

Single-stranded DNA-binding Protein Recruits DNA Polymerase V to Primer Termini on RecA-coated DNA^{*[S]}

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Translesion DNA synthesis (TLS) by DNA polymerase V (polV) in *Escherichia coli* involves accessory proteins, including RecA and single-stranded DNA-binding protein (SSB). To elucidate the role of SSB in TLS we used an *in vitro* exonuclease protection assay and found that SSB increases the accessibility of 3' primer termini located at abasic sites in RecA-coated gapped DNA. The mutant SSB-113 protein, which is defective in protein-protein interactions, but not in DNA binding, was as effective as wild-type SSB in increasing primer termini accessibility, but deficient in supporting polV-catalyzed TLS. Consistently, the heterologous SSB proteins gp32, encoded by phage T4, and ICP8, encoded by herpes simplex virus 1, could replace *E. coli* SSB in the TLS reaction, albeit with lower efficiency. Immunoprecipitation experiments indicated that polV directly interacts with SSB and that this interaction is disrupted by the SSB-113 mutation. Taken together our results suggest that SSB functions to recruit polV to primer termini on RecA-coated DNA, operating by two mechanisms: 1) increasing the accessibility of 3' primer termini caused by binding of SSB to DNA and 2) a direct SSB-polV interaction mediated by the C terminus of SSB.

Translesion DNA synthesis is a DNA damage tolerance mechanism in which replication blocks caused by DNA lesions are relieved by specialized DNA polymerases proficient in synthesizing DNA across lesions (1). In *Escherichia coli* this reaction is catalyzed primarily by the Y family DNA polymerases encoded by the *umuDC* operon (DNA polymerase V (polV)²) and the *dinB* gene (DNA polymerase IV (polIV)) (2–5). The *in vitro* reconstitution of TLS with purified proteins indicated that both RecA and single-stranded DNA-binding protein (SSB) are required for polV-catalyzed TLS (6–9). RecA, the main recombinase in *E. coli*, is also the main activator of the SOS stress response via its ability to promote the autocleavage of the LexA

repressor, which is the negative regulator of all SOS genes. RecA has two additional roles in TLS: (a) It activates UmuD' by promoting its autocleavage to UmuD', which then binds to UmuC to form the UmuD'₂C complex polV (1). (b) A direct role in TLS which involves the recruitment of UmuD' to DNA (10). RecA promotes these activities in its DNA-bound form, which consists of a nucleoprotein filament formed by its cooperative binding to ssDNA (11).

SSB is an essential protein in *E. coli*, involved in replication, recombination and repair. Functional homologs of SSB are present in all organisms, and their importance is well illustrated by the occurrence of viral SSBs (12–15). *E. coli* SSB is a 75kDa tetramer composed of four identical subunits. It binds ssDNA strongly via a ssDNA-binding domain located at its N terminus, and interacts with a variety of proteins via its C terminus (15–20). A detailed kinetic analysis showed that SSB had a dramatic effect on TLS by polV across an abasic site (21), however, under some conditions SSB was found to be stimulatory rather than essential for *in vitro* TLS (22). We used an *in vitro* reconstituted TLS assay system to study the mechanism by which SSB exerts its effect on TLS. We report here that SSB has a dual role in polV-catalyzed TLS: 1) it increases the accessibility of the primer-template near a template lesion on RecA-coated DNA, and 2) it directly interacts with UmuC. This suggests that SSB functions in the recruitment of polV to the primer-template-lesion site on RecA-coated DNA, an early stage in the initiation of TLS.

EXPERIMENTAL PROCEDURES

Proteins—Proteins used in this study were purified as described in the respective references: RecA (23); *E. coli* SSB mutant proteins H55Y (SSB-1), H55K, and P176S (SSB-113) (24); polV (MBP-UmuC and UmuD' purified separately) (7, 8); *E. coli* SSB (25, 26), with an additional purification step of a Mono P column; the β subunit of polIII (27). Human simplex virus type 1 (HSV) ICP8 was a gift from I. Robert Lehman (Stanford University School of Medicine), and the γ complex and human RPA were gifts from Michael O'Donnell (The Rockefeller University, New York). T4 gene 32 protein, *E. coli* DNA pol I Klenow fragment, and *E. coli* exonuclease III were from Amersham Biosciences. Bovine serum albumin, proteinase K, calf intestine alkaline phosphatase, creatine kinase, and Asp700 were from Roche Applied Science; Restriction endonucleases, T4 DNA ligase, T4 polynucleotide kinase, were from New England Biolabs, T7 gp6 exonuclease was from Amersham Biosciences, and S1 nuclease was from Promega. T4 gp32 and ICP8

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[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Tables S1–S3.

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² The abbreviations used are: polV, polymerase V; polIV, polymerase IV; SSB, single-stranded DNA-binding protein; HSV, herpes simplex virus; MBP, maltose-binding protein; ssDNA, single-stranded DNA; TLS, translesion DNA synthesis; RPA, replication protein A.

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min. Reaction products were treated with proteinase K, restricted, and fractionated by urea-PAGE as described for the TLS assay.

Restriction Nuclease Protection Assay—The restriction nuclease protection assay measured the modulation of the restriction of the gap-lesion plasmid by RecA and SSB. The relevant restriction sites are shown in Fig. 3A. The reactions were performed as described for the TLS assay with the following changes. The DNA substrates used were not radiolabeled, and reactions were carried out with neither dNTPs nor polV, and with either RsaI (0.04–0.06 unit/ μ l) or MspA II (1.6 units/ μ l), RecA (2–25 μ M), *E. coli* SSB (0.2–1 μ M as tetramers), and an ATP-regenerating system, as described above. As a control, in addition to the gap-lesion substrate, plasmid pFGP21 was used. This is a control covalently closed plasmid, which is similar to GP21E, except that it is fully double-stranded and contains no lesion (31). Reactions were carried out at 37 °C as following: RecA and the DNA substrate (GP21E or pFGP21, each at 4 nM) were preincubated in the assay buffer for 2 min. When present, SSB was added at this stage. The reactions were further incubated for 4 min, after which the restriction nuclease was added, and the reaction was incubated at for up to 6 min 37 °C. Reactions were terminated by the addition of 1% SDS loading buffer, heat-inactivated for 5 min at 70 °C, and fractionated by agarose gel electrophoresis in the presence of ethidium bromide. Reaction products were visualized under UV light.

