Biochemical Characterization of Interactions between DNA Polymerase and Single-stranded DNA-binding Protein in Bacteriophage RB69

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The organization and proper assembly of proteins to the primer-template junction during DNA replication is essential for accurate and processive DNA synthesis. DNA replication in RB69 (a T4-like bacteriophage) is similar to those of eukaryotes and archaea and has been a prototype for studies on DNA replication and assembly of the functional replisome. To examine protein-protein interactions at the DNA replication fork, we have established solution conditions for the formation of a discrete and homogeneous complex of RB69 DNA polymerase (gp48), primer-template DNA, and RB69 single-stranded DNA-binding protein (gp32) using equilibrium fluorescence and light scattering. We have characterized the interaction between DNA polymerase and single-stranded DNA-binding protein and measured a 60-fold increase in the overall affinity of RB69 single-stranded DNA-binding protein (SSB) for template strand DNA in the presence of DNA polymerase that is the result of specific protein-protein interactions. Our data further suggest that the cooperative binding of the RB69 DNA polymerase and SSB to the primer-template junction is a simple but functionally important means of regulatory assembly of replication proteins at the site of action. We have also shown that a functional domain of RB69 single-stranded DNA-binding protein suggested previously to be the site of RB69 DNA polymerase-SSB interactions is dispensable. The data from these studies have been used to model the RB69 DNA polymerase-SSB interaction at the primer-template junction.

The replisome is a dynamic macromolecular machine that must constantly respond to changes within the cell that may affect the rate and accuracy of DNA replication (1). The complex topology of the chromosome, together with the fact that DNA is constantly accessed for transcription, recombination, and repair, suggests that the replication of DNA cannot be static, but rather must maintain a great deal of structural flexibility (1). The interaction of the replicative DNA polymerase with its cognate SSB is an example of this dynamic process. During DNA replication, ssDNA is cooperatively and non-specifically bound by the SSB family of proteins to protect template strand DNA from nucleases and facilitate the removal of adventitious secondary structures (2). The length of available template strand during lagging strand synthesis can be variable and depends, in part, on the particular organism and its metabolic state (3). The problem of how to efficiently bind available ssDNA is solved by the highly cooperative and non-templated specific interaction of the SSB family proteins for single-stranded DNA. In contrast, the interaction of the replicative DNA polymerase is, of necessity, confined to the primer-template junction. The polymerase has a high affinity for this unique structure over either ssDNA or double-stranded DNA (4, 5). The processivity of the polymerase is further enhanced by the sliding clamp family of proteins that tether the polymerase to the DNA (6, 7). Taken together, the SSB, DNA polymerase, and sliding clamp (once the sliding clamp has been loaded) are able to extend DNA at nearly in vivo rates (8), and thus, efficient DNA replication depends on making specific protein-protein contacts within the context of the expanding DNA replication bubble. We have determined conditions that allow us to accurately measure the association of a replicative DNA polymerase for its cognate SSB and apply this information to our understanding of the functional replisome.

T4 bacteriophage has proven to be a productive system to study DNA replication because of its simplicity and organizational similarity to eukaryotic and archaeanal systems (9, 10). Bacteriophage RB69 is closely related to T4, and thus, insights from the well characterized T4 system are readily applicable to RB69. RB69 and T4 DNA polymerases (gp43) share 74% amino acid similarity and are members of the pol family of replicative DNA polymerases (11). Structural studies on the T4/RB69 system have been successful in determining high resolution x-ray structures for the DNA polymerase (gp43) (12–14), sliding clamp (gp45) (13, 15), and SSB (gp32) (16) and provide an excellent starting point for the study of replisome assembly.

In bacteriophage T4, specific association of gp32 with gp43 has been qualitatively demonstrated using affinity chromatography of radiolabeled crude extracts (17, 18). Limited proteolysis has shown that the T4/RB69 gp32 can be divided into three distinct domains. The N-terminal B-domain (residues 1–21) is essential for the cooperative binding of RB69 SSB to ssDNA. Loss of this domain reduces affinity for ssDNA by over a thousand-fold (19). The C-terminal A-domain (residues 255–301 for T4 and residues 255–299 for RB69) is highly acidic and mediates interactions with other proteins involved in DNA replication, recombination, and repair (18, 20). The core domain (residues 22–254) is the DNA-binding domain and has the same intrinsic affinity as the intact protein for short single-stranded DNA (19). Based upon proteolysis studies performed in the presence or absence of DNA, it has been suggested that T4 SSB undergoes a conformational change upon binding to ssDNA.

