MOLECULAR AND CELLULAR BIOLOGY, July 1997, p. 3876–3883 Vol. 17, No. 7
0270-7306/97/$04.00+0

Denaturation of the Simian Virus 40 Origin of Replication Mediated by Human Replication Protein A

CRISTINA IFTODE AND JAMES A. BOROWIEC*

Department of Biochemistry and Kaplan Comprehensive Cancer Center, New York University Medical Center, New York, New York 10016

Received 27 January 1997/Returned for modification 31 March 1997/Accepted 23 April 1997

The initiation of simian virus 40 (SV40) replication requires recognition of the viral origin of replication (*ori*) by SV40 T antigen, followed by denaturation of *ori* in a reaction dependent upon human replication protein A (hRPA). To understand how origin denaturation is achieved, we constructed a 48-bp SV40 "pseudo-origin" with a central 8-nucleotide (nt) bubble flanked by viral sequences, mimicking a DNA structure found within the SV40 T antigen-*ori* complex. hRPA bound the pseudo-origin with similar stoichiometry and an approximately fivefold reduced affinity compared to the binding of a 48-nt single-stranded DNA molecule. The presence of hRPA not only distorted the duplex DNA flanking the bubble but also resulted in denaturation of the pseudo-origin substrate in an ATP-independent reaction. Pseudo-origin denaturation occurred in 7 mM $MgCl_2$, distinguishing this reaction from Mg^{2+}-independent DNA-unwinding activities previously reported for hRPA. Tests of other single-stranded DNA-binding proteins (SSBs) revealed that pseudo-origin binding correlates with the known ability of these SSBs to support the T-antigen-dependent origin unwinding activity. Our results suggest that hRPA binding to the T antigen-*ori* complex induces the denaturation of *ori* including T-antigen recognition sequences, thus releasing T antigen from *ori* to unwind the viral DNA. The denaturation activity of hRPA has the potential to play a significant role in other aspects of DNA metabolism, including DNA repair.

A key step during the initiation of chromosomal DNA replication is the denaturation of the replication origin to generate two DNA replication forks. Study of prokaryotic and eukaryotic model systems indicates that both *trans*-acting protein factors and discrete *cis*-acting elements within DNA play essential roles as the duplex DNA becomes unwound and the nucleotide bases are exposed. Although the critical components required for DNA helicase loading during replication initiation have been established for various systems, the mechanism by which protein and DNA interact to achieve this goal is poorly understood.

The initiation and elongation phases of eukaryotic DNA replication have been productively studied with simian virus 40 (SV40) (19, 39). The early stages of SV40 initiation are particularly simple in that only two protein factors are needed to denature the viral origin of replication (*ori*), the viral large tumor antigen (T antigen) (12), and human replication protein A (hRPA) (11, 45, 46). In an ATP-dependent reaction, T antigen forms a double hexamer over *ori* with each hexamer covering one *ori* half (5, 9, 10, 25) and each hexamer forming face- and strand-specific contacts with the underlying *ori* flank (34). T-antigen binding induces *ori* structural transitions, including the melting of 8 bp within the essential early palindrome (EP) region (6, 33). Denaturation of *ori* occurs upon the addition of hRPA to the T antigen-*ori* complex (14, 43). The DNA is then unwound bidirectionally from *ori* by the DNA helicase activity of T antigen (8, 38, 47). T antigen apparently remains as a double hexamer during unwinding such that two single-stranded DNA (ssDNA) loops are extruded from the complex (37, 42). Nascent strand synthesis takes place on the exposed template.

Biochemical and genetic studies of eukaryotic RPA indicate that it plays a critical role not only in DNA replication but also in DNA repair and recombination and RNA transcription (7, 23, 24, 31, 36, 40). Similar to RPA in other eukaryotes, hRPA is a heterotrimer with 70-, 29-, and 11-kDa subunits (11, 46). Although the three hRPA subunits exist as a complex in the S phase, their different localization during mitosis indicates that subunit association is regulated throughout the cell cycle (32). hRPA binding to ssDNA evidently proceeds through a multistep pathway in which the hRPA first binds 8 nucleotides (nt) of DNA unstably (the $hRPA_{8nt}$ complex) (2, 3) and then aligns along the DNA to contact 30 nt (the $hRPA_{30nt}$ complex) (2, 3, 22). Stable complex formation in the latter mode is accompanied by significant changes in hRPA structure as suggested by increased phosphorylation of the p29 subunit by DNA-dependent protein kinase, alterations in appearance when visualized by electron microscopy, and altered sensitivity of hRPA to protease cleavage (3, 15). The correlation between the hRPA recognition of 8 nt of ssDNA and the 8 nt of melted DNA within the T antigen-*ori* complex raises the possibility that hRPA induces *ori* denaturation by transiting from the $hRPA_{8nt}$ to the $hRPA_{30nt}$ complex.

To examine the role of hRPA in *ori* denaturation, we tested the interaction of hRPA with a "pseudo-origin" (PO-8) substrate. This DNA molecule contains a central 8-nt ssDNA region flanked by duplex DNA, thus resembling the structure induced by T antigen within the EP element of *ori*. The PO-8 substrate was bound by hRPA with an affinity approaching that found for a similar length of ssDNA. hRPA binding distorted the duplex structure of PO-8 and resulted in denaturation of a significant fraction of the substrate pool. These results suggest that hRPA plays a dynamic role during the initiation of SV40 replication by extending the DNA bubble formed by T antigen.

* Corresponding author. Mailing address: Department of Biochemistry and Kaplan Comprehensive Cancer Center, New York University Medical Center, 550 First Ave., New York, NY 10016. Phone: (212) 263-8453. Fax: (212) 263-8166. E-mail: borowj01@mcrcr.med.nyu.edu.