Immunoprecipitation Assay—Immunoprecipitation reactions were performed by incubating the indicated proteins (UmuC-MBP, 9.5 μ g; MBP, 9.5 μ g; SSB, 13 μ g; and SSB-113, 13 μ g) at 4 °C for 30 min, in a buffer containing 25 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.1% Nonidet P-40, and 0.1% bovine serum albumin, in a final volume of 100 μ l. A monoclonal antibody against MBP (1 μ g, New England Biolabs) diluted in 200 μ l of binding buffer was then added to each reaction mixture, for an additional incubation of 1 h at 4 °C. Finally, 50 μ l (50% v/v) protein A resin (Amersham Biosciences) was added to the reaction mixtures, for an additional incubation of 1 h. The protein A resins were subsequently washed 6 times with 1 ml of binding buffer. Resin-bound proteins were eluted by boiling in 25 μ l of a solution containing 100 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 0.2% bromophenol blue and fractionated by 10% SDS-PAGE. The presence of SSB in the gel was assayed by immunoblotting with rabbit polyclonal anti-SSB antibodies (32), followed by goat-anti-rabbit antibodies linked to horseradish peroxidase (1:20,000, Jackson ImmunoResearch Laboratories, Inc). SuperSignal West Pico Chemiluminescent Substrate (Pierce) was used for detecting horseradish peroxidase on immunoblots.

RESULTS

The SSB-1 and SSB H55K Mutants Are Defective in polV-catalyzed TLS—The *in vitro* TLS assay used in this study, which was previously developed in our laboratory (7, 8, 29), is based on a gapped plasmid carrying a site-specific synthetic abasic site in the ssDNA region, and an internal ³²P label near the primer terminus. Upon addition of polV, RecA, and SSB, DNA synthesis initiates from the 3' primer terminus, and continues up to and across the abasic site. To facilitate the analysis of TLS prod-

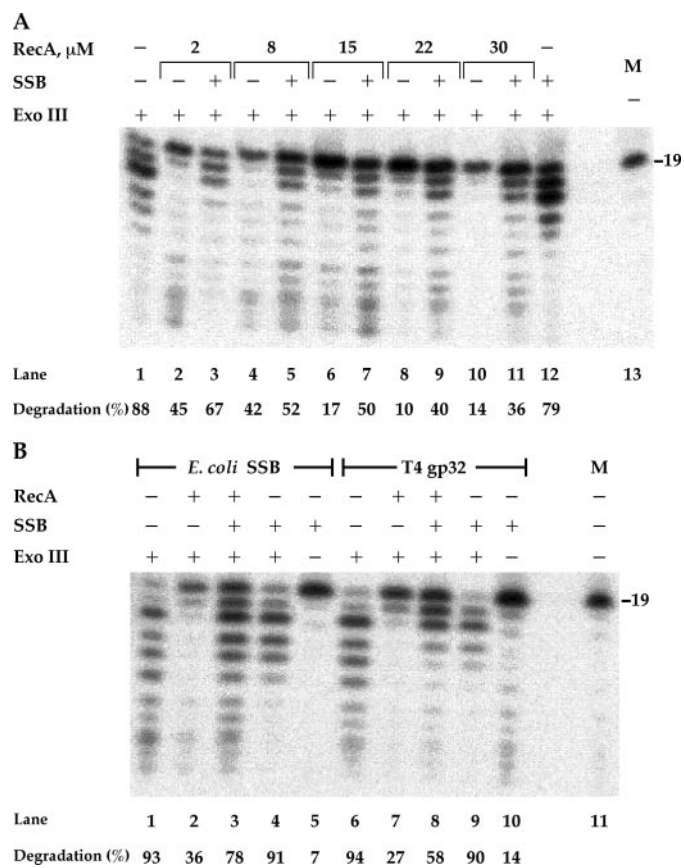


FIGURE 2. Effect of RecA and SSB on primer accessibility measured by an exonuclease-protection assay. A, primer excision reactions with exonuclease III were carried out at 37 °C, for 6 min, in the presence of a creatine-based ATP-regenerating system, as described under "Experimental Procedures." The indicated RecA concentrations were used, and when present, SSB was at 100 nM tetramers. At the end of the reaction, the products were processed and analyzed as in a standard TLS reaction. Lane 13 contains a 19-mer as a marker for the non-degraded primer. B, an exonuclease protection assay was performed as described in A, with 2 μ M of RecA, and 1 μ M of T4 gp32 instead of *E. coli* SSB, where indicated.

ucts, the DNA is extracted from the reaction mixture and restricted to produce a set of short radiolabeled oligonucleotides, representing uninitiated primers, replication arrest at the lesion, and TLS products. Analysis of the products obtained in this manner allows the quantification of three variables as follows: (a) synthesis, defined as all extended products out of all available primers; (b) bypass, defined as all synthesis products longer than 30 nucleotides, out of all primers that were extended (the lesion is at position 30 with respect to the MspAII cleavage site; Fig. 1A); and (c) TLS, defined as all products longer than 30 nucleotides, out of all available primers.