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(21). Current models suggest the conformational changes during binding facilitate T4 SSB interactions with other proteins in replication, recombination, and repair.

To date, quantitative studies on the interaction between DNA polymerase and SSB protein have been limited because of difficulties in establishing conditions that favor formation of a discrete homogeneous complex (22). The recent determination of co-crystal structures of RB69 polymerase-primer-template DNA complex (13, 14) and core domain of T4 SSB (16) have allowed us to design conditions and DNA substrates to form a discrete RB69 DNA polymerase, a primer-template DNA, and RB69 SSB complex for quantitative analysis. The composition and stoichiometry of the complexes were validated using light scattering. Once we had established solution conditions that favored formation of a 1:1:1 RB69 DNA polymerase-SSB-primer-template DNA complex, we were then able to study the interactions among RB69 DNA polymerase, RB69 SSB, and primer-template DNA by equilibrium fluorescence methods where the tryptophan signal from the RB69 DNA polymerase and SSB is quenched upon binding to the DNA. We have found that the protein-protein cooperativity between RB69 DNA polymerase and SSB causes a roughly 60-fold increase of affinity of SSB to the adjoining single-stranded template region. These data have allowed us to construct testable models for the formation of the DNA polymerase-SSB interaction as they occur in replication forks of all organisms.

**EXPERIMENTAL PROCEDURES**

**Materials**

Enzymes were purchased from New England Biolabs, and chemicals were from either Sigma or Fluka. Chromatography resins were purchased from Whatman and Amersham Biosciences. The ssDNA cellulose column was prepared in the laboratory using the protocols of Alberts and Herrick (23). Oligonucleotides were purchased from Integrated DNA Technologies.

**Methods**

**Cloning and Overexpression of RB69 Gene 32**—The original clone encoding RB69 gene 32 (pLIA8) was a gift from Dr. J. Karam (Tulane University School of Medicine). pLIA8 was not efficient in making the RB69 SSB, because an upstream autogenous regulatory element was present in the clone (24). gp32 was subcloned into the overexpression vector pK3C0 without the native 5′ autoregulatory sequences and used to transform *Escherichia coli* AR120 (25). In *toto*, three different RB69 SSB expression constructs were engineered for this work: the intact protein (gp32), a truncation mutant without the N-terminal domain (gp32-B, residues 22–299), and a mutant without the C-terminal domain (gp32-A, residues 1–253). The clone for expression of gp32-A was generated by changing the codon for Val-254 to a stop codon on intact gene 32 using the QuikChange site-directed mutagenesis kit (Stratagene).

**Expression and Purification of RB69 gp43 exo** and gp32 Proteins—Clones for the overexpression of RB69 DNA polymerase and an exonuclease mutant were the generous gift of Dr. J. Karam (Tulane University School of Medicine). RB69 DNA polymerase exo was purified in a similar manner as described previously (26). Intact RB69 gp43, gp32-gp32-A, and gp32-B were purified in a manner similar to intact T4 SSB (25) using ssDNA cellulose affinity column. RB69 gp43 core protein was produced from trypsin digestion of gp32-A protein (19) and purified using ssDNA cellulose affinity column (26). The proteins were flash-frozen in liquid nitrogen and stored in 10 mM Tris-Cl, pH 7.5, 10 mM NaCl, 1 mM EDTA, 3 mM dithiothreitol, and 5% (v/v) glycerol.