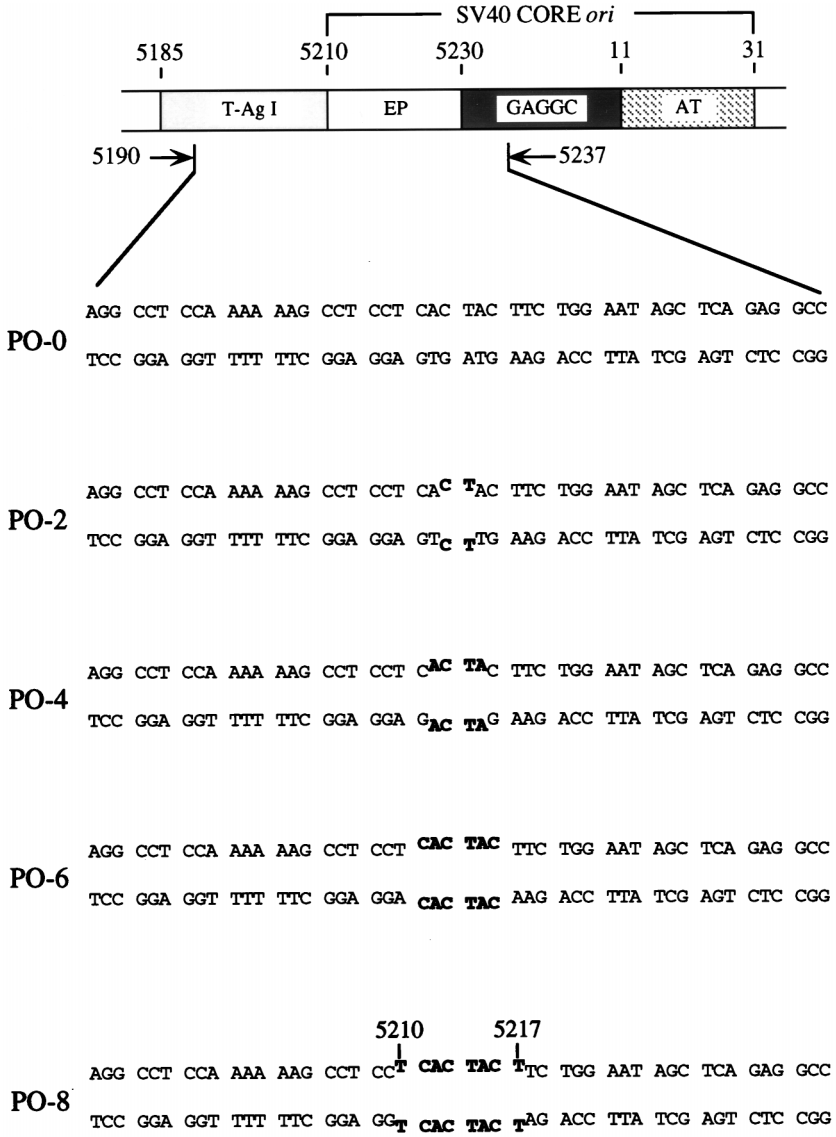


FIG. 1. Pseudo-origin DNA substrates used to test hRPA binding. Five DNA substrates were constructed from sequences (bp 5190 to 5237) within and adjacent to the SV40 origin of replication. The pseudo-origin sequences include a partial T-antigen binding site I (T-Ag I), the EP element, and a partial T-antigen recognition element (GAGGC). The AT-rich element (AT) was not included. The substrates differed in the size of the central ssDNA region: PO-0, completely duplex; PO-2, 2-nt bubble; PO-4, 4-nt bubble; PO-6, 6-nt bubble; PO-8, 8-nt bubble. The top-strand sequence was identical for all substrates. The bottom strand contained various lengths of noncomplementary bases (i.e., they were identical to the top-strand bases) to generate the desired bubble length.

The enlargement of the ssDNA bubble denatures the T-antigen recognition sequences, releasing the T-antigen DNA helicase to unwind the viral DNA.

MATERIALS AND METHODS

Preparation of the DNA substrates. The pseudo-origin substrates were constructed by annealing either fully or partially complementary oligonucleotides (Oligos Etc., Wilsonville, Oreg.) to generate 48-bp DNA molecules with central ssDNA regions of 0, 2, 4, 6, or 8 nt. Oligonucleotide sequences are given in Fig. 1. Prior to annealing, either the top- or bottom-strand oligonucleotides were 5′ ^{32}P labeled with T4 polynucleotide kinase (Boehringer Mannheim) to a specific activity of approximately 1×10^6 to 2×10^6 cpm/pmol. The annealing reaction mixtures (100 μl), containing 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 5 pmol each of the desired top and bottom strands, were heated to 90°C and then slowly cooled to room temperature over a period of 2 h. The 5′-^{32}P-labeled top or bottom strands were used for the ssDNA substrates.

hRPA and other SSBs. hRPA was isolated from 0.6×10^{11} HeLa cells (Cell Culture Center, Minneapolis, Minn.) as previously described (21), with a Mono Q-Sepharose column used in the last purification step. hRPA purity was analyzed by silver staining of sodium dodecyl sulfate–12% polyacrylamide gels and found to be approximately 98% pure. Other ssDNA-binding proteins (SSBs) were generously provided by Kenneth Marians (EcoSSB), I. Robert Lehman (HSV ICP8), Ronald T. Hay (AdDBP), and Charles Richardson (T7 gp2.5). Bacteriophage T4 gene 32 protein (T4 gp32) was purchased from Boehringer Mannheim Biochemicals.

Pseudo-origin binding reactions. Binding reaction mixtures (20 μl) containing 30 mM HEPES (pH 7.8), 7 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 mg of bovine serum albumin per ml, 0.5 to 3 pmol of hRPA, and 0.05 to 0.3 pmol of DNA substrate were incubated for 20 min at 37°C. Identical conditions were used in binding reactions testing other SSBs. When protein-DNA complexes were cross-linked, glutaraldehyde was subsequently added to a final concentration of 0.1% and the reaction mixture was incubated for an additional 15 min at 37°C. Protein-DNA complexes were separated by electrophoresis through a nondenaturing 5% polyacrylamide gel (29:1 acrylamide/bisacrylamide ratio) and visualized by autoradiography. To detect ssDNA that was generated as a result of hRPA binding,

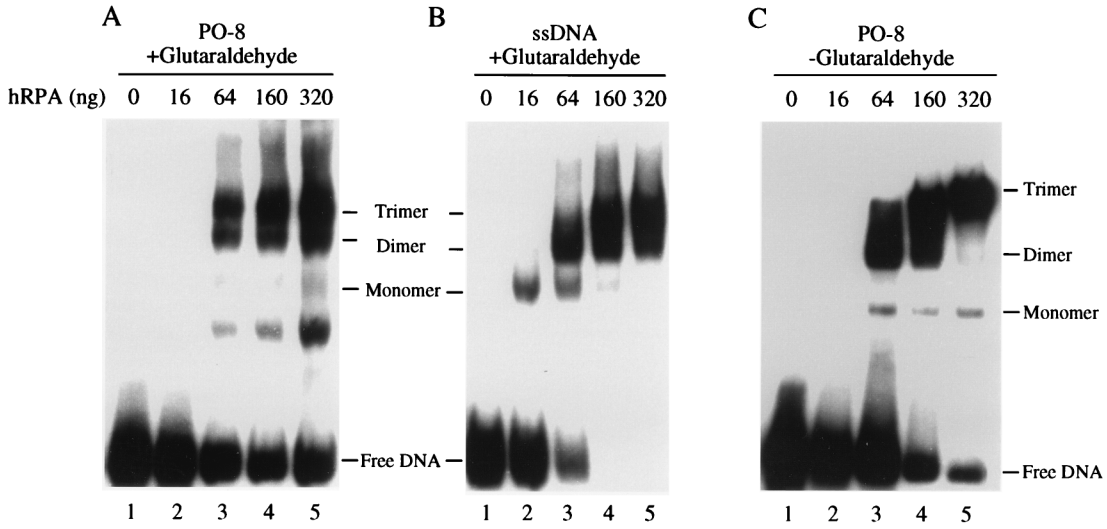


FIG. 2. hRPA binds with high affinity to the PO-8 substrate. The ^{32}P-labeled PO-8 (A and C) or 48-nt ssDNA (B) substrates (0.16 pmol) were incubated with increasing levels of hRPA (as indicated). In panels A and B, glutaraldehyde was employed to cross-link the hRPA-DNA complexes as described in Materials and Methods. Complexes were separated by electrophoresis through a native 5% polyacrylamide gel and autoradiographed. Complexes were identified as containing a monomer, dimer, or trimer of hRPA from previous analyses of hRPA-ssDNA complex formation (2, 3). The position of free DNA is also indicated.

reaction products were separated on nondenaturing 10% polyacrylamide gels (29:1 acrylamide/bisacrylamide ratio) at 8 V/cm by using 45 mM Tris, 45 mM boric acid, and 1 mM EDTA as the running buffer.

Nitrocellulose DNA-binding assay. Each SSB (1 pmol) was incubated with the 5′-^{32}P-labeled ssDNA or PO-8 substrate (0.15 pmol) under the binding conditions described above, in the absence of glutaraldehyde. The reaction mixtures were passed through 0.45-μm-pore-size nitrocellulose filters (type HA; Millipore) and then washed three times with binding buffer. In order to reduce nonspecific binding of DNA, filters were pretreated with alkali (28) in the following sequence: 0.5 M NaOH for 20 min, distilled water for 5 min, 0.1 M Tris-HCl (pH 7.5) for 40 min, and binding buffer for 30 min. Radioactivity retained on the air-dried filters was determined by liquid scintillation counting.

