ATP Cross-Linked to *Escherichia coli* Single-Strand DNA-Binding Protein Can Be Utilized by the Catalytic Center of Primase as Initiating Nucleotide for Primer RNA Synthesis on Phage G4oric Template†

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ABSTRACT: We report a new observation of the role of *Escherichia coli* single-strand DNA binding protein (SSB) in synthesis of primer RNA (pRNA) catalyzed by *E. coli* primase on the SSB-coated phage G4oric template. Using a set of ATP priming substrates with reactive groups attached to the 5′ γ-phosphate on different length "arms", we have demonstrated that, in the primase/SSB/G4oric pRNA synthesis complex, ATP cross-linked to both primase and SSB could be equally utilized as initiating nucleotide for pRNA synthesis. The distance between SSB surface and α-phosphorus of the priming substrate was estimated to be less than 7 Å. ATP cross-linked to primase and SSB can be further elongated in the presence of other NTPs, giving almost identical patterns of covalently attached pRNAs of up to 12 nucleotides in length. The regions of primase and SSB with cross-linked ATP that can be used for pRNA synthesis are, therefore, arranged in a similar way relative to the active center of pRNA synthesis. The pRNA covalently linked to SSB was localized, mapping between Met48 and Trp88. This observation raises the possibility that SSB may play an active role in the initiation of pRNA synthesis in this system.

The *Escherichia coli* single-strand DNA-binding protein (SSB) plays an essential role in DNA replication. However, whether SSB is just a passive structural scaffold protein functioning to sequester single-strand DNA or whether or it is involved in some aspects of DNA metabolism is unclear.

In the *E. coli* single-strand DNA (ss-DNA) phage systems that have been used extensively to study the mechanism of action of *E. coli* primase, SSB is an essential component (*1, 2*). On phage G4 template, primase initiates primer RNA (pRNA) synthesis at the T residue of a single specific 5′ CTG 3′ sequence which is in a region of the ss-DNA region called the G4oric. Primase will not synthesize pRNA unless the oric ss-DNA is bound with SSB (*4*), and recently we have shown that a specific SSB/G4oric ss-DNA structure is required for primase to bind and synthesize pRNA (*5, 6*).

The single specific 5′ CTG 3′ pRNA initiation sequence of G4oric has enabled us to use specific ATP affinity labeling (*7, 8*) to identify amino acids at the catalytic center of the primase (*9*). In this method, ATP with a reactive group attached at the terminal 5′ γ-phosphate are used as first substrate in a template-dependent pRNA synthesis reaction. Activating the 5′ reactive group cross-links the ATP to target amino acids. [α-^{32}P]GTP is then added as second substrate. Only ATP covalently attached to amino acids inside the active center for pRNA synthesis can be elongated with radioactive GTP by the catalytic site of primase. This produces a pppA^{32}pG dimer which is covalently attached to the protein. ATP cross-linked to primase outside the active center for pRNA synthesis will not be labeled by addition of the [α-^{32}P]GTP. Interestingly, when we mapped the catalytic center of primase we observed that when ATP reagents with cross-linking arms longer than 12 Å were used, SSB was also labeled with the pppA^{32}pG dinucleotide.

We have now extended these observations and demonstrate that on the G4oric template, ATP cross-linked to SSB can participate in the pRNA chain synthesis by the catalytic site of *E. coli* primase. The surface of SSB containing the cross-linked ATP maps a region between Met48 and Trp88 and is within 5−7 Å of the initiating substrate binding site of primase. The amount and the size distribution of pRNA synthesized from ATP covalently attached to the catalytic center of primase and ATP covalently attached to the surface of SSB are very similar. This suggests that SSB may be part of the active center for pRNA synthesis of pRNA in the primase/SSB/G4oric priming system.

MATERIALS AND METHODS

Materials. [α-^{32}P]GTP (3000 Ci/mmol) was purchased from New England Nuclear. R199/G4oric viral ss-DNA was prepared as described in ref *10*. The structure and synthesis of the 5′ modified ATP derivatives, reagents F and G, are described in refs *8* and *11*. Thrombin was purchased from Sigma.

Plasmids and Proteins. Primase was prepared by inducing *E. coli* BL21 cells containing the overexpressing primase plasmid pGNG1 (*10*) with 0.4 mM IPTG (*12*) and purified

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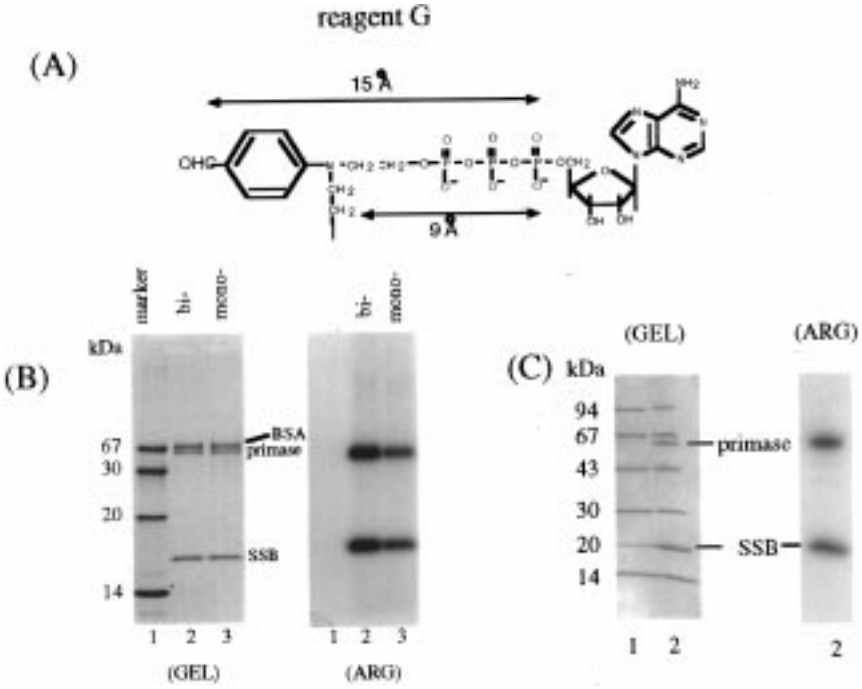