First we examined whether the SSB-1 mutant can support *in vitro* polV-catalyzed TLS. The SSB-1 mutant causes a temperature-sensitive phenotype *in vivo*, as well as UV sensitivity (33, 34). *In vitro* analysis revealed that the SSB-1 mutation causes a defect in tetramerization of the protein, which is likely to be responsible for its phenotype (35). Analysis of TLS with polV, RecA, and the SSB-1 protein revealed strong reductions in both synthesis and bypass by polV (Fig. 1B). Consistently, analysis of the SSB H55K mutant protein, known to be more defective than SSB-1 in tetramerization *in vitro* (24), showed even lower extents of initiation and bypass by polV (Fig. 1B). These results

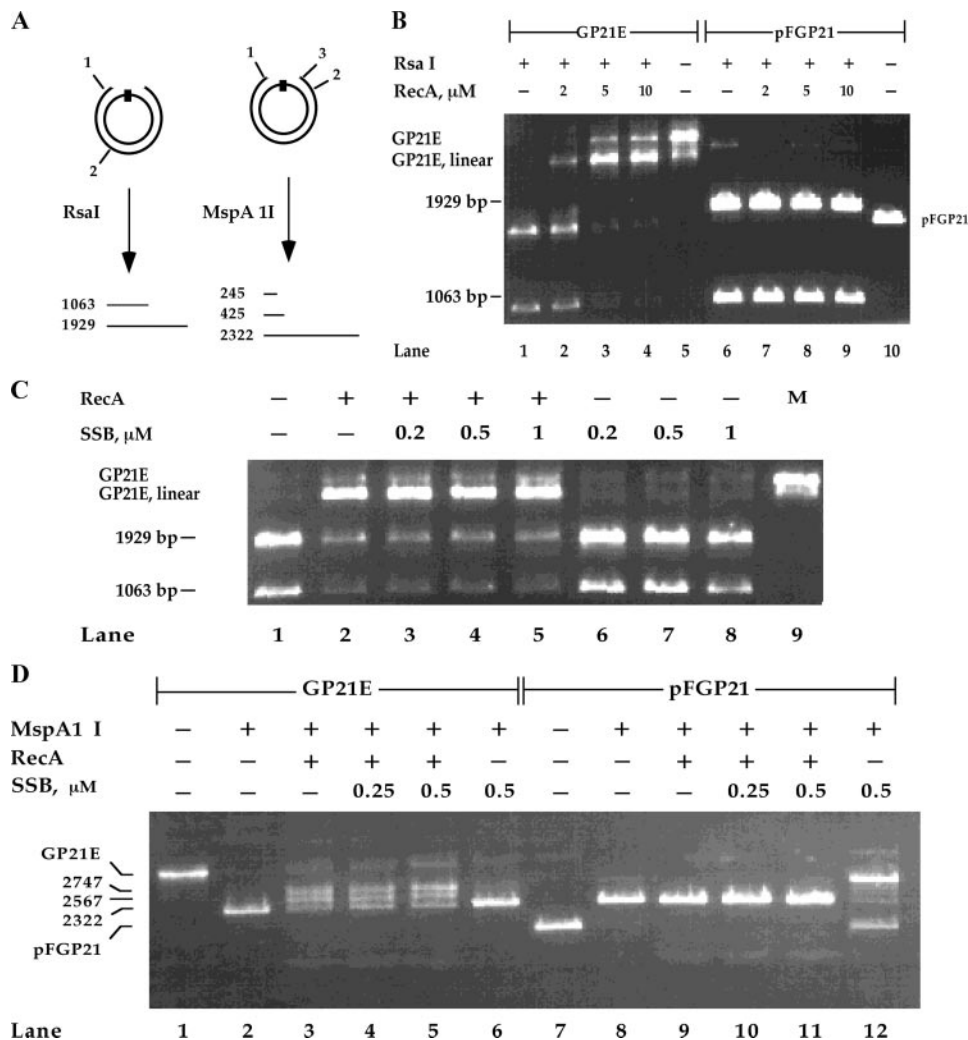


FIGURE 3. Effect of RecA on restriction nuclease cleavage of a gap-lesion plasmid. *A*, the predicted products of GP21E restriction with RsaI and MspA1I. RsaI sites on GP21E (*left panel*) are located 78 (1) and 1141 (2) bp upstream to the primer terminus. Cleavage of GP21E by RsaI yields two fragments, at the indicated lengths. MspA1I sites on GP21E (*right panel*) are located 19 (1), 2341 (2), and 2586 (3) bp upstream to the primer terminus. Cleavage of GP21E by MspA1I yields three fragments at the indicated lengths. *B*, cleavage of the gapped DNA by RsaI in the presence of RecA. The gapped plasmid GP21E and plasmid pFGP21 were each preincubated at 37 °C for 6 min in the presence of RecA, at the indicated concentrations. RsaI was then added for a further incubation of 5 min at 37 °C. Reaction mixtures were fractionated on a 0.8% agarose gel, as described under "Experimental Procedures." *C*, cleavage of the gapped DNA by RsaI in the presence of RecA and SSB. The gap-lesion plasmid GP21E was preincubated at 37 °C for 2 min in the presence of RecA and further preincubated for 4 min upon the addition of SSB. RsaI was then added as described in *B*. When present, RecA was at 5 μ M. Reaction products were obtained as described in *B*. *D*, cleavage of the gapped plasmid by MspA1I in the presence of RecA and SSB. Gapped plasmid GP21E and plasmid pFGP21 were each preincubated at 37 °C in the presence of RecA and SSB. Gapped plasmid GP21E and plasmid pFGP21 were each preincubated at 37 °C for 2 min in the presence of RecA and SSB. MspA1I was then added for a further incubation of 5 min at 37 °C. When present, RecA was at 25 μ M. Reaction products were obtained as described in *B*.

suggest that SSB is required in its tetrameric form during polV-catalyzed TLS.

