Catalysis of Strand Annealing by Replication Protein A Derives from Its Strand Melting Properties*

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Eukaryotic DNA-binding protein replication protein A (RPA) has a strand melting property that assists polymerases and helicases in resolving DNA secondary structures. Curiously, previous results suggested that human RPA (hRPA) promotes undesirable recombination by facilitating annealing of flaps produced transiently during DNA replication; however, the mechanism was not understood. We designed a series of substrates, representing displaced DNA flaps generated during maturation of Okazaki fragments, to investigate the strand annealing properties of RPA. Until cleaved by FEN1 (flap endonuclease 1), such flaps can initiate homologous recombination. hRPA inhibited annealing of strands lacking secondary structure but promoted annealing of structured strands. Apparently, both processes primarily derive from the strand melting properties of hRPA. These properties slowed the spontaneous annealing of unstructured single strands, which occurred efficiently without hRPA. However, structured strands without hRPA displayed very slow spontaneous annealing because of stable intramolecular hydrogen bonding. hRPA appeared to transiently melt the single strands so that they could bind to form double strands. In this way, melting ironically promoted annealing. Time course measurements in the presence of hRPA suggest that structured single strands achieve an equilibrium with double strands, a consequence of RPA driving both annealing and melting. Promotion of annealing reached a maximum at a specific hRPA concentration, presumably when all structured single-stranded DNA was melted. Results suggest that displaced flaps with secondary structure formed during Okazaki fragment maturation can be melted by hRPA and subsequently annealed to a complementary ectopic DNA site, forming recombination intermediates that can lead to genomic instability.

Replication protein A (RPA) is a single-stranded DNA-binding protein that participates in multiple processes in eukaryotes, including DNA replication, DNA repair, and recombination (1). RPA was first identified as a protein required for simian virus 40 (SV40) replication in vitro (2–4). It is known to bind and protect single-stranded DNA (ssDNA) from nucleases, assist other proteins in the unwinding of double-stranded DNA (dsDNA), and prevent the formation of stable hairpins during DNA processing (5–7).

Human RPA is a stable heterotrimeric complex consisting of subunits of 70, 32, and 14 kDa. The core of this trimeric protein readily binds to ssDNA sequences with a defined 5′–3′ polarity (8, 9). RPA contains six DNA-binding domains (DBD) distributed among the three subunits. The RPA70 subunit has the highest affinity for ssDNA with four DBDs, whereas the two smaller subunits have one DBD each (10, 11). Additionally, the N-terminal domain of RPA70 (DBD F) has been shown to be important for binding and melting double-stranded DNA, and for helix destabilization of short DNA strands (12). Current models of RPA binding suggest that the domains actually used for binding are contingent on the length of the ssDNA to which the RPA is bound (13–15). Structural studies have shown that two DBDs of RPA70 (DBD A and DBD B) can interact with an 8-nt segment of ssDNA and that longer DNAs interact with additional domains. The DBD located on RPA32 (DBD D) is thought to bind to the 3′-end of the ssDNA only in the highest affinity binding mode when all four domains are utilized, although a mutation in the D site does not affect RPA binding or function in vivo (15–17). Consequently, the role of DBD D in binding is unclear. DBD D has also been shown to bind the 3′-hydroxyl of a partial duplex (such as a primer-template junction), whereas the RPA70C domain is bound to the 5′ ssDNA overhang of the other DNA strand in the duplex.

Interactions between RPA and helicases promote strand unwinding (5, 18–20). Through various protein interactions, RPA may be directly placed on ssDNA after it emerges from a helicase complex to prevent the separated strands from reannealing or forming any secondary structure. The strand unwinding activities of yeast Srs2 and human RECQ1 helicases both benefit from the addition of RPA (21, 22). Also, RPA has been shown to stimulate the unwinding activity of the Werner and Bloom syndrome helicases on long duplexes by directly interacting with the proteins (5, 6, 23, 24). Yeast RPA has also been shown to stimulate Pif1 helicase activity and aid in unwinding of both DNA-DNA substrates and DNA-RNA hybrids (25).

The analogous single-stranded DNA-binding protein from Escherichia coli has been shown to remove secondary structure
from ssDNA at high magnesium concentrations, allowing RecA to form presynaptic complexes, which ultimately facilitate strand annealing and exchange (26, 27). Similarly, two studies showed that yeast RPA can stimulate RAD52-catalyzed annealing of ssDNA (28, 29). In the first, RPA was found to reduce ssDNA with secondary structure during presynaptic complex formation, inhibiting nonproductive binding of RAD52 to those regions and thus streamlining its activity (28). In the second, the addition of RPA stimulated RAD52-catalyzed annealing of long plasmid (2961 nt) DNA. The authors suggested that the role of RPA was to reduce secondary structure of the DNA so that it would be susceptible to the annealing and ultimately exchange activities of RAD52 (29).

In DNA replication, Okazaki fragments are processed by a pathway in which the 5'-end region of each fragment is displaced into a single-stranded flap for nucleolytic removal of the RNA primer (30, 31). It has been hypothesized that some flaps become long enough to be coated by RPA (30, 32, 33). When we tested flap substrates with RPA-coated flaps, we were surprised to find that the RPA promoted annealing of the flap to other single-stranded template DNA (34). This result suggested that RPA alone can support rather than suppress recombination. The current study is focused on determining the conditions under which RPA can promote strand annealing.