FIGURE 1: ATP cross-linked to SSB can be used as a substrate for pRNA synthesis in the primase/SSB/ss-DNA G4oric system. (A) Structure of reagent G (*11*). (B) The complete pRNA synthesis mixture containing primase, SSB, BSA, and the G4oric ss-DNA template was incubated with reagent G, either in the monofunctional or in the bifunctional mode, and [α-^{32}P]GTP added as second substrate. Both primase and SSB were approximately equally labeled. Left panel, Coomassie Blue-stained gel; right panel, autoradiograph of the same gel. (C) A protein mixture (1 μg) containing approximately equimolar amounts of phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (34 kDa), trypsin inhibitor (20 kDa), and α-lactoglobulin (14 kDa) was added to the synthesis reaction as carrier protein instead of BSA. Left panel, Coomassie Blue-stained gel; right panel, autoradiograph of the same gel.

by passing a 35−50% $NH_4(SO_4)_2$ fraction of a cell lysate through a FPLC Mono Q column. The peak fractions contained less than 1% contaminating proteins and no detectable DNase or RNase activity. SSB was purchased from U.S. Biochemicals. The GST-primase and GST-SSB fusion proteins were prepared from BL21 cells infected with pGEX-2TK plasmid (Pharmacia) containing the primase and SSB genes (to be published). Absorption to Glutathione Sepharose 4B and cleavage of the primase and SSB moieties from the fusion proteins with thrombin followed the procedures described in the Pharmacia manual received with the plasmids.

pRNA Synthesis Reaction. Conditions for pRNA synthesis on the R199/G4oric ss-DNA template are described in ref *13*. Ten picamoles of primase (0.7 μg), 20 pmol of SSB tetramer (1.5 μg), 0.13 pmol of R199/G4oric ss-DNA (0.3 μg), and 30 pmol of bovine serum albumin (BSA) (2 μg) were incubated with 100 μM ATP; CTP, GTP, and UTP (each at 20 μM); and 20 μCi of [α-^{32}P]GTP (3000 Ci/mmol, DuPont, New England Nuclear) for 10 min at 30 °C in a 20 μL volume containing 20 mM Tris-HCl pH 7.5, 8 mM DTT, 8 mM $MgCl_2$, and 4% w/v sucrose (pRNA synthesis buffer).

pRNA Synthesis with Modified ATP Reagents. Modified ATP reagents were used as first substrate in a normal pRNA synthesis reaction in the absence of other NTP's. After treatment with $NaBH_4$ to stabilize the cross-link of the ATP to the target amino acid, [α-^{32}P]GTP was added as second substrate. This resulted in a pppA^{32}pG radioactive labeled dinucleotide covalently attached to catalytic center of primase. The exact protocol depended upon the particular ATP reagent used and whether the reagent was cross-linked to proteins in a complete pRNA synthesis reaction mixture or cross-linked to individual proteins before adding them to the pRNA synthesis reaction.

Labeling Proteins with Reagent G. Reagent G is a bifunctional ATP reagent with an aldehyde group attached to the terminal phosphate and an alkylating group attached to the aromatic nitrogen (Figure 1A). It can be used in a monofunctional mode with only the alkylating group active or in a bifunctional mode with both the aldehyde and alkylating groups active. For the bifunctional mode, 20 pmol (1.5 μg) of SSB tetramer, 0.13 pmol (0.3 μg) of R199/G4oric ss-DNA, 30 pmol (2 μg) of BSA, and 10 pmol (0.7 μg) of primase were incubated with 1 mM reagent G for 10 min at 30 °C in 20 μL of pRNA synthesis buffer (see above). The aldehyde group of the reagent G cross-linked to Lys residues in proteins. $NaBH_4$ was then added to a final concentration of 1 mM, and the mixture was incubated for a further 10 min at 30 °C. This stabilized the cross-link of the aldehyde group with Lys residues and activated the alkylating group which attacked Cys, His, Lys, Tyr, Ser, and Thr residues in descending order. Amounts of 25 μCi of [α-^{32}P]GTP (3000 Ci/mmol) was then added, and the mixture was incubated for a further 10 min at 30 °C to allow pRNA synthesis to proceed. To use reagent G in the monofunctional mode, 1 mM $NaBH_4$ was added to the pRNA synthesis mixture prior to adding reagent G. This immediately destroyed activity of the aldehyde group and activated the alkylating group. After activation with $NaBH_4$, the alkylating group decays with a half-life of 5 min at 30 °C. After pRNA synthesis, the reaction was stopped by adding $^1/_5$ volume of denaturing mixture (5% SDS, 5% DTT, 50% glycerol, and 0.05% bromophenol blue). The labeled products were analyzed on a 4−20% SDS−polyacrylamide gel.

To label SSB alone, primase and R199/G4oric ss-DNA were omitted from the first step of the reaction so that only SSB was exposed to reagent G and $NaBH_4$. Primase, BSA, R199/G4oric ss-DNA, and [α-^{32}P]GTP were added after the $NaBH_4$ treatment. A similar protocol was used to label primase alone.

The amount of radioactive label transferred to primase and SSB was calculated by excising radioactive protein bands from the polyacrylamide gel and counting them in a liquid scintillation spectrometer. From these data, the efficiency of the reaction was calculated as pmol of [α-^{32}P]GMP incorporated per pmol of template DNA. The relative amounts of label transferred to primase and SSB in each reaction was measured by making a digital image of the SDS−polyacrylamide gel using a PhosphorImager screen (Molecular Dynamics) and scanning the screen with ScanMaker III scanner (Microtek) and quantitating the image using ImageQuant program (Molecular Dynamics). Alternatively, an X-ray film exposure of the gel was scanned and similarly quantitated.