SSB Increases the Accessibility of the 3' Primer Terminus in RecA-coated Gap-lesion Plasmids—The RecA nucleoprotein filament was proposed to target polV to the damaged DNA, most likely through its interaction with UmuD'. However, as the RecA filament extends to the double-stranded DNA region at the 3' primer terminus, it might interfere with the binding of polV to the primer terminus. Indeed, high concentrations of RecA were found to inhibit TLS in our system (36). To directly examine the effects of RecA and SSB on primer accessibility, we utilized an exonuclease protection assay, based on the ability of the *E. coli* exonuclease III to excise the primer terminus in the 3'→5'

direction. As can be seen in Fig. 2A, exonuclease III excised the primer strand, as indicated by the appearance of radiolabeled oligonucleotides shorter than 19 nucleotides (Fig. 2A, lane 1). Pre-formation of a RecA nucleoprotein filament with increasing concentrations of RecA caused increasingly stronger inhibition of the 3'→5' excision of the primer terminus by exonuclease III (Fig. 2A, lanes 2, 4, 6, 8, and 10). Taking the fraction of the degraded primers as a measure of exonuclease activity, excision decreased from 88% in the absence of RecA down to 10–14% in its presence (Fig. 2A, lanes 2, 4, 6, 8, and 10). The addition of SSB partially relieved this inhibition, causing up to a 3- to 4-fold increase in primer excision at 15–30 μ M RecA (Fig. 2A). SSB alone had a minor effect on the excision by exonuclease III (Fig. 2A, lane 12).

A Heterologous SSB Also Increases the Accessibility of the Primer Terminus in RecA-coated DNA—To examine whether SSB exerts its effect via its ability to bind ssDNA, we examined the ability of a heterologous SSB, the phage T4 gp32, to increase the accessibility of the primer terminus in RecA-coated DNA. To that end we performed the exonuclease protection assay with RecA-coated gapped plasmid as before, but with T4 gp32 instead of SSB. As can be seen in Fig. 2B the addition of RecA to GP21E caused a decrease in primer excision by exonuclease III, as shown above, from 94% to 27% (Fig. 2B, lanes 6 and 7, respectively). Addition of T4 gp32 partially relieved this inhibition, leading to 58% primer degradation (Fig. 2B, lane 8). T4 gp32 itself had a

marginal effect on the activity of exonuclease III and did not contain contaminating exonuclease activity (lanes 9 and 10, respectively). The ability of T4 gp32 to relieve the RecA inhibition of exonuclease III activity is generally similar to that of *E. coli* SSB (Fig. 2, A and B (lanes 1–5)), suggesting that their effect is at the DNA level, mediated primarily by their ssDNA binding activity. The observed mild activity difference between the two SSBs may stem from their different ssDNA binding modes. We also examined the effect of RPA, the human SSB, on primer accessibility in the presence of RecA, using the same assay. In contrast to the *E. coli* SSB and phage T4 gp32, human RPA was essentially unable to relieve the RecA inhibition of exonuclease activity (supplemental Table

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S1). Thus not every SSB can relieve RecA inhibition of 3' primer accessibility.

SSB Does Not Cause Total Disassembly of the RecA Nucleo-protein Filament—To examine whether SSB causes a major disassembly of the RecA nucleofilament, or rather a more local change at the primer-template-lesion region, we used a restriction nuclease-protection assay. As shown in Fig. 3A, the restriction nuclease RsaI has two cleavage sites, located 78 and 1141 bp upstream to the primer terminus. Incubation of RsaI with pFGP21, an intact control plasmid, which mimics filled and ligated GP21E, yielded the two predicted DNA fragments of 1929 and 1063 bp (Fig. 3B, lane 6). Addition of RecA at concentrations of 2–10 μM had no effect on RsaI cleavage (Fig. 3B, lanes 7–9), as expected. This demonstrates that RecA, when not bound to DNA, did not inhibit RsaI. When the assay was conducted with the gap-lesion plasmid GP21E, the digestion pattern changed. In the absence of RecA, the gapped plasmid was fully cleaved by RsaI, yielding two DNA fragments (Fig. 3B, lane 1; the migration of the larger fragment is slightly faster than the control, because it is partially single-stranded). However, upon the addition of RecA, cleavage of GP21E by RsaI was inhibited, such that at 5 and 10 μM RecA the large majority of cleavage products consisted of singly cut GP21E (Fig. 3B, lanes 3 and 4). Additionally, uncleaved GP21E was also observed (Fig. 3B, lanes 3 and 4), suggesting that both restriction sites were blocked for RsaI digestion. As the only difference between GP21E and pFGP21 is the single-stranded DNA region, these results indicate that a nucleoprotein RecA-ssDNA filament formed at the gapped region enabled RecA to continuously cover the double-stranded DNA region adjacent to the gap in the 5'→3' direction, covering both RsaI sites. Addition of increasing amounts of SSB had essentially no effect on the digestion of GP21E by RsaI, as demonstrated in Fig. 3C (lanes 2–5). These results suggest that SSB did not cause an overall disassembly of the RecA nucleoprotein filament. It is noteworthy that SSB itself did not affect the activity of RsaI (Fig. 3C, lanes 6–8).

The RsaI site closest to the 3' primer terminus is located 78 bases upstream from it. To study the effect of SSB on the RecA filament closer to the 3' primer terminus, another restriction endonuclease, MspAII, was employed. As schematically shown in Fig. 3A, MspAII has three restriction sites on GP21E, located 19, 2341 and 2586 bp upstream to the primer terminus (Fig. 3A, right scheme, 1–3, respectively). The predicted products of MspAII digestion are shown in Fig. 3A, as well. Full digestion of GP21E in the presence of MspAII is demonstrated in Fig. 3D, lane 2, where only the largest fragment obtained is shown. In the presence of RecA, in addition to the fully digested DNA product, which was 2322 bp long, several longer products were obtained, including the closed circular plasmid (Fig. 3D, lane 3). These fragments resulted from inhibition of MspAII cleavage, leading to partial digestion. A similar digestion pattern was obtained upon the addition of SSB to RecA-containing reactions (Fig. 3D, lanes 4 and 5). SSB itself did not affect digestion by MspAII (Fig. 3D, lane 6). Digestion of the control fully double-stranded pFGP21 by MspAII was unaffected by the presence of RecA (Fig. 3D, lanes 8 and 9), or RecA and SSB (Fig. 3D, lanes 10 and 11). However, SSB itself caused an electrophoretic shift (Fig. 3D, lane 12), consistent with the previously reported binding of