EXPERIMENTAL PROCEDURES

Materials—All oligonucleotides were obtained commercially from Integrated DNA Technologies (Coralville, IA). Radionucleotide [γ-32P]ATP (3000 Ci/mmole) was purchased from PerkinElmer Life Sciences, and the T4 polynucleotide kinase (labeling grade) was purchased from Roche Applied Science. All other reagents were of the best available commercial grade.

Enzyme Expression and Purification—Recombinant human RPA was purified as described previously (35).

Oligonucleotide Substrates—Oligomer sequences are listed in Table 1. Sequences with the secondary structure required to inhibit strand annealing were designed with the help of the mfold software (version 3.2) by Zuker and Turner, available on the World Wide Web (36). Using standard procedures, substrates T1 and T2 were radiolabeled at the 5'-end with [γ-32P]ATP and T4 polynucleotide kinase. The radiolabeled strands were purified on 15% denaturing polyacrylamide gels containing 7 m urea.

Secondary Structures—All oligomers were analyzed using the mfold software (36). The folding free energy change (ΔG) for each oligomer was calculated based on parameters set forth in previous work (37, 38) at salt concentrations of 40 mM KCl and 4 mM MgCl2 and an ambient temperature of 37 °C. A positive free energy change predicts that the oligomer should not fold into consistent structures beyond random coils. Such oligomers are described here as having no secondary structure. Only the hydrogen-bonding configuration of the lowest free energy structure was considered for each oligomer, although in many cases there were other predicted structures with higher free energies.

Enzyme Assays—Reactions were performed in buffer containing 30 mM HEPES (pH 7.5), 5% glycerol, 40 mM KCl, 0.1 mg/ml bovine serum albumin, and 4 mM MgCl2. Enzyme stocks were diluted in 30 mM HEPES (pH 7.5), 5% glycerol, 40 mM KCl, and 0.1 mg/ml bovine serum albumin. Each reaction contained 5 fmol of radiolabeled template (T1 or T2) and 20 fmol (C1, C2, C3, C4, C5 or F1) or 10 fmol (C6) of unlabeled complementary strand in a 20-μl reaction mixture with varying amounts of the enzymes as indicated in the figure legends. Reactions were initiated by mixing the labeled template and unlabeled complementary strand followed immediately by the addition of the enzyme. All assays were incubated at 37 °C for the time specified in the figure legend. Reactions were stopped by the addition of 0.25 volumes of helicase dyes (30% glycerol, 50 mM EDTA, 0.9% SDS, 0.25% bromphenol blue, and 0.25% xylene cyanole) and immediately run on a native 8% polyacrylamide gel at 25 watts for 3 h. After vacuum drying, each gel was visualized and quantitated using a GE Healthcare PhosphorImager and analyzed using ImageQuant version 5.0 software from Molecular Dynamics. In all studies, the quantitated amounts of substrates and products were utilized to calculate the percentage of product formation from the product/(product + substrate) ratio. This method allows for the correction of any loading errors among lanes. The graphed data points were calculated by quantifying the pixel density of each band and subtracting the measurable background pixel density. The percentage of strand-annealing activity was calculated as the amount of labeled DNA in the final annealed product band divided by the total amount of DNA from all bands in the gel lane. All experiments were done in triplicate, and the error bars on each graph represent one S.D. in both directions.

In most assays, 10–250 fmol of RPA was found to be sufficient for demonstrating strand annealing activities. At 50 fmol, RPA is approximately equimolar to the recombination intermediate complex because of all of the excess DNA needed to ensure that all (or almost all) of the labeled strand is in complex. Since each assay contained ~50 fmol of single-stranded DNA, 100 fmol of RPA was used in all nontitration assays to maintain the 2:1 RPA/ssDNA ratio.

RESULTS

We previously acquired evidence that RPA can accelerate the annealing of DNA strands (34). Our current work is an investigation of the conditions and substrate structures that support RPA-catalyzed strand annealing.

Design of Oligonucleotide Substrates to Assess the Strand Annealing Properties of RPA—We first questioned whether the efficiency of RPA-catalyzed strand annealing correlates with the amount of structure in the single strands prior to annealing. We designed single-stranded oligomers with different folding structures that can anneal in various pairs (Table 1). The folding program mfold (version 3.2) was used to predict the free energy change (ΔG) for conversion of each strand from random coil to its lowest free energy folded structure. The hydrogen-bonding patterns of the lowest energy folding structures of those oligonucleotides are shown (Fig. 1). Oligomers with a positive ΔG are more likely to form a random coil than any consistent secondary structure and are thus designated as unstructured. Strands are designated as either templates (T) or complementary (C) strands, in which each C strand is comple-
Catalysis of Strand Annealing by RPA