Copper-phenanthroline footprinting. For 5-phenyl-1,10-phenanthroline-copper (PPC) footprinting reactions, 0.75 to 1.5 pmol of hRPA was incubated with 0.15 pmol of PO-8 substrate (5′ ^{32}P labeled on the top or bottom strand) under binding conditions identical to those described above (in the absence of glutaraldehyde). Cleavage reactions were as described by Sigman et al. (35). Following hRPA–PO-8 complex formation, 2 μl of a 1 mM 5-phenyl-1,10-phenanthroline–0.23 mM $CuSO_4$ solution and 2.5 μl of 58 mM 3-mercapto-propionic acid were added, and the reaction mixture was incubated for 5 min at 37°C. Cleavage reactions were quenched by addition of 2.5 μl of 28 mM 2,9-dimethyl-1,10-phenanthroline. Following phenol-chloroform extraction (1:1, vol/vol) and ethanol precipitation, DNA samples were separated by electrophoresis through a denaturing 8% polyacrylamide gel (19:1 acrylamide/bisacrylamide ratio) and autoradiographed.

RESULTS

High-affinity binding of hRPA to an SV40 pseudo-origin. A DNA substrate containing a structure resembling that found within the ATP-dependent T antigen-*ori* complex (6, 33) was constructed. This pseudo-origin substrate (PO-8) was prepared from two partially complementary oligonucleotides that anneal to generate an 8-nt bubble flanked by 20 bp of duplex DNA on each side (Fig. 1). The molecule was constructed from viral DNA sequences found in the early half of *ori* and adjacent elements, except for the 8 nt of noncomplementary sequence on the bottom strand used to generate a bubble. The top-strand bubble sequence is thus identical to that occurring in the melted EP element of the T antigen-*ori* complex.

The ability of hRPA to bind the ^{32}P-labeled PO-8 substrate was tested. As hRPA has been demonstrated to have an intrinsic Mg^{2+}-independent DNA-unwinding activity (13, 41), binding reactions were performed in the presence of 7 mM $MgCl_2$ to prevent this reaction. Moreover, this Mg^{2+} concentration supports the SV40 DNA replication reaction (for example, see reference 44). Addition of hRPA to the PO-8 substrate resulted in efficient hRPA–PO-8 complex formation (Fig. 2A). The highest levels of protein tested (320 ng) caused more than half of the PO-8 substrate to be bound. Most prominent were complexes that comigrated with ssDNA complexes containing hRPA dimers and trimers (Fig. 2B) identified in our previous studies (2, 3). Under these conditions, no ssDNA was produced (see below). Binding to a 48-nt ssDNA substrate (Fig. 2B) was somewhat more efficient, with the affinity estimated to be approximately fivefold greater as determined by densitometric analysis of the autoradiographs (data not shown). Thus, hRPA binds with high affinity to a DNA structure similar to that induced in *ori* by T antigen.

The PO-8 complexes also appear to contain hRPA dimers and trimers because, in the presence of glutaraldehyde, the DNA substrate has relatively minor effects on the mobility of complexes bound by identical molecules of hRPA (2). For example, pseudo-origin substrates containing 2 to 8 nt of ssDNA have similar ratios of hRPA complexes even though the total amounts of complexes that are formed differ (see Fig. 3 and below). Moreover, on larger PO-8 substrates (i.e., 60 bp), in which ssDNA production (see below) is greatly reduced, the pattern and migration of hRPA complexes resembles that detected with the 48-bp PO-8 substrate (data not shown).

The patterns of complexes on the PO-8 and ssDNA substrates were similar, with two exceptions. First, PO-8 complexes bound by a single hRPA monomer were less abundant than the analogous complex formed on ssDNA (compare lanes 2 and 3 of Fig. 2A and B), indicating that hRPA binds PO-8 cooperatively. Interestingly, our previous binding studies indicated that hRPA binding in the 8-nt mode was significantly cooperative (2), suggesting that binding of the PO-8 substrate requires initial $hRPA_{8nt}$ complex formation. Second, a novel complex was observed only with the PO-8 substrate that migrated faster than monomeric hRPA complexes. Although this band cross-reacted with anti-hRPA antibodies, it was not observed with all preparations of hRPA.

Because these binding experiments were performed using glutaraldehyde to stabilize the hRPA-DNA complexes, hRPA binding to the PO-8 substrate was repeated in the absence of a

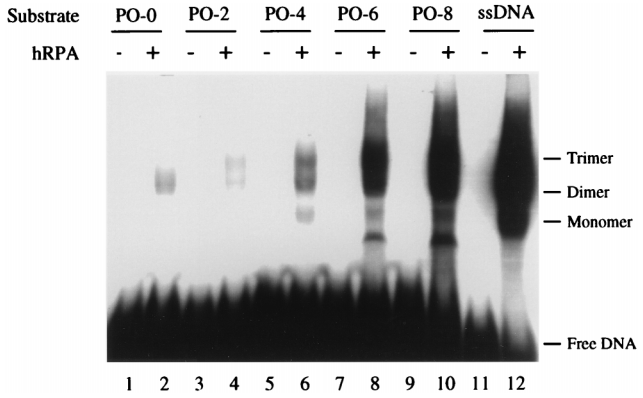
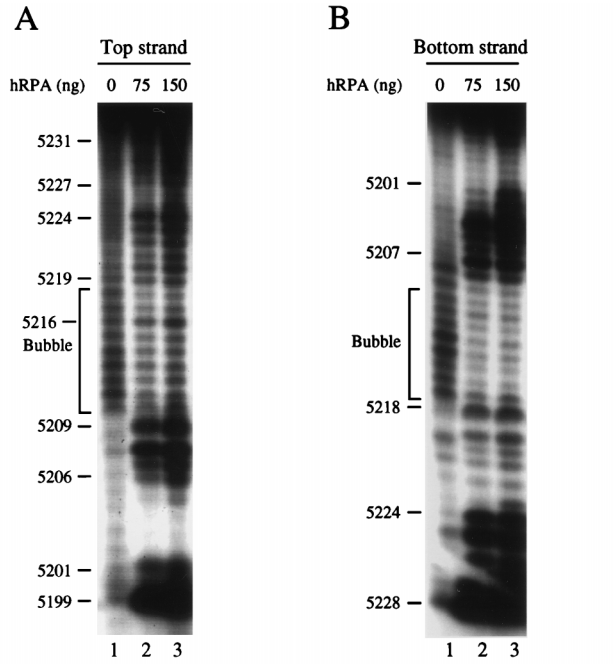


FIG. 3. Efficient hRPA–pseudo-origin complex formation requires at least 6 nt of ssDNA. hRPA (50 ng) was incubated with various ^{32}P-labeled DNA substrates (0.05 pmol): PO-0 (lanes 1 and 2), PO-2 (lanes 3 and 4), PO-4 (lanes 5 and 6), PO-6 (lanes 7 and 8), PO-8 (lanes 9 and 10), and a 48-nt ssDNA control (the PO-8 bottom strand) (lanes 11 and 12). Following incubation, the reaction mixtures were subjected to glutaraldehyde cross-linking and separated by electrophoresis through a native 5% polyacrylamide gel and visualized by autoradiography. The positions of the hRPA-DNA complexes and free DNA are indicated on the right side of the panel.