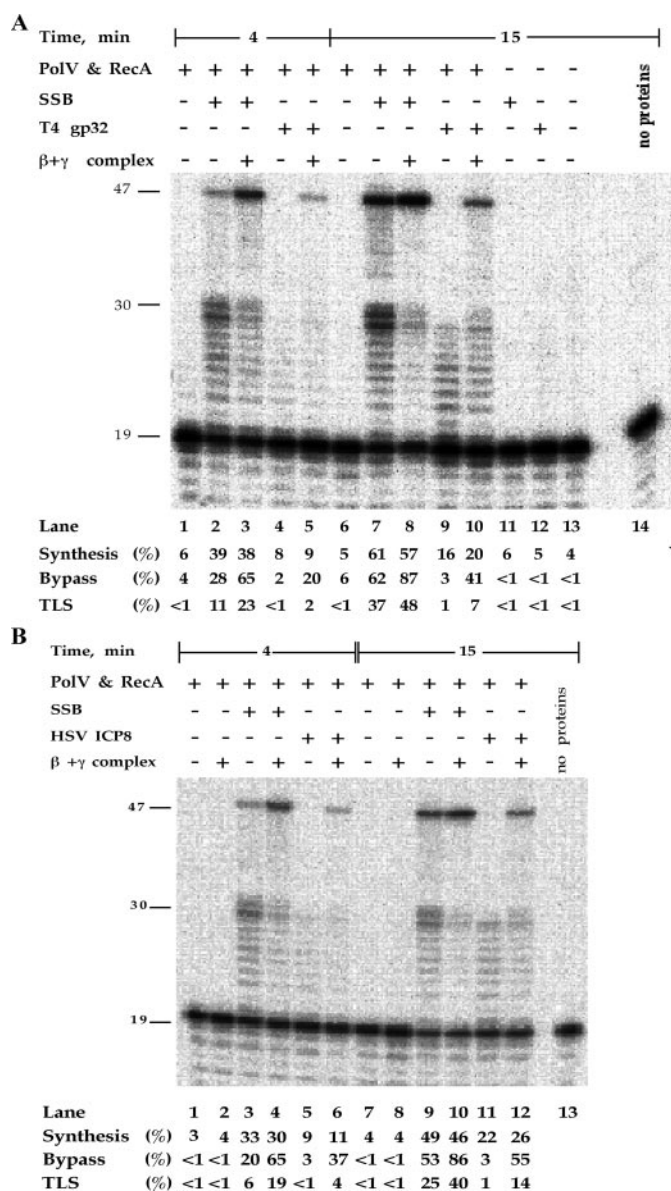


FIGURE 4. PolV-catalyzed TLS in the presence of heterologous SSBs. A, TLS in the presence of T4 gp32. The reactions were performed as described under "Experimental Procedures," in the presence of polV and RecA and 1 μM T4 gp32 (lanes 4–5 and 9–10), or 100 nM SSB tetramers (lanes 2–3 and 7–8). When present, the β subunit was at 40 nM (as dimers) and the γ complex was at 20 nM. Lanes 11–13 are control reactions, to determine the purity of *E. coli* SSB, T4 gp32, and HSV ICP8, respectively, from contaminating DNA synthesis activities. Analysis of reaction products was performed as described in the legend to Fig. 1. Lane 14 contains a 19-mer primer as a marker for non-replicated DNA. B, TLS in the presence of HSV ICP8. Reactions were performed as described under "Experimental Procedures," in the presence of polV and RecA and 180 nM HSV ICP8 (lanes 5–6 and 11–12) or 100 nM SSB (lanes 3–4 and 9–10). When present, the β subunit was at 40 nM (as dimers) and the γ complex was at 20 nM. Reaction products were obtained as described in the legend to Fig. 1. Lane 13 contains a 19-mer primer as a marker for non-replicated DNA (19-mer).

SSB to supercoiled DNA (37). The finding that SSB is unable to relieve the RecA inhibition at sites flanking the primer terminus and beyond indicate that SSB acts in a significantly local manner, presumably by exposing the primer terminus to enzymatic activity.

Heterologous SSBs Support TLS by polV—If the function of SSB in TLS involves increasing the accessibility of the 3' primer

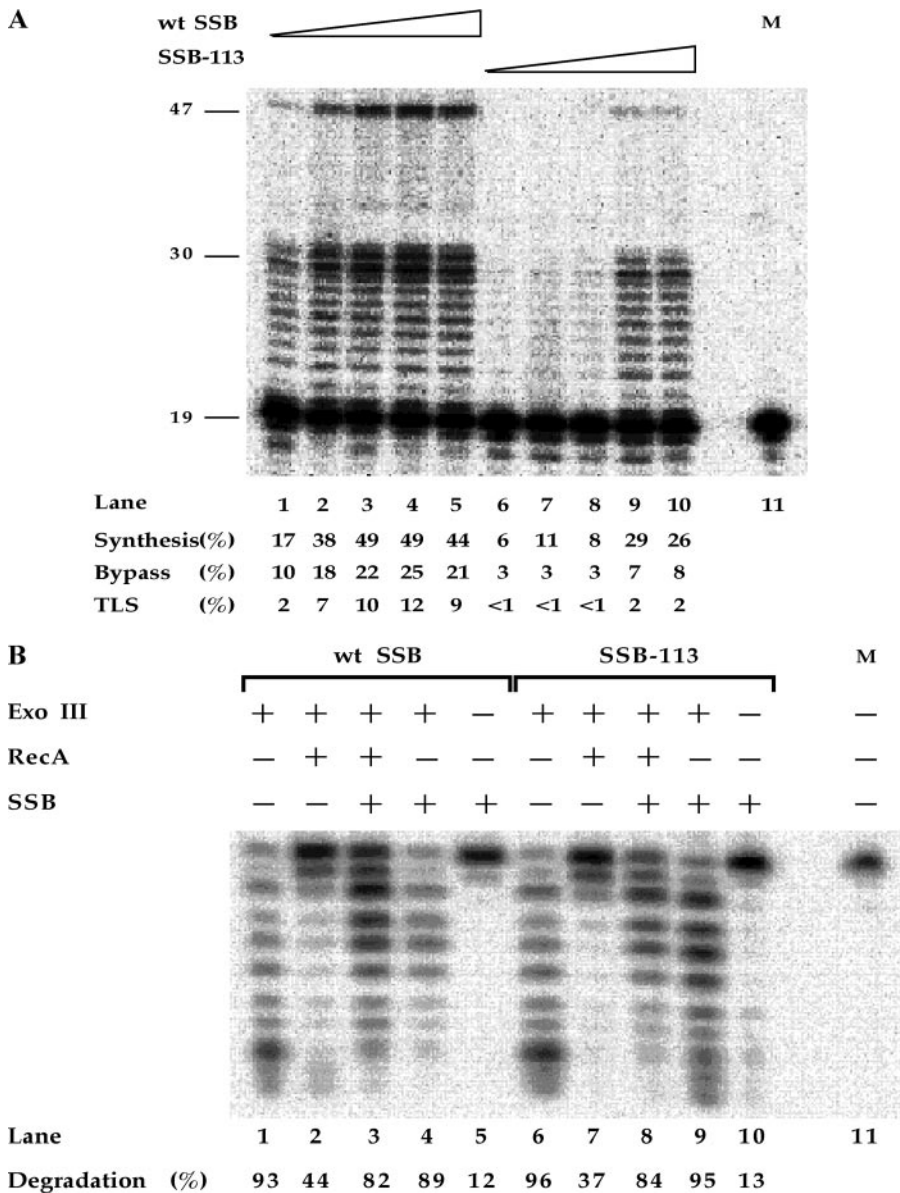


FIGURE 5. Analysis of the effect of SSB-113 on TLS. A, polV-catalyzed TLS in the presence of SSB-113. Reactions were performed in the presence of polV, RecA, and 25, 50, 100, 200, or 400 nM wt SSB or SSB-113, at 37 °C for 5 min, as described under "Experimental Procedures." Reaction products were analyzed as described in the legend to Fig. 1. Lane 11 contains a 19-mer primer as a marker for unreplicated DNA. B, effect of SSB-113 on primer accessibility to exonuclease III. Reactions were performed as described under "Experimental Procedures," with 2 μ M RecA, and 100 nM wt SSB or SSB-113. Degradation products were obtained as described in the legend to Fig. 2. Lane 11 contains a 19-mer as a marker for the non-degraded primer.

terminus, heterologous SSBs, which exhibit a similar property, might be able to substitute for *E. coli* SSB in the *in vitro* TLS reaction. Fig. 4A shows the results of a TLS experiment performed with polV, RecA, and T4 gp32. As expected, in the absence of any SSB, polV and RecA did not show any DNA synthesis activity (Fig. 4A, lane 6). Upon addition of T4 gp32, primer extension by polV was observed up to the abasic site, but not beyond it (Fig. 4A, lanes 4 and 9). T4 gp32 alone did not show any significant primer extension activity (Fig. 4A, lane 12). Because the β subunit sliding clamp and the γ complex clamp loader were previously shown to stimulate the TLS activity of polV (22, 38, 39), we examined their effect on TLS in the presence of T4 gp32. Remarkably, upon the addition of these

processivity proteins polV, in the presence of T4 gp32, was able to significantly bypass the abasic site, as evident from the extension of the primer to a length of 47 nucleotides (Fig. 4A, lanes 5 and 10). Thus, T4 gp32 can support polV-catalyzed TLS in the absence of SSB, although it is less effective than *E. coli* SSB (Fig. 4A, compare lanes 5 to 3, and lanes 10 to 8).

A similar experiment was performed with ICP8, the HSV single-stranded DNA-binding protein, yielding remarkably similar results (Fig. 4B). DNA synthesis up to the abasic site, but not beyond it, was observed when ICP8 was present in the reaction mixture instead of SSB (Fig. 4B, lanes 5 and 11). When the β subunit sliding clamp and the γ complex clamp loader were added, significant bypass across the abasic site by polV was observed in the presence of ICP8 (Fig. 4B, lanes 6 and 12). Similar to the reaction in the presence of T4 gp32 protein, TLS was less effective when *E. coli* SSB was replaced by ICP8. We also examined the effect of human RPA on polV-catalyzed TLS. In contrast to the experiments with T4 gp32 and HSV ICP8, only marginal TLS was observed by polV in the presence of RPA (supplemental Table S2), consistent with its inability to increase accessibility of the 3' primer terminus (supplemental Table S1).

SSB Acts in TLS via a Second Mechanism, Additional to ssDNA Binding—The findings that heterologous SSBs stimulated bypass to a lesser extent than *E. coli* SSB raised the possibility that the latter acts by

a mechanism additional to ssDNA binding. To address the possibility that this mechanism involves specific protein-protein interactions, we utilized the SSB-113 mutant protein, which was previously shown to be defective in interactions with other proteins (16, 19), while maintaining normal ssDNA binding activity (40). A gel mobility shift assay confirmed that our SSB-113 protein maintained wild-type-like ssDNA binding activity (supplemental Table S3). The effects of SSB-113 on both TLS and primer accessibility were examined. Fig. 5A shows the results of a TLS assay, in which wt SSB was replaced with the SSB-113 protein. TLS activity by polV was greatly reduced when the SSB-113 replaced wild-type SSB (Fig. 5A, compare lanes 6–10 to lanes 1–5).