**TABLE 1**

| Oligonucleotide Sequences (5′–3′) |
|----------------------------------|
| Unannealed regions are in boldface type. |

| Oligonucleotide | Sequence |
|----------------|----------|
| Radiolabeled templates | TCG AGA CCT TCG TTT CCA AGT AAA ACG AGC GCC AGT GCT AGC GTA CAA TAC GAC |
| T1 57-mer | TCG AGA CCT TCG TTT CCA AGT AAA ACG AGC GCC AGT GCT AGC GTA CAA TAC GAC |
| T2 57-mer | TGG AAA AAA AAA AAA AAG TGG AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA GGA AGG |
| Complementary primers | GTC GTA TGG TAC GCT AGC ACT GCT GCC GCT GTP TTA CTT GGA ACA AGA GGT CTC GA |
| C1 57-mer | GTC GTA TGG TAC GCT AGC ACT GCT GCC GCT GTP TTA CTT GGA ACA AGA GGT CTC GA |
| C2 47-mer | CAC TGG CGG TCG TTT TAC TCG GTA AAG CTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TT |
| C3 37-mer | CAC TGG CGG TCG TTT TAC TCG GTA AAG CTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TT |
| C4 20-mer | CTT GGA AAC AGA GGT CTC GA |
| C5 17-mer | CAC TGG CGG TCG TTT TAC |
| C6 57-mer | CCT TCC TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT CCA |

| Flaps | CAC TGG CGG TCG TTT TAC GCT GGT GAC TGG GAA AAC CAC CCG TCC CGA CGC CAC CTC CTG |
| F1 63-mer |

Two oligonucleotides that bind near the center of T1 were created. C5 is an 18-nt oligomer lacking secondary structure and is thus predicted to remain in the random coil configuration. Under the reaction conditions employed, it binds rapidly to T1 in the absence of RPA. Flap F1 contains the same 18 nt as C5 but has an additional 45 nt that are not complementary to T1 in the absence of RPA. Flap F1 contains the same 18 nt as C5 but has an additional 45 nt that are not complementary to T1 in the absence of RPA. Flap F1 was created to test the effect of secondary structure in the longer strand. Two different oligonucleotides were synthesized; T1 is a 57-nt oligomer that folds into a hairpin containing a stem loop, and T2 is a 57-nt oligomer expected to remain a random coil based on a ΔG of −2.95 kcal/mol.

RPA Can Promote Strand Annealing—The addition of RPA caused a progressive increase in the level of F1-T1 strand annealing as measured by the creation of the dsDNA complex (Fig. 2B). In the absence of RPA, F1 bound very slowly to T1 in our assay (Fig. 2A, lanes 1–5). Strand annealing in the absence of RPA was linear, with a rate of 0.026% of the radiolabeled template annealed/min over the entire 60 min. Linearity is anticipated, since the measurement was made under initial rate conditions in which the concentration of starting substrates does not change substantially during the course of the reaction. Annealing of C5 with T1, representing the same annealing sequence, but without structure, also occurred linearly, but at a rate of 0.934%/min, or 36 times faster than T1-F1 annealing (Fig. 2, C and D). These results demonstrate the inhibitory effect of secondary structure in the longer strand. They also show no evidence of spontaneous unannealing.

The rate of F1-T1 annealing increased with the amount of RPA added, accumulating in the highest rate of 0.452% of the radiolabeled template annealed/min over the first 15 min with 250 fmol of RPA, a greater than 17-fold increase (Fig. 2A, lanes 6–10).

Evidence that RPA Strand Annealing Derives in Part from Its Strand Melting Properties—We compared the effect of RPA on the rate of annealing of template T1 to the structured flap F1 and to structureless complementary strand C5. A basal level of 31% of the labeled T1 DNA annealed to C5 during the 15-min period in the absence of RPA (Fig. 3, lane 1), whereas only 0.2% annealed to F1 (Fig. 3, lane 6). Titration of RPA lowered the annealed amount of the T1-C5 complex to 8.6% at 250 fmol, a decrease of 3.5-fold (Fig. 3, lanes 2–5). At the same time, titration of RPA raised the annealing level of the T1-F1 complex to 7.6% at 250 fmol, an increase of 3.5-fold (Fig. 3, lanes 7–10). This suggests that RPA strand annealing/melting reaches an equilibrium of −8% annealed for the 18-nt segment that is annealing. A reasonable interpretation of this result is that RPA most effectively promotes annealing of highly structured substrates, such as T1-F1, that would otherwise anneal at a very slow rate.

However, RPA also has a strand melting activity that must be operating during the annealing reaction. T1 and C5 anneal rapidly in the absence of RPA, presumably because they have little interfering structure (see Fig. 1). However, 250 fmol of RPA is a very effective inhibitor of the rapid annealing reaction. It is notable that the level of annealing at 250 fmol of RPA becomes similar in both T1-F1 and T1-C5 annealing (Fig. 3B), and we found that RPA can unanneal both T1-F1 and T1-C5 preformed complexes (data not shown). The behavior of the system is consistent with the interpretation that RPA is displaying two activities, melting and annealing. When rapid spontaneous annealing is possible, the primary observable activity of RPA is melting. The amount of annealed strands at 250 fmol of RPA is reflective of the opposing reactions. RPA accelerated the poor annealing of the more structured strands (Fig. 2A, lanes 21–30).

At high RPA, both strand pairs behaved similarly, consistent with the interpretation that RPA allows the structured strands to anneal as if they had little structure. This would occur if melting of the structure in the unannealed strands promoted the annealing reaction.