**Design of Primer-template DNA for Complex Formation**—The design of a primer-template DNA to form a complex with RB69 DNA polymerase and SSB was based on the crystal structure of RB69 DNA polymerase bound to a primer-template DNA (13) and biochemical studies on the site size of T4 SSB binding to ssDNA (27) (Table I). Oligonucleotides were purified by ion-pairing reverse phase chromatography (C4 resin; Vydac, Hesperia, CA). Primer-template DNAs were annealed by mixing equal molar amounts of primer and template strands in 10 mM Tris-Cl, pH 7.5, 0.1 mM EDTA (TE) heated to 80 °C and allowed to slow cool to 20 °C prior to use.

**Light Scattering**—A miniDAWN three-angle light-scattering photometer (Wyatt Technology, Santa Barbara, CA) with a 30-milliwatt quartz fluorimetric cuvette containing an active magnetic stirrer at 20 °C and a 0.4-mm band-pass for both excitation and emission monochromators. Initial protein concentrations were 1 or 2 μM in 10 mM Tris-Cl, pH 7.5, 150 mM NaCl in the cuvette. Small aliquots (2 to 10 μl) of the corresponding DNA solution in TE buffer were added to the sample cuvette, and TE buffer was added to the reference cuvette. After mixing for 4 min, the spectrometer made ten measurements, and the average was taken as one data point. The effects of dilution and photobleaching were corrected by data from the reference cuvette. Nucleic acids absorb excitation light at 295 nm, and it was necessary to correct for this inner filter effect using an n-acetyltryptophan amide (NATA) calibration titration (30). Because there is no appreciable interaction between NATA and nucleic acids, decrease in NATA fluorescence is a consequence of inner filter effect. After correcting for photobleaching, dilution, and inner filter effects, the titration curves were fitted using the program DynaFit (BioKin Ltd., Pullman, WA) (31). In DynaFit, users give the reaction mechanisms, which are translated by the program into the underlying systems of mathematical equations by using the theory of matrices (32). The data are then fitted using least squares regression.

**RESULTS**

**RB69 DNA Polymerase-SSB-Primer-template DNA Complex Formation**—To analyze the formation of the RB69 DNA polymerase-SSB-primer-template DNA complex, it was necessary to find conditions that support a discrete 1:1:1 stoichiometry. Light scattering was used to assess complex stoichiometry by an accurate determination of their molecular mass and by analysis of their contents by SDS-PAGE and UV-visible spectrophotometry. As shown in Table II, RB69 DNA polymerase exo-, gp32-B, and gp32 core protein were well behaved monomer species whereas intact gp32 and gp32-A were highly polydisperse, consistent with their known ability to self-aggregate (33). We estimated the molecular mass of the RB69 DNA polymerase exo- primer-template DNA complex to be 122.2 kDa, which was 2.2% away from the theoretical molecular mass of 119.6 kDa. Finally, we determined the molecular mass for the complexes in the presence of different RB69 SSB proteins (Table III). Because some aggregation was always seen in the front shoulder (less than 5% of the total material), the middle of the peak was selected for molecular mass calculations. Under the conditions used (10 mM Tris-Cl, pH 7.5, 150 mM NaCl), the RB69 DNA polymerase-SSB-primer-template DNA complex was monodisperse (Fig. 1) and displayed a molecular mass of about 130 kDa. Molecular mass estimates for com-
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Monodispersity and accuracy of molecular mass of DNA polymerase: $p/t + 12$ complexes determined by light scattering in the presence or absence of RB69 SSB

| Protein    | Theoretical mass (kDa) | Experimental mass (kDa) | Apparent Error | Monodispersitya |
|------------|------------------------|-------------------------|---------------|-----------------|
| BSA standard | 66                     | 67.9                    | 2.9           | 1.004 ± 0.046   |
| Polymerase exo- | 104.6                  | 104.8                   | 0.2           | 1.005 ± 0.040   |
| gp32        | 33.1                   | 55.0                    | ND3          | 1.064 ± 0.229   |
| gp32-B      | 31.1                   | 31.0                    | -0.3         | 1.006 ± 0.045   |
| gp32-A      | 28.2                   | 152.1                   | ND           | 1.221 ± 0.307   |
| gp32 core   | 26.2                   | 26.7                    | 1.9          | 1.029 ± 0.084   |

Table II

Monodispersity and accuracy of molecular mass of individual proteins determined by light scattering

The whole peak was selected to calculate molecular mass and monodispersity.

| Protein        | Theoretical mass (kDa) | Experimental mass (kDa) | Apparent Error | Monodispersity |
|----------------|------------------------|-------------------------|---------------|----------------|
| gp32 core      | 26.3                   | 26.7                    | 1.9          | 1.029 ± 0.084   |

A value of 1 means absolutely monodisperse. Values greater than 1 indicate higher polydispersity.