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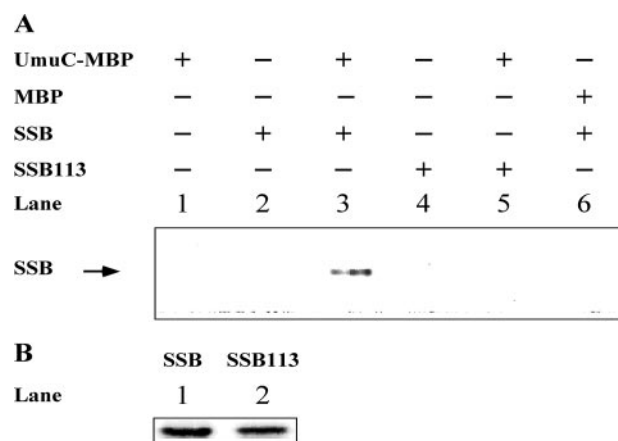


FIGURE 6. Immunoprecipitation analysis of polV-SSB interaction. *A*, lanes 1–6 contain immunoprecipitation reactions performed by the incubation of the indicated proteins at 4 °C for 30 min, as described in “Experimental Procedures.” A monoclonal anti-MBP antibody was then added to each reaction mixture, and incubated for 1 h at 4 °C. Finally, a protein A-coated resin was added to the reaction mixtures, followed by an additional incubation of 1 h. Resin-bound proteins were eluted, fractionated by SDS-PAGE, and analyzed by Western blot analysis using anti-SSB antibodies. *B*, lanes 1 and 2 contain purified SSB (30 ng) and purified SSB-113 (30 ng), respectively, as loading markers (19 kDa).

Next we examined the ability of SSB-113 to relieve the inhibition exerted by RecA on the degradation activity of exonuclease III. Remarkably, the SSB-113 protein was as effective as wild-type SSB in relieving the inhibition exerted by RecA (Fig. 5*B*, compare lanes 1–3 to lanes 6–8). Thus, although the SSB-113 protein had wild-type activity in increasing accessibility of the primer, it was still deficient in supporting TLS by polV.

SSB Interacts with polV—It was previously shown in our laboratory that the plasmid-encoded polV homolog, pol RI (MucA₂B), interacts with SSB (41). Based on the homology between the two polymerases, we hypothesized that SSB also interacts with polV. To examine this possibility, we took an immunoprecipitation approach. UmuC, the catalytic subunit of polV, fused to MBP (MBP-UmuC) was incubated with SSB, after which a monoclonal anti-MBP antibody was added. The antibody was then immobilized on protein A-conjugated beads, and unbound proteins were washed off. The immobilized proteins were solubilized, fractionated by SDS-PAGE, and the gel was blotted with anti-SSB antibodies. As can be seen in Fig. 6*A*, SSB was found bound to the beads when it was incubated with MBP-UmuC (Fig. 6*A*, lane 3). No binding was observed when either of the two proteins was incubated alone (Fig. 6*A*, lanes 1 and 2), or when the MBP tag was incubated with SSB (Fig. 6*A*, lane 6). Noteworthy, when the mutant SSB-113 protein was incubated with MBP-UmuC no binding was observed (Fig. 6*A*, lane 5), implying that this mutation reduces the binding affinity of SSB to polV. The control experiment with SSB-113 alone showed no binding, as anticipated (Fig. 6*A*, lane 4). Similar amounts of SSB and SSB-113 proteins were loaded, as demonstrated by the loading control experiment (Fig. 6*B*).

DISCUSSION

E. coli polV requires several accessory proteins for its bypass activity (1, 3, 42). A key accessory protein is the RecA protein, which was shown to be required for *in vitro* TLS, in addition to

its other known functions in the SOS response (1). RecA forms a nucleoprotein filament on DNA and was originally proposed to help recruit polV to DNA via its interaction with the UmuD' subunit of polV (10). Interestingly, increased concentrations of RecA were found to inhibit polV-catalyzed TLS in our *in vitro* system (36), most likely by covering the primer-template-lesion region, and hindering the access of polV to the primer terminus (30). This does not appear to be merely a matter of modulating *in vitro* assay conditions, because RecA is present at a very high cellular concentration of ~50 μM under SOS conditions (43). The data presented above suggest that SSB relieves this inhibition and functions to target polV to the primer terminus. SSB was reported to be required for TLS across an abasic site by polV (8, 9). Subsequent studies have shown that polV and RecA can promote TLS in the absence of SSB when oligonucleotide substrates were used (44), or when low RecA concentrations, well below the concentration measured in SOS-induced cells, were employed (22). However, under conditions that are more consistent with the cellular ones with respect to DNA size and RecA concentration, SSB is essential for efficient TLS by polV (21, 30, 45).

Two characteristics of SSB are important in its function in TLS: (*a*) It is able to increase the accessibility of the primer terminus to other proteins, as indicated by the exonuclease protection assay. This is mediated most likely via the ability of SSB to bind the template DNA at the site of the primer-lesion junction and locally remodel the RecA filament or promote its local dissociation. This mechanism is supported by the known ability of SSB to strongly bind ssDNA (15) and by the fact that the heterologous ssDNA-binding protein T4 gp32 had similar effects on primer accessibility, as presented in this study. This binding seems to be highly localized, because it did not cause a global collapse of the RecA nucleoprotein filament. (*b*) It interacts with other protein participants in the TLS reaction. This mechanism is supported by the decreased TLS activity observed for SSB-113 and by the observed direct interaction between SSB and polV, as revealed by the immunoprecipitation assay.

The study of the SSB-113 mutant was most revealing, because this mutation separated between two functions of SSB in TLS. The binding of SSB-113 to ssDNA is similar to that of wt SSB, as previously reported (40), and confirmed here. Still, despite the fact that it maintains wild-type ssDNA binding, it is deficient in supporting polV-catalyzed TLS *in vitro*. The SSB-113 mutation, located in the C terminus of SSB, was shown to affect the binding of SSB to other proteins (16, 19, 20, 46, 47). Its reported inherently disordered state may contribute to the plasticity in binding to its partners (48, 49). The observed direct interaction of SSB with polV, via its UmuC subunit, may assist in the recruitment of polV to the primer-template at the site of a lesion. This interaction may be further enhanced by the RecFOR proteins, which are needed for efficient polV-promoted TLS (45, 50–52).