Attempting to Simulate the Effects of RPA with Heat—We reasoned that if RPA promotes annealing through strand melting, increased temperature should have an equivalent effect on annealing; increased temperature should reduce the dependence of annealing on RPA, because heat can also destabilize structure that could interfere with annealing of single strands. The reaction temperature was increased for annealing of 57-nt T1 and 18-nt C5 in the absence of RPA (Fig. 4A). We found that RPA inhibited strand annealing of the 57-nt template and 18-nt complementary oligomer in the same manner regardless of increased temperature. Without RPA, the level of strand annealing increased with temperature, from 9.8% at 25 °C to 21.4% at 37 °C to 43.4% at 50 °C (Fig. 4A, lanes 1, 7, and 13). We interpreted the result to mean that the increased temperature destabilized intramolecular hydrogen bonds in T1, allowing it to bind C5. With this strand pair, the addition of RPA did not
simulate the increase in temperature. The addition of RPA at 50 °C caused the level of strand annealing to decrease 13-fold, from 43.4% in the absence of RPA to 3.3% in the presence of 250 fmol of RPA. This suggests that RPA was either melting the dsDNA T1-C5 complex or preventing it from forming. RPA had a negative effect on annealing at both low and high concentration, showing that it is very effective at disrupting the T1-C5 complex (Fig. 4B). Clearly, with this strand pair, RPA and increased temperature had different effects, most likely because the highest temperature employed did not hinder annealing or melt the double-stranded product.

Annealing of T1 and 57-nt C1 in the absence of RPA displayed a similar positive response to temperature. As with the T1-C5 annealing, the level of strand annealing rose more than 34-fold with the increase in temperature, from 1.9% at 25 °C to 65.6% at 50 °C (Fig. 4A, lanes 19 and 31). Unlike with the previous strand pair, the addition of RPA augmented the effect of the temperature increase, raising the amount of strand annealing to 9.5% at 25 °C and 81% at 50 °C. This suggests that RPA and increased temperature had additive effects on breaking the intramolecular hydrogen bonds that stabilize T1 and C1 secondary structure. However, unlike with the 18-nt C5, the addition of RPA increased strand annealing from 14.9% in the absence of RPA to 40% in the presence of 100 fmol of RPA at 37 °C. This implies that RPA and increased temperate collaborated in removing secondary structure from T1 and C1 to promote annealing. However, once T1 and C1 were annealed, RPA had difficulty melting the resulting 57 nt/57 nt dsDNA structure like it could the 18 nt/57 nt T1-C5 structure because of the additional 75-kcal/mol difference in free energy. The similar and additive effects of RPA and temperature with the longer strand are consistent with the interpretation that RPA promotes annealing by melting secondary structure in the reactant strands.

We further compared effects of temperature and RPA by annealing flap F1 to T1. This strand pair utilized the same 18 nt that comprise C5 for annealing but with the addition of a 47-nt flap to give the C strand secondary structure (Fig. 4E). At 25 °C, the annealing pattern looked very similar to that which occurred using C1, suggesting that the secondary structure of the single strands initially inhibited annealing but the presence of RPA partially alleviated the interfering structure (Fig. 4F). Increasing the temperature from 25 to 37 °C raised annealing to a high of 10.5% at 100 fmol of RPA. A further increase in temperature shifted the annealing pattern higher, since in the absence of RPA the temperature is enough to relieve secondary structure. Interestingly, at 250 fmol of RPA, there was a reduction in annealing, reminiscent of the pattern seen using 18-nt C5; however, the resulting drop was much less than that seen with C5. Some nucleotides in the noncomplementary part of F1 can potentially form bonds with T1, contributing extra binding stability compared with the T1-C5 pair. We conclude that, as seen with C1, F1 secondary structure inhibited annealing until RPA or heat could reduce that structure. However, as seen with

FIGURE 1. Free-forming secondary structures of strands used in this study. Using mfold, version 3.2, all oligomers were analyzed to determine the free energy change (ΔG, in kcal/mol) for the conversion of each strand from a random coil to its lowest free energy folded structure. Oligomers with a positive ΔG are more likely to form a random coil than any consistent secondary structure and are thus designated as unfolded.
18-nt C5 but not with 57-nt C1, the 18 nt did not anneal with enough stability to keep the resulting double-stranded product from being melted in the presence of high amounts of RPA.

RPA Hinders the Annealing of Short Strands and Facilitates Annealing of Long Strands—For strand pairs that do not form flaps on annealing, the hypothesis that RPA displays both annealing and strand melting properties.

**FIGURE 2.** RPA assists in the annealing of structured single-stranded DNA. A, the annealing of the radiolabeled template T1* to either 18-nt complementary strand C5 or 63-nt flap substrate F1. The top band is the annealed complex, T1*-C5 on the left and T1*-F1 on the right. The bottom band is the unannealed T1*. The strand annealing reactions were performed at 37 °C for 0, 5, 15, and 30 min. B, points are plotted as the percentage of labeled substrate in the annealed complex versus time (min). C, 18-nt C5; ○, 63-nt F1.

**FIGURE 3.** RPA strand annealing derives from its strand melting property. A, the annealing of the radiolabeled template T1* to either 18-nt complementary strand C5 or 63-nt flap substrate F1. The top band is the annealed complex, T1*-C5 on the left and T1*-F1 on the right. The bottom band is the unannealed T1*. The strand annealing reactions were performed at 37 °C for 15 min with 0, 25, 50, 100, and 250 fmol of RPA. B, the addition of RPA causes a proportional change in the level of strand annealing as measured by the creation of the dsDNA complex. Points are plotted as the percentage of labeled substrate in the annealed complex versus amount of RPA in the reaction. ■, 18-nt C5; ○, 63-nt F1.

