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Mechanisms of DNA Binding and Regulation of *Bacillus anthracis* DNA Primase

Subhasis B. Biswas^{*}, Eric Wydra, and Esther E. Biswas

Department of Molecular Biology, University of Medicine & Dentistry of New Jersey, Stratford, NJ 08084

Abstract

DNA primases are pivotal enzymes in chromosomal DNA replication in all organisms. In this article, we report unique mechanistic characteristics of recombinant DNA primase from *Bacillus anthracis* (*B. anthracis*). The mechanism of action of *B. anthracis* DNA primase (DnaG_{BA}) may be described in several distinct steps as follows. Its mechanism of action is initiated when it binds to single-stranded DNA (ssDNA) in the form of a trimer. Although DnaG_{BA} binds to different DNA sequences with moderate affinity (as expected of a mobile DNA binding protein), we found that DnaG_{BA} bound to the origin of bacteriophage G4 (G4ori) with approximately eight-fold higher affinity. DnaG_{BA} was strongly stimulated (≥ 75 -fold) by its cognate helicase, DnaB_{BA} during RNA primer synthesis. With the G4ori ssDNA template, DnaG_{BA} formed short (≥ 20 nucleotides) primers in the absence of DnaB_{BA}. The presence of DnaB_{BA} increased the rate of primer synthesis. The observed stimulation of primer synthesis by cognate DnaB_{BA} is thus indicative of a positive effector role for DnaB_{BA}. By contrast, *E. coli* DnaB helicase (DnaB_{EC}) did not stimulate DnaG_{BA} and inhibited primer synthesis to near completion. This observed effect of *E. coli* DnaB_{EC} is indicative of a strong negative effector role for heterologous DnaB_{EC}. We conclude that DnaG_{BA} is capable of interacting with DnaB proteins from both *B. anthracis* and *E. coli*; however, between DnaB proteins derived from these two organisms, only the homologous DNA helicase (DnaB_{BA}) acted as a positive effector of primer synthesis.

Chromosomal DNA replication in *E. coli* requires many proteins and enzymes that work in unison to execute the initiation, elongation and termination stages of DNA synthesis (1-3). At the origin of *E. coli* replication, *oriC*, DnaA protein initiates the process through localized melting of the origin and subsequent recruitment of other replication proteins to form a large nucleoprotein complex (4-6). DnaB protein joins the DnaA•*oriC* complex and unwinds the duplex DNA at the partially melted origin. The entry of DnaB protein at *oriC* requires the complex formation of DnaB protein with DnaC protein. The DNA binding activity of DnaB

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^{*}To whom correspondence should be addressed: Subhasis B. Biswas, Department of Molecular Biology, University of Medicine & Dentistry of New Jersey, Stratford, NJ 08084. Telephone: 856-566-6270; FAX: 856-566-6291; E-mail: subhasis.biswas@umdnj.edu..

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is enhanced by its association with DnaC; by contrast, its ATPase activity is attenuated (7-10). With the help of DnaA, DnaC guides DnaB to the DnaA•*oriC* complex.

In the replication of *E. coli* RK2 plasmid DNA, DnaA plays a similar role in delivering DnaB•DnaC complex to the replication origin of the RK2 plasmid and activating the helicase activity with plasmid-encoded TrfA protein (11-13). Incidentally, during the replication of bacteriophage λ , the phage-encoded λ P protein binds to DnaB protein, shuts off its ATPase activity, and guides it to the activated λ origin (8,14,15). The activated λ origin is created by phage-encoded λ O protein (the functional equivalent of DnaA protein) binding to the λ origin (14-17). A comparison of the two replication systems clearly indicates that DnaB protein plays a crucial role in determining the fate of replication at each origin (8). The presence of DnaB protein appears to be sufficient for other replication proteins, such as primase and DNA polymerase III holoenzyme to follow and join the complex afterwards.