ND, not determined. Error was not calculated, because the protein is highly polydisperse.

Monodispersity and accuracy of molecular mass of DNA polymerase: $p/t + 12$ complexes determined by light scattering in the presence or absence of RB69 SSB

Table III

| gp32 type used in the complex | Theoretical mass (kDa) | Experimental mass (kDa) | Apparent Error | Monodispersity |
|------------------------------|------------------------|-------------------------|---------------|----------------|
| None                         | 119.6                  | 122.2                   | 2.2           | 1.016 ± 0.028   |
| gp32                         | 152.7                  | 128.2                   | -15.4         | 1.001 ± 0.024   |
| gp32-B                       | 150.6                  | 137.6                   | -8.6          | 1.004 ± 0.062   |
| gp32-A                       | 147.8                  | 133.1                   | -9.9          | 1.001 ± 0.021   |
| gp32 core                    | 145.7                  | 140.6                   | 3.5           | 1.009 ± 0.012   |

Polymerase exo- alone, the DNA polymerase

Interestingly, whereas intact gp32 and gp32-A were polydisperse alone, the DNA polymerase

 complexes with these proteins were monodisperse (Table III) and further confirm that only one RB69 SSB was accommodated onto the template DNA. We also performed the same experiments using 10 mM Tris-Cl, pH 7.5, 10 mM MgCl$_2$ as a buffer but found that the complexes were more polydisperse, presumably because of nonspecific aggregation at low ionic strength (data not shown).

Determination of Binding Affinity of RB69 DNA Polymerase to Primer-template DNA—To assay whether the interactions between RB69 DNA polymerase and SSB change the affinity of the polymerase to primer-template DNA, we determined the binding affinity of RB69 DNA polymerase to primer-template DNA by titration of 2 $\mu$m RB69 DNA polymerase exo- with $p/t + 12$ primer-template DNA. Fig. 2 shows the saturation binding curves of DNA polymerase to primer-template DNA under different salt concentrations generated by measuring the percentage change in protein fluorescence as a function of DNA concentration.

Under low salt conditions (50–100 mM NaCl), the binding is too tight to allow an accurate estimate of $K_d$; however, we can estimate the stoichiometry of binding to be 1:1 using the break point estimated from the initial and final slopes of the binding curve (19). The dissociation constant for RB69 DNA polymerase to primer-template DNA at 10 mM Tris-Cl, pH 7.5, 100 mM NaCl was estimated to be greater than 2 nM, in good agreement with previous studies (4, 5) on other polymerases. To obtain an accurate measurement of binding, it was necessary to increase the concentration of NaCl to 150 mM. Data were fitted in Dynafit, and the calculated dissociation constant for RB69 DNA polymerase to primer-template DNA at 10 mM Tris-Cl, pH 7.5, 150 mM NaCl was 124 ± 16 nM. In addition, we noted that the maximum change in fluorescence quenching decreased as a function of salt concentration. As a consequence all our studies were performed at 150 mM NaCl.

Determination of Binding Affinity of RB69 SSB to $T_6$ DNA—We also measured the affinity of RB69 SSB to a short ssDNA. By measuring the individual affinities of RB69 DNA polymerase and SSB to their substrates, we were then able to determine their cooperativity when bound together on the same DNA. Bacteriophage T4 gp32 has been a prototype for protein-nucleic acid interactions, and the RB69 homolog also proved amenable to physicochemical analysis. The site size of RB69 SSB to ssDNA and maximum fluorescence quenching were determined by titrating 2 $\mu$m RB69 SSB intact protein with poly(dT) DNA. The site size was determined under stoichiometric binding conditions to be $n = 6 ± 1$ nucleotides (Data not shown), in good agreement with the previous studies (27) on T4 SSB. The dissociation constant for gp32-B to $T_6$ DNA at 10 mM Tris-Cl, pH