18-nt C5 but not with 57-nt C1, the 18 nt did not anneal with enough stability to keep the resulting double-stranded product from being melted in the presence of high amounts of RPA.

RPA Hinders the Annealing of Short Strands and Facilitates Annealing of Long Strands—For strand pairs that do not form flaps on annealing, the hypothesis that RPA displays both annealing and strand melting properties was supported by the data from Figure 2. The strand annealing reactions were performed at 37 °C for 0, 5, 15, and 30 min. D, points are plotted as the percentage of labeled substrate in the annealed complex versus time (min). ■, 18-nt C5.
melting activities predicts that RPA would be most effective at catalyzing the annealing of a template strand to progressively longer complementary strands. This prediction is based on two expected properties of longer strands. First, the longer complementary (C) strand would anneal with greater stability to the template strand, resisting the melting activity of RPA (as suggested by data in Fig. 4, C and D). Moreover, the longer C strand would be more likely to have a greater negative free energy of folding, allowing it to respond more to the annealing function of the RPA. To test this idea, we measured the influence of RPA on the annealing rates of T1 to progressively longer complementary strands. Complementary oligomers C4 through C1 are 20, 37, 47, and 57 nt long, respectively. Their sequences are all complementary to the 5′-end of T1, and they anneal progressively further toward the T1 3′-end. As expected for nearly any progressively longer series of oligomers, the predicted negative free energies of folding were calculated to increase with length (Fig. 1).

In the absence of RPA, we found that, over a 15-min time course, C4 exhibited the greatest rate of strand annealing (24%), with the values for C1, C2, and C3 predictably less (11, 5, and 4%, respectively). This suggests that in the absence of RPA, the secondary structure of the complementary oligomers influences the rate of strand annealing, with the smaller, less structured strands annealing at a faster rate than the larger oligomers having more structure (Fig. 5A).

When the same measurements were made with RPA, one phenomenon was evident; high levels of RPA promoted more effective annealing of longer complementary oligomers. The annealing of the shorter oligomers achieved a plateau or declined in value at 100 fmol of RPA and beyond, but the amount of annealing of the longest oligomer continued to rise throughout the titration (Fig. 5B). A likely explanation is that the forward reaction, requiring melting of structure of the single strands, is effectively catalyzed by RPA. However, the back melting reaction is suppressed when the double-stranded product becomes so long that its helix is very stable.

**Does RPA Promote Better Annealing of Longer Strands Independent of Structure?**—Results to this point indicate that RPA promotes annealing of long strands by melting intramolecular

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**FIGURE 4. Comparing the effects of temperature and RPA on strand annealing.**

A, the annealing of the radiolabeled template T1* to complementary strand C5 and flap substrate F1. The strand annealing reactions were run for 15 min at 25, 37, or 50 °C with 0, 10, 25, 50, 100, and 250 fmol of RPA. The top band is the annealed complex, and the bottom band is the unannealed T1*.

B, points are plotted as the percentage of labeled substrate in the annealed complex versus RPA. Shown is C5 annealing to T1* at 25 °C ( ), 37 °C ( ), and 50 °C (dotted line).

C, D, C1 annealing to T1* at 25 °C ( ), 37 °C ( ), and 50 °C (dotted line).

E, F, F1 annealing to T1* at 25 °C ( ), 37 °C ( ), and 50 °C (dotted line).
bonds, but once a long strand intermolecular annealing occurs, it is stable in the presence of RPA. Moreover, RPA decreases the annealing rate of shorter segments by creating an annealing/melting equilibrium.

To determine whether the strand annealing that we see in the presence of RPA with a long complementary oligomer is structure-dependent, length-dependent, or both, we created a new set of ssDNA oligomers that lack secondary structure. When designing the substrates, we chose to anchor long central adenine (oligomer T2) and thymine (oligomer C6) runs with flanking unique nucleotides. This deterred secondary structure from forming while avoiding slippage or bubble intermediates. An RPA titration utilizing T2 and C6 was sampled at 5 min (Fig. 6A). We used a lower level of complementary strand here than in other experiments so that annealing in the absence of RPA would not be absolutely complete. Predictably, in the absence of RPA, 81% of the radiolabeled T2 annealed to C6, presumably because no secondary structure was present to inhibit annealing (Fig. 6A, lane 1). In the presence of progressively more RPA, strand annealing dropped almost 4-fold down to a low of 22% annealed at 250 fmol (Fig. 6B). The results suggest that RPA will generally slow annealing of unstructured strands.

Like T1 and C1, template T2 and its complementary substrate, C6, are both 57 nt in length; however, T2 and C6 have a much lower GC content than T1 and C1 (14% versus 50.9%, respectively). Because of this, the T2/C6 product is expected to have a lesser double-stranded bonding stability than T1-C1. We considered the possibility that the amount of the T2/C6 product in the annealing reaction is decreased, because RPA could readily carry out the reverse melting reaction. However, we found that when T2/C6 was preannealed, it was not at all melted by concentrations of RPA used in the annealing reaction (Fig. 6A, lanes 7–12). Evidently, RPA slows annealing by preventing the forward reaction, possibly by destabilizing partially annealed intermediates, but once a certain length threshold is reached (somewhere between 18 and 57 nt), the dsDNA product is too stable for RPA to melt.