After DnaC or λ P protein departs from the origin complex, DnaG primase is recruited to the replication fork through a transient protein-protein interaction with DnaB in the origin (5). Unlike holoenzyme, DnaG can initiate and elongate short RNA primers, which are then extended by the holoenzyme (18-21). DnaG synthesizes RNA primer or prime only once to initiate leading strand DNA synthesis. However, it must prime repeatedly on the lagging strand and these primers lead to the synthesis of Okazaki fragments (22). It has been demonstrated that primase initiates *in vivo* Okazaki fragment synthesis of *E. coli* chromosome and G4-bacteriophage. Initiation predominantly occurs from unique regions containing CTG trinucleotide regions (20,21). DNA primases are very specific in choosing priming sites during the initiation event, but its specificity is reduced significantly in the presence of DnaB, which allows primase to synthesize multiple primers on the lagging strand of the replication fork (18). Kinetic analysis suggest that *E. coli* primase acting alone is the slowest RNA polymerase with an *in vitro* rate of approximately one primer per second, which may be accelerated by the addition of DnaB protein (19,21,23,24). It is thus highly likely that DnaB•DnaG complex formation triggers lagging strand primer synthesis.

In the *E. coli* replication fork, primase acts distributively; for example, the frequency of primer synthesis increases with the concentration of primase (25,26). The average length of the Okazaki fragments decreases correspondingly because the length of Okazaki fragments is inversely proportional to the frequency of primer synthesis. Primase binding to a ssDNA template may therefore play an important role in both the rate and the extent of primer synthesis. Even though primase exists primarily as a monomer in solution, we have previously shown that it forms a multimeric-primase•DNA complex in the absence of DnaB_{EC} (20,21).

Despite well-known diversity among prokaryotic organisms, studies on DNA replication in prokaryotes generally have focused only on *E. coli* and its bacteriophages. *E. coli* is a gram-negative bacterium; however, most of the emerging pathogens are gram-positive and the DNA replication process in this class of prokaryotes remains poorly understood (27). It is possible that the replication enzymes and proteins in gram-positive prokaryotes would have significantly different characteristics than those found in *E. coli*. The genomes of a large number of gram-positive pathogens, including *B. anthracis*, have been completely sequenced (28,29). Though it is still not possible to grow these bacteria in large scale and purify their replication proteins, biochemical analysis of their replication processes is now feasible due to the ability to clone and express their individual replication protein components. Previous studies of gram-positive DNA primase from *Staphylococcus aureus* were hampered due to the fact that recombinant protein expressed by *E. coli* is completely insoluble and can only be studied as glutathione S-transferase (GST) fusion protein with a large GST appendage (30, 31). In contrast to *E. coli* primase, DNA primase purified from high temperature bacterium, *Bacillus stearothermophilus*, (DnaG_{BS}) appear to form a stable complex with its cognate

DnaB_{BS} (32). This complex contains three DnaG_{BS} per DnaB hexamer. Bailey et. al. resolved the X-ray structure of a complex of DnaB_{BS} with the helix binding domain of DnaG_{BS} at 2.9 Å resolution and it demonstrated that the two proteins formed a complex with 6:3 ratio (33).

The present study demonstrates that the replicative DNA primase from gram-positive bacteria exhibits novel mechanisms of action, rarely observed for other classes of bacterial primases.

MATERIALS & METHODS

Nucleic Acids, Enzymes, Oligonucleotides and Other Reagents

Ultra pure ribonucleotides were obtained from GE Biosciences (Piscataway, NJ) and were used without further purification. [α -³²P] UTP was obtained from Perkin Elmer Inc. (Boston, MA). All other chemicals used to prepare buffers and solutions were reagent grade and were purchased from Fisher Chemical Company (Pittsburgh, PA). Ion exchange resins (POROS-Q, and POROS-S) were from Applied Biosystems Inc. (Foster City, CA). Preparative HPLC gel filtration column, Bio-Sil TSK250 (21.5 mm × 60 cm), was obtained from BioRad Inc. (Hercules, CA) and was used in a Biocad 20 HPLC system from Applied Biosystems Inc. Analytical HPLC gel filtration column (7.8 mm × 30 cm) was from Tosoh Biosciences LLC (Montgomeryville, PA).