Fig. 1. The molecular weight distribution of DNA polymerase exo- (a), gp32-B (b), DNA polymerase exo- and $p/t + 12$ complex (c), and DNA polymerase exo-, $p/t + 12$, and gp32-B complex (d) across the gel filtration elution peak showing that they are monodisperse in 10 mM Tris-Cl, pH 7.5, 150 mM NaCl. The chromatographic peaks are values from the refractive index detector, and the points are the weight-averaged molecular mass across the chromatographic peak for each species tested.

Fig. 2. Fluorescence titration curves for the binding of $p/t + 12$ to RB69 DNA polymerase exo- under different salt concentrations. $F_p$ is the fluorescence quenched and $F_0$ is the starting fluorescence. All measurements were at 20 °C at a protein concentration of 2 $\mu$m. The maximum percentage fluorescence quenching decreases as the salt concentration increases, and all fluorescence experiments were performed at 150 mM NaCl.
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7.5, 150 mM NaCl was very weak, 10.9 ± 1.7 μM (data not shown), consistent with previous studies using T4 gp32 (19).

The cis and trans DNA Binding Experiments—We designed experiments to determine the cooperativity between RB69 DNA polymerase and SSB when bound to the same (cis) or different (trans) DNA strands (Fig. 3). In the cis experiment, equimolar ratios of RB69 DNA polymerase and SSB bind to a single contiguous primer template. If the presence of RB69 DNA polymerase at the primer-template junction increases the affinity of SSB for the template strand, the increase in binding through protein-protein interactions can be readily measured. In the trans experiment, the template strand is broken into a shorter primer-template junction, which only RB69 DNA polymerase will bind, and a six-nucleotide DNA, which RB69 SSB will bind. Because no significant association has been found between RB69 DNA polymerase and SSB in solution in the absence of DNA, the experiments serves as a control for the proteins when they are bound in trans to separate DNA strands. To simplify our analysis of the fluorescence data, we used a truncation fragment of gp32 (gp32-B) that binds with strands. To simplify our analysis of the fluorescence data, we used a truncation fragment of gp32 (gp32-B) that binds with single contiguous primer template. If the presence of RB69 DNA polymerase binds to ssDNA about 100 times more weakly (10.9 \(\mu\)M) and as proposed in our model, suggests DNA polymerase would bind preferentially at the primer-template junction whereas gp32-B would associate with available single-stranded template DNA (Fig. 3). Under identical solution conditions, 1 μM RB69 DNA polymerase and gp32-B were titrated with p/t+12 DNA or p/t+\(T_e\) DNA (see Table I and Fig. 3). The saturation binding curves are shown in Fig. 4A. As expected, in the cis situation, the interaction between RB69 DNA polymerase and SSB increased the affinity of SSB to ssDNA, which appeared as a tighter binding regime, whereas in the trans experiment, two independent binding processes were displayed as a two-phase curve. The mechanism and their associated fits to our data are shown in Fig. 4B. The overall calculated dissociation constant for RB69 DNA polymerase/gp32-B to primer-template DNA at 10 mM Tris-Cl, pH 7.5, 150 mM NaCl was 353 ± 25 nM, similar to the affinity of DNA polymerase alone to primer-template DNA (124 ± 16 nM). In the trans experiment, we were readily able to fit the data assuming there were two independent binding processes in the cuvette, and the calculated dissociation constant for RB69 DNA polymerase to primer-template DNA was 139 ± 14 nM and for RB69 gp32-B to \(T_e\) DNA was 20.0 ± 0.4 μM. These data are in good agreement with the dissociation constant for individual binding processes, 124 ± 16 nM and 10.9 ± 1.7 μM, respectively, and support the proposed mechanism for the trans experiment. The strength of the RB69 DNA polymerase-SSB interaction can be estimated from the difference in apparent association of SSB to available single-stranded template DNA. At 10 mM Tris-Cl, pH 7.5, 150 mM NaCl, the presence of RB69 DNA polymerase results in a 57-fold increase in SSB affinity for ssDNA as a result of DNA polymerase:SSB cooperativity.