We had hypothesized that catalysis of annealing is primarily the result of disruption of intramolecular bonds in the single strands by RPA and not any intrinsic ability of RPA to promote strand interaction. The demonstration that RPA slows annealing of long unstructured strands, without a significant back reaction, is consistent with this hypothesis.

DISCUSSION

In this study, we report that RPA has a strand annealing property that acts in conjunction with its known strand melting property. By designing a series of complementary oligonucleotides that vary either in length or amount of secondary structure or both, we were able to investigate the specific conditions necessary for RPA strand annealing and how changing these
conditions could alter the equilibrium between strand annealing and strand melting.

The effects of RPA on strand interaction were dependent on the folding structure of the tested strands. Generally, single strands that lacked structure annealed rapidly. With such substrates, the presence of RPA slowed annealing. Higher levels of RPA slowed annealing progressively more. Single strands predicted to have more structure (i.e., a greater negative free energy of folding) annealed slowly in the absence of RPA. In these cases, the presence of higher levels of RPA caused a progressive acceleration of annealing. These observations suggested that RPA can promote strand annealing by transient melting of secondary structure in the single strands so that nucleotides are available for intermolecular interactions.

Measurement of annealing versus RPA concentration, in a reaction in which annealing was accelerated by RPA (Fig. 4), showed that the proportion of annealed strands reached a plateau. This suggests that RPA promotes an equilibrium between folded single strands and the double-stranded product. In such an equilibrium, RPA-catalyzed annealing would increase until the concentration of double strands reached a level at which the RPA-catalyzed melting reaction rate matched the annealing rate. F1 has secondary structure allowing RPA to promote the forward reaction. A clearly apparent equilibrium is achieved with the T1-F1 pair, presumably because either the higher levels of RPA hinder the forward annealing reaction or because the annealed region is sufficiently short that there is an efficient melting back reaction (see 37 and 50 °C after 15 min with 100–250 fmol of RPA in Fig. 4F). For other substrates, this equilibrium level would vary, depending on the length and GC content of the two oligomers annealing. Longer oligomers or a higher GC content would favor a higher percentage of annealed products at equilibrium.

In general, the longer the strands of genomic ssDNA involved in an annealing reaction, the more secondary structure they should contain. Consequently, the longer the segments, the more likely their annealing will be accelerated by RPA. Once longer segments are annealed, it will be more difficult than with shorter segments for RPA to reverse the annealing reaction. Based on our results, once the negative free energy of annealing achieves a certain value, annealing goes to completion in the presence of RPA. This resulted in a switch from RPA inhibition to RPA facilitation of annealing of ssDNA oligomers between 20 and 37 nt in length (Fig. 5B), but presumably this threshold will vary depending on nucleotide composition, temperature, and ionic strength.

We also attempted to distinguish whether length, structure or both influenced RPA-promoted annealing. The annealing of the short segments we tested (T1 and C5) was inhibited by RPA (Fig. 3). Moreover, the annealing of longer segments lacking structure (T2 and C6) was also inhibited (Fig. 6). We interpreted this to suggest that RPA promotes annealing primarily by melting of structure in the starting strands. RPA was highly effective at inhibiting the annealing of short strands. This observation is consistent with the expectation that the melting activity of RPA is most effective when it can both prevent formation of annealing intermediates and melt the short strand-containing double-stranded products once they are formed.

**FIGURE 6.** The effect of RPA on annealing of strands is dependent on length. A, comparing the annealing and melting of two complementary unstructured oligomers. Prior to the experiment, T2* and C6 were either unannealed (lanes 1–6) to show strand annealing or annealed (lanes 7–12) to show strand melting. The top band is the annealed complex T2*-C6. The bottom band is the unannealed T2*. The strand annealing reactions were performed at 37 °C for 5 min with the following amounts of RPA: 0, 10, 25, 50, 100, and 250 fmol. The addition of 250 fmol of unlabeled T1 at the completion of the experiment discouraged reannealing. B, the addition of RPA causes a proportional change in the level of strand annealing as measured by the creation of the dsDNA complex but fails to melt the complex once it forms. Points are plotted as the percentage of labeled substrate in the annealed complex versus amount of RPA in the reaction. ◆, RPA strand annealing of initially unannealed ssDNA oligomers T2* and C6; ◇, RPA strand melting of initially annealed dsDNA complex T2*-C6.
Catalysis of Strand Annealing by RPA

Does RPA have an intrinsic strand annealing activity in addition to its ability to promote annealing by unfolding single strands? Because it has multiple DNA binding domains (16, 17), it has been hypothesized that a single RPA trimer may be able to bind to two strands of ssDNA simultaneously (16). RPA has multiple binding modes with the core DNA binding domains RPA70A and RPA70B, both of which are necessary and sufficient for high affinity binding. Once bound, RPA70AB facilitates binding of other DNA binding domains, including RPA70C and RPA32D, forming a stable ssDNA-RPA complex. The N-terminal domain of RPA70 (RPA70F) has been shown to play a role in DNA melting, suggesting that multiple conformations may exist with different DNA structures (12). Additionally, it has been shown that binding domains RPA70C and RPA32D can bind to a ssDNA/dsDNA junction with a 3’-OH and a 5’ ssDNA overhanging strand (17). It has been proposed that junction binding by RPA70C and RPA32D leaves RPA70AB free to bind to a different ssDNA strand (16). However, our results showed that the annealing of long strands lacking structure (T2 and C6) was inhibited by RPA. We also showed that these same strands, once annealed, were sufficiently stable that they were not melted by RPA. This indicates that RPA does not have the ability to directly accelerate the forward annealing reaction. The only property that our results reveal is melting.