The data presented here propose that SSB and RecA are trading roles during the cellular response to DNA damage that blocks replication. When the replication fork encounters an unrepaired UV lesion, it stops. At this stage the ssDNA in the fork region is bound to SSB, which fulfills an essential role in

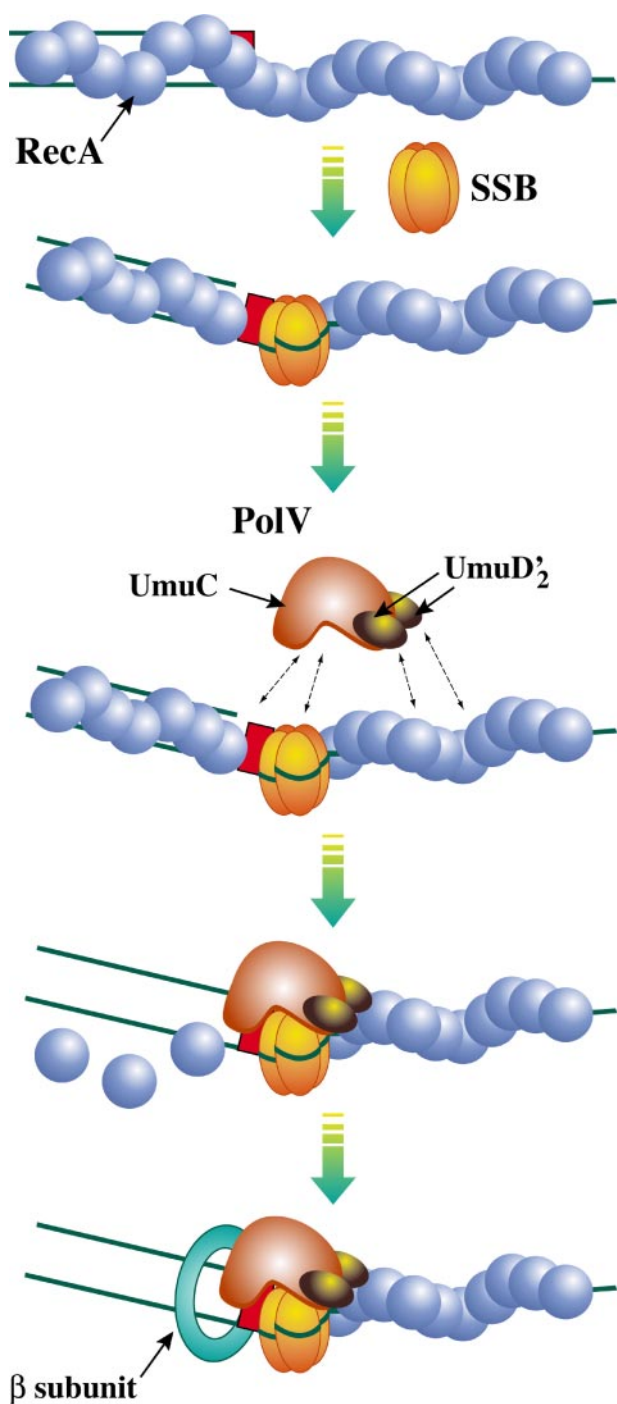


FIGURE 7. A model describing the role of SSB in polV-catalyzed TLS. Binding of SSB to the primer-template-lesion junction on RecA-coated DNA exposes the 3' primer terminus. The interactions between SSB and UmuC, the catalytic subunit of polV, targets it to the primer, possibly aided by the UmuD'₂-RecA interactions. Final positioning of polV is guided by its interaction with the primer-template and binding of the β subunit DNA-sliding clamp. The red rectangle represents the lesion. The dashed arrows represent interaction between the UmuD' subunits of polV and RecA, between UmuC and SSB, and between polV and the primer-template. See text for details.

melting out secondary structures in DNA, and preparing it for replication. However, once replication stops, the exposed state of the ssDNA wrapped around SSB, poses a threat to its integrity, making it vulnerable to breakage. This situation is ameliorated by the RecA protein, which is induced to large amounts in

the cell, and displaces SSB, covering the ssDNA with a shielding sleeve. This stable RecA nucleoprotein filament acts to induce the SOS response and, under the appropriate conditions, promotes homologous recombination. Remarkably, for TLS to occur, SSB returns and, via its ability to bind ssDNA, interacts with polV thus enabling the initiation of TLS.

It was recently proposed that the RecA filament acts in trans, namely from another DNA region (or another molecule) to promote TLS (53). That study was performed in the absence of SSB, despite earlier observations from the same laboratory showing the importance of SSB in TLS (21). Our results do not contradict the results reported by Goodman and coworkers, but they do support a simpler model that does not invoke action in trans. According to our proposed model (Fig. 7), the RecA filament is formed when replication arrests at a lesion, as described above, and provides a protective and regulatory platform for subsequent events. It promotes the autocleavage of LexA leading to induction of the SOS regulon, and it promotes homologous recombination repair, whenever possible as an inherently error-free tolerance mechanism. After UmuC and UmuD are induced, UmuD is processed to UmuD' and polV is formed. SSB binds to the primer-template-lesion junction, recruits polV via its interaction with UmuC, and is perhaps aided by the interaction between UmuD' and RecA (Fig. 7). This reaction may be stimulated by the RecFOR proteins (45).

The *ssb* gene is essential, and therefore only conditional mutants exist. These *ssb-1* and *ssb-113* mutants exhibited UV sensitivity defective UV mutagenesis and defects in induction of the SOS response (34, 54, 55). It was originally suggested that the primary defect in these mutants is in the induction of the SOS response (56). However, this does not preclude also a direct role in TLS, as presented here.

In conclusion, SSB stimulates polV-catalyzed TLS by two independent mechanisms, which are genetically separated by the *ssb-113* mutation: (a) Its ssDNA binding activity increases the accessibility of 3' primer terminus, enabling binding of polV. This may involve either local remodeling or local dissociation of the RecA filament (2). (b) Its direct interaction with UmuC, mediated by its C terminus, helps recruit polV to the DNA.

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