Labeling with Reagent F. Reagent F is identical with reagent G except that it does not have the alkylating group but only the aldehyde group. It therefore attacks only Lys residues. Conditions for labeling with reagent F were the same as described for reagent G in the bifunctional reaction.

pRNA Chain Extension from Protein Cross-Linked ATP. Reagent H was used to analyze products of pRNA chain extension from ATP covalently attached to proteins. The bond linking reagent H and the target Lys residue can be acid hydrolyzed so that the covalently attached pRNA products can be cleaved from the proteins and their sizes analyzed on a polyacrylamide gel. The reaction mixture and incubation conditions for reagent H were the same as those described for reagent G, except that the incubation time was longer (60 min; reagent H has a half-life of 60 min at 30 °C) and treatment with $NaBH_4$ was omitted because it was not needed to stabilize the cross-link. An amount of 25 μCi of [α-^{32}P]GTP (3000 Ci/mmol) and 25 μCi of [α-^{32}P]UTP (3000 Ci/mmol) were added immediately after cross-linking, and the mixture was incubated for a further 15 min at 30 °C. ATP, CTP, GTP, and UTP (each at 1 mM) were then added, and the mixture was incubated for 30 min to extend the cross-linked pRNA into longer chains.

Cross-Linking of Reagent G to Primase and SSB That Is Bound to Glutathione Sepharose 4B. Primase and SSB were expressed as GST fusion proteins as described above. Five milliliters of a lysate derived from approximately 100 mL of induced cells was absorbed with 0.1 mL of Glutathione Sepharose 4B for 30 min at room temperature. The Sepharose was washed four times with 0.5 mL of cold PBS buffer using Eppendorf tubes centrifuged at 2000 revolutions per min for 1 min, followed by a single wash with pRNA synthesis buffer and resuspension in 50 μL of the same buffer. Amounts of 5 μL of 5 mM reagent G and 5 μL of 10 mM $NaBH_4$ were added, and the mixture was incubated for 20 min at 30 °C. After incubation, the Sepharose was washed twice with 0.5 mL of cold pRNA synthesis buffer and once with 0.5 mL of cold PBS and resuspended in 50 μL of PBS. A volume of 2.5 μL of thrombin (100 units) was added, and the mixture was incubated at 22 °C for 60 min to cleave primase and SSB moieties from the GST fusion proteins. After digestion, the Sepharose was removed by centrifugation (Eppendorf, 14 000 revolutions per min for 2 min). The amount of primase and SSB recovered was assayed with a Bio-Rad protein assay kit. The purity of the protein was estimated from the Coomassie Blue staining after separation on a 4−20% SDS−polyacrylamide gel. Glycerol (10%) was added to the purified protein, which was then stored at −70 °C.

Complete Digestion of Labeled Protein with CNBr. [α-^{32}P]GTP-labeled SSB or primase was separated from unincorporated radioactivity and other reaction components by SDS−polyacrylamide gel electrophoresis. The radioactive protein bands were excised from the gel. These were washed with water; the gel slices were crushed, and the radioactive protein was eluted by incubation in 0.3% SDS (w/v). The protein was recovered by freeze-drying in a Savant Speed Vac apparatus. To digest the protein, the residue was dissolved in 70% formic acid containing 0.3 M CNBr and incubated at 20 °C for 18 h. Five volumes of water was then added, and the mixture was freeze-dried as before. The residue was dissolved in NOVEX Tricine sample buffer supplemented with 1% 2-mercaptoethanol, and the digestion products were separated on a 16% NOVEX Tricine polyacrylamide gel. The dried gel was autoradioagraphed on Kodak AR film.

Partial Digestion of Labeled Protein with CNBr and N-Bromosuccinimide (NBS). To digest labeled SSB with CNBr under conditions of "single-hit", it was first purified by SDS−PAGE and eluted as described above. The 0.3% SDS eluate was concentrated in vacuo; $^1/_{20}$ volume of 1 M HCl and $^1/_{20}$ volume of 1 M CNBr were added, and the mixture was incubated at 20 °C. Aliquots were taken at time intervals, and the cleavage reaction was stopped by addition of $^1/_3$ volume of a stop solution (500 mM Tris-HCl, pH 8.5, 5% 2-mercaptoethanol, 50% glycerol, and 0.1% bromophenol blue). The reaction products were analyzed on a 8−16% gradient polyacrylamide gel.

To digest labeled SSB with NBS under single-hit conditions, the gel-purified labeled SSB was dissolved in 60 μL of 0.1 M sodium formate, pH 4.0. *N*-Bromosuccinimide (NBS) was added to 20 μL aliquots of labeled SSB to a final concentration of 0.1 and 0.3 mM. After 20 min at 20 °C, the reaction was stopped as described above, and the cleavage products were analyzed by gel electrophoresis.

RESULTS

In previous experiments to map amino acids at the catalytic center of primase using ATP reagents with aldehyde cross-linking groups attached to the 5′ γ-phosphate, we observed that a surface of SSB was within 12−15 Å of the site of phosphodiester synthesis on the template DNA (*9*). To extend this observation, we have now used a more reactive reagent, reagent G, which is bifunctional with an aldehyde group attached to the 5′ γ-phosphate of ATP and an alkylating substituent attached to the aromatic nitrogen atom (Figure 1A). The aldehyde group can react with primary amines such as the ε-amino group of Lys, yielding a Schiff base which can be stabilized with $NaBH_4$. The alkylating group is nonreactive, but can be activated by reduction with $NaBH_4$ to stably interact with Cys, His, Lys, Tyr, Ser, and Thr residues, in descending order. Reagent G, therefore, can be used either in a bifunctional mode with both the alkylating

and the aldehyde group active (adding $NaBH_4$ later) or in the monofunctional mode with only the alkylating group active ($NaBH_4$ added immediately, to inactivate the aldehyde group and activate the alkylating group). The aldehyde and the alkylating groups are 15 and 9 Å, respectively, from the α-phosphorus atom of ATP.