The A-domain of RB69 SSB Is Not Essential for Interaction between DNA Polymerase and gp32—It has been postulated that the A-domain of gp32 was the binding site for RB69 DNA polymerase (18). If this is true, the cooperative RB69 DNA polymerase-SSB interactions should be abolished by the removal of the A-domain. To test this hypothesis, we performed our DNA polymerase-SSB cooperativity studies using core gp32 (without the A-domain). To our surprise, we obtained a curve that demonstrated tighter DNA polymerase-SSB interaction than we observed with gp32-B protein (Fig. 4A). The calculated dissociation constant for RB69 DNA polymerase/gp32 core to primer-template DNA at 10 mM Tris-Cl, pH 7.5, 150 mM NaCl was 48.6 ± 7.7 nM. This is about a 350-fold increase in affinity from protein-protein interactions and is 7-fold higher than observed for RB69 DNA polymerase/gp32-B. The dissociation constants are summarized in Tables IV and V. We also obtained a saturation curve for RB69 DNA polymerase/gp32 intact protein binding to primer-template DNA and for RB69 DNA polymerase: gp32-A binding to primer-template DNA. Interestingly, the gp32 and gp32-B proteins showed similar cooperativity (−80-fold),
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TABLE IV

| Protein           | DNA ligand | Dissociation constant (nm) |
|-------------------|------------|----------------------------|
| Polymerase exo⁻   | p/t + 12   | 124 ± 16                   |
| gp32-B            | Tₚ         | 10900 ± 1730               |
| Pol exo⁻ and gp32-B | p/t + 12  | 353 ± 25°                  |
| Pol exo⁻ and gp32-B | p/t + 6/Tₚ | SSB:DNA = 139 ± 14         |
| Pol exo⁻ and gp32 core | p/t + 12 | 48.6 ± 7.7°                |

* Data were fitted using single binding process as the mechanism, e.g., pol + SSB + p/t + 12 = pol:SSB:p/t + 12.

TABLE V

| Protein           | DNA ligand | Dissociation constant (nm) |
|-------------------|------------|----------------------------|
| gp32-B alone      | Tₚ         | 10900 ± 1730               |
| gp32-B and Pol exo⁻  | p/t + 12  | 48.8 ± 1.8°                |
| gp32-B and Pol exo⁻  | p/t + 6/Tₚ | 20000 ± 403                |
| gp32 core and Pol exo⁻  | p/t + 12 | ND*                        |

* Data were fitted using two independent binding processes as the mechanism, e.g., pol + SSB + p/t + 12 = pol:SSB:p/t + 12 and SSB + p/t + 12 = SSB:p/t + 12.  
** ND, not determined. The binding is too tight to be accurately fitted by DynaFit.

whereas gp32 core and gp32-A consistently showed stronger polymerase:SSB cooperativity (~350-fold).

**DISCUSSION**

The specificity and strength of protein-DNA and protein-protein contacts within the replisome are critical for its correct assembly and function. Even a minimal replisome in T4/RB69 consists of at least five proteins: DNA polymerase, sliding clamp, single-stranded DNA-binding protein, the primase, and the helicase. Each organism has proteins fulfilling analogous conserved roles throughout DNA replication, although eukaryotes typically have more subunits (10, 34). Although there have been several very good qualitative studies of protein-protein interactions within replication, quantitative analysis has proven to be more difficult (3, 35). Typically, this is because the assembly of the replisomal proteins is quite weak and dissociates readily in the absence of DNA. Based on the crystal structure of RB69 DNA polymerase-primer-template DNA complex and the biochemical studies on the site size of T4 SSB core protein, we have found solution conditions that support formation of a homogeneous complex of RB69 DNA polymerase, SSB, and primer-template DNA. Our results show that the cooperativity between RB69 DNA polymerase and SSB increases the affinity of SSB to the single-stranded region on the template strand about 60-fold.