Buffers

Lysis buffer was: 25 mM Tris-HCl, (pH 7.9), 10 % sucrose and 250 mM NaCl. Buffer A was 25 mM Tris-HCl, (pH 7.5), 5 mM MgCl₂, 10% glycerol, 5 mM DTT and NaCl as indicated. Buffer B contained 20 mM Tris-HCl (pH 7.5), 5 mM MgCl₂ and 10% glycerol, and KCl as indicated.

Oligonucleotides

G4ori: 5'-GCCGTCCCTACTGCAAAGCCAAAAGGA-3';

Fl-G4ori: 5'-FAM- GCCGTCCCTACTGCAAAGCCAAAAGGA-3';

Sixtymer: 5'-GGGGTCTCACGACGTTGTAAAACGACTGCAGCCGTTGTCGAGC TCGGTACCC GGGGTAGGA-3'.

Cloning and Expression of DnaG_{BA}

DnaG_{BA} gene was amplified by PCR using genomic DNA from *Bacillus anthracis* strain 9131, obtained as a gift from Dr. Theresa M Koehler of the University of Texas Houston Health Science Center, Houston, TX [28]. This ORF codes for a 598 amino acid polypeptide with a predicted molecular weight of 68.3 kDa. The amplified gene was inserted in a pET30a vector (Novagen Inc., Madison, WI) under the control of a T7 promoter (pET30a-DnaG_{BA} recombinant plasmid).

Purification of DnaG_{BA}

DnaG_{BA} was purified from *E. coli* BL21(DE3)RIL strain (Stratagene Inc., La Jolla, CA) harboring pET30a-DnaG_{BA} plasmid. *E. coli* cells were grown in 8 liter batches in 2xYT media containing 50 µg/ml kanamycin, 12 µg/ml chloramphenicol at 37°C to an OD₆₀₀ of 0.4-0.6 and 1-isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added to a final concentration of 0.25 mM. The cells were grown for an additional twelve hours at 12-13 ° and then harvested by centrifugation at 8000 rpm for 10 minutes at 4 °C. The cell pellet was resuspended in lysis buffer (25 mM Tris.HCl, pH 7.9, and 10% sucrose) and frozen at -80°C, until further use. Cells were thawed on ice, adjusted to pH 8.0 with 1 M Tris base, and lysed using 0.25 mg/ml

lysozyme, 5 mM MgCl₂, 5 mM spermidine•HCl, and 2.5 mM DTT by incubating on ice for 60 minutes. This is followed by five-minute incubation at 37 °C. The mixture was homogenized on ice followed by centrifugation. The lysate was centrifuged at 19,000 rpm for 30 minutes and 4°C. The supernatant was precipitated overnight using 0.25 g/ml ammonium sulfate on ice followed by centrifugation. The precipitate was collected by centrifugation at 18,000 rpm for 30 minutes and 4°C. The protein pellet was resuspended in buffer A (Fraction II).

DnaG_{BA} protein was first purified by POROS-Q anion exchange chromatography (Applied Biosystems Inc., Foster City, CA). The salt concentration of DnaG_{BA} Fraction II was adjusted to the conductivity of buffer A₂₅ (Buffer A with 25 mM NaCl) by diluting with buffer A₀. The protein was then passed through a 5 ml POROS-Q column equilibrated with buffer A₂₅. DnaG_{BA} protein was eluted with a 100 ml gradient from A₂₅ to A₃₀₀. The DnaG_{BA} fractions, identified by SDS-PAGE, were pooled (Fraction III) and bound to a 5 ml POROS-S column equilibrated with buffer A₁₀₀. DnaG_{BA} protein was eluted with a gradient of buffers A₁₀₀ and A₅₀₀. The fractions were analyzed by SDS-PAGE. The DnaG_{BA} containing fractions were pooled and concentrated by ultra-filtration using Millipore YM30 membrane. Finally, it was purified to homogeneity by HPLC gel filtration using a BioRad TSK gel filtration column (2.1×60 cm). Purified DnaG_{BA} was ≤99% pure. Protein homogeneity was determined by SDS-PAGE.

RNA Primer Synthesis Assay

RNA primer synthesis was carried out essentially as described by Stayton and Kornberg (21), with minor modifications. Briefly, the typical 25-μl reaction mixture contained 20 mM Tris-HCl (pH 7.5), 8 mM dithio-threitol (DTT), 4% (w/v) glycerol, 80 μg/ml bovine serum albumin, 8 mM MgCl₂, GTP, and CTP each at 100 μM, 0.5 mM ATP, 20 μM of UTP, 0.125 μCi [α -³²P] UTP, 5 pmol of ssDNA template with enzymes as indicated. Samples were incubated at 30 °C for 15 minutes unless otherwise indicated. Reactions were stopped by chilling on ice and purified by passing through a micro gel filtration column. Purified primed template was ethanol precipitated with 40 μg of glycogen and 3-volume 100% ethanol and incubated at -80 °C. Pellets were air-dried and resuspended in 8 μl of formamide loading buffer (95% formamide, 20 mM EDTA, 0.01% bromophenol blue and 0.01% xylene cyanol).

RNA Primer Analysis

RNA primers were analyzed by denaturing polyacrylamide gel electrophoresis. Samples were heated at 95 °C for 2 min and loaded immediately onto a 20% polyacrylamide sequencing gel (15 × 50 cm, 0.4 mm thick) containing 7 M Urea and 1 × TBE (89 mM Tris-borate, pH 8.3, 2.5 mM EDTA). Electrophoresis was carried out for 4 h at 55 W in 1 × TBE buffer. Gels were dried and exposed to Fuji RX film at -80 °C for 12-24 h.

Electrophoretic Mobility Shift DNA Binding Assay (EMSA)

EMSA was carried out as described earlier (19,34). Briefly, reactions were carried out in a 25 μl final volume of binding buffer (25 mM Tris-HCl (pH 7.5), 10% (v/v) glycerol, 0.1 mg/ml BSA, 10 mM MgCl₂ and 5 mM DTT) containing 200 pg of ³²P labeled oligonucleotide, 100 ng poly(dI.dC), and protein as indicated. The binding reactions were allowed to proceed for specified time, after which 2 μl of 0.1% bromophenol blue in loading buffer was added. A fraction (85%) of the reaction mixtures were immediately loaded on to a 4→8% polyacrylamide gel containing 1X TBE. Electrophoresis was carried out at 30 mA; the gel was dried and autoradiographed. Band quantitations were carried out by scintillation counting.

Steady-State Anisotropy Measurement

Equilibrium DNA binding in solution was measured by fluorescence anisotropy (35-37). Fluorescence measurements were performed on a steady-state photon-counting spectrofluorometer, PC1, from ISS Instruments (Champaign IL) equipped with a Hamamatsu R928P photomultiplier tube. Excitation and emission slits were adjusted at 8 nm and 4 nm, respectively.

Fluorescein-labeled G4ori oligonucleotide, Fl-G4ori, was used as fluorescence anisotropy probes. The oligonucleotide was diluted in buffer B to a concentration of 6 nM and titrated with DnaG_{BA} in the concentration range of 0.1 nM to 1 μM. The samples were excited at 488 nm and the fluorescence anisotropy was measured at 540 nm (38), where minimal variation in the total fluorescence intensity was observed. Fluorescence intensities were measured for 3 × 10 seconds and averaged. Anisotropy values were expressed as millianisotropy or mA, which is equal to the anisotropy divided by 1000. The standard deviation for the anisotropy values was <5 mA. The total fluorescence intensity did not change significantly with increase in protein concentration. Therefore, fluorescence lifetime changes or the scattered excitation light did not affect anisotropy measurements. The interaction of DnaG_{BA} with labeled oligonucleotide can be represented as follows:

Where R is the ligand, i.e., fluorescent labeled oligonucleotide; and P is the protein or DnaG_{BA} in this case. As shown earlier (39), the equilibrium dissociation constant, K_d can be further defined as the DnaG_{BA} concentration at which half of the ssDNA molecules in protein-DNA complex.

DNA-Binding Data Analysis

The anisotropy protein titration data or EMSA data were fitted to equation 2 using non-linear regression analysis. The data points were fitted to an equation for sigmoidal semi-logarithmic plot using Prism 5.0 (GraphPad Software Inc. CA) presented in equation 2.

$$Y = Y_{\text{MIN}} + \left[(Y_{\text{MAX}} - Y_{\text{MIN}}) / \left(1 + 10^{(\text{Log EC}_{50} - X)N_{\text{APP}}} \right) \right] \quad (\text{Equation 2})$$

where, Y represents the value of DNA binding, Y_{MIN} and Y_{MAX} are the values at the bottom and top plateaus of the plots respectively. EC_{50} is the X value (or enzyme concentration) at 50% DNA binding (or K_d) and N_{APP} is the Hill coefficient, which represents the number of molecules of protein (DnaG_{BA}) bound to a single molecule of ssDNA or the composition of the DnaG_{BA} • ssDNA complex.

RESULTS

DNA Primase Gene of *B. anthracis*

The DNA primase gene of *B. a* (BAS4195) was identified by BLAST search of annotated sequenced genome of *B. anthracis* Stern strain (28,29). BAS4195 was the only gene in *B. anthracis* genome that is homologous to *E. coli* dnaG gene. The gene encodes for a polypeptide of 598 amino acid residues with a deduced molecular weight of 68.3 kDa.

Significant Homology was Observed in the N-terminus of DnaG_{BA}

The amino acid sequence of the polypeptide was compared with the sequences of a number of gram-positive (*Bacillus cereus*, & *Staphylococcus aureus*) and gram-negative (*E. coli* and

Salmonella enterica) DNA primases. Multiple-alignment of these sequences is shown in Figure 1. Extensive homology of DnaG_{BA} was observed within the family of DNA primases from gram-positive bacteria and to a lesser degree with the gram-negative primases. Overall, high degrees of homology were observed in three regions (Figure 1). All three of these regions are located at the N-terminal. Amino acid residues 1-320, which constitute the major part of the primase core domain with 25% identity and 45% similarity; amino acid residues 1-80 which is the location of the zinc-finger domain (residues 5-103) displayed ~36% identity and 35% similarity. A second area of homology was observed between the residues 186-254 with 41% identity and ~41% similarity. A third area of homology was observed between the residues 264 and 320 with 25% identity and ~49% similarity.

Unlike the N-terminus of primase, homology in the C-terminus between amino acid residues 321 and 599 was rather minimal and contained significant gaps in some of the sequences particularly in the gram-negative sequences. Interestingly, DnaG_{SA} of *Staphylococcus aureus* contained a unique seven amino acid insertion starting at residue 459 (Figure 1) that was not present in any of the other primases discussed here. The C-terminus of primase is known to be involved in the interaction with its cognate DnaB helicase and this interaction is pivotal in the efficient and rapid priming of the lagging strand of the replication fork as well as movement of the replisome (40-42). Thus, the lack of homology in this region could help explain the specificity of helicase-primase interaction. A motif search indicated that a putative DnaB helicase-binding domain (or helicase-binding motif) in DnaG_{BA} is located at amino acids 466-591. Homology in this domain among these five DnaG proteins is significantly low: 5% identity and ~20% similarity (Figure 1). Therefore, it is likely that the lack of homology in the C-terminal domain among bacterial primases makes the interaction species specific for cognate primase-helicase pairs. Although this assumption is logical, it has not been rigorously examined.

Anthrax Primase, DnaG_{BA}, is Monomeric in Solution

Previous attempts to purify DNA primase from gram-positive bacteria, *Staphylococcus aureus*, (DnaG_{SA}) led to protein that is insoluble. It is soluble only as a glutathione S-transferase (GST) fusion protein and removal of GST-moiety leads to the precipitation of cleaved primase (30). Studies with GST-DnaG_{SA} fusion protein showed lack of stimulation with cognate DnaB protein (30,31). Interestingly, our earlier attempts to purify DnaG_{BA} as His₆-fusion protein using Ni²⁺-affinity chromatography also produced an inactive protein (data not shown). Therefore, in order to understand the mechanism of action of gram-positive DNA primases, it was necessary to purify it in its native form without any modification in its polypeptide sequence.

Therefore, we expressed DnaG_{BA} in its native polypeptide sequence, without any N- or C-terminal fusion, in *E. coli*. Surprisingly, unmodified recombinant DnaG_{BA}, expressed in *E. coli*, was highly soluble unlike that observed with other DnaG_{SA}. Because of its high solubility it could be purified by a simple combination of chromatographic procedures. The final stage in the purification was high performance gel filtration chromatography (Figure 2A). This step removed all of the remaining impurities. It should also be noted that whenever a recombinant protein (like DnaG_{BA}) is over expressed, often some aggregated proteins are produced during expression and purification. The aggregates co-purify with the target protein in ion exchange columns and in general, cannot be distinguished in SDS-PAGE. In HPLC gel filtration, the aggregated proteins fractionate in the void volume (>300 kDa), which is the case here (fractions 30-34, Figure 2A). The aggregated form of DnaG_{BA} did not have any enzymatic activity and therefore, was not pursued further. Thus, HPLC gel filtration completely separated purified biologically active primase (major peak, fractions 40-46, Figure 2A) from impurities as well as aggregated proteins. Since we were studying the oligomeric state of DnaG_{BA} in DNA-

protein complex formation, it was important to determine that the preparation was free from aggregated protein. SDS-PAGE analysis of the purified active DnaG_{BA} indicated approximately ~99% purity (Figure 3C). This DnaG_{BA} peak eluted from the column at an elution volume that is similar to that of BSA (68 kDa). Thus, DnaG_{BA} appeared to be a monomer in solution. The protein fractions were first assayed by EMSA using a ³²P labeled Sixtymer oligonucleotide as probe. Active fractions, centered on the peak, were pooled and used in our studies.

DnaG_{BA} Bound ssDNA with Moderate Affinity

The first step in the mechanism of primer synthesis by DNA primases is its binding to ssDNA and formation of the DnaG_{BA}•ssDNA complex. As DNA primases prime the entire lagging strand of the genome, we anticipated that it would be able to bind to a variety of sequences with varying affinities. Therefore, we examined ssDNA binding by DnaG_{BA} using EMSA analysis. The EMSA results for ssDNA binding by DnaG_{BA} fractionated by gel filtration is presented in Figure 2B. Fractions containing DnaG_{BA} (fractions 40-46) bound the Sixtymer oligonucleotide and produced a ³²P-containing band that appeared to correspond to DnaG_{BA}•Sixtymer complex. Thus, recombinant DnaG_{BA} appeared to be biologically active in terms of its ability to form a stable DnaG_{BA}•oligonucleotide complex that could be separated by gel electrophoresis. The SDS-PAGE analysis of purified DnaG_{BA} is shown in Figure 2C.

We have tested a variety of oligonucleotide sequences that may bind DnaG_{BA} with higher affinity. However, all of the synthetic sequences that were tested gave very similar binding affinities comparable to that described above. In addition, we explored several DNA replication origin sequences from bacteria to bacteriophages (1). Surprisingly, origin sequence derived from a single-stranded bacteriophage G4 DNA appeared to bind with higher affinity. An EMSA titration of DnaG_{BA} with a 27bp G4ori oligonucleotide is presented in Figure 3A. Titration was carried out in a standard assay containing 0-1.6 µg DnaG_{BA}. An increase in the formation of DnaG_{BA}•G4ori complex was observed. At high primase concentrations, multiple protein-DNA complex formation appeared which was likely due to the process leading to the primase trimer formation. In order to determine quantitatively the nature of this ssDNA binding, we determined the ³²P-content in each band by scintillation counting and plotted against the log of molar concentrations of DnaG_{BA} to create a binding isotherm (Figure 3B). The K_d value determined from the plot was $3.35 \pm 0.69 \times 10^{-8}$ M and with Sixtymer oligonucleotide, K_d increased significantly and was eight-fold higher, $2.47 \pm 0.34 \times 10^{-7}$ M. The Hill slope of this plot, N_{APP}, determines the numbers of protein molecules that are associating with a single ligand or in this case, oligonucleotide. The Hill slope, as determined from this plot, was 2.76 ± 0.3 and this value can be equated to 3. Hill slope remained unaltered with the Sixