MOLECULAR AND CELLULAR BIOLOGY, June 1994, p. 3993–4001
0270-7306/94/$04.00+0

Vol. 14, No. 6

Human Replication Protein A Binds Single-Stranded DNA in Two Distinct Complexes

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Received 25 October 1993/Returned for modification 10 January 1994/Accepted 22 March 1994

Human replication protein A, a single-stranded DNA (ssDNA)-binding protein, is a required factor in eukaryotic DNA replication and DNA repair systems and has been suggested to function during DNA recombination. The protein is also a target of interaction for a variety of proteins that control replication, transcription, and cell growth. To understand the role of hRPA in these processes, we examined the binding of hRPA to defined ssDNA molecules. Employing gel shift assays that "titrated" the length of ssDNA, hRPA was found to form distinct multimeric complexes that could be detected by glutaraldehyde cross-linking. Within these complexes, monomers of hRPA utilized a minimum binding site size on ssDNA of 8 to 10 nucleotides (the $hRPA_{8-10nt}$ complex) and appeared to bind ssDNA cooperatively. Intriguingly, alteration of gel shift conditions revealed the formation of a second, distinctly different complex that bound ssDNA in roughly 30-nucleotide steps (the $hRPA_{30nt}$ complex), a complex similar to that described by Kim et al. (C. Kim, R. O. Snyder, and M. S. Wold, Mol. Cell. Biol. 12:3050–3059, 1992). Both the $hRPA_{8-10nt}$ and $hRPA_{30nt}$ complexes can coexist in solution. We speculate that the role of hRPA in DNA metabolism may be modulated through the ability of hRPA to bind ssDNA in these two modes.

The replication, recombination, and repair of the cellular genetic information entail interconversion of DNA between the duplex and single-stranded forms. Study of nucleic acid enzymology has shown that single-stranded DNA (ssDNA) generated within cells is invariably associated with protein factors, often ssDNA-binding proteins (SSBs). SSBs combine with the ssDNA to form protein-DNA complexes that maintain the unwound state and are more active as substrates, for example, in DNA replication (12). A clear understanding of the architecture of SSB-ssDNA complexes is required to comprehend the function of SSB in DNA metabolism and determine how these processes are regulated within the cell.

Eukaryotic SSBs with defined roles in DNA enzymology have recently been isolated, one of which, from human cells, is termed human replication protein A (hRPA). hRPA, a heterotrimer with subunits of 68, 29, and 14 kDa, was initially isolated as a factor from primate cells required to support simian virus 40 (SV40) DNA replication in vitro (24, 42, 43). The factor was identified as an SSB by various criteria including the high affinity of hRPA for ssDNA (24, 42, 43) and ability to stimulate human DNA polymerase α, an essential eukaryotic replication factor (22, 31). hRPA is phosphorylated in a cell-cycle specific manner, suggesting that DNA replication and other hRPA-mediated events may be modulated through regulation of hRPA activity (18, 21, 25). Moreover, recent studies have shown that hRPA specifically interacts with a number of transcription factors, suggesting that these factors regulate DNA replication, in part, through hRPA (20, 27, 35). In addition to its defined role in SV40 DNA replication, hRPA is also a required factor in an in vitro system reconstituting nucleotide excision repair of damaged DNA (13, 14).

The isolation of proteins similar to hRPA from a variety of sources indicates that RPA is highly conserved in eukaryotic cells. Brill and Stillman isolated a factor from *Saccharomyces cerevisiae*, yRPA, by its ability to replace hRPA in the T-antigen-mediated DNA unwinding reaction from the SV40 origin of replication (7). yRPA was also shown to be identical to a yeast factor that stimulated a DNA strand exchange reaction with purified yeast proteins (28). RPA homologs have recently been isolated from *Xenopus* (1) and calf thymus (3) cells. The cloning of each of the three RPA genes from human (22, 23, 41) and yeast (8, 28) cells reveals significant homologies between each pair of corresponding genes at the amino acid level. Null mutations of each gene in yeast cells are lethal, with a terminal phenotype consistent with a required role in chromosomal DNA replication.

The largest subunit of hRPA is the primary ssDNA-binding subunit, although it is unable to substitute for the heterotrimeric hRPA in the SV40 replication reaction (22, 32, 45). This subunit is also sufficient for interactions with the DNA polymerase α-DNA primase complex (19) and can specifically bind regulatory factors for transcription and cell growth including VP16, the bovine papillomavirus type 1 E2 protein, and the tumor suppressor protein p53 (20, 27, 35). Monoclonal antibodies directed against the 29-kDa subunit inhibit the hRPA-mediated stimulation of DNA polymerase α activity, suggesting direct interactions between the polymerase and the middle hRPA subunit as well (23, 32). The cell cycle-dependent phosphorylation of hRPA is detected on this 29-kDa subunit (18, 21, 25). The function of the small 14-kDa subunit is unknown. The intact heterotrimeric hRPA, as opposed to any single subunit, is required for detectable interactions with the SV40 large T antigen (19).

The interaction of hRPA (33) and yRPA (2) with ssDNA has been recently examined. hRPA binds to ssDNA with an association constant in the range of 10^9 M^{-1}, though with a 50-fold higher affinity for polypyrimidine sequences compared with polypurine sequences. Kim et al. also determined that hRPA bound to ssDNA noncooperatively with a binding site size of approximately 30 nucleotides (nt) (33). The binding properties of yRPA differ in that the binding site size of yRPA was found to be in the range of 90 to 100 nt and it bound ssDNA with a high degree of cooperativity (ω, 10^4 to 10^5 [2]).

Because of the critical roles of hRPA in nucleic acid

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enzymology, we further characterized the binding of hRPA to defined ssDNA templates. Surprisingly, we determined that hRPA bound ssDNA using a step size of approximately 8 to 10 nt (the $hRPA_{8-10nt}$ complex). By alterations of binding and assay conditions, a second distinct hRPA-ssDNA complex, similar to that described by Kim et al. (33), that utilized a step size of approximately 30 nt (the $hRPA_{30nt}$ complex) was detected. Both complexes can be detected simultaneously in solution. These results show that hRPA binds ssDNA in at least two modes, a flexibility that has the potential to regulate nucleic acid metabolism.

MATERIALS AND METHODS

Preparation of hRPA-binding substrates. $d(GACT)_{20}$, $d(GACT)_{11}G$, and $d(T)_{80}$ ssDNA oligonucleotides were synthesized with an Applied Biosystems 380B DNA synthesizer. The oligonucleotides were labeled at the 5′ end with polynucleotide kinase and [γ-^{32}P]ATP to a specific activity of 1×10^6 to 3×10^6 cpm/pmol. Intermediate sizes of each oligonucleotide were prepared by digesting the end-labeled oligonucleotides with the 3′→5′ *Crotalus durissus* phosphodiesterase (Boehringer Mannheim). The digestion reaction mixtures (6 μl) contained 2 to 80 μg of phosphodiesterase per ml (depending on the final average DNA size needed), 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA and were incubated for 5 min at 37°C. No exogenous Mg^{2+} was added to the reaction mixture. The pool of labeled oligonucleotides was separated on an 8% acrylamide denaturing gel and visualized by autoradiography. After the size of each species was ascertained, the desired bands were excised from the gel and isolated by the standard crush-and-soak method (39).

Isolation of hRPA. hRPA was isolated according to the procedure of Kenny et al. (32). Briefly, cytosolic extracts of HeLa cells (60 liters at 10^6 cells/ml) were precipitated by the addition of ammonium sulfate to 35% saturation (the AS35 fraction). The AS35 fraction was passed through a single-stranded DNA cellulose column (U.S. Biochemical) equilibrated with BN buffer (20 mM Tris-HCl [pH 7.5], 10% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol, and 0.01% Nonidet P-40) containing 0.5 M NaCl. After extensive washing with this same buffer, bound proteins were eluted with BN buffer containing 2 M NaCl. hRPA was further purified by fast protein liquid chromatography with a mono-Q Sepharose column (Pharmacia). After this step, the hRPA was >95% pure by densitometric analysis of silver-stained sodium dodecyl sulfate-polyacrylamide gels.

Gel shift analysis. The ssDNA oligonucleotides (40 fmol; 5′ ^{32}P labeled) of predetermined lengths were incubated with hRPA (50 to 100 ng) in reaction mixtures (20 μl) containing 30 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) (pH 7.8), 7 mM $MgCl_2$, 0.5 mM dithiothreitol, and 0.1 mg of bovine serum albumin per ml for 15 min. To detect the $hRPA_{8-10nt}$ complex, reaction mixtures were incubated at 37°C and then cross-linked with 0.1% (final concentration) glutaraldehyde for 15 min at 37°C. To detect the $hRPA_{30nt}$ complex, reaction mixtures were incubated at 25°C and no glutaraldehyde was used. At the completion of each incubation, reaction mixtures were immediately subjected to native gel electrophoresis. The $hRPA_{8-10nt}$ complexes were separated on a gradient gel containing 5 to 20% acrylamide (acrylamide/bisacrylamide ratio, 80:1) at 12 V/cm by using 50 mM Tris and 50 mM glycine (pH 8.8, adjusted with HCl) as the running buffer. The $hRPA_{30nt}$ complexes were separated on a 5% polyacrylamide gel (acrylamide/bisacrylamide ratio, 80:1) at 12 V/cm by using an electrophoresis buffer that contained 89 mM Tris, 89 mM boric acid, and 2 mM EDTA. Our gradient gel conditions did not allow detection of the $hRPA_{30nt}$ complex, a result of these complexes dissociating in the Tris-glycine gel buffer. The gradient gel conditions were found to provide better resolution of the $hRPA_{8-10nt}$ complexes relative to the nongradient gel.

Nitrocellulose filter binding. An oligonucleotide pool, containing 5′-^{32}P-labeled molecules ranging in size from 1 to 45 nt, was generated by the digestion of a 5′-labeled $d(GACT)_{11}G$ by *C. durissus* phosphodiesterase (Boehringer Mannheim). Standard binding reaction mixtures (20 μl) containing 40 mM creatine phosphate (di-Tris salt, pH 7.8), 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 150 pg of the oligonucleotide pool, and 50 to 100 ng of hRPA were incubated for 15 min at 37°C. The protein-DNA complexes were then fixed with glutaraldehyde at 37°C as described above. The reaction mixture was passed through a nitrocellulose filter, and the effluent was collected. The effluent was directly loaded onto a denaturing 19% polyacrylamide gel, and the separated reaction products were detected by autoradiography. The amount of DNA in the effluent, for each size of ssDNA, was quantitated with an LKB Ultrascan XL laser densitometer.

Gel filtration chromatography. hRPA-ssDNA binding reactions were identical to that used for the gel shift assays (above) except that the reaction mixture was increased proportionally to 100 μl. The hRPA-ssDNA reaction mixtures were chromatographed through a Sephacryl S-300 column (90 by 1 cm; Pharmacia). The chromatography buffer contained 30 mM HEPES (pH 7.8), 7 mM $MgCl_2$, 0.5 mM dithiothreitol, and 10% glycerol. Molecular weight markers (Sigma) were used to construct the standard curve. The elution volumes of the RPA-ssDNA complexes were determined by assaying fractions for ^{32}P.

RESULTS

The ability of hRPA to bind ssDNA molecules of various lengths was examined by a gel shift assay. We initially tested oligonucleotides with a $d(GACT)_n$ sequence because these molecules would likely form no stable secondary structure under our reaction conditions. Moreover, as all four nucleotides are present, the single-stranded $d(GACT)_n$ DNA would more closely resemble "natural" DNA than homopolymeric sequences. DNA molecules that were 20, 30, and 45 nt in length were tested.

Increasing amounts of hRPA were incubated with each of these ssDNA molecules, labeled on the 5′ end with ^{32}P. The hRPA-ssDNA complexes were then cross-linked with glutaraldehyde and analyzed by a gel shift assay (Fig. 1). Although hRPA bound with weak affinity to the smallest DNA, two distinct complexes were found at each level of hRPA tested (complexes 1 and 2; lanes 1 to 4). As the ssDNA length was increased to 30 nt, the amount of complex 2 formed was increased and a new, more slowly migrating complex (complex 3) was detected (lanes 5 to 8). With the largest DNA tested, ssDNA binding was more efficient compared with the smaller DNA molecules and at least one additional complex was formed. Thus, hRPA can form multiple complexes on ssDNA as a function of ssDNA length.

To determine the binding site size of an hRPA monomer on ssDNA more clearly, we employed the following novel methodology. An ssDNA molecule, 45 nt in length and labeled with ^{32}P on the 5′ end, was incubated with *C. durissus* phosphodiesterase. Treatment by this 3′-to-5′ exonuclease generates a pool of labeled ssDNA molecules that differ by single nucleotide steps. The pool was subjected to denaturing gel electro-

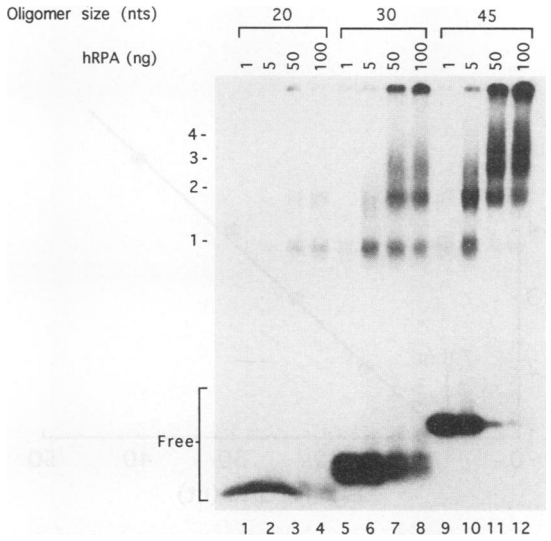

FIG. 1. Binding of hRPA to ssDNA molecules of various lengths. hRPA was incubated with 5′-^{32}P-labeled oligonucleotides that were 20 [d(GACT)$_5$] (lanes 1 to 4), 30 [d(GACT)$_7$GA] (lanes 5 to 8), and 45 [d(GACT)$_{11}$G] (lanes 9 to 12) nt in length. The amounts of hRPA used were 1 (lanes 1, 5, and 9), 5 (lanes 2, 6, and 10), 50 (lanes 3, 7, and 11), and 100 (lanes 4, 8, and 12) ng. Following incubation at 37°C for 15 min, the hRPA-ssDNA complexes were cross-linked with 0.1% glutaraldehyde. The protein-DNA complexes were separated by electrophoresis through a native gel containing a 5 to 20% gradient of acrylamide and visualized by autoradiography. The positions of complexes 1 to 4 are indicated on the left, as is the free (unbound) ssDNA.

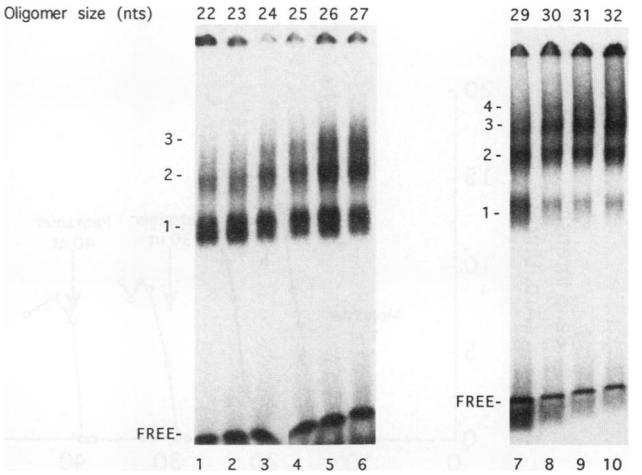

FIG. 2. Formation of distinct hRPA-ssDNA complexes as a function of the ssDNA length. ssDNA oligonucleotides, each labeled on the 5′ end with ^{32}P, were incubated with 50 ng of hRPA for 15 min at 37°C and then cross-linked with 0.1% glutaraldehyde. The protein-DNA complexes were separated by electrophoresis through a native gel containing a 5 to 20% gradient of acrylamide and visualized by autoradiography. The oligonucleotides tested were from 22 to 27 nt (lanes 1 to 6) or from 29 to 32 nt (lanes 7 to 10) in length. hRPA-ssDNA complexes containing a monomer, dimer, trimer, or tetramer of hRPA and the position of the free DNA are indicated on the left of each panel.

phoresis and autoradiography to visualize the oligonucleotide products (e.g., see Fig. 5A). A number of individual bands, each of which corresponded to an oligonucleotide of defined length, were isolated from the gel. These oligonucleotide species, ranging in size from 5 to 45 nt, were then individually used as substrates for hRPA binding in a gel shift assay. As longer ssDNA molecules potentially contain more binding sites for hRPA, we determined the ssDNA length that allowed the binding of an additional hRPA molecule.

hRPA binds ssDNA with a site size of 8 to 10 nt. We examined the binding of hRPA to d(GACT)$_n$ oligonucleotides that ranged in size from 22 to 27 nt. Binding of hRPA to the 22-nt ssDNA molecule gave rise to two observable complexes (complexes 1 and 2; Fig. 2, lane 1). As the length of the DNA was increased from 22 to 27 nt, a novel hRPA-ssDNA complex was observed at a minimum length of approximately 24 nt (complex 3; lane 3). The intensity of this species increased as the DNA was lengthened to 27 nt (lane 6). We similarly examined the binding of hRPA to ssDNA molecules that ranged in size from 29 to 32 nt. Complex 3 was the largest detectable complex that formed on a 29-nt ssDNA molecule (Fig. 2, lane 7). As the length of the oligonucleotide was increased, a new species was detected at approximately 30 to 31 nt (complex 4; lanes 8 and 9).

Complexes 1 to 4 appear to correspond to ssDNA molecules containing monomers, dimers, trimers, and tetramers of hRPA. To verify this, we determined the approximate molecular weights of the hRPA-ssDNA complexes by gel filtration chromatography. hRPA was incubated with increasing lengths of ^{32}P-labeled ssDNA, ranging in size from 20 to 75 nt. Each reaction mixture was chromatographed through a Sephacryl S-300 column, and the approximate molecular weight for each pool of hRPA-ssDNA complexes was calculated (Fig. 3). Increases in the ssDNA length led to an increase in the average molecular weight of these complexes. Using a 30-nt ssDNA molecule, we estimated that an average of approximately three molecules of hRPA were bound, consistent with the gel shift data.

As gel filtration alone cannot be used to determine molecular weights with high accuracy (40), we determined the multimeric state of hRPA bound to ssDNA by a second

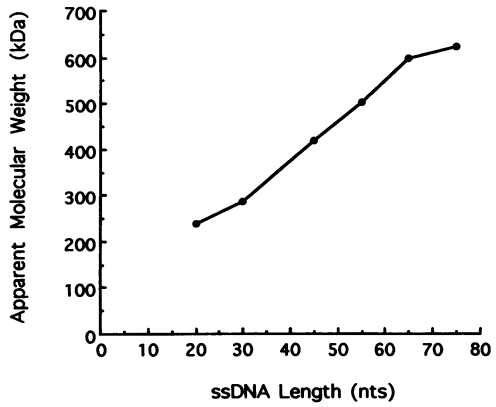

FIG. 3. Size determination of the hRPA-ssDNA complexes. Various lengths of 5′-^{32}P-labeled oligonucleotides were incubated with hRPA, and the hRPA-ssDNA complexes were cross-linked with glutaraldehyde. The reaction mixtures were then subjected to gel filtration chromatography using a Sephacryl S-300 resin, and the radioactivity in each fraction was determined. The average molecular weight of each hRPA-ssDNA pool was determined by comparison to molecular weight standards. The approximate molecular weight of each hRPA-ssDNA pool is plotted as a function of the length of ssDNA used as a substrate.

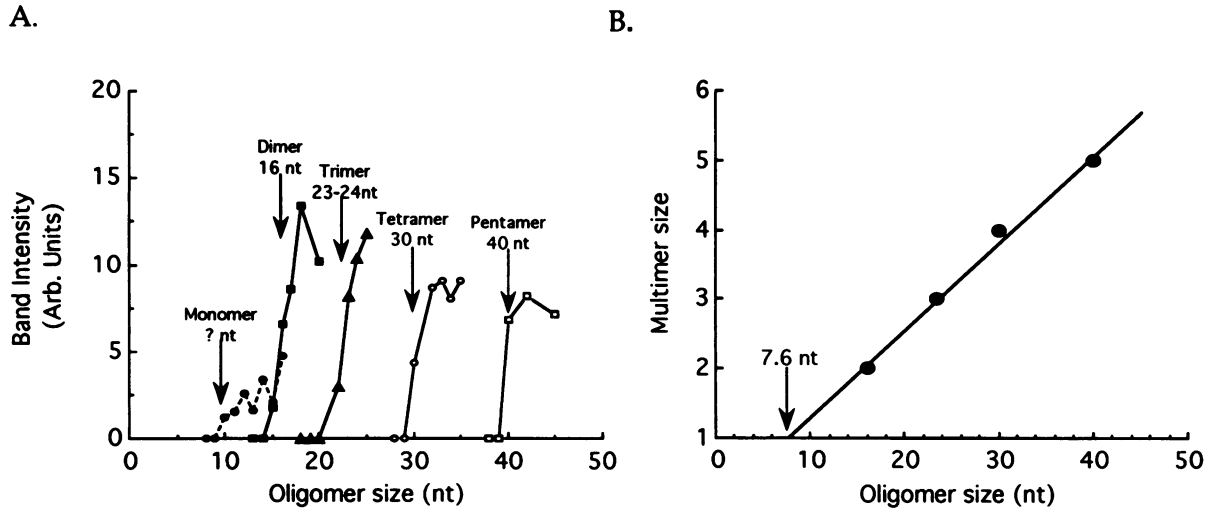

FIG. 4. Determination of the minimum binding site size of hRPA. (A) Various lengths of 5′-^{32}P-labeled oligonucleotides were incubated with hRPA, and the production of these complexes was assayed by gel retardation as shown in Fig. 2. The intensities of bands corresponding to hRPA-ssDNA complexes containing dimers, trimers, tetramers, and pentamers of hRPA were quantitated by densitometric scanning of the autoradiographs. The intensity of these bands, expressed in arbitrary (Arb.) units, is graphed as a function of the ssDNA length at which the complexes were first detected in significant quantities. (B) The length of ssDNA which produced 50% of the maximal complex level, in the initial rising portion of each curve, was determined for hRPA-ssDNA complexes containing dimers, trimers, tetramers, and pentamers of hRPA. The number of hRPA molecules in each complex is graphed as a function of the calculated ssDNA length. Extrapolation of the line to the position of an hRPA monomer indicates that hRPA requires a minimum of 7.6 nt to bind to ssDNA.

procedure. ^{3}H-hRPA was incubated in the presence or absence of ^{32}P-ssDNA. Electrophoresis and fluorography of ^{3}H-hRPA alone using native gel conditions revealed that a single monomeric band comigrated with complex 1 of Fig. 2. Determinations of the relative molar amounts of ^{3}H-hRPA and ^{32}P-ssDNA in complexes 2 to 4 were consistent with the hypothesis that these complexes contain two, three, and four molecules of hRPA bound per ssDNA molecule, respectively (data not shown). Therefore, these data indicate that ssDNA "length titrations" can be employed to determine the minimum ssDNA lengths that allow transitions to larger hRPA-ssDNA complexes.

A number of binding reactions were performed with hRPA and ^{32}P-ssDNA molecules with lengths from 5 to 45 nt, and the complexes were separated by native gel electrophoresis. Autoradiographs of these gels were densitometrically scanned to quantitate the intensity of bands that corresponded to dimers, trimers, tetramers, or pentamers of hRPA on the ssDNA. We plotted the average band intensity of each hRPA-ssDNA complex as a function of the length of the substrate DNA (Fig. 4A). Each curve is shown only in the ssDNA length range in which both the complex was first seen and the amount of the complex was increasing sharply. Above these intervals, the amount of each hRPA-ssDNA complex was found to plateau, or increase slightly because of the higher affinity of hRPA for longer ssDNA (Fig. 1). The length of ssDNA that produced a half-maximal level of complex formation, in the sharply increasing portion of the curve, was determined. Clear transitions were found for the formation of each hRPA-ssDNA complex that contained two to five hRPA molecules. Interestingly, when we used ssDNA molecules that were shorter than 15 nt, only a very low level of hRPA-ssDNA complexes was found.

The hRPA multimer size (i.e., dimer, trimer, etc.) was plotted against the ssDNA length that allowed half-maximal complex formation (Fig. 4B). The resulting plot was linear and extrapolated through the origin (i.e., setting the multimer size to 0 results in an oligonucleotide size of −0.8 nt). Thus, we conclude that each hRPA monomer, from monomer through pentamer, binds to the ssDNA with an equivalent step size in this size range of ssDNA. Moreover, we calculate the minimum binding site size of an hRPA monomer within the multimeric complexes to be 7.6 nt, or approximately 8 nt.

We similarly examined the binding site size of hRPA on ssDNA molecules that contained homopolymeric or alternate heteropolymeric sequences. A similar binding site size was determined, suggesting that DNA sequence did not significantly affect the gross interactions of hRPA with ssDNA in this complex (data not shown). We did find, though, that a fraction of our hRPA preparations resulted in slightly larger values of the hRPA monomer site size (~10 nt) using the same approach. While the cause for these slight differences is not known, these studies indicate that the minimum binding site size of hRPA in multimeric hRPA-ssDNA complexes is likely to be in the range of 8 to 10 nt. For purposes of comparison, we term this hRPA-ssDNA entity the $hRPA_{8-10nt}$ complex.

To verify that this technique can accurately determine the binding site size of SSB proteins, the binding of *Escherichia coli* SSB and bacteriophage T4 gp32 to ssDNA was also examined. *E. coli* SSB was seen to bind ssDNA in steps of approximately 30 nt, while gp32 bound ssDNA in approximately 5-nt steps (data not shown). While the binding site size of SSB is dependent upon a number of variables including ionic strength, our reaction conditions would predict a binding site size of 35 nt (9). The published monomer site size for gp32 is between 5 and 10 nt (30, 34, 38). Thus, these experiments indicate that use of a length titration for the determination of the binding site size is valid.

Cooperative binding of hRPA in the 8- to 10-nt complex. We next determined the minimum length of ssDNA that was stably bound by hRPA using a nitrocellulose filter binding assay. Rather than using ssDNA molecules of a specific size, we used

a pool of labeled oligonucleotides, ranging in size from 1 to 45 nt, that was generated by phosphodiesterase treatment of a 45-nt d(GACT)$_n$ ssDNA. The labeled oligonucleotide pool was incubated in either the presence or the absence of hRPA, treated with glutaraldehyde, and then passed through a nitrocellulose filter to adsorb the hRPA-ssDNA complexes. The flowthrough from each reaction was subjected to denaturing gel electrophoresis and autoradiography (Fig. 5A). Examination of the autoradiograph revealed that ssDNA in the small size class was not efficiently retained on the filter. To quantitate the differential binding of hRPA to these ssDNA molecules, the autoradiograph was subjected to densitometric analysis. We plotted the fraction of ssDNA flowthrough in the absence and presence of hRPA as a function of DNA length (Fig. 5B). Only those ssDNA molecules longer than 15 to 16 nt were efficiently bound by hRPA.

These data suggest that hRPA binds to ssDNA in a cooperative manner by the following reasoning. As shown above, the minimum binding site size of hRPA on ssDNA was determined to be approximately 8 nt. hRPA exists as a monomer in solution (32) and, from our gel shift assays, appears to bind in monomeric steps. With the gel shift assay (above), the minimum length of ssDNA that allowed stable binding of an hRPA dimer was approximately 16 nt. As the ssDNA binding affinity of hRPA dimers and other higher-order complexes appears to be significantly greater than the binding of an isolated monomer, our data suggest that interactions between hRPA monomers on ssDNA can significantly stabilize hRPA binding. This conclusion is strengthened by our observations that the affinity of hRPA for ssDNA is strongly dependent on the length of the DNA substrate (e.g., see Fig. 1).

We examined the possibility that our glutaraldehyde-cross-linking protocol gave rise to altered hRPA molecules that abnormally bound to ssDNA. hRPA was treated with glutaraldehyde for various times and then incubated with ssDNA. Even at the shortest time tested (60 s), significant amounts of hRPA-ssDNA complexes were not detected (data not shown). We conclude, therefore, that glutaraldehyde modification does not produce hRPA with altered binding characteristics.

Because glutaraldehyde abolishes the ssDNA-binding activity of hRPA, classical determination of the association constant and ω (degree of cooperativity) for hRPA bound in the 8- to 10-nt complex is quite challenging. Glutaraldehyde is able to trap potentially unstable hRPA-ssDNA intermediates, yet also causes dissociation of a fraction of the hRPA-ssDNA complexes. Thus, accurate determination of the binding parameters of hRPA in the hRPA$_{8-10nt}$ complex cannot be achieved by standard approaches. In that our assays identify the minimum ssDNA lengths that are required to form distinct hRPA-ssDNA complexes, the dissociation of a fraction of these complexes does not affect the interpretation of our results.

hRPA binds ssDNA in two distinct complexes. Kim et al. (33) have examined the binding site size of hRPA using a somewhat similar approach. Interestingly, these investigators found that hRPA bound ssDNA without significant cooperativity and had a binding site size of approximately 30 nt. As these hRPA characteristics differ significantly from those we described for the hRPA$_{8-10nt}$ complex, we investigated the cause of this discrepancy. Differences between our binding protocols and those described by Kim et al. (33) were tested for their effect on hRPA binding to ssDNA. In the absence of glutaraldehyde, we did not detect any hRPA-ssDNA complexes using our Tris-glycine electrophoresis buffer. By replacing our electrophoresis buffer with a Tris-borate buffer described by Kim et al. (33), hRPA-ssDNA complexes were

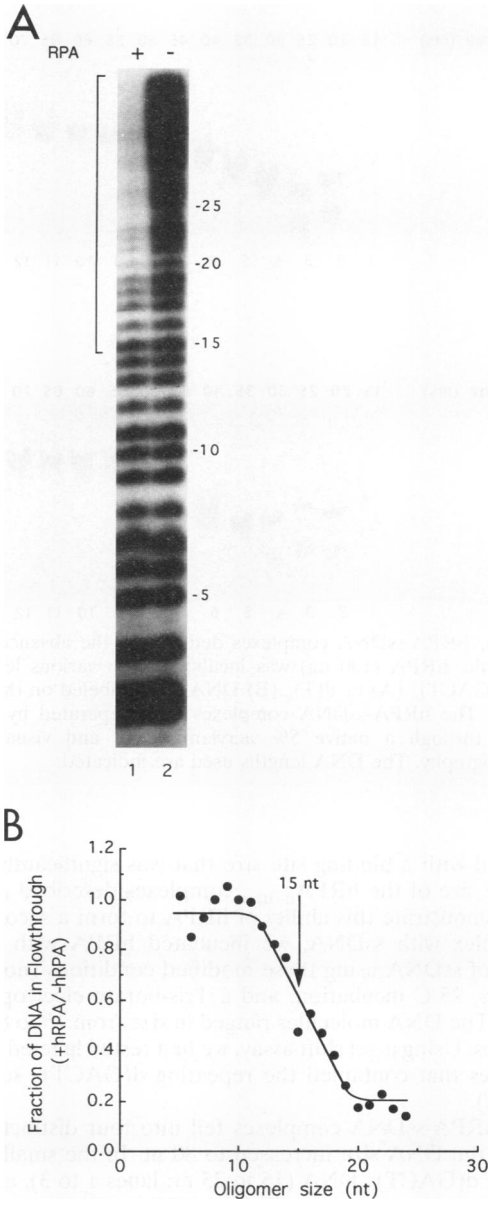

FIG. 5. Determination of the minimum length of DNA required to form stable hRPA-ssDNA complexes. (A) A pool of 5′-^{32}P-labeled ssDNA molecules that ranged in length from 1 to 45 nt was incubated in either the presence (lane 1) or the absence (lane 2) of 100 ng of hRPA. At the completion of the incubation, the hRPA-ssDNA complexes were cross-linked with glutaraldehyde and the reaction mixture was passed through a nitrocellulose filter to adsorb the hRPA-ssDNA complexes. The effluent was collected and subjected to denaturing gel electrophoresis and autoradiography to visualize the nonbound DNA molecules. Markers indicating the ssDNA length (in nucleotides) are indicated on the right. The portion of the DNA pool retained by hRPA on the filter is indicated by a bracket on the left. (B) Autoradiographs similar to that in panel A were subjected to densitometric analysis. The average intensity of each band in lanes 1 and 2 was determined (from two separate experiments). The fraction of ssDNA in the flowthrough in the presence of hRPA was determined and plotted as a function of the ssDNA length. The plot indicates that ssDNA shorter than 15 or 16 nt was not efficiently retained on the filter by hRPA.

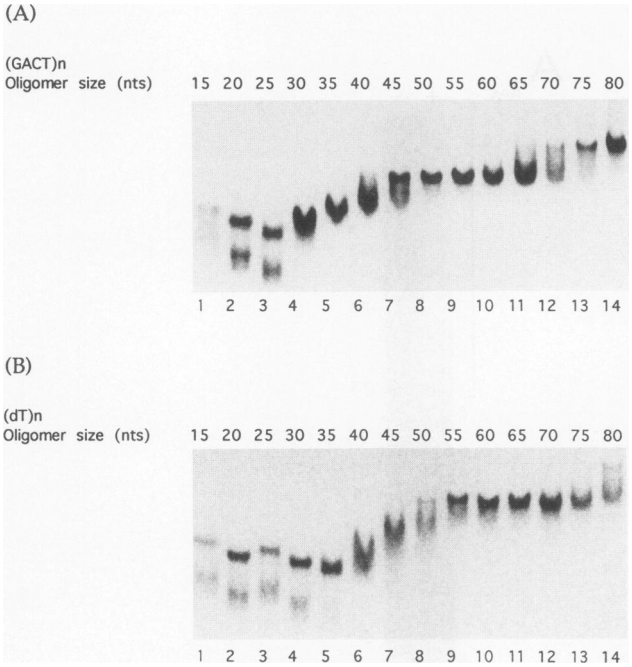

FIG. 6. hRPA-ssDNA complexes detected in the absence of glutaraldehyde. hRPA (100 ng) was incubated with various lengths of either d(GACT)$_n$ (A) or d(T)$_n$ (B) DNA, each labeled on the 5′ end with ^{32}P. The hRPA-ssDNA complexes were separated by electrophoresis through a native 5% acrylamide gel and visualized by autoradiography. The DNA lengths used are indicated.

produced with a binding site size that was significantly larger than the size of the hRPA$_{8-10nt}$ complexes described above.

To demonstrate this ability of hRPA to form a second type of complex with ssDNA, we incubated hRPA with various lengths of ssDNA using these modified conditions (no glutaraldehyde, 25°C incubation, and a Tris-borate electrophoresis buffer). The DNA molecules ranged in size from 15 to 80 nt, in 5-nt steps. Using a gel shift assay, we first tested labeled ssDNA molecules that contained the repeating d(GACT)$_n$ sequence (Fig. 6A).

The hRPA-ssDNA complexes fell into four distinct transitions as the DNA size increased to 80 nt. In the smallest size range of d(GACT)$_n$ DNA (15 to 25 nt; lanes 1 to 3), a similar pattern of two or three distinct hRPA-ssDNA complexes increased in mobility as the DNA length increased. As the ssDNA length increased from 30 to 45 nt of DNA (lanes 4 to 7), a single more diffuse complex that migrated more slowly as a function of DNA length was observed. From 45 to 60 nt of ssDNA, we found a single complex with an electrophoretic mobility independent of DNA length (lanes 7 to 10). This complex was replaced by a more slowly migrating complex, again with a constant mobility, in the range of 65 to 80 nt (lanes 11 to 14). In the DNA size range of 45 to 80 nt, the transition between these two complexes occurred with a step size of approximately 30 nt (i.e., note the similar patterns of hRPA-ssDNA complexes, although shifted in their electrophoretic mobility, in Fig. 6A, lanes 7 [45 nt] and 13 [75 nt]). For comparative purposes, we term these entities hRPA$_{30nt}$ complexes.

Kim et al. (33) showed that hRPA bound with highest affinity to polypyrimidine ssDNA compared with polypurine or other mixed-sequence oligonucleotides. We therefore similarly examined the binding of hRPA to d(T)$_n$ DNA (Fig. 6B). As was observed for hRPA complex formation on d(GACT)$_n$ DNA, four distinct transitions in hRPA-d(T)$_n$ complexes were noted as the DNA length increased from 15 to 80 nt. Below 35 nt, increases in DNA length caused a similar increase in the electrophoretic mobilities of two or three hRPA-ssDNA complexes. From 35 to 55 nt, a more diffuse complex that migrated more slowly with each 5-nt addition to the d(T)$_n$ DNA length was detected. As the DNA length increased from 55 to 75 nt, the hRPA-ssDNA complexes became more distinct and were not notably affected by DNA length. For the largest DNA examined (80 nt), a small amount of a second, more slowly migrating complex was observed. Surprisingly, while the general patterns of hRPA complexes formed on all DNA sequences tested were similar, transitions on d(T)$_n$ DNA required approximately 10 additional nt relative to the d(GACT)$_n$ DNA to be detected. Interestingly, hRPA binds to polypyrimidine DNA with higher affinity than to other DNA sequences. Thus, the higher affinity for polypyrimidine DNA by hRPA may result from the first hRPA$_{30nt}$ complex using relatively more ssDNA, yielding a greater number of hRPA-ssDNA contacts.

We examined the relationship between the hRPA$_{8-10nt}$ and hRPA$_{30nt}$ complexes. In dual reactions, hRPA was incubated with labeled d(GACT)$_n$ DNA molecules that differed in size by 5-nt steps and ranged in length from 20 to 80 nt. One of the two parallel reactions was subjected to glutaraldehyde cross-linking, and the second reaction was not cross-linked. Each pair of reaction products were then subjected to electrophoresis in adjacent lanes to directly compare the reaction products (Fig. 7). As the length of the ssDNA increased to 40 nt, the hRPA complex formed in the absence of glutaraldehyde proceeded through multiple transitions similar to those described above (Fig. 6A). At approximately 40 nt, a stable hRPA$_{30nt}$ complex was produced (lane 9). In this same range of d(GACT)$_n$ ssDNA lengths, four distinct hRPA$_{8-10nt}$ complexes were observed. On the 40-nt ssDNA, we observed that the major hRPA$_{8-10nt}$ complex was an hRPA dimer, although minor amounts of hRPA trimers and tetramers were also seen (lane 10). Essentially no complex corresponding to an hRPA$_{8-10nt}$ monomer was found on the 40-nt ssDNA. This first stable hRPA$_{30nt}$ complex was found to comigrate with an hRPA$_{8-10nt}$ trimer (e.g., compare lanes 11 and 12).

As the ssDNA length was increased to 70 nt, the glutaraldehyde-independent hRPA$_{30nt}$ complex underwent a transition to a more slowly migrating complex that corresponded to an hRPA$_{30nt}$ dimer. Over the same range, a large increase in the amount of hRPA$_{8-10nt}$ tetramers was noted. Higher-order hRPA$_{8-10nt}$ complexes including hRPA pentamers and, to a lesser extent, hexamers were also detected. Thus, it appears that higher-order multimers of the hRPA$_{8-10nt}$ complex and monomers and dimers of the hRPA$_{30nt}$ complex can coexist in solution.

DISCUSSION

A glimpse of recent literature indicates that hRPA can directly interact with a variety of transcriptional transactivators, the tumor suppressor protein p53 (20, 27, 35), and required elements of the eukaryotic replication machinery (19). As the presence of p53 or various transactivators can selectively repress or enhance DNA replication, these studies suggest that DNA replication and other ssDNA-dependent processes may be modulated either through alterations in the architecture of hRPA-ssDNA complexes or through conformational changes of hRPA bound to ssDNA. Our finding that

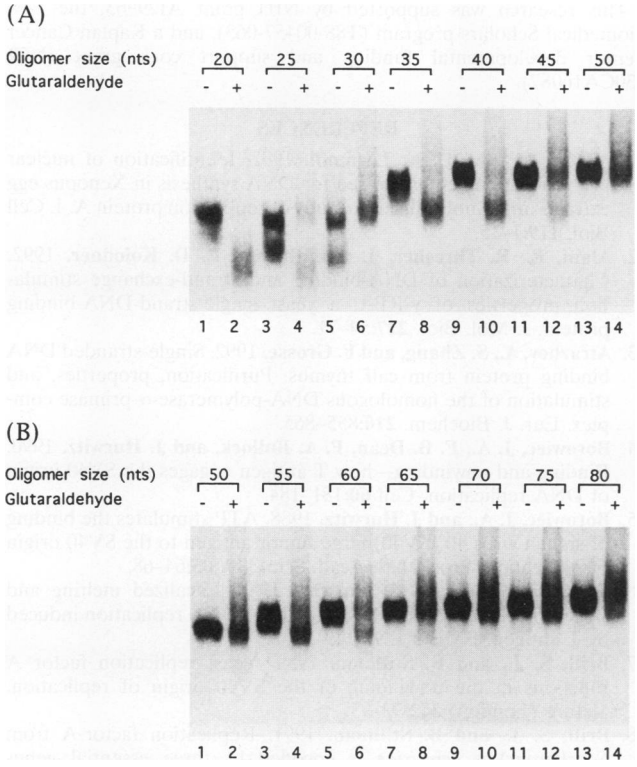

FIG. 7. Comparison of the hRPA-ssDNA complexes formed in the presence and absence of glutaraldehyde. In duplicate reactions, hRPA (100 ng) was incubated with various lengths of d(GACT)$_n$ DNA. One of the two reaction mixtures was cross-linked with 0.1% glutaraldehyde, and then both reaction products were electrophoresed through a native 5% acrylamide gel and visualized by autoradiography. The presence (+) or absence (−) of glutaraldehyde and the lengths of ssDNAs used are indicated.

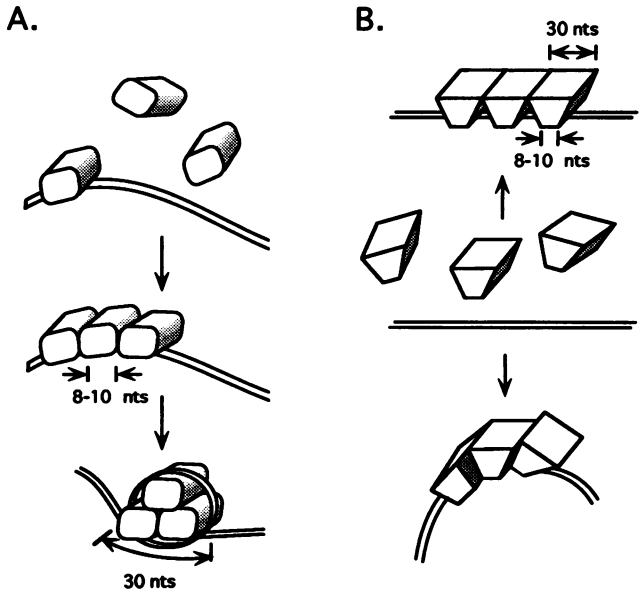

FIG. 8. Two models that suggest a relationship between the hRPA$_{8-10nt}$ and hRPA$_{30nt}$ complexes. In model A, hRPA monomers bind to ssDNA with each monomer utilizing a binding site size of 8 to 10 nt. An undetermined number of these monomers then coalesce to form a multimeric hRPA-ssDNA complex that requires approximately 30 nt of ssDNA. The model is for diagrammatic purposes only and is not meant to suggest either that a specific number of hRPA monomers are contained within the hRPA$_{30nt}$ complex or that the ssDNA is bound to hRPA in a specific topological form, such as being looped around the hRPA core. In model B, a single hRPA monomer has the potential to bind to ssDNA with a binding site size of either 8 to 10 nt (top) or 30 nt (bottom).

hRPA can bind ssDNA in two distinct modes therefore potentially provides a control point for the modulation of DNA metabolism.

The first hRPA-ssDNA complex, which we term the hRPA$_{8-10nt}$ complex, requires a relatively small binding site and appears to bind the ssDNA cooperatively. In contrast, hRPA can also bind ssDNA in a complex that requires a substantially larger binding site of approximately 30 nt that we define as the hRPA$_{30nt}$ complex. A complex that is very similar to hRPA$_{30nt}$ has been previously reported and found to bind ssDNA in a noncooperative manner (33).

The finding that SSBs bind ssDNA in distinct complexes has precedence. *E. coli* SSB binds ssDNA in multiple complexes, distinguished by the binding site size, that are interconvertible by salt concentration and buffer composition (e.g., see reference 9). The filamentous bacteriophage Pf1 gene 5 protein has two distinct modes of ssDNA binding which appear to result from the occupancy of either one or two DNA-binding sites on a gene 5 homodimer by ssDNA (11). Other SSB proteins have somewhat similar properties (10, 29). Our findings are somewhat surprising, however, in that the two complexes formed by hRPA are different both in the site size and cooperativity of binding and are present simultaneously in solution.

What is the relationship between the hRPA$_{8-10nt}$ and hRPA$_{30nt}$ complexes? One possibility is that a number of hRPA monomers, each binding to 8 to 10 nt of ssDNA, coalesce to form a distinct multimeric hRPA-ssDNA complex that covers roughly 30 nt of DNA (Fig. 8A). This notion is supported by the data from Fig. 7, in which the first stable hRPA$_{30nt}$ complex comigrates with an hRPA$_{8-10nt}$ trimer (compare lanes 11 and 12). Weighing against this possibility is the study of Alani et al. showing distinct monomeric complexes of yRPA on ssDNA (2), even though the binding characteristics of yRPA and hRPA appear to differ (33).

An alternate possibility is that an hRPA monomer has the intrinsic ability to bind ssDNA in two distinct modes. As depicted in Fig. 8B, free hRPA could bind ssDNA in either 8- to 10- or 30-nt steps. We also raise the possibility that the hRPA$_{8-10nt}$ complex represents a significant intermediate in the pathway of hRPA$_{30nt}$ complex formation. This model may reflect a cellular need for hRPA to bind ssDNA in a nonsingular manner during DNA replication, recombination, or repair. For example, only short stretches of ssDNA may be available during the initiation of replication (see below) that precludes hRPA binding in the hRPA$_{30nt}$ mode. Our ability to detect either the hRPA$_{8-10nt}$ or the hRPA$_{30nt}$ complex, rather than mixed complexes, in each gel shift assay indicates that glutaraldehyde is able to "trap" the hRPA$_{8-10nt}$ complex while selectively destabilizing the hRPA$_{30nt}$ form. The two proposed models of hRPA binding are currently being tested.

By limiting the length of ssDNA bound by hRPA, we appear to detect the earliest stages of hRPA$_{30nt}$ complex formation. In Fig. 6, we demonstrated that the electrophoretic mobility of the hRPA-ssDNA complexes increased in the range of 15 to 25 or 15 to 35 nt (Fig. 6A, lanes 1 to 3, and B, lanes 1 to 5, respectively). The fastest-migrating complexes were formed on intermediate lengths of ssDNA that neither produced the first

stable complex nor were the smallest DNA molecules observed to bind hRPA. The binding of additional hRPA monomers or the presence of longer DNA would be expected to decrease the mobility of the hRPA-ssDNA complex. Thus, the changes in migration are more likely indicative of transitions in the conformation of the hRPA-ssDNA complex that are dependent on ssDNA length. In this view, these fastest-migrating complexes reflect $hRPA_{30nt}$ intermediates with a compact arrangement of protein and DNA. As the DNA length is increased beyond this point (i.e., 45 to 55 nt), a decrease in the mobility of the hRPA-ssDNA complex was detected, suggesting that formation of the first stable $hRPA_{30nt}$ complex entails either an expansion or an elongation of the complex.

These striking changes in complex mobility, as a function of ssDNA length, were observed only for the formation of the first $hRPA_{30nt}$ complex and were not visually apparent for the formation of subsequent $hRPA_{30nt}$ complexes. The mass and conformation of the first $hRPA_{30nt}$ complex have the potential to minimize the detection of similar conformational changes during the formation of the second and third $hRPA_{30nt}$ complexes on the ssDNA. Because hRPA appears to bind ssDNA cooperatively in the $hRPA_{8-10nt}$ complex, the presence of one prebound $hRPA_{30nt}$ complex on the ssDNA can easily be envisaged to influence the binding of subsequent $hRPA_{30nt}$ complexes so that intermediates in the hRPA-ssDNA complex formation are less apparent.

The binding site size data have interesting implications concerning the mechanism of replication initiation from the SV40 origin of replication (*ori* [4]). It has been shown that the viral T antigen binds to *ori* in an ATP-dependent reaction (5, 16, 17, 36) that generates structural transitions in two regions within *ori*. The transition in one of these two regions results from the melting of 8 bp (6, 37). As the addition of hRPA or other heterologous SSBs induces the complete denaturation of *ori* (7, 15, 31, 44), it has been proposed that the binding of hRPA to this ssDNA region is a required step in the denaturation of *ori*.

In support of this hypothesis, we find a close correlation between the ssDNA-binding site size for the $hRPA_{8-10nt}$ complex and the size of the melted bubble within the T-antigen–*ori* complex, each approximately 8 nt. Furthermore, our data suggest that hRPA in the 8-nt complex requires two molecules of protein to bind stably to ssDNA. Thus, while a single monomer of hRPA would bind the melted region within *ori* with weak affinity, the binding of additional molecules of hRPA would significantly strengthen this interaction. As such binding would propagate the melted region within *ori*, the cooperative binding of hRPA in the $hRPA_{8-10nt}$ mode may be a key element in the complete denaturation of *ori*. Other investigators have found that hRPA can actively unwind DNA in an ATP-independent reaction under low-salt-concentration conditions (26). Upon the complete denaturation of *ori*, the availability of longer regions of ssDNA would allow hRPA binding in the $hRPA_{30nt}$ complex.

The finding that hRPA binds ssDNA in at least two distinct modes suggests that this protein has a more dynamic role in DNA metabolism than the mere stabilization of ssDNA and the activation of ssDNA as a template for DNA polymerases. We anticipate that future work will undoubtedly reveal further surprises concerning the role of hRPA in DNA metabolism.

ACKNOWLEDGMENTS

We thank Dhruba SenGupta and Thomas Gillette for constructive comments during the course of this project.

This research was supported by NIH grant AI29963, the Pew Biomedical Scholars program (T88-00457-063), and a Kaplan Cancer Center developmental funding and support core grant (NCI P30CA16087).

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