Studies on the SSB family of proteins suggest their function in DNA replication is conserved across all organisms. In addition to being essential to DNA replication, recombination, and repair, they are among the first proteins that assemble onto the DNA for these diverse processes and recruit other proteins to the site of action. A direct interaction between SSB and replicative DNA polymerase has also been proposed for eukaryotes. In eukaryotes, the SSB protein is replication protein A (RPA), a heterotrimeric protein shown to act as a common “touchpoint” (36) for the assembly of the pol³ replication complex. Our studies show that binding in cis to the same DNA strand is facilitated by protein-protein interactions and thus promotes proper assembly of proteins to the primer-template junction. Immunoaffinity chromatography has shown that DNA pol³ from calf thymus and the p70 subunit of RPA associate with several other proteins involved in DNA replication (37), and RPA is also directly responsible for the loading of replication factor C and pol³ onto primer-template DNA in a coordinated manner (36). These data are consistent with the scenario we found in our studies in which RB69 DNA polymerase and SSB directly and coordinately assemble at the primer-template junction.

More specifically for the RB69/T4 system, it has been hypothesized that the C-terminal A-domain of RB69 SSB recruits proteins essential for replication, recombination, and repair (18, 21) via protein-protein interactions. Current models suggest that in the DNA-free state of RB69 SSB, the A-domain is at least partially buried within the ssDNA-binding site (21, 38, 39). Upon binding to ssDNA, the A-domain is exposed and can make interactions more readily with other proteins (38, 40, 41). Hurley et al. (18) purified a C-terminal region of the T4 SSB (residues 213–301) and immobilized it on an agarose matrix. They found that this affinity column was able to retain DNA polymerase and several other replication and recombination proteins from T4 whole cell extract. If the A-domain is the domain responsible for interaction with DNA polymerase, the DNA polymerase-primer-template DNA:SSB core complex should not display any cooperativity between DNA polymerase and SSB core in our studies. Surprisingly, we observed about 7-fold higher cooperativity for this complex than with gp32-B protein (which has the A-domain). This implies that the A-domain on RB69 SSB is not the site for binding to DNA polymerase. On the contrary, it slightly hinders the interaction between RB69 DNA polymerase and SSB. Our studies support a model for DNA polymerase-SSB interaction in the RB69/T4 system, in which the A-domain partially folds back into the DNA-binding pocket, blocking the binding site for DNA polymerase (Fig. 5). Upon binding to ssDNA, the A-domain is displaced, and the binding site for DNA polymerase is exposed to make interactions with DNA polymerase. The experiments performed by Hurley et al. (18) used a peptide that includes not only the A-domain of T4 SSB but also 42 extra amino acids (residues 213–255) on the core domain, and this may account for differences between our studies. Because the crystal structure of T4 gp32 core bound to DNA shows that these 42 amino acids form a large surface on the back of the DNA-binding
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DNA, the DNA polymerase and SSB will not associate to form protein-protein association predicts that, in the absence of overall effect of increasing the affinity of SSB for ssDNA proximally to DNA polymerase, the interaction surface with DNA polymerase may be inaccessible and therefore the "false signal" that gp32 is bound to DNA and is available for DNA replication. In the intact gp32, the binding site for DNA polymerase is protected by the A-domain and is exposed only when it is bound to ssDNA.

We have found that specific protein-protein cooperativity between RB69 DNA polymerase and its SSB (gp32) has the overall effect of increasing the affinity of SSB for ssDNA proximal to the DNA polymerase by about 60-fold. This modest protein-protein association predicts that, in the absence of DNA, the DNA polymerase and SSB will not associate to form unproductive "pseudoreplicas" at their in vitro concentrations. Conversely, the cooperativity assures that when both proteins are bound to the same DNA strand, they will productively associate to facilitate accurate and processive DNA synthesis. Previous studies have suggested that SSB from bacteriophage T4 and E. coli are able to diffuse along ssDNA, and therefore the "search" for either another SSB or DNA polymerase is confined to two dimensions (42). The protein-protein cooperativity exhibited by DNA polymerase and SSB furthers the view that the dynamic character of the replisome is the product of subtle and finely tuned interactions at the molecular level. It is our expectation that these organizing principles will be seen throughout DNA replication and in all organisms.

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