When reagent G was used as the first substrate in the normal primase/SSB/G4oric pRNA synthesis reaction and cross-linked to the proteins, adding [α-^{32}P]GTP as second substrate resulted in synthesis of a pppA^{32}pG dinucleotide which was covalently attached to both primase and SSB (Figure 1B). This indicated that ATP attached to primase or SSB could participate in the template-directed pRNA synthesis. Labeling of primase and SSB were similar when reagent G was used in both the bifunctional and monofunctional modes. The ratio of label transfer to primase and SSB was 1.00:1.04 and 1.00:0.94 in the samples shown in lanes 1 and 2, respectively. This confirmed that in the primase/SSB/G4oric complex, ATP covalently cross-linked to the catalytic center of primase and covalently cross-linked to SSB could be used as a substrate to initiate pRNA synthesis. It should be noted that in the experiment (Figure 1B) mentioned above, BSA was not labeled. When ATP reagent was added to the pRNA synthesis reaction mixture, it could potentially cross-link to all proteins present (i.e., primase, SSB, and BSA). The absence of radioactive transfer labeling of BSA suggested that ATP attached to BSA could not be extended with [α-^{32}P]GTP by the catalytic center of primase. As a further control, BSA was replaced with a mixture of six different proteins ranging in size from 14 to 94 kDa. None of these proteins was labeled (Figure 1C), again indicating the specificity of labeling of primase and SSB.

To quantitate the label transfer, the proteins were separated on a polyacrylamide gel and, after staining and autoradiography, the radioactive bands were excised from the gel and counted in a liquid scintillation spectrometer. Knowing the specific activity of the [α-^{32}P]GTP and the concentration of reactants present in the mixture, the number of [α-^{32}P]GMP molecules incorporated per DNA template was calculated. Summing the radioactive counts attached to primase and SSB, we calculated that 18.2% of the template molecules were used in the synthesis reaction. This amount varied from 10 to 25%, depending upon the cross-linking conditions but are in the range for template molecules used in the normal pRNA synthesis reaction with unmodified ATP. Similar efficiencies of incorporation of cross-linkable ATP analogues into RNA by RNA polymerase have been reported (*7*).

To establish unequivocally that ATP attached to SSB could provide the first substrate in pRNA synthesis, SSB, primase, and BSA were individually cross-linked with reagent G. After cross-linking, the samples were dialyzed to remove free unreacted reagent G. Template DNA and the two unmodified proteins were then added, and the mixture was incubated with [α-^{32}P]GTP to label the cross-linked ATP. As can be seen in Figure 2, when reagent G was cross-linked to primase in the absence of SSB and BSA, only primase was labeled in the pRNA synthesis reaction (lane 2). Similarly, when reagent G was cross-linked to SSB in the absence of primase and BSA, only SSB was labeled by pRNA synthesis (lane 4). The absence of a background of labeling of the unmodified proteins in these reactions indicated that there were no other sources of first substrate available except for

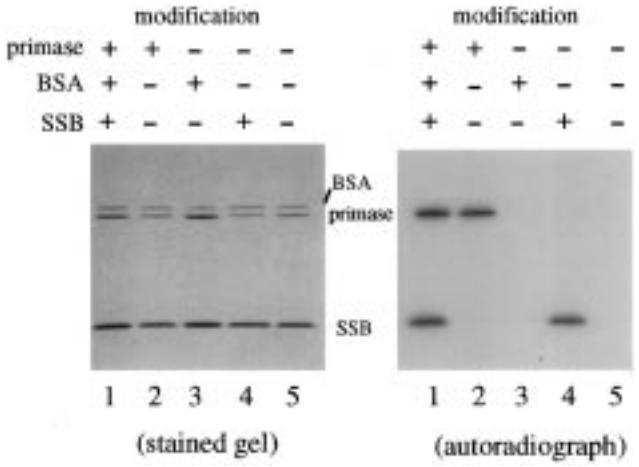

FIGURE 2: Label transfer when reagent G is pre-attached to either SSB or primase. Primase, BSA, and SSB were incubated, either alone or together, with reagent G and $NaBH_4$ for 10 min at 30 °C in a total volume of 20 μL. The reaction mixtures were then transferred to a Pierce microdialyzer and dialyzed at room temperature for 20 min against a large volume of pRNA synthesis buffer. As much as possible of the reaction mixture was recovered (usually 50−75%); template DNA, [α-^{32}P]GTP, and the unmodified proteins were added, and the mixture was incubated for a further 20 min at 30 °C to effect pRNA synthesis. Lane 1, all proteins were reacted with reagent G and $NaBH_4$ before dialysis; lane 2, only primase; lane 3, only BSA; lane 4, only SSB; and lane 5, no proteins were reacted with reagent G and $NaBH_4$ before dialysis. Left panel, Coomassie Blue-stained gel; right panel, autoradiograph of the same gel. Plus signs indicate which proteins were present during reaction with reagent G, and minus signs indicate which protein was added later after dialysis.

ATP cross-linked to the modified protein. When reagent G was cross-linked to BSA and the modified BSA used a source of ATP in the synthesis reaction, no label transfer to primase, SSB, or BSA took place (lane 3). This again indicated that ATP attached to BSA could not enter the catalytic center of the primase/SSB/G4oric complex to become a substrate for pRNA synthesis. Two control reactions containing either all three modified proteins or all nonmodified proteins are shown in lanes 1 and 5, respectively. The ratio of label transfer to primase and SSB in lane 1 is 1.00:1.34. This experiment confirmed the results mentioned above and further demonstrated that the SSB-attached ATP, like the primase-attached ATP, can be used as a source of the first substrate by the catalytic site of primase for the synthesis of pRNA on G4oric template.

Preattachment of Reagent G to Column-Immobilized SSB. To further test whether ATP attached to SSB could participate in the pRNA synthesis, we used GST fusion proteins of primase and SSB and cross-linked reagent G to them when they were immobilized on Glutathione Sepharose 4B. This enabled us to affinity purify primase and SSB after attaching reagent G. Primase and SSB fusion proteins were adsorbed to Glutathione Sepharose 4B and reacted with reagent G in the presence of $NaBH_4$. The adsorbed protein was washed, and then the primase and SSB moieties were cleaved from the bound GST fusion protein with thrombin. The modified primase and SSB were then used in the normal pRNA synthesis reaction together with the appropriate unmodified components. Figure 3 shows the Coomassie-stained gel and an autoradiograph of the radioactive labeling. The modified SSB (lanes 2 and 4) migrated noticeably more slowly than unmodified SSB (lanes 1 and 3). It was calculated from

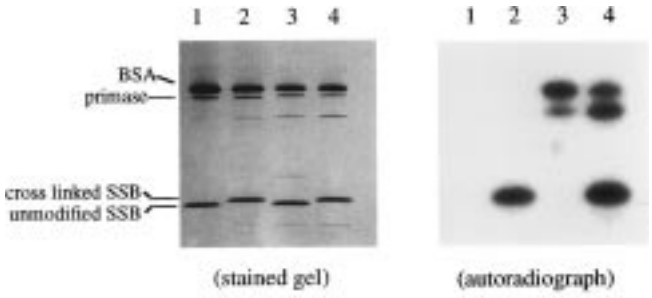

FIGURE 3: pRNA synthesis with affinity purified, modified primase and SSB. Primase and SSB were expressed from pGEX-2TK plasmids as GST fusion proteins and adsorbed to Glutathione Sepharose 4B. A small aliquot of Sepharose 4B containing the adsorbed fusion protein was incubated with reagent G and $NaBH_4$ for 10 min at 30 °C. After extensive washing to remove free reagent G, the primase and SSB moieties were cleaved from the adsorbed fusion proteins with thrombin. After quantitation, the cleaved modified primase and SSB were used directly in pRNA synthesis reactions in various combinations with unmodified primase and SSB. Lane 1, unmodified primase and unmodified SSB; lane 2, modified SSB and unmodified primase; lane 3, modified primase and unmodified SSB; and lane 4, modified primase and modified SSB. Left panel, Coomassie Blue-stained gel; right panel, autoradiograph of the same gel.

the migrations that the difference in molecular mass of the modified and unmodified SSB was approximately 1000 Da, which corresponded to the addition of one reagent G molecule. The modified primase migrated slightly more slowly than the unmodified primase, which can be seen if lanes 1 and 2 are compared with lanes 3 and 4. These changes in migration confirmed that the proteins were modified by addition of reagent G. When modified SSB and unmodified primase were used in the pRNA synthesis reaction, SSB was labeled and primase was not labeled (lane 2). When modified primase and unmodified SSB were used, only primase was labeled (lane 3). The two radioactive bands in lane 3 are primase and the P47 kDa N-terminal fragment that was cleaved from primase by thrombin (primase has an inefficient thrombin cleavage site between the N-terminal P47 and C-terminal P16 domains which can be seen in the Coomassie-stained gel). When both modified primase and modified SSB were used for pRNA synthesis, both were labeled (lane 4). When unmodified primase and unmodified SSB were used for pRNA synthesis, neither protein was labeled with [α-^{32}P]GTP (lane 1).

This experiment again confirmed the conclusion that ATP covalently cross-linked to SSB could be used by the catalytic site of primase as the source of first substrate for pRNA synthesis on a G4oric DNA template.

The Labeled Cross-Linked ATP on SSB Is within 7 Å of the Site of Phosphodiester Synthesis. To determine how close the face of SSB was to the site of phosphodiester synthesis on G4oric DNA template, we used derivatives of reagent G with shorter cross-linking arms (Figure 4A). These reagents had the same aldehyde and alkylating reactive groups as reagent G, but instead of being attached to ATP, were attached to ADP (G2) and AMP (G1). This shortened the reactive arms by 2 Å per phosphate group, so that the alkylating cross-linking arm of reagents G2 and G1 were 7 and 5 Å away from the α-phosphate atom, respectively, and the aldehyde group 13 and 11 Å away, respectively.

When reagent G2 was used under bifunctional conditions in the complete pRNA synthesis reaction so that SSB and

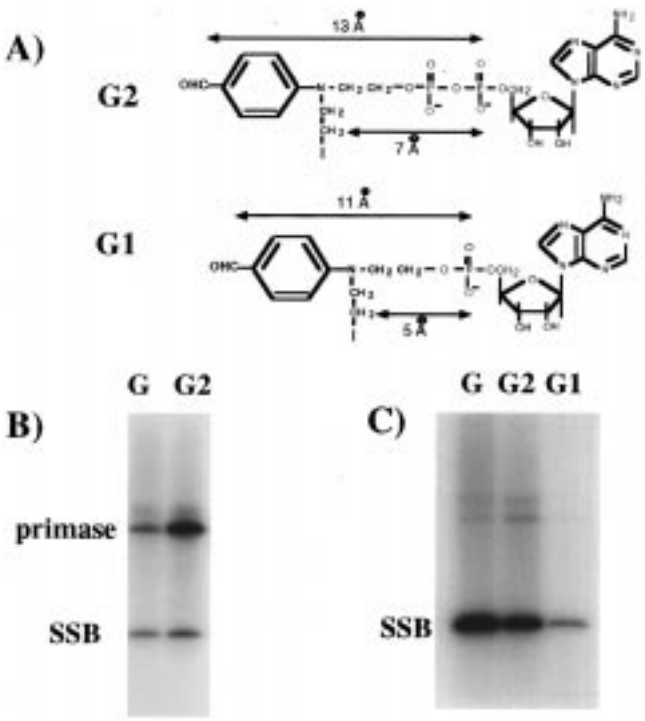

FIGURE 4: Labeling of primase and SSB with derivatives of reagent G with shorter cross-linking arms. (A) Structure of the ADP (G2) and AMP (G1) derivatives of reagent G (*11*). (B) In lanes 1 and 2, reagents G and G2 were used under bifunctional cross-linking conditions in the complete pRNA synthesis reaction. (C) SSB was incubated with reagents G, G2, and G1 and $NaBH_4$ for 15 min, and then primase and template DNA were added with [α-^{32}P]GTP; the mixture was incubated for a further 15 min at 30 °C. The proteins were separated on a 4−20% gradient polyacrylamide gel and autoradiographed.

primase were exposed to the reagent in the same complex, both primase and SSB were labeled, as with reagent G. However, reagent G2 labeled primase more than SSB (Figure 4B). The ratio of labeling of primase to SSB with reagent G was 1.00:1.14 and with G2 was 1.00:0.70. When reagents G, G2, and G1 were cross-linked to SSB in the absence of primase and in the presence of $NaBH_4$ [i.e., only the alkylating arm (9, 7, and 5 Å, respectively) was active] and the modified SSB used in the pRNA synthesis reaction as the source of first substrate, AMP attached to SSB by all three reagents could participate in pRNA synthesis (Figure 4C). Relative labelings of SSB by G, G2, and G1 were 1.00, 0.78, and 0.21, respectively [as these samples were not dialyzed to remove undecayed reagent, there is a slight background (5%) of labeled primase].

We interpret these results to mean that in the primase/SSB/R199G4 complex, a surface of SSB is within 5 to 7 Å of the site of catalysis on the DNA template.

pRNA Synthesized from ATP Covalently Attached to Primase and SSB Are Similar. The approximately equal intensity of label transfer to primase and SSB observed when reagent G was used in the complete pRNA synthesis reaction (Figures 1 and 2) suggested that ATP attached to primase and to SSB behaved similarly in template-directed pRNA synthesis. This might be expected if the surface of SSB formed part of the active center for pRNA synthesis on the template DNA and would predict that ATP cross-linked to primase and SSB would be extended by continued synthesis to give a similar population of cross-linked pRNA molecules.

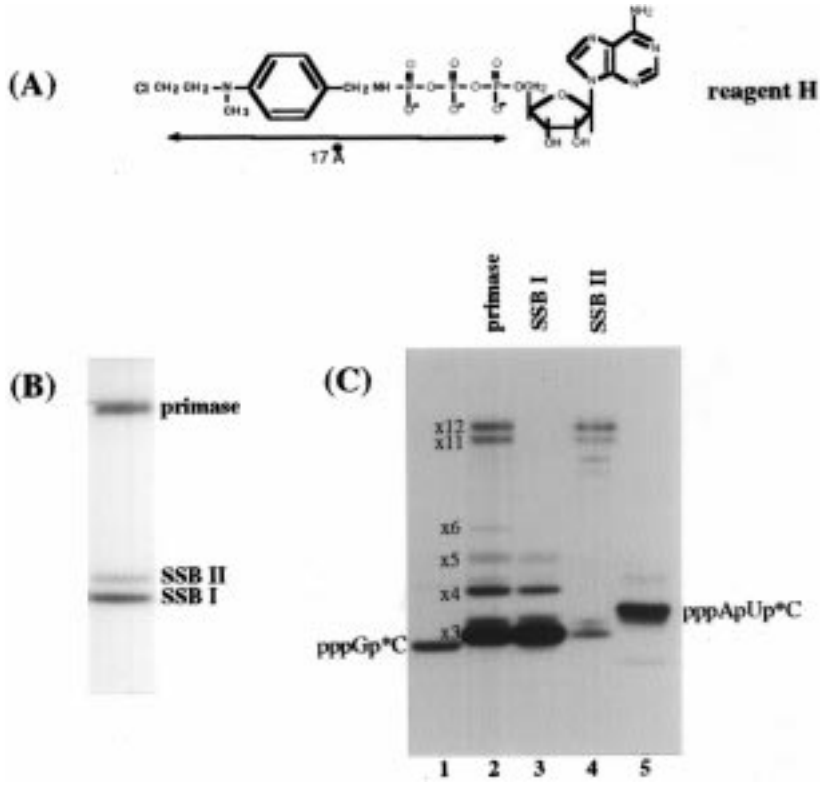

FIGURE 5: Size distribution of pRNA synthesized from ATP attached to primase and SSB in the same pRNA synthesis reaction. (A) Structure of reagent H. (B) The complete pRNA synthesis reaction mixture was incubated with reagent H for 60 min at 30 °C. An amount of 25 μCi each of [α-^{32}P]GTP and [α-^{32}P]UTP were then added and the incubation was continued for another 15 min. ATP, CTP, GTP, and UTP (each at 1 mM) were then added, and the mixture was incubated for a further 30 min to extend the tethered pRNA. Labeled primase and SSB were separated on a 16% SDS−polyacrylamide gel, and the wet gel was autoradiographed. (C) Labeled primase and the upper and lower SSB bands were excised from the gel; the protein was eluted, and the covalent link between the Lys and the ATP residue was cleaved with 10 mM HCl at 37 °C for 60 min. The released pRNA was recovered by ethanol precipitation and separated on a 23% polyacrylamide gel containing 7 M urea. The gel was dried and autoradiographed. pppG^{32}pC and pppApU^{32}pC were used as small size markers. These were synthesized by abortive RNA synthesis using RNA polymerase (*11*). [γ-^{32}P]ATP end-labeled oligonucleotides (12−30 nt) were used as larger size markers (not shown).

To examine this reaction, a different ATP derivative, reagent H (Figure 5A), was used. This reagent has a reactive group that cross-links to nucleophilic amino acid residues with a phosphoramide (P−N) bond which can be acid-hydrolyzed to release the attached pRNA chains. The cross-linking arm of reagent H is 17 Å in length. Reagent H was incubated with the complete primase/SSB/R199G4oric mixture, so that both the SSB and primase were cross-linked in the same reaction. [α-^{32}P]GTP and [α-^{32}P]UTP were added for 10 min, followed by a chase unlabeled NTP. ([α-^{32}P]-UTP was added to the reaction as well as [α-^{32}P]GTP because the longer pRNA chains contain several U residues). When the radioactive proteins were separated on a 16% SDS−polyacrylamide gel, both primase and SSB were labeled. The ratio of labeling of primase to SSB was 1.00: 1.25 (Figure 5B), which was similar to the results of cross-linking with reagent G. This suggested that the two reagents had a similar affinity to proteins in this system. Interestingly, both labeled primase and SSB gave two closely migrating bands, with less label in the upper band (Figure 5B). The two SSB bands were excised separately from the gel, but the two primase bands migrated too closely to be individually excised and were cut out as a single band. pRNA was cleaved from the eluted proteins and analyzed on a 23% polyacrylamide−7 M urea gel (Figure 5C). The size distribution of pRNA attached to SSB and primase was identical, except that the pRNA penta-, tetra-, and trinucleotide chains were attached to the smaller SSB band (SSB I) and the longer pRNA chains (11 and 12 nt plus a ladder of smaller species) were attached to the larger SSB band (SSB II). The slower migration of the upper SSB band was therefore due to attachment of the longer pRNA chains.

This experiment demonstrated that there was no difference in the size distribution of pRNA synthesized from ATP attached to either primase or SSB and that pRNA chains up to 12 nt in length could be synthesized from ATP attached to both of them. ATP attached to both SSB and primase, therefore, appeared to participate equally in the active center where phosphodiester bond synthesis takes place on the G4oric template DNA.

Mapping the Cross-Linking Site on SSB. To investigate which part of the surface of SSB participated in the active center during pRNA synthesis, we mapped the location of the cross-linked pppA^{32}pG dinucleotide by the complete and partial chemical cleavage. First we used reagent F, which is similar to reagent G in that it has the 15 Å aldehyde Lys-specific cross-linking group, but does not have the alkylating group. Then we used reagent G in the bifunctional mode which has a larger target area as it attacks not only Lys residues, but also Cys, His, Ser, Tyr, and Thr residues. These reagents were cross-linked to SSB or primase before assembling the G4oric complex.

Purified labeled primase and SSB were digested to completion with CNBr (cleaves at Met residues), and the

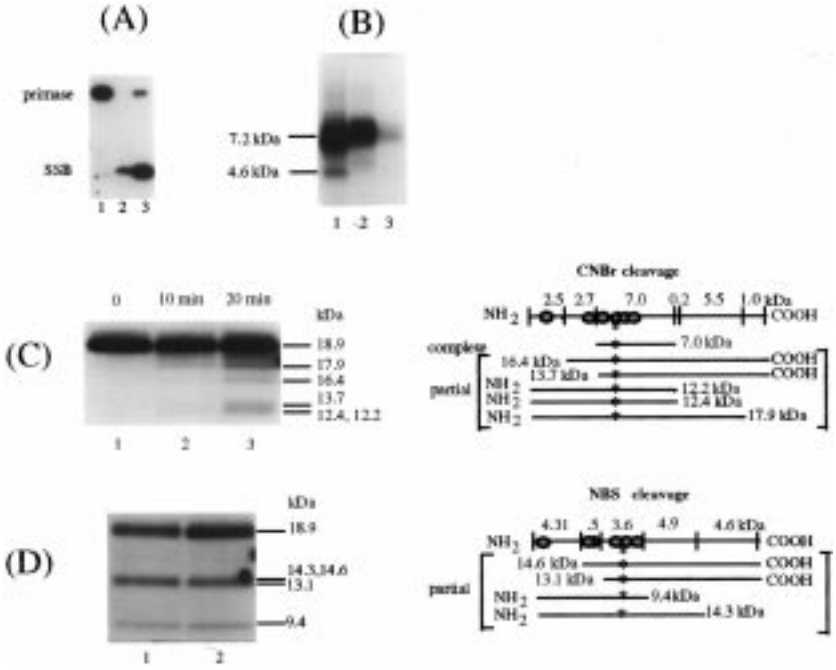

FIGURE 6: Mapping of the amino acids in SSB cross-linked to the pppA[32]pG dinucleotide by reagents F and G. The position of the cross-linked amino acids in SSB was determined by the chemical cleavage mapping procedures (*8*) that had been used to map the catalytic center of primase (*9*). (A) Primase (lane 1) and SSB (lane 2) were cross-linked with reagent F in the absence of the other reaction components and then were labeled with [α-^{32}P]GTP. SSB was also cross-linked with reagent G before labeling (lane 3). (B) Purified labeled primase and SSB were digested to completion with CNBr, and the digestion products were separated on a 16% Tricine polyacrylamide gel. The starting materials for digestion were the following: lane 1, primase labeled using reagent F; lane 2, SSB labeled using reagent G; and lane 3, SSB labeled using reagent F. Sizes of the digestion fragments were measured using fluorescent protein size markers and by referring to the known sizes of the primase 7.2 and 4.6 kDa CNBr fragments (*9*). (C) The location of the cross-linked Lys residues in reagent F labeled SSB was mapped by single-hit cleavage with CNBr. Left panel, autoradiograph of the labeled fragments after single-hit digestion of labeled SSB with CNBr; right panel, map of the CNBr cleavage sites and position of Lys residues (filled circles) on SSB together with all partial digestion fragments that would be derived from a single-hit from both ends of SSB, assuming the 7.0 kDa CNBr fragment contained the cross-linked Lys residue (star). Sizes of the cleavage fragments in kDa are given above the map. The sizes of the theoretically labeled fragments are shown next to the experimental gel autoradiograph in the left panel. (D) Labeled SSB was digested with *N*-bromosuccinimide (cleaves at Trp residues) under single-hit conditions. Left and right panels are as above. The amino acid sequence of SSB was taken from ref *23*.

digestion products were separated by SDS−polyacrylamide electrophoresis. Primase labeled using reagent F gave two radioactive CNBr fragments, which migrated at approximately 7.2 and 4.6 kDa, respectively (Figure 6B, lane 1), as previously reported (*9*). CNBr digestion of SSB labeled using reagent G or reagent F gave a single labeled fragment migrating close to the 7.2 kDa primase CNBr fragment (Figure 6B, lanes 2 and 3). This radioactive SSB fragment corresponded to a 7.0 kDa SSB CNBr fragment (see the CNBr cleavage map shown in Figure 6C). The next largest SSB CNBr fragment is 5.5 kDa in size and does not contain Lys residues, the exclusive target of reagent F. The 7.0 kDa SSB fragment spans amino acids 49−110, and contains four Lys residues (Lys49, Lys62, Lys73, and Lys87) that would be targets for both reagents F and G. His55, Ser68, Ser75, Ser92, Tyr78, and Tyr97 would be additional targets for reagent G.

To confirm that the Lys residues cross-linked with reagent F were in the 7.0 CNBr fragment, their position was mapped by single-hit CNBr digestion. In this method, the smallest labeled fragment corresponds to the smallest single-hit partial digestion product from either the N- or C-terminus (*8*). The size of this fragment defines the end from which it is derived. The pattern of larger labeled fragments consists of a mixture of single-hit peptides from both the N- and C-termini. The results are given in Figure 6C. The patterns of radioactive partial cleavage products (left panel) fitted the theoretical patterns (right panel) derived under the assumption that the target amino acids resided within the 7.0 kDa CNBr fragment. After single-hit cleavage with CNBr, the smallest labeled fragments were a pair migrating at approximately 12.2 and 12.4 kDa (Figure 6C, left), which corresponded to the smallest partial N-terminal digestion product containing the 7.0 kDa CNBr fragment (Figure 6C, right). If the labeled SSB was digested with *N*-bromosuccinimide (NBS), which cleaves at Trp residues (*14*) under single-hit conditions, the smallest labeled cleavage fragment was 9.4 kDa. This placed the cross-linked amino acids on the N-terminal side of Trp88 (Figure 6D). The target area of reagent F must therefore be within the region spanned by Met48 and Trp88.

DISCUSSION

The approximately equal label transfer to primase and SSB when reagent G was cross-linked to the primase and SSB indicates that the surface of SSB must also be very close to the site of catalysis. The shorter arm derivatives of reagent G were less efficient in providing the first substrate for pRNA synthesis when they were cross-linked to SSB than when cross-linked to primase. This indicates that the surface of SSB is further away from the site of catalysis than the involved regions of primase, but within 5 to 7 Å of the active site of pRNA synthesis. In our earlier study of the catalytic center of primase, we showed that three amino acids from different regions of primase were within 3 Å of the site of

catalysis. These are His43 of the N-terminal zinc finger motif, Lys241 of catalytic center, and Lys528, which is close to the C-terminus. The site of pRNA synthesis on the template DNA must therefore be bounded by four different protein surfaces, three regions of primase, and one of SSB.

Involvement of surfaces of two different polypeptide chains in the active site for template-directed pRNA synthesis has a precedent in *E. coli* RNA polymerase. In this case, the site for initiating substrate binding and RNA synthesis resides on the interface of σ and β subunits. By using the same set of cross-linking derivatives as that used in our present study, it was demonstrated that the σ subunit was 7 Å away from the α-phosphorus atom of the initiating substrate (*11*). Both the σ subunit of RNA polymerase and SSB therefore bind to DNA close to the initiation site of RNA synthesis in these systems. The σ has a newly recognized site (TWW_{434}IRQAITRSIAD) which has been shown by crystallography (*15*) to bind to ss-DNA in the open −10 promoter complex. Interestingly, the SSB DNA binding site involves Trp54 and Phe60 which is in a similar sequence TEW_{54}HRVVLF$_{60}$GKLAE (*16*, *17*). SSB and the σ subunit of RNA polymerase may therefore have similar roles in establishing the active center of phosphodiester synthesis on the template DNA.

SSB is required for most aspects of DNA metabolism that involve transient single-stranded DNA states, and the direct interaction of SSB with some critical proteins that recognize DNA has been reported. In the ϕX174 primosome system, where SSB is required for DNA replication, PriB protein, which recognizes the specific primosome assembly site (pas) on the ss-DNA, directly interacts with SSB (*18*). In the recF recombination pathway, SSB directly interacts with RecF protein to form a RecF/RecO/SSB complex (*19*), and in the DNA repair, where SSB is required for efficient base excision and exonuclease I has been shown to interact in vivo with SSB (*20*). SSB is also required for the replication of R-plasmids and has been shown to be involved in the pRNA synthesis step (*21*). The newly recognized F*rpo* single-stranded DNA promoter that directs RNA polymerase to initiate transcription at a specific site requires SSB to position the polymerase in a stem−loop structure (*22*), which is similar to that described for primase on the G4oric. These observations, together with our report of the involvement of SSB in pRNA synthesis on G4oric, suggest in some aspects of DNA metabolism SSB may play a more active role in DNA than the passive structural role that is normally assigned to it.

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