 3). For all mutants, the calculated masses were similar to those predicted from the amino acid sequence and were consistent with the mutants forming heterotrimeric complexes. All of the mutant complexes had a frictional coefficient consistent with an elongated shape similar to rhRPA. In the case of rhRPA·70Δ1−236, the shape was more elongated than rhRPA. These results suggest that in these four mutants, the deletion of extensive regions of RPA70 did not cause major disruptions of the RPA complex.

DNA-Binding Properties of Mutant RPA Complexes. Previously, gel mobility shift assays have been used to obtain apparent association constants for RPA binding ssDNA (Kim *et al.*, 1992, 1994; Kim & Wold, 1995; Gomes & Wold, 1995). This assay has the advantages that it allows binding isotherms to be determined rapidly with small mounts of protein under conditions close to those used for SV40 DNA replication. Oligodeoxythymidine 30 residues in length [oligo$(dT)_{30}$] was incubated with increasing concentrations of wild-type or mutant RPA, and the resulting protein·DNA complexes were separated by electrophoresis on agarose gels. With rhRPA, a single band of altered mobility was observed at saturation (data not shown; Kim *et al.*, 1992). Similarly, a single complex of altered mobility was observed with rhRPA·70Δ1−112, rhRPA·70Δ1−168, or rhRPA·70Δ1−236 (data not shown). In contrast, no band of altered mobility was observed with rhRPA·70Δ1−382 even at high protein concentrations, indicating that this mutant was unable to bind to ssDNA (data not shown). Binding isotherms for each mutant were determined (Figure 3) and apparent binding constants calculated (Table 4). The affinities of rhRPA·70Δ1−112 and rhRPA·70Δ1−168 for oligo$(dT)_{30}$ were equivalent to that of wild-type rhRPA while the apparent binding constant for rhRPA·70Δ1−236 was ∼1/300 that of rhRPA (Figure 3 and Table 4). These data indicate that rhRPA·70Δ1−112 and rhRPA·70Δ1−168 contain the entire ssDNA-binding domain of RPA70 while deletion of the N-terminal 236 amino acids partially disrupts the binding domain.

We confirmed the binding properties of the RPA mutants using an independent technique. We have shown previously that when RPA binds to ssDNA, its intrinsic fluorescence is reduced or "quenched" (Kim *et al.*, 1994; Kim & Wold, 1995). This quenching makes it possible to directly examine the binding of RPA to ssDNA (Lohman & Mascotti, 1992). In these studies, wild-type or mutant RPA complexes were titrated with oligo$(dT)_{30}$ in the presence of different concentrations of NaCl. The percent quenching observed is then plotted versus the concentration of oligo$(dT)_{30}$, and the resulting binding isotherm fit to the Langmuir equation for bimolecular interactions (Kim *et al.*, 1994). High concentrations of RPA (∼100 nM) must be used in order to obtain a measurable fluorescent signal. To obtain equilibrium binding at such high concentrations of RPA, salt is added to reduce the affinity of RPA for ssDNA. In these studies, the fluorescence of wild-type and mutant RPA was monitored in the presence of 1.5 and 2 M NaCl. Under these conditions, equilibrium binding of wild-type RPA was observed (data not shown).

The binding constants determined by fluorescence quenching are shown in Table 4. As observed in the gel mobility shift assays, the binding affinities of rhRPA·70Δ1−112 and rhRPA·70Δ1−168 were identical to that of rhRPA (Table 4). The affinity of rhRPA·70Δ1−236 was low enough that it was not possible to obtain a complete fluorescence binding isotherm under these conditions. Note that the apparent binding constants determined for RPA are significantly lower than those observed in the gel mobility shift experiments because of the high salt concentration present in these assays. Thus, the binding constants determined by fluorescence cannot be directly compared to those obtained by gel mobility shift assay. However, the results from both assays were consistent, demonstrating that the affinities of rhRPA, rhRPA·70Δ1−112, and rhRPA·70Δ1−168 for oligo$(dT)_{30}$ were identical to each other and that the affinity of rhRPA·70Δ1−236 was several orders of magnitude lower.

Previously we have characterized the binding affinity of two C-terminal deletion mutants, RPA70Δ442-616 (ΔC442) and RPA70Δ327-616 (ΔC327), under slightly different conditions (Gomes & Wold, 1995). Both of these mutants do not form a complex with the 32- and 14-kDa subunits of RPA and exist as individual, soluble polypeptides. To allow direct comparison of their binding constants to the N-terminal deletion mutants, we reexamined the binding of these deletion mutants to oligo$(dT)_{30}$ by both gel mobility shift and fluorescence quenching assays. These experiments confirm our previous results and showed ΔC327 has a significant defect in DNA-binding activity and that ΔC442 binds ssDNA with high affinity (Figure 3, Table 4). In both assays, the affinity of ΔC442 for oligo$(dT)_{30}$ was approximately 1/20 that of wild-type RPA (Figure 3, Table 4).

SV40 Replication Activity. We examined if the RPA70 N-terminal deletion mutant complexes could substitute for wild-type rhRPA in SV40 replication. A series of replication assays were performed in which all the components were provided either as purified proteins or as partially purified

Table 3: Hydrodynamic Properties[a]

proteins	RPA	ΔN112	ΔN168	ΔN236	ΔN382
Stokes radius (Å)	52.7[b]	49.5 ± 0.5	44.2 ± 0.5	63.3 ± 0.5	40.1 ± 0.5
sedimentation coefficient (S)	5.3[b]	3.7 ± 0.1	4.0 ± 0.1	3.6 ± 0.1	3.2 ± 0.1
calculated mass (kDa)	~117[b]	~78	~74	~96	~55
predicted mass (kDa)	111[b]	98	93	86	69
frictional coefficient (f/f_o)	1.6[b]	1.7	1.6	2.1	1.6

[a] Sedimentation coefficient and Stokes radius were determined as described previously by sedimentation on a 15−35% glycerol gradient and chromatography on a Superose 6 column (HR5/30-Pharmacia) (Kim *et al.*, 1994). Mass and frictional coefficients were calculated using the method of Siegal and Monty (1966). Predicted mass was based upon the amino acid sequence derived from the DNA sequence. [b] Values from Kim *et al.* (1994).

Table 2: Purification of Mutant Complexes

	total protein (mg)			
proteins	ΔN112	ΔN168	ΔN236	ΔN382
soluble proteins	840	467	186	75
Affi-Gel blue	80	104	57	4.8
HAP	10	7.6	8	4.2
MonoQ	6.7	4.2	6.8	2.4

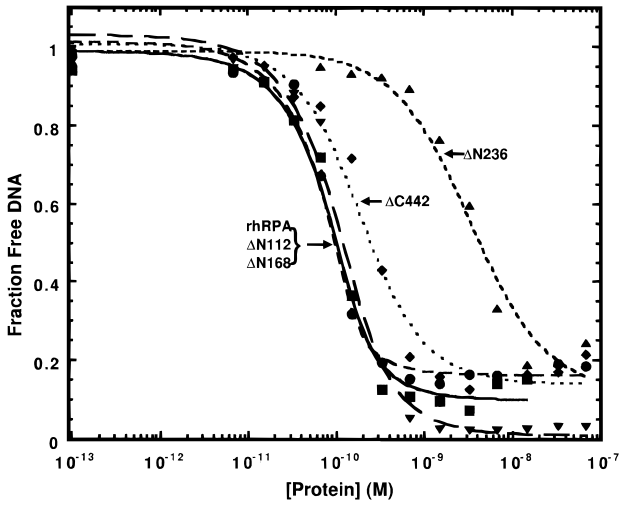

FIGURE 3: ssDNA-binding activity of deletion mutants. Gel mobility shift DNA-binding assays were performed as described previously (Kim *et al.*, 1992) with slight modifications. rhRPA (squares, solid line), ΔN112 (inverted triangles, long dashes), ΔN168 (circles, medium dashes), ΔC442 (diamonds, dots), and ΔN236 (triangles, short dashes) were incubated with 2 fmol of labeled oligo(dT)$_{30}$ in 15 μL of 1× FBB for 20 min at 25 °C in the presence of 750 ng of BSA. (BSA was added to these assays in order to make the conditions more like those used for SV40 replication.) Binding reactions were brought to a final concentration of 4% glycerol and 0.01% bromophenol blue and separated on a 1% agarose gel in 0.1× TAE (4 mM Tris−acetate, 0.1 mM EDTA) at 250 V/cm for 1.5 h. The gels were then dried on DE81 paper, and radioactive bands were visualized by autoradiography. The radioactivity in each band was quantitated using a Packard Instant Imager. The data were analyzed by nonlinear least-squares fitting to the Langmuir binding equation using Kaleidagraph (Abelbeck Software) as described previously (Kim *et al.*, 1994; Gomes & Wold, 1995). Best-fit curves are shown.

fractions. Wild-type rhRPA complex or individual mutant complexes were then added, and DNA synthesis was measured (Figure 4). DNA synthesis in these reactions was absolutely dependent on both T antigen and functional RPA. When rhRPA was substituted with either rhRPA·70Δ1−112 or rhRPA·70Δ1−168, DNA synthesis was observed; however, the level of DNA synthesis supported was approximately half that observed with rhRPA (Figure 4A). In reactions in which rhRPA and either rhRPA·70Δ1−112 or rhRPA·70Δ1−168 were mixed, the amount of synthesis observed was the average of the levels of synthesis observed when the two proteins were used alone. This suggests that rhRPA·70Δ1−112 and rhRPA·70Δ1−168 compete with rhRPA to support SV40 replication and that the defect in the mutants is not dominant (data not shown). Throughout these studies, the levels of synthesis obtained with rhRPA·70Δ1−112 or rhRPA·70Δ1−168 were between 50 and 75% of wild-type rhRPA. This lower level of activity was also observed in assays containing suboptimal levels of T antigen (data not shown). The time course of replication with rhRPA·70Δ1−168 was identical to that of rhRPA except that the level of synthesis was reduced throughout (Figure 4C). This demonstrated that the reduced activity of the mutants was not due to a slower rate of initiation. We conclude that the N-terminus, residues 1−168, of RPA70 is not required for SV40 DNA replication *in vitro* but that this region is important for maximal replication activity.

Table 4: DNA Binding Activities of RPA Mutants

	apparent association constants for oligo(dT)$_{30}$ (M^{-1})		
	GMSA	fluorescence quenching	
NaCl added (M)	0	1.5	2.0
rhRPA	1.3e11 ± 1e11	1.7e7 ± 0.2e7	8.5e6 ± 0.5e6
rhRPA·70Δ1−112	9.6e10 ± 2.1e10	1.8e7 ± 0.1e7	8.2e6 ± 0.2e6
rhRPA·70Δ1−168	9.8e10 ± 8.8e10	1.9e7 ± 0.2e7	9.3e6 ± 0.4e6
rhRPA·70Δ1−236	4.3e7 ± 4.3e7	ns	ns
ΔC442	7.0e9 ± 6.0e9	6.2e5 ± 0.2e4	ns
ΔC327	1.3e8 ± 0.7e8	ns	nd

[a] GMSA titrations of oligonucleotides with indicated proteins were carried out as described in Figure 3. Fluorescence quenching was carried out in filter-binding buffer containing the indicated concentration of NaCl as described previously (Kim & Wold, 1995). Approximately 100 nM rhRPA or the indicated mutant complex was titrated with oligo(dT)$_{30}$. The resulting quenching curves were corrected for dilution and photobleaching, and analyzed by nonlinear least-squares fitting to the Langmuir binding equation as described in Figure 3. All apparent association constants shown are the average of multiple independent determinations. e, the exponent of 10 (e.g., 1e11 is 1×10^{11}); ns, saturation of quenching was not observed; nd, not determined.

We also examined the ability of rhRPA·70Δ1−236 and rhRPA·70Δ1−382 to support SV40 DNA replication. Both of the deletion mutants were unable to support SV40 replication even when present at high concentrations (Figure 4B). When rhRPA·70Δ1−236 or rhRPA·70Δ1−382 was added in combination with rhRPA, the mutants did not inhibit the ability of rhRPA to support SV40 replication (Figure 4B). These results were expected since these mutant complexes have significant defects in ssDNA-binding activity. We conclude that residues 168−616 are sufficient for maintaining RPA−ssDNA and RPA−protein interactions essential for RPA function in SV40 DNA replication.

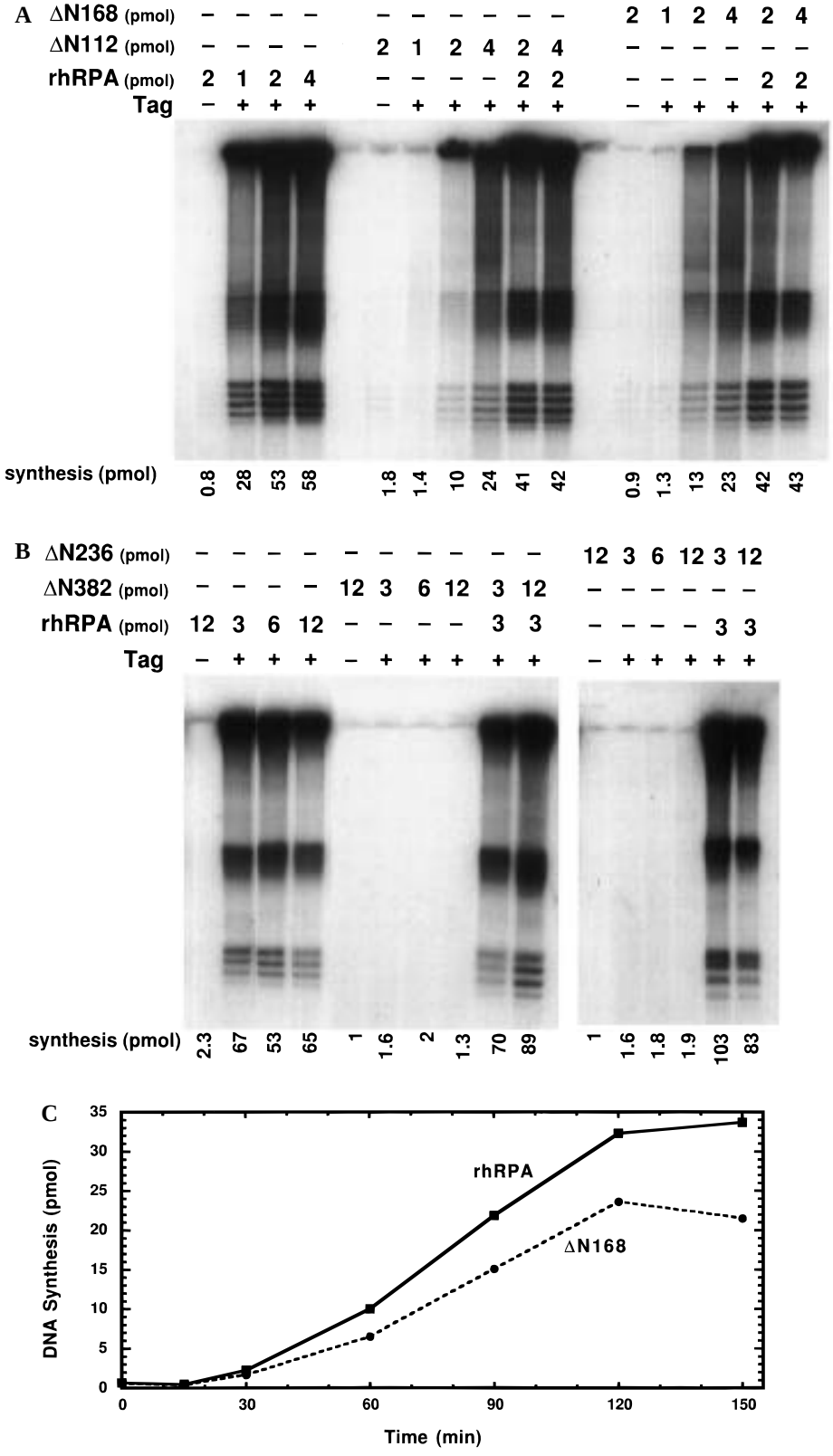

FIGURE 4: Replication activity of deletion mutant complexes. SV40 replication assays were performed using a mixture of purified and partially purified protein fractions as described previously (Henricksen *et al.*, 1994). Each reaction contained 2.24 μg of CF II (contains DNA polymerases α and δ and RF-C), 5.6 μg of fraction CF IBC [contains proliferating nuclear cell antigen and protein phosphatase 2A (Wold *et al.*, 1989; Henricksen *et al.*, 1994)], 1.5 units of topoisomerase I (Promega Corp.), and the indicated amounts of rhRPA or individual deletion mutants. Plasmid pUC.HSO (Wold *et al.*, 1987), which contains the SV40 origin of replication on a 198 bp *Hin*dIII to *Sph*I fragment, was used as the template for these reactions. Synthesis was quantitated by TCA precipitation, and the products were analyzed by electrophoresis on a 1% agarose gel in 1× TAE, followed by autoradiography; 0.7 μg of T antigen was added where indicated. (A) Titration of either rhRPA, ΔN112, or ΔN168. (B) Titration of either rhRPA, ΔN236, or ΔN382. Total picomoles of synthesis obtained in each assay is indicated. (C) Times course of replication with rhRPA (squares, solid line) or ΔN168 (circles, dashed line). Complete replication reactions (250 μL) containing either rhRPA or ΔN168 were incubated at 37 °C. At the times indicated, 25 μL aliquots were removed, and the products present were analyzed as described above. Total DNA synthesis at each time point is shown.

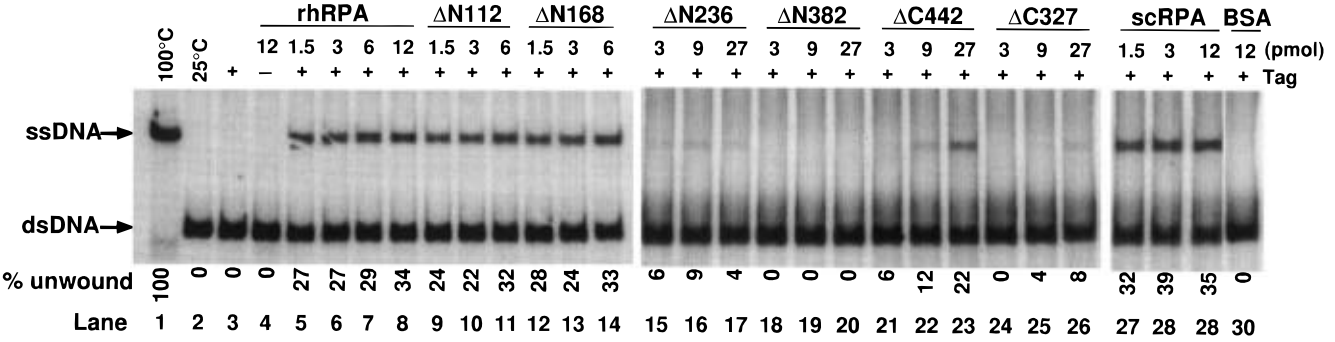

FIGURE 5: Stimulation of T antigen-dependent unwinding of the SV40 origin. Unwinding assays were performed as described previously (Virshup & Kelly, 1989). Increasing amounts of the indicated protein were incubated with 300 ng of T antigen and 0.6 ng of a radioactively labeled, 226 bp fragment from pUC·HSO. [This HindIII to *Bam*HI fragment contains the SV40 origin of replication (Kim *et al.*, 1992).] The reaction mixture was incubated at 37 °C for 30 min. The reaction was terminated by the addition of 2× stop solution containing 2% SDS, 1 mM EDTA, and 1 mg/mL proteinase K followed by incubation at 37 °C for 30 min. The reaction mixtures were brought to a final concentration of 8% glycerol and 0.008% bromophenol blue and separated on an 8% polyacrylamide gel in 1× TBE (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA) at 100 V/cm for 5 h. The gels were then dried on DE81 paper, and radioactive bands were localized by autoradiography. The arrows indicate the position of single-stranded (ssDNA) and double-stranded (dsDNA) DNA. Radioactivity in ssDNA and dsDNA was quantitated using a Packard Instant Imager and the percentage of DNA unwound determined (% unwound). Data from two gels are shown; identical results were obtained in multiple independent experiments.

T Antigen-Dependent, Origin-Dependent Unwinding Activity. During initiation of SV40 DNA replication, T antigen binds to the SV40 origin and, in the presence of RPA, catalyzes localized unwinding of the origin sequences (Dean *et al.*, 1987; Wold *et al.*, 1987; Dodson *et al.*, 1987; Wold & Kelly, 1988). A defect in the ability of a deletion mutant to stimulate unwinding would severely compromise its ability to support SV40 DNA replication; therefore, we tested the ability of the N-terminal mutant complexes and other RPA70 deletion mutants to stimulate T antigen-dependent unwinding. In these assays, a 226 base pair ^{32}P-labeled DNA fragment containing the SV40 origin was incubated at 37 °C for 30 min with T antigen and wild-type or mutant RPA. The products were then separated on an 8% polyacrylamide gel. Under these conditions, the single- and double-stranded forms of the origin fragment have different mobilities and are easily distinguished (Figure 5, lanes 1 and 2). Neither T antigen nor rhRPA alone supports unwinding of the origin fragment (Figure 5, lanes 3, 4, 30); however, in the presence of both proteins, ssDNA was observed, indicating that localized unwinding had occurred (lanes 5−8). All proteins that bind with affinity equivalent to wild-type RPA: rhRPA, rhRPA·70Δ1−112 or rhRPA·70Δ1−168, and the RPA homologue from *Saccharomyces cerevisiae* (scRPA), stimulated unwinding (Figure 5, lanes 9−14, 27−29). ΔC442 supported unwinding; however, more that 10-fold higher concentrations of ΔC442 were needed for significant unwinding (Figure 5, compare lanes 5−8 and 21−23). These results are consistent with the conclusion (Table 4) that the affinity of ΔC442 for ssDNA is more that an order of magnitude lower than wild-type rhRPA. Mutants with low DNA-binding activity, rhRPA·70Δ1−236 and ΔC327, supported barely detectable levels of unwinding and then only at high protein concentrations (Figure 5, lanes 15−17, 24−26). No unwinding was detected with rhRPA·70Δ1−382 which does not bind ssDNA (Figure 5, lanes 18−20). We conclude that the ability to stimulate T antigen-dependent unwinding directly correlated with the strength of ssDNA−DNA binding activity of these mutants.

Phosphorylation of RPA Deletion Mutants. The 32-kDa subunit of RPA (RPA32) becomes phosphorylated during SV40 replication *in vitro*. This phosphorylation is dependent upon DNA-dependent protein kinase (Pan *et al.*, 1994; Brush *et al.*, 1994) and is strongly stimulated by the presence of single-stranded DNA (Fotedar & Roberts, 1992; Henricksen *et al.*, 1994; Henricksen & Wold, 1994). The phosphorylated forms of RPA32 can be detected by a change in mobility of RPA32 on a 15% SDS−polyacrylamide gel (Dutta & Stillman, 1992; Henricksen & Wold, 1994). To determine the role of RPA70 in the phosphorylation of RPA32, the deletion mutant complexes were tested for the ability to become phosphorylated under replication conditions. Wild-type rhRPA or mutant complexes were incubated under replication conditions in the presence or absence of single-stranded M13 DNA. In some reactions, CF IBC (which contains protein phosphatase 2A) was left out to enhance the level of phosphorylation of RPA32. After incubation, the proteins were separated on a 15% polyacrylamide gel, and the level of phosphorylation was examined by immunoblot analysis using monoclonal antibodies to RPA32. All of the mutant RPA complexes except rhRPA·70Δ1−382 were multiply phosphorylated on RPA32 to a similar extent as the wild-type RPA complex (Figure 6). This phosphorylation reaction was strongly stimulated by the presence of ssDNA and by the absence of protein phosphatase 2A (compare minus ssM13 and minus CF IBC reactions to complete reactions, Figure 6). In contrast, little or no phosphorylation of rhRPA·70Δ1−382 was observed under all conditions examined. The ability of the various mutants to be phosphorylated correlated with the presence of ssDNA-binding activity; thus, rhRPA·70Δ1−236 which bound ssDNA (albeit with significantly reduce affinity) was phosphorylated while rhRPA·70Δ1−382 which had no detectable ssDNA-binding activity was not. We conclude that the association of RPA with ssDNA is important for phosphorylation but that the actual affinity of RPA for ssDNA was not critical for this reaction. These results also indicate that deletion of up to 236 amino acids from the N-terminus of RPA70 does not cause perturbations in the conformation of RPA32 that affect its phosphorylation.

DISCUSSION

In these studies, we describe the generation and characterization of a series of N-terminal deletion mutants of

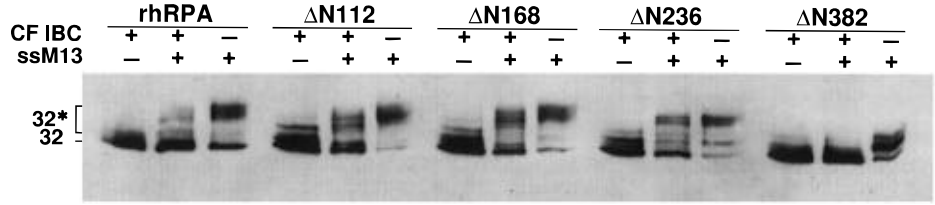

FIGURE 6: Phosphorylation status of mutant complexes. Kinase assays were performed as described previously (Henricksen *et al.,* 1994). Three picomoles of rhRPA or the indicated mutant complexes was incubated at 37 °C for 1 h with ssM13 under replication conditions. Cellular replication proteins were supplied as partially purified protein fractions. Each reaction contained 2.24 μg of CFII (contains DNA polymerases α and δ and RF-C) and where indicated 3.4 μg of CFIBC (contains proliferating nuclear cell antigen and protein phosphatase 2A; Wold *et al.,* 1989; Henricksen *et al.,* 1994). The reaction mixture was then separated on a 15% SDS−polyacrylamide gel, transferred onto nitrocellulose, and probed with monoclonal antibody to RPA32. The positions of RPA32 (32) and the phosphorylated forms of RPA32 (32*) are indicated.

RPA70 (Figure 1). These mutants have provided a number of new insights into the structure and function of RPA70. Previously, we have suggested that the C-terminal domain of RPA70 mediates interactions between RPA70 and the other subunits of RPA and that it is a major determinant of subunit solubility (Henricksen *et al.,* 1994; Gomes & Wold, 1995). The data presented here are consistent with this hypothesis. All of the N-terminal deletion mutants characterized were able to form stable, soluble heterotrimeric complexes with RPA32 and RPA14. The finding that RPA70Δ1−382 formed a stable, heterotrimeric complex demonstrated that in addition to being necessary, the C-terminal one-third of RPA70 is sufficient for formation of the RPA complex.

Comparison of the ssDNA-binding activity of these mutants indicated that the N-terminal boundary of the DNA-binding domain is near residue 168. We have shown previously that the N-terminal ~170 residues of RPA70 are a separate structural domain (Gomes *et al.,* 1996), so this functional boundary coincides with a structural domain boundary in RPA70. Previous C-terminal deletion analysis (Gomes & Wold, 1995) mapped the C-terminal boundary to near residue 442; however, these studies show that the ssDNA-binding activity of ΔC442 while high was actually reduced relative to wild-type RPA. This suggests that the C-terminal boundary of the DNA-binding domain of RPA70 is not precisely defined and that residues between 442 and 616 could be involved in interactions with DNA. Alternatively, this region could be involved in maintaining the structure of the DNA-binding domain, or, since ΔC442 does not form a complex with RPA32 and RPA14, the smaller two subunits of RPA could be involved in modulating RPA interactions with ssDNA. Additional experiments will be needed to distinguish between these possibilities. We conclude that the DNA-binding domain of RPA70 is located between residues 168 and approximately 442. These data also suggest that the residues responsible for interactions with ssDNA are dispersed throughout the DNA-binding domain because deletions from either side first reduce and then eliminate ssDNA-binding activity (Table 4 and Gomes and Wold, 1995). Based on these results, the DNA-binding domain can be modeled as being composed of three regions: a core region (residues 236−327) which is essential for ssDNA binding and two other regions (residues 168−236 and 327−~442) which are both required for high-affinity binding.

Whenever deletions are made in a protein, they have the potential to perturb protein structure either globally or locally, or to cause indirect effects on protein activity. Several lines of evidence suggest that our results are not due to such effects. Hydrodynamic analysis of these mutants indicated that there were no global perturbations of their structure. In addition, the structure of several of these mutants has been examined by partial proteolysis and found to be similar to that of wild-type RPA (Gomes *et al.,* 1996). It is more difficult to rule out deletions causing local or indirect effects, but most major conclusions from these studies are supported by more than one deletion mutant (e.g., rhRPA·70Δ1−112 and rhRPA·70Δ1−168). In addition, all of these studies are consistent with previous deletion and structure/function analysis (Henricksen *et al.,* 1994; Gomes & Wold, 1995; Gomes *et al.,* 1996).

Both rhRPA·70Δ1−112 and rhRPA·70Δ1−168 support SV40 DNA replication *in vitro*, however, at a lower level than wild-type RPA. We conclude that the N-terminal domain is not required for SV40 replication. However, the reduced activity of these mutants suggests that this domain is participating in DNA replication. This conclusion is supported by genetic evidence; the N-terminus of the RPA70 homologue in *S. cerevisiae* is essential for growth, and mutations in this domain can cause defects in DNA replication (and/or repair and recombination) (Longhese *et al.,* 1994; Firmenich *et al.,* 1995). Since residues 1−168 are not involved in interactions with oligonucleotides, this domain probably participates in RPA−protein interactions. Direct biochemical studies have shown that the N-terminal domain of RPA70 interacts with SV40 T antigen (K. A. Braun, Z. He, C. J. Ingles, and M. S. Wold, unpublished data); however, replication with rhRPA·70Δ1−112 and rhRPA·70Δ1−168 was not further reduced at suboptimal levels of T antigen. This suggests that interactions with T antigen were not limiting in our reactions. A second protein that could interact with this domain is DNA polymerase α/primase complex. It has been shown previously that a monoclonal antibody to RPA70 that inhibits RPA stimulation of DNA polymerase α binds to the N-terminal domain (Kenny *et al.,* 1990; Gomes *et al.,* 1996). Thus, the reduced activity of rhRPA·70Δ1−112 and rhRPA·70Δ1−168 could be due to an inability of these mutants to interact appropriately with DNA polymerase α. Additional studies are underway to determine the function of the N-terminal domain of RPA70.

The DNA-binding activity of these deletion mutants precisely correlated with their ability to stimulate T antigen-dependent unwinding. We conclude that the high-affinity ssDNA-binding activity of RPA is essential for and is the primary requirement for stimulation of T antigen-dependent unwinding. Since unwinding was observed with ΔC442, we

also conclude that RPA32 and RPA14 are not required for this reaction. These subunits also are not able to support unwinding in the absence of ssDNA-binding activity as evidenced by results with rhRPA·70Δ1−382. These conclusions appear to contradict the conclusions made by Lee and Kim based on the characterization of a mutated RPA complex containing a C-terminal deletion of RPA32 (Lee & Kim, 1995). This mutant complex was shown to have high-affinity ssDNA-binding activity, a reduced ability to interact with T antigen, and a decreased ability to support T antigen-dependent unwinding (Lee & Kim, 1995). From these data, Lee and Kim concluded that RPA−T antigen interactions were essential for unwinding and initiation of DNA replication (Lee & Kim, 1995). However, the low levels of unwinding observed in these studies made it impossible to determine the level of activity of the mutant complex relative to wild-type RPA. In addition, ssDNA binding was examined under stoichiometric binding conditions which only allow the estimation of a lower limit for binding affinity. From such assays, it cannot be determined whether the ssDNA-binding affinity of this mutant complex is lower, equal to, or higher than wild-type RPA. Therefore, it is not possible to compare Lee and Kim's results to those described here. Our data would predict that the mutant complex characterized by Lee and Kim has a binding affinity lower than that of wild-type RPA.

Our model of unwinding is consistent with all other previous studies. Other single-stranded DNA-binding proteins (SSBs) including the yeast homologue of RPA, *E. coli* SSB, and adenovirus DNA-binding protein can substitute for RPA during T antigen-dependent unwinding (Wold *et al.*, 1987; Brill & Stillman, 1989; Kenny *et al.*, 1989; Melendy & Stillman, 1993). The only SSB tested that did not support unwinding was T4 gene 32 protein (Kenny *et al.*, 1989) which has a very different mode of binding to ssDNA than that of RPA and the other SSBs examined (Revzin, 1990). None of the other SSBs (including the yeast homologue of RPA) supported efficient primer synthesis by DNA polymerase α (Kenny *et al.*, 1989; Collins & Kelly, 1991; Murakami *et al.*, 1992; Melendy & Stillman, 1993). This suggests that ssDNA-binding activity is sufficient for stimulation of unwinding but additional functions (e.g., RPA−protein interactions) are required for initiation.

RPA32 normally becomes phosphorylated during SV40 DNA replication *in vitro*. This hyperphosphorylation of RPA32 was also observed with all mutant RPA complexes that had detectable ssDNA-binding activity including rhRPA·70Δ1−236 which has a ssDNA-binding activity more than 3 orders of magnitude lower than wild-type RPA. In contrast, the mutant complex rhRPA·70Δ1−382, which has no detectable ssDNA-binding activity, was not hyperphosphorylated *in vitro*. These data could be interpreted to suggest that residues 236−382 of RPA70 are necessary for efficient phosphorylation of the RPA32; however, we believe that this interpretation is unlikely because RPA32 in a RPA32/RPA14 complex can be readily phosphorylated in the absence of RPA70 by purified DNA-dependent protein kinase (Henricksen and Wold, unpublished results). Instead, we believe that RPA32 must be associated with ssDNA to be efficiently phosphorylated. This is in contrast to high-affinity ssDNA-binding activity being required for stimulation of T antigen unwinding (see above). It has been shown previously that phosphorylation of RPA occurs preferentially when RPA is bound to ssDNA (Fotedar & Roberts, 1992; Henricksen *et al.*, 1994; Henricksen & Wold, 1994). Recently we have shown that RPA undergoes a conformational change upon binding to ssDNA and proposed that the increase in phosphorylation observed upon ssDNA binding is due to this conformational change (Gomes *et al.*, 1996). Thus, the ability of these mutants to become phosphorylated suggests that high-affinity binding is not required for a DNA-induced conformational change in RPA.

On the basis of these and previous studies (Gomes & Wold, 1995; Gomes *et al.*, 1996), RPA70 is composed of at least three structural/functional domains: an N-terminal domain of unknown function (residues 1−168), a central DNA-binding domain (residues 168−∼442), and a C-terminal domain required for complex formation (residues ∼442−616). The ∼18-kDa N-terminal domain exists as a structurally distinct domain (Gomes *et al.*, 1996) and is not involved in ssDNA binding or required for SV40 DNA replication *in vitro* (see above). The other two functional domains may be part of a single 52-kDa structural domain (Gomes *et al.*, 1996). The central DNA-binding domain is required for interactions with ssDNA. The C-terminus of RPA70 is necessary for formation of the heterotrimeric RPA complex, and residues 382−616 are sufficient for interactions with RPA32 and RPA14. All three functional domains may be involved in RPA−protein interactions.

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