We previously analyzed the propensity of single-stranded flaps created during Okazaki fragment maturation to participate in illegitimate recombination (34). Eukaryotic Okazaki fragment processing has been proposed to occur via two pathways. In the first pathway, short flaps up to eight nucleotides in length are processed by the flap endonuclease FEN1, which cleaves at the base of the flap and generates a nick for ligation (39–41). In the second pathway, longer flaps are generated and then bound by RPA, which inhibits FEN1 cleavage (29, 30). However, RPA stimulates Dna2 nuclease/helicase, which cleaves the long flaps to create shorter flaps that can no longer be bound by RPA (32, 42, 43). FEN1 can then finish processing the shorter flaps. Genetic evidence that Dna2 participates in Okazaki fragment processing (44–46) and biochemical reconstructions showing that some flaps escape FEN1 to become long (47) indicate that cells process a portion of Okazaki fragments through the long-flap pathway.

Since RPA has been described to melt DNA helices, we anticipated that although it inhibits FEN1 cleavage, it would protect against the participation of long flaps in undesirable recombination. Instead, we found that RPA could promote strand annealing and subsequent recombination (34). Our current results show that RPA-coated flaps of a variety of lengths could be precursors to recombination, suggesting a need for Dna2 helicase to eliminate these flaps before recombination occurs.

In earlier work, we found that flaps with stable secondary structure inhibit cleavage by FEN1 (48). We suggested that formation of secondary structure allows flaps to become long (43). Binding of RPA would then relieve the structure to allow loading of Dna2 for efficient processing. If this interpretation is correct, it provides a reason why the long flap pathway has been retained, although it can lead to genome instability.

A model for processing structure-containing flaps, based on the above considerations, is shown in Fig. 7. As the flap is generated, it forms secondary structure that prevents cleavage by FEN1. RPA then binds to the flap and melts the secondary structure, but cleavage by FEN1 is still inhibited by the presence of the RPA (32). At this point, the desirable pathway is processing of the flap by Dna2 and then FEN1 to an intact strand as originally proposed by Seo and co-workers (32). Alternatively, the flap is available for recombination. The promptly formed secondary structure of the flap would have made the flap resistant to the annealing process to initiate recombination. However, the presence of the RPA would melt that structure, allowing the flap to readily equilibrate with a homologous ectopic site. The initial ectopic annealing interaction, if involving only a small number of nucleotides, would be briefly susceptible to the
melting activity of RPA. However, once the region of annealing had spread to 40–50 nt, the recombination intermediate would resist the melting activity of RPA and proceed to a stable recombination product.

RPA might also act to suppress recombination by stimulating Dna2 to rapidly process long flaps as shown in yeast (32, 49). RPA has also been shown to stimulate sealing of nicks by human DNA ligase I (50, 51). However, it is clear that RPA does not insulate long single-stranded flaps from participating in recombination. This situation creates the need for other enzymes that can dissociate recombination intermediates. We previously provided biochemical evidence that BLM helicase can disrupt replication flap interactions at ectopic sites before they can be ligated to form permanent recombination products (34). Hallmarks of BLM−/− cells include hyperrecombination, elevated levels of sister chromatid exchange, and accumulation of replication intermediates, all of which could derive in part from illegitimate recombination initiated by Okazaki flaps (52–54). These results would suggest that one cellular role of BLM is to suppress recombination by opposing RPA-catalyzed recombination.

The ability of RPA to directly promote annealing of Okazaki flaps or of any single strands has not previously been described. However, known properties of RPA are consistent with this finding. RPA was shown to bind to 5′ single-stranded flaps and to the 5′ arm of a fork structure (9). RPA has been shown to participate in Okazaki fragment processing in yeast, acting as a facilitator of the ordered activities of Dna2 and then Fen1 (32, 49). RPA had also been found to promote the strand annealing function of Rad52, mediating Rad51-independent recombination in yeast (55, 56).

Another ssDNA/dsDNA-binding protein, HIV-1 nucleocapsid protein (NC), has been shown to facilitate annealing of complementary oligonucleotides (57). NC aids complementary nucleic acid annealing and exchange, which helps strand transfer during retroviral reverse transcription. NC melts nucleic acids by binding to secondary structures, such as bulges and internal loops, and then induces conformational rearrangements into more stable structures by decreasing the kinetic barrier between single-stranded DNA or RNA and double-stranded configurations of these nucleic acids (58–62). NC also increases the local concentration of complementary nucleic acid strands, resulting in aggregation and a greater likelihood of annealing (59). With the ability to melt single strands and promote exchange into double strands and the potential capacity to aggregate DNA by means of multiple bind sites, the properties of RPA are similar.

In conclusion, we report that RPA has the capacity to accelerate the annealing of single strands of DNA into a double-stranded product. RPA is effective at promoting annealing of structured single strands but opposes the annealing of unstructured single strands. It is most effective at accelerating annealing long single strands. One reason is that long strands usually have a greater negative free energy of folding than shorter strands, so that they require RPA-catalyzed unfolding for rapid annealing. A second reason is that once annealed, long strands resist the melting activity of RPA. RPA has multiple binding sites for DNA, suggesting the ability to aggregate strands to promote annealing. However, we could not acquire direct evidence that RPA has an intrinsic ability to catalyze annealing. Okazaki fragments are processed through a mechanism involving creation and cleavage of 5′ DNA flaps. Some resist cleavage, possibly because they have secondary structure, so that they become long enough to bind RPA. The presence of RPA promotes the ability of structured flaps to engage in recombination. It is likely that the cell has evolved mechanisms, such as the activity of BLM, to resist this effect.

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REFERENCES
1. Wold, M. S. (1997) Annu. Rev. Biochem. 66, 81–92
2. Fairman, M. P., and Stillman, B. (1988) EMBO J. 7, 1211–1218
3. Wobbe, C. R., Weissbach, L., Borowiec, J. A., Dean, F. B., Murakami, Y., Bullock, P., and Hurwitz, J. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 1834–1838
4. Wold, M. S., and Kelly, T. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 2523–2527
5. Brosh, R. M., Jr., Li, J. L., Kenny, M. K., Karow, I. K., Cooper, M. P., Kurekattil, R. P., Hickson, I. D., and Bohr, V. A. (2000) J. Biol. Chem. 275, 23500–23508
6. Brosh, R. M., Jr., Orren, D. K., Nehlin, J. O., Ravn, P. H., Kenny, M. K., Machwe, A., and Bohr, V. A. (1999) J. Biol. Chem. 274, 18341–18350
7. Yuzhakov, A., Kelmaz, Z., Hurwitz, J., and O’Donnell, M. (1999) EMBO J. 18, 6189–6199
8. de Laat, W. L., Appeldoorn, E., Sugasawa, K., Weterings, E., Jaspers, N. G., and Hoeijmakers, J. H. (1998) Genes Dev. 12, 2598–2609
9. Itoide, C., and Borowiec, J. A. (2000) Biochemistry 39, 11709–11791
10. Daughdrill, G. W., Ackerman, J., Isern, N. G., Botuyan, M. V., Arrowsmith, C., Wold, M. S., and Lowry, D. F. (2001) Nucleic Acids Res. 29, 3270–3276
11. Gao, H., Cervantes, R. B., Mandell, E. K., Otero, J. H., and Lundblad, V. (2007) Nat. Struct. Mol. Biol. 14, 208–214
12. Lao, Y., Lee, C. G., and Wold, M. S. (1999) Biochemistry 38, 3974–3984
13. Bastin-Shanower, S. A., and Brill, S. J. (2001) J. Biol. Chem. 276, 36446–36453
14. Bochkareva, E., Belegu, V., Korolev, S., and Bochkarev, A. (2001) EMBO J. 20, 612–618
15. Bochkareva, E., Korolev, S., Lees-Miller, S. P., and Bochkarev, A. (2002) EMBO J. 21, 1855–1863
16. Fanning, E., Klimovich, V., and Nager, A. R. (2006) Nucleic Acids Res. 34, 4120–4137
17. Pestryakov, P. E., Khlimankov, D. Y., Bochkareva, E., Bochkarev, A., and Lavrik, O. I. (2004) Nucleic Acids Res. 32, 1894–1903
18. Machwe, A., Lozada, E. M., Xiao, L., and Orr, D. K. (2006) BMC Mol. Biol. 7, 1
19. Garcia, P. L., Liu, Y., Jiricny, J., West, S. C., and Jancsak, P. (2004) EMBO J. 23, 2882–2891
20. Opresko, P. L., Laine, J. P., Brosh, R. M., Jr., Seidman, M. M., and Bohr, V. A. (2001) J. Biol. Chem. 276, 44677–44687
21. Cui, S., Arosio, D., Doherty, K. M., Brosh, R. M., Jr., Falaschi, A., and Vindigni, A. (2004) Nucleic Acids Res. 32, 2158–2170
22. Van Komen, S., Reddy, M. S., Krejci, L., Klein, H., and Sung, P. (2003) J. Biol. Chem. 278, 44331–44337
23. Doherty, K. M., Sommers, J. A., Gray, M. D., Lee, J. W., von Kobbe, C., Thoma, N. H., Kureeckattil, R. P., Kenny, M. K., and Brosh, R. M., Jr. (2005) J. Biol. Chem. 280, 29494–29505
24. Shen, J. C., Lao, Y., Kamath-Loeb, A., Wold, M. S., and Loeb, L. A. (2003) Mech. Ageing Dev. 124, 921–930
25. Boule, J. B., and Zakian, V. A. (2007) Nucleic Acids Res. 35, 5809–5818
26. Kowalczykowski, S. C., and Krupp, R. A. (1987) J. Mol. Biol. 193, 97–113
27. Muniyappa, K., Chaner, S. L., Tsang, S. S., and Radding, C. M. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 2757–2761