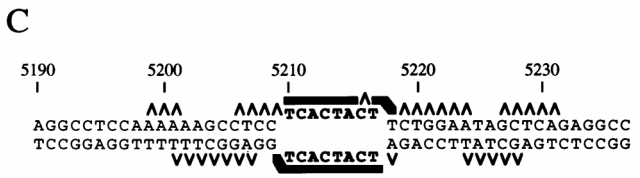


FIG. 4. hRPA-dependent distortion of PO-8 structure detected by copper-phenanthroline footprinting. The PO-8 substrate (0.15 pmol), 5′ ^{32}P labeled either on the top (A) or bottom (B) strand, was incubated with increasing levels of hRPA (as indicated) in the absence of glutaraldehyde. Following binding, the reaction mixtures were treated with PPC as described in Materials and Methods. Reaction products were separated by electrophoresis through a denaturing 8% polyacrylamide gel and autoradiographed. The locations of the bubble and various sequence positions are indicated on the left of each panel. (C) Compilation of the PPC footprinting pattern. Protection from PPC cleavage is indicated by solid bars, while hyperreactivity is indicated by carets.

cross-linking agent. hRPA–PO-8 complex formation was more efficient in the absence of glutaraldehyde, with >95% of the PO-8 substrate bound at the highest level of hRPA (Fig. 2C). Complexes containing a single hRPA monomer were again only weakly observed under these conditions. These data indicate that non-cross-linked complexes formed between heterotrimeric hRPA and the PO-8 substrate are sufficiently stable to withstand the electrophoretic conditions used for the gel shift assay.

Pseudo-origin substrates with bubble lengths ranging from 0 to 8 nt, differing in 2-nt increments (Fig. 1), were tested to determine the minimum bubble length supporting hRPA binding. hRPA bound with weak affinity to DNA molecules that were completely duplex (PO-0) or contained 2 (PO-2) or 4 (PO-4) nt of ssDNA (Fig. 3). In contrast, hRPA bound with highest affinity to molecules with bubble lengths of 6 (PO-6) or 8 (PO-8) nt, with densitometric scanning of the autoradiographs indicating >10-fold-stronger binding to these molecules than to the PO-0 substrate (Fig. 3, lanes 8 and 10). hRPA weakly binds ssDNA molecules shorter than 15 nt (2, 22), and we found, similarly, that incubation of hRPA with a 12-nt ssDNA molecule produced only a small level of glutaraldehyde-dependent hRPA-DNA complexes (data not shown). Taken together, these observations suggest that high-affinity binding of hRPA to the pseudo-origin substrates requires a minimum of 6 nt of ssDNA and additional DNA provided by the flanking elements. Matsunaga et al. (27) recently reported that hRPA can also bind DNA substrates containing longer ssDNA bubbles (12 and 30 nt).

hRPA complex formation was tested with substrates having longer flanking duplex regions and at reduced temperatures (e.g., 0°C). hRPA bound these longer substrates, albeit with reduced affinity, and binding was able to occur at the low temperatures (19a). These observations rule out the possibility that the increased bubble length merely destabilized the duplex structure of PO-8, thereby allowing efficient hRPA complex formation.

hRPA–PO-8 complex formation is accompanied by duplex DNA distortion. The effect of hRPA–PO-8 complex formation on PO-8 structure was examined by copper-phenanthroline footprinting (35, 49). PPC binds 2 to 3 bp within the minor groove of double-stranded DNA (dsDNA), from which it becomes oxidized to generate a reactive species that nicks one of the two strands. Subtle distortion of the duplex DNA altering minor-groove width or accessibility can be detected with PPC.

The PO-8 substrate, 5′ ^{32}P labeled on either the top (Fig. 4A) or bottom (Fig. 4B) strand, was incubated with two different levels of hRPA. PPC cleavage of the PO-8 substrate in the absence of hRPA showed enhanced nicking of the top and bottom strands of the ssDNA bubble compared to flanking duplex regions (Fig. 4A and B, lanes 1). Addition of hRPA resulted in the moderate protection of a 9-nt top-strand region encompassing the bubble (nt 5210 to 5218), with the exception of a single hyperreactive site at nt 5216 (Fig. 4A, lanes 2 and 3). Protection was also observed over bottom-strand sequences between nucleotides 5209 and 5217 within the bubble. We interpret these results to indicate that hRPA binds both the top and bottom strands of the bubble, most likely with one hRPA molecule to each strand.

The presence of hRPA caused various regions in the duplex DNA flanks to become strongly hyperreactive to the PPC probe. On the top strand, two adjacent hyperreactive patches were noted on each side of the bubble: nt 5199 to 5201 and nt

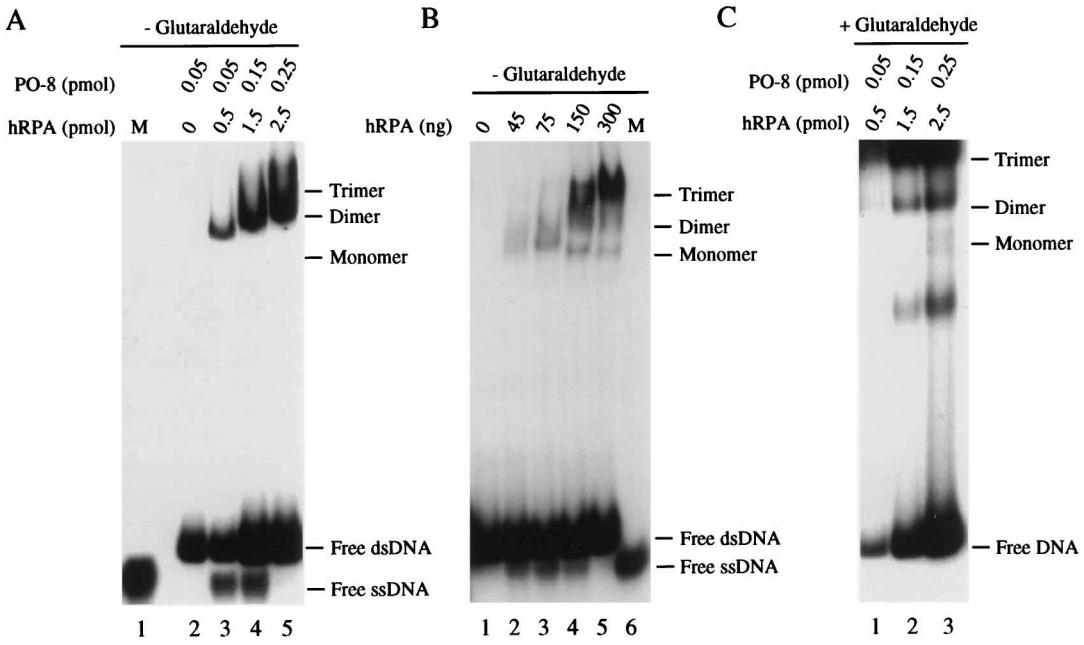


FIG. 5. Denaturation of the PO-8 substrate mediated by hRPA. (A) Various amounts of the ^{32}P-labeled PO-8 substrate and hRPA (as indicated) were incubated in the absence of glutaraldehyde as described in Materials and Methods. Following incubation, the reaction products were separated by electrophoresis through a native 10% polyacrylamide gel and autoradiographed. (B) Increasing amounts of hRPA (as indicated) were titrated against a constant level of the ^{32}P-labeled PO-8 substrate (0.15 pmol) in the absence of glutaraldehyde. Reaction products were detected as described for panel A. (C) Various amounts of the ^{32}P-labeled PO-8 substrate and hRPA (as indicated) were incubated and then cross-linked with glutaraldehyde. Reaction products were detected as described for panel A. The locations of hRPA-DNA complexes, free dsDNA, and free ssDNA are shown on the right of each panel. The ssDNA marker (M) is the ^{32}P-labeled top strand of the PO-8 substrate.

5206 to 5209 in the duplex region on the 5′ side, and nt 5219 to 5224 and nt 5227 to 5231 on the 3′ side. In contrast, two bottom-strand patches hyperreactive to PPC were seen (nt 5201 to 5207 and nt 5224 to 5228), each located opposite the top-strand doublets (compiled in Fig. 4C). A single hyperreactive site at nt 5218 was also detected on the bottom strand. The hyperreactivity of these sites increased slightly with the addition of more hRPA. Thus, the hRPA-dependent changes in PPC cleavage indicate that hRPA binding distorts the DNA structure of PO-8 over a 30-nt region.

Although we observed that hRPA can denature the PO-8 substrate (see below), our data suggests that the PPC hyperreactive sites are not a result of this denaturation. PPC cleavage of a single-stranded top strand in the absence of hRPA was uniform without regions of hyperreactivity, and this cleavage was inhibited by the addition of hRPA (data not shown). These hyperreactive sites also do not appear to result from a more limited melting of the PO-8 DNA, because potassium permanganate footprinting (for example, see reference 6) of the hRPA–PO-8 complexes did not reveal significant reactivity at these sites (data not shown). Therefore, our data indicate that hRPA binding to the PO-8 substrate causes a conversion of flanking duplex regions to a non-B-DNA structure. As the formation of hRPA–PO-8 complexes led to an increase in PPC cleavage, it is possible that hRPA binding causes an increase in minor-groove width that facilitates PPC binding and subsequent nicking.

hRPA denatures the PO-8 substrate. The ability of hRPA to denature the PO-8 substrate was tested. With a constant molar ratio of protein to DNA (10:1), various amounts of hRPA and the PO-8 substrate (5′ ^{32}P labeled on the top strand) were incubated under standard binding conditions in the absence of glutaraldehyde. Reaction products were then separated by gel electrophoresis to detect ssDNA. A novel band that migrated below the pseudo-origin substrate (Fig. 5A, lanes 3 and 4) was observed. Comigration of this novel band with a single-stranded top-strand marker (Fig. 5A, lane 1) established its identity as free ssDNA. Densitometry of the autoradiographs indicate that 15 to 25% of the available substrate became denatured. Notably, the generated ssDNA was detected only by using lower levels of protein and DNA (Fig. 5A, lanes 3 and 4). Increases in the concentration of protein and DNA caused the disappearance of the free ssDNA even while increasing the total amounts of hRPA–PO-8 complexes. We also titrated hRPA against a constant level of DNA, and similarly, denaturation of the substrate was observed only at low to intermediate levels of protein (Fig. 5B, lanes 2 to 4). It seems likely that ssDNA is still generated by hRPA binding to the pseudo-origin but that use of higher concentrations of hRPA causes complete retention of generated ssDNA into shifted complexes. More generally, our data indicates that hRPA can denature the substrate.

Denaturation was not detected when glutaraldehyde was used to cross-link the protein-DNA complexes, regardless of the protein-DNA concentrations used (Fig. 5C). Thus, it appears that glutaraldehyde traps more-weakly-associated hRPA-ssDNA complexes, so that free unwound product is not observed. This observation is consistent with previous studies showing the ability of glutaraldehyde to trap a class of transient hRPA-ssDNA complexes (2, 3).

SSBs that support the *ori* unwinding reaction also bind PO-8. SSBs other than hRPA have been found to support the T-antigen-mediated *ori*-dependent DNA-unwinding reaction. Those SSBs functional in the DNA-unwinding reaction are the *Escherichia coli* SSB (EcoSSB), the herpes simplex virus ICP8 (HSV ICP8) and the adenovirus DNA-binding protein (Ad-DBP) (20). In contrast, the bacteriophage T4 gene 32 protein (gp32) and T7 gene 2.5 protein (gp2.5) did not allow the production of unwound DNA from *ori* (18, 20). The ability of these SSBs to bind the PO-8 substrate was examined.

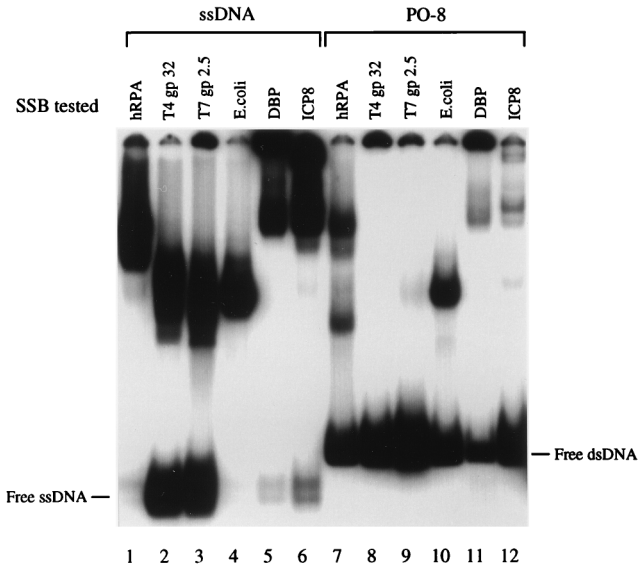


FIG. 6. A subclass of SSBs can bind the PO-8 substrate. Various SSBs were incubated with 0.05 pmol of the 48-nt ^{32}P-labeled bottom strand of the PO-8 substrate (ssDNA; lanes 1 to 6) or the ^{32}P-labeled PO-8 substrate (lanes 7 to 12) and then cross-linked with glutaraldehyde. The reaction products were separated by electrophoresis through a native 10% polyacrylamide gel and autoradiographed. The positions of free dsDNA and ssDNA are indicated on the right and left, respectively. *E. coli*, EcoSSB; DBP, AdDBP; ICP8, HSV ICP8.

The PO-8 substrate and PO-8 top strand (i.e., a ssDNA control) were each incubated with an equivalent amount of SSB (50 ng), and the reaction products were cross-linked with glutaraldehyde. As expected, all tested SSBs bound the ssDNA substrate, although their binding affinity varied (Fig. 6, lanes 1 through 6). When the PO-8 substrate was tested, high-affinity binding was noted by three proteins—hRPA, EcoSSB, and AdDBP (Fig. 6, lanes 7, 10 and 11)—while an intermediate level of binding was noted for HSV ICP8 (Fig. 6, lane 12). Densitometric analysis indicated that 45, 43, 57, and 18% of the available PO-8 substrate was bound into a discrete complex by hRPA, EcoSSB, AdDBP, and HSV ICP8, respectively. In the case of AdDBP, a significant amount of the substrate was retained in the electrophoretic wells, likely a result of glutaraldehyde trapping weak AdDBP-ssDNA complexes, leading to a reduction in the amount of unbound PO-8 substrate. Each SSB–PO-8 complex migrated similarly to the SSB-ssDNA complexes. In contrast, only weak binding by the gp32 and gp2.5 proteins was detected This was determined by densitometric analysis to be <0.5 and 4.5%, respectively, of the available PO-8 substrate. Similar results were found with constant molar ratios of SSB to PO-8 substrate (data not shown).

Because glutaraldehyde may in some manner alter the interaction of these SSBs with DNA, we tested SSB-DNA complex formation in the absence of cross-linking by nitrocellulose filter binding (Table 1). The presence of each SSB (1 pmol) caused the retention of 21 to 76% of the ssDNA substrate to the filter, with the T4 and T7 proteins least able to form detectable complexes. Addition of other amounts of the gp32 and gp2.5 proteins (up to 10-fold-higher levels of protein) did not greatly increase substrate retention, with a maximum of 30% of substrate bound for either SSB (data not shown). Use of the PO-8 substrate showed that hRPA was most efficient in retaining the substrate on the filter (56% of the substrate bound), followed by EcoSSB (37%), HSV ICP8 (13%), and AdDBP (9%). The same amount (1 pmol) of T4 gp32 or T7 gp2.5 did not lead to detectable PO-8 retention to the filter.

TABLE 1. Binding of SSBs to ssDNA and the PO-8 substrate[a]

SSB tested	Bound ssDNA substrate (%)	Bound PO-8 substrate (%)
hRPA	76.0	56.1
EcoSSB	79.2	37.2
HsV ICP8	72.4	12.6
AdDBP	58.6	8.8
T4 gp32	29.3	<0.1
T7 gp2.5	21.7	<0.1

[a] As measured by retention on nitrocellulose filters. Each SSB (1 pmol) was incubated with 0.15 pmol of the ^{32}P-labeled PO-8 or ssDNA substrate (the PO-8 top strand) under standard binding conditions. Reaction mixtures were then filtered through nitrocellulose membranes as described in Materials and Methods. Binding of each substrate is expressed as the percentage of input DNA after subtraction of nonspecific binding.

Thus, the ability of hRPA to associate with the pseudo-origin structure is not unique to hRPA, because heterologous SSBs can also bind. Moreover, we find a correlation between the known ability of an SSB to support the *ori*-dependent DNA-unwinding reaction and PO-8 binding activity.

DISCUSSION

A key feature of the initiation of DNA replication is the denaturation of origin DNA and the loading of DNA helicase molecules onto the nascent DNA forks. The SV40 system is one of the simplest models for analyzing this process in that only two proteins (T antigen and hRPA) are required in addition to the *ori*-containing DNA molecule. One special characteristic of the *ori* denaturation reaction is that T antigen converts from a sequence-specific DNA-binding protein to a sequence-independent DNA helicase. This change in activity is somewhat paradoxical, as T antigen must bind with sufficient affinity to form a stable complex with *ori* yet disrupt these contacts to commence DNA unwinding. Our data indicates that hRPA mediates this reaction by enlarging the 8-nt bubble present in the T antigen-*ori* complex. The duplex T antigen recognition elements are thus rendered single-stranded, and T antigen is released from static contacts with the *ori* DNA.

Previous data led us to postulate that hRPA binds ssDNA in a multistep pathway that entails initial recognition of 8 nt of DNA (the $hRPA_{8nt}$ complex) prior to formation of a more stable complex covering 30 nt (the $hRPA_{30nt}$ complex) (3). Stable binding of the PO-8 substrate is consistent with the ability of hRPA to productively interact with 8 nt of ssDNA, although our finding of efficient hRPA–PO-6 complex formation indicates that recognition of 6 nt of ssDNA by hRPA is also possible. A transition in hRPA binding from the 8- to the 30-nt mode would lead to the formation of additional stabilizing contacts between protein and DNA. We hypothesize that the observed PO-8 denaturation is driven by the favorable free energy change occurring upon conversion of the $hRPA_{8nt}$ complex to the $hRPA_{30nt}$ complex.

Our previous binding studies showed that significant hRPA binding to ssDNA in the 8-nt mode required at least two hRPA monomers (2). We similarly found that hRPA–PO-8 complexes also contained two or more hRPA monomers, suggesting that cooperative hRPA-hRPA interaction is required to achieve stable PO-8 binding. Because both strands of the bubble were protected from PPC cleavage in the presence of hRPA, it is possible that one monomer binds to each strand of the bubble with physical interaction occurring between their respective DNA-binding surfaces. Alternatively, hRPA molecules may bind adjacently upon the PO-8 substrate, for exam-

ple, with one hRPA molecule binding to the ssDNA bubble and the second to the flanking duplex DNA. In support of this view, RPA can bind duplex DNA elements within transcriptional control regions in a sequence-specific manner (24, 36, 40). However, because hRPA did not significantly protect the duplex DNA from PPC cleavage (Fig. 4), binding of hRPA to this DNA would likely be more transient than the hRPA binding to the bubble.

PPC probing of hRPA–PO-8 complexes revealed the presence of significant DNA distortion within the flanking duplex DNA regions. This distortion does not appear to be a result of DNA melting, because the flanking DNA was not significantly reactive to a potassium permanganate probe (for example, see reference 6). Thus, the hRPA-mediated distortion of PO-8 structure is apparently caused by conversion of the duplex DNA to a non-B-DNA structure which likely precedes and facilitates PO-8 denaturation. This DNA distortion could be caused by hRPA binding to the bubble, which destabilizes the duplex structure of the flanking DNA. As described above, a second view is that the hRPA directly interacts with the duplex DNA in a manner that alters the DNA conformation.

DNA-unwinding activities related to the PO-8 denaturation reaction have been previously reported. hRPA causes the denaturation of *Saccharomyces cerevisiae ARS* elements when these are present on supercoiled plasmids, perhaps by hRPA binding to transiently formed ssDNA regions (26). Calf thymus RPA can unwind lengthy (350-nt) DNA molecules annealed to M13 ssDNA in a Mg^{2+}-free reaction (13). Similarly, Knippers and coworkers found unwinding of linear dsDNA by hRPA in a reaction prevented by 5 mM Mg^{2+} (41). In that we found denaturation of the pseudo-origin substrate to occur with 7 mM $MgCl_2$, it is unclear whether the latter findings represent an alternate mode of RPA action or, perhaps, that the pseudo-origin substrate is better suited to being denatured by RPA under conditions that support SV40 DNA replication.

The ability of various SSBs (hRPA, EcoSSB, HSV ICP8, AdDBP, T4 gp32, and T7 gp2.5) to support the T-antigen-dependent *ori* unwinding reaction (18, 20) was found to correlate with their efficiency in binding the PO-8 substrate. More generally, our data suggest that the active SSBs (i.e., hRPA, EcoSSB, HSV ICP8, and AdDBP) catalyze *ori* denaturation through productive interaction with the single-stranded EP bubble in the ATP-dependent T antigen-*ori* complex. Helix-destabilizing properties have been reported for AdDBP (30, 50) and HSV ICP8 (4) which may contribute to their activity in the *ori* denaturation reaction. EcoSSB has not been reported to denature dsDNA (29), but substrates similar to the pseudo-origin have not been tested.

These and previous results support the following model for viral *ori* denaturation during the initiation of SV40 DNA replication (Fig. 7). In an ATP-dependent reaction, T antigen binds *ori* using numerous protein-DNA contacts in the central GAGGC region, causing the melting of 8 bp within the EP element and distortion of the AT tract. T antigen forms contacts predominantly with one DNA strand and one helical face in the duplex flanks (34), indicating that hRPA binds only one strand via the helical face not bound by T antigen. Initial recognition of the ssDNA within the EP structure occurs with an $hRPA_{8nt}$ complex. In possible combination with other hRPA molecules, hRPA binding is stabilized by conversion to the $hRPA_{30nt}$ form concomitant with denaturation of at least 30 nt of *ori* DNA, including the central GAGGC recognition element. As T antigen is unable to recognize the GAGGC repeats when present on ssDNA (1), the T-antigen hexamer on the early *ori* half is released from these sequence-specific contacts. Either by the binding of additional hRPA molecules or by further denaturation of *ori* facilitated by DNA distortion in the AT tract, the late-half hexamer is released. Bidirectional unwinding occurs in the form of a T-antigen double hexamer, in which dsDNA enters the complex and two strands of ssDNA are spooled out (37, 42).

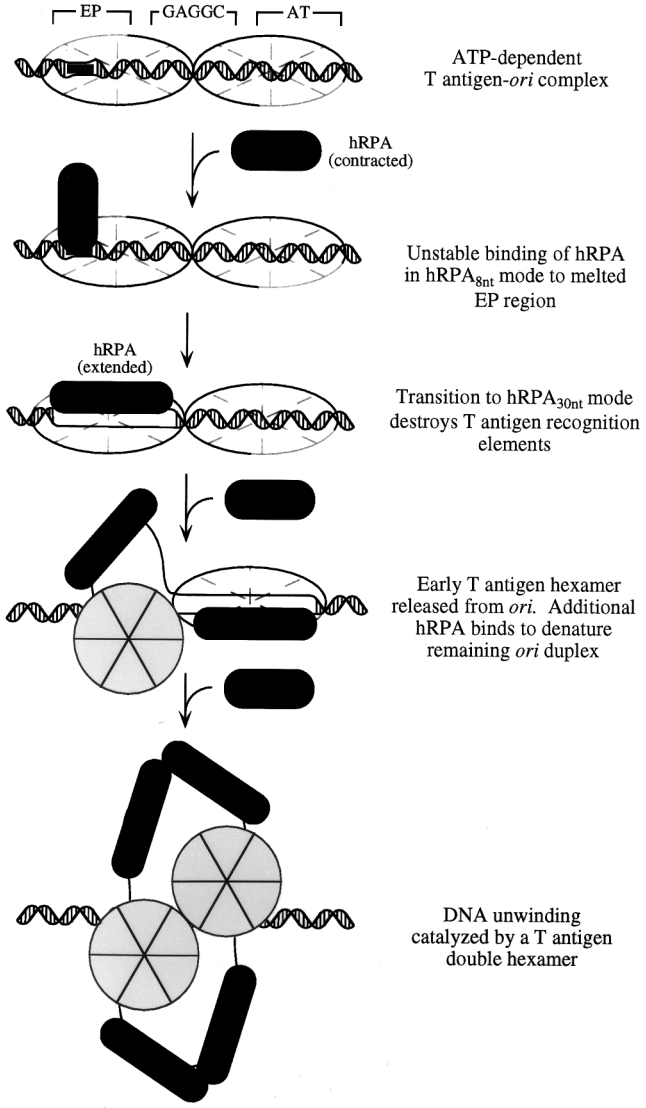


FIG. 7. Model for the denaturation of the SV40 *ori* mediated by hRPA. See the text for details.

The denaturation activity of hRPA has the potential to play critical roles in other DNA metabolic reactions. For example, nucleotide excision repair leads to the generation of an ~30-nt gap caused by excision of the damaged DNA strand (17). DNA damage has been shown to be cooperatively bound by the hRPA and XPA proteins (16), both of which are essential for nucleotide excision repair in reconstituted cell-free systems (7, 48). It is therefore conceivable that hRPA, in combination with other factors, catalyzes the formation of an ~30 nt ssDNA bubble that is a substrate for the repair endonucleases. Limited DNA denaturation from a localized site also occurs in other DNA transactions, and it will be of interest to determine whether hRPA has a more general activity in facilitating these reactions.

ACKNOWLEDGMENTS

We thank Kenneth Marians (Memorial Sloan-Kettering Cancer Center), I. Robert Lehman (Stanford University School of Medicine), Ronald T. Hay (University of St. Andrews, United Kingdom), and Charles Richardson (Harvard Medical School) for generously supplying EcoSSB, HSV ICP8, AdDBP, and gp2.5, respectively, and Jerry Hurwitz for communicating unpublished observations. We thank Natalia Smelkova, Eda Kapsis, Thomas Gillette, Leonard Blackwell, and Elizabeth Maranville for constructive comments during the course of this project and for critical reading of the manuscript.

This research was supported by NIH grant AI29963 and Kaplan Cancer Center Developmental Funding and Kaplan Cancer Center Support Core grant NCI P30CA16087.

REFERENCES

1. **Auborn, K. J., R. B. Markowitz, E. Wang, Y. T. Yu, and C. Prives.** 1988. Simian virus 40 (SV40) T antigen binds specifically to double-stranded DNA but not to single-stranded DNA or DNA/RNA hybrids containing the SV40 regulatory sequences. J. Virol. **62:**2204–2208.
2. **Blackwell, L. J., and J. A. Borowiec.** 1994. Human replication protein A binds single-stranded DNA in two distinct complexes. Mol. Cell. Biol. **14:** 3993–4001.
3. **Blackwell, L. J., J. A. Borowiec, and I. A. Mastrangelo.** 1996. Single-stranded-DNA binding alters human replication protein A structure and facilitates interaction with DNA-dependent protein kinase. Mol. Cell. Biol. **16:**4798–4807.
4. **Boehmer, P. E., and I. R. Lehman.** 1993. Herpes simplex virus type 1 ICP8: helix-destabilizing properties. J. Virol. **67:**711–715.
5. **Borowiec, J. A., and J. Hurwitz.** 1988. ATP stimulates the binding of simian virus 40 (SV40) large tumor antigen to the SV40 origin of replication. Proc. Natl. Acad. Sci. USA **85:**64–68.
6. **Borowiec, J. A., and J. Hurwitz.** 1988. Localized melting and structural changes in the SV40 origin of DNA replication induced by T-antigen. EMBO J. **7:**3149–3158.
7. **Coverley, D., M. K. Kenny, M. Munn, W. D. Rupp, D. P. Lane, and R. D. Wood.** 1991. Requirement for the replication protein SSB in human DNA excision repair. Nature (London) **349:**538–541.
8. **Dean, F. B., P. Bullock, Y. Murakami, C. R. Wobbe, L. Weissbach, and J. Hurwitz.** 1987. Simian virus 40 (SV40) DNA replication: SV40 large T antigen unwinds DNA containing the SV40 origin of replication. Proc. Natl. Acad. Sci. USA **84:**16–20.
9. **Dean, F. B., M. Dodson, H. Echols, and J. Hurwitz.** 1987. ATP-dependent formation of a specialized nucleoprotein structure by simian virus 40 (SV40) large tumor antigen at the SV40 replication origin. Proc. Natl. Acad. Sci. USA **84:**8981–8985.
10. **Deb, S. P., and P. Tegtmeyer.** 1987. ATP enhances the binding of simian virus 40 large T antigen to the origin of replication. J. Virol. **61:**3649–3654.
11. **Fairman, M. P., and B. Stillman.** 1988. Cellular factors required for multiple stages of SV40 DNA replication *in vitro*. EMBO J. **7:**1211–1218.
12. **Fanning, E., and R. Knippers.** 1992. Structure and function of simian virus 40 large tumor antigen. Annu. Rev. Biochem. **61:**55–85.
13. **Georgaki, A., B. Strack, V. Podust, and U. Hübscher.** 1992. DNA unwinding activity of replication protein A. FEBS Lett. **308:**240–244.
14. **Goetz, G. S., F. B. Dean, J. Hurwitz, and S. W. Matson.** 1988. The unwinding of duplex regions in DNA by the simian virus 40 large tumor antigen-associated DNA helicase activity. J. Biol. Chem. **263:**383–392.
15. **Gomes, X. V., L. A. Henricksen, and M. S. Wold.** 1996. Proteolytic mapping of human replication protein A: evidence for multiple structural domains and a conformational change upon interaction with single-stranded DNA. Biochemistry **35:**5586–5595.
16. **He, Z., L. A. Henricksen, M. S. Wold, and C. J. Ingles.** 1995. RPA involvement in the damage-recognition and incision steps of nucleotide excision repair. Nature (London) **374:**566–569.
17. **Huang, J.-C., D. L. Svoboda, J. T. Reardon, and A. Sancar.** 1992. Human nucleotide excision nuclease removes thymine dimers from DNA by incising the 22nd phosphodiester bond 5′ and the 6th phosphodiester bond 3′ to the photodimer. Proc. Natl. Acad. Sci. USA **89:**3664–3668.
18. **Hurwitz, J.** Personal communication.
19. **Hurwitz, J., F. B. Dean, A. D. Kwong, and S.-H. Lee.** 1990. The *in vitro* replication of DNA containing the SV40 origin. J. Biol. Chem. **265:**18043–18046.

19a. **Iftode, C., and J. A. Borowiec.** Unpublished data.

20. **Kenny, M. K., S.-H. Lee, and J. Hurwitz.** 1989. Multiple functions of human single-stranded-DNA binding protein in simian virus 40 DNA replication: single-strand stabilization and stimulation of DNA polymerases α and δ. Proc. Natl. Acad. Sci. USA **86:**9757–9761.
21. **Kenny, M. K., U. Schlegel, H. Furneaux, and J. Hurwitz.** 1990. The role of human single-stranded DNA binding protein and its individual subunits in simian virus 40 DNA replication. J. Biol. Chem. **265:**7693–7700.
22. **Kim, C., B. F. Paulus, and M. S. Wold.** 1994. Interactions of human replication protein A with oligonucleotides. Biochemistry **33:**14197–14206.
23. **Longhese, M. P., P. Plevani, and G. Lucchini.** 1994. Replication factor A is required in vivo for DNA replication, repair, and recombination. Mol. Cell. Biol. **14:**7884–7890.
24. **Luche, R. M., W. C. Smart, T. Marion, M. Tillman, R. A. Sumrada, and T. G. Cooper.** 1993. *Saccharomyces cerevisiae* BUF protein binds to sequences participating in DNA replication in addition to those mediating transcriptional repression (*URS1*) and activation. Mol. Cell. Biol. **13:**5749–5761.
25. **Mastrangelo, I. A., P. V. C. Hough, J. S. Wall, M. Dodson, F. B. Dean, and J. Hurwitz.** 1989. ATP-dependent assembly of double hexamers of SV40 T antigen at the viral origin of DNA replication. Nature (London) **338:**658–662.
26. **Matsumoto, K., and Y. Ishimi.** 1994. Single-stranded-DNA-binding protein-dependent DNA unwinding of the yeast *ARS1* region. Mol. Cell. Biol. **14:** 4624–4632.
27. **Matsunaga, T., C.-H. Park, T. Bessho, D. Mu, and A. Sancar.** 1996. Replication protein A confers structure-specific endonuclease activities to the XPF-ERCC1 and XPG subunits of human DNA repair excision nuclease. J. Biol. Chem. **271:**11047–11050.
28. **McEntee, K., G. M. Weinstock, and I. R. Lehman.** 1980. recA protein-catalyzed strand assimilation: stimulation by *Escherichia coli* single-stranded DNA-binding protein. Proc. Natl. Acad. Sci. USA **77:**857–861.
29. **Meyer, R. R., and P. S. Laine.** 1990. The single-stranded DNA-binding protein of *Escherichia coli*. Microbiol. Rev. **54:**342–380.
30. **Monaghan, A., A. Webster, and R. T. Hay.** 1994. Adenovirus DNA binding protein: helix destabilising properties. Nucleic Acids Res. **22:**742–748.
31. **Moore, S. P., L. Erdile, T. Kelly, and R. Fishel.** 1991. The human homologous pairing protein HPP-1 is specifically stimulated by the cognate single-stranded binding protein hRP-A. Proc. Natl. Acad. Sci. USA **88:**9067–9071.
32. **Murti, K. G., D. C. He, B. R. Brinkley, R. Scott, and S.-H. Lee.** 1996. Dynamics of human replication protein A subunit distribution and partitioning in the cell cycle. Exp. Cell Res. **223:**279–289.
33. **Parsons, R., M. E. Anderson, and P. Tegtmeyer.** 1990. Three domains in the simian virus 40 core origin orchestrate the binding, melting, and DNA helicase activities of T antigen. J. Virol. **64:**509–518.
34. **SenGupta, D. J., and J. A. Borowiec.** 1994. Strand and face: the topography of interactions between the SV40 origin of replication and T-antigen during the initiation of replication. EMBO J. **13:**982–992.
35. **Sigman, D. S., M. D. Kuwabara, C.-H. B. Chen, and T. W. Bruice.** 1991. Nuclease activity of 1,10-phenanthroline-copper in study of protein-DNA interactions. Methods Enzymol. **208:**414–433.
36. **Singh, K. K., and L. Samson.** 1995. Replication protein A binds to regulatory elements in yeast DNA repair and DNA metabolism genes. Proc. Natl. Acad. Sci. USA **92:**4907–4911.
37. **Smelkova, N. V., and J. A. Borowiec.** Submitted for publication.
38. **Stahl, H., P. Dröge, and R. Knippers.** 1986. DNA helicase activity of SV40 large tumor antigen. EMBO J. **5:**1939–1944.
39. **Stillman, B.** 1994. Smart machines at the DNA replication fork. Cell **78:**725–728.
40. **Tang, C.-M., A. E. Tomkinson, W. S. Lane, M. S. Wold, and E. Seto.** 1996. Replication protein A is a component of a complex that binds the human metallothionein IIA gene transcription start site. J. Biol. Chem. **271:**21637–21644.
41. **Treuner, K., U. Ramsperger, and R. Knippers.** 1996. Replication protein A induces the unwinding of long double-stranded DNA regions. J. Mol. Biol. **259:**104–112.
42. **Wessel, R., J. Schweizer, and H. Stahl.** 1992. Simian virus 40 T-antigen DNA helicase is a hexamer which forms a binary complex during bidirectional unwinding from the viral origin of DNA replication. J. Virol. **66:**804–815.
43. **Wiekowski, M., M. W. Schwarz, and H. Stahl.** 1988. Simian virus 40 large T antigen DNA helicase. J. Biol. Chem. **263:**436–442.
44. **Wobbe, C. R., F. Dean, L. Weissbach, and J. Hurwitz.** 1985. *In vitro* replication of duplex circular DNA containing the simian virus 40 DNA origin site. Proc. Natl. Acad. Sci. USA **82:**5710–5714.
45. **Wobbe, C. R., L. Weissbach, J. A. Borowiec, F. B. Dean, Y. Murakami, P. Bullock, and J. Hurwitz.** 1987. Replication of simian virus 40 origin-containing DNA *in vitro* with purified proteins. Proc. Natl. Acad. Sci. USA **84:**1834–1838.
46. **Wold, M. S., and T. Kelly.** 1988. Purification and characterization of replication protein A, a cellular protein required for *in vitro* replication of simian virus 40 DNA. Proc. Natl. Acad. Sci. USA **85:**2523–2527.
47. **Wold, M. S., J. J. Li, and T. J. Kelly.** 1987. Initiation of simian virus 40 DNA replication *in vitro*: large-tumor-antigen- and origin-dependent unwinding of the template. Proc. Natl. Acad. Sci. USA **84:**3643–3647.
48. **Wood, R. D., P. Robins, and T. Lindahl.** 1988. Complementation of the xeroderma pigmentosum DNA repair defect in cell-free extracts. Cell **53:** 97–106.
49. **Yoon, C., M. D. Kuwabara, R. Law, R. Wall, and D. S. Sigman.** 1988. Sequence-dependent variability of DNA structure. Influence of flanking sequences and fragment length on digestion by conformationally sensitive nucleases. J. Biol. Chem. **263:**8458–8463.
50. **Zijderveld, D. C., and P. C. van der Vliet.** 1994. Helix-destabilizing properties of the adenovirus DNA-binding protein. J. Virol. **68:**1158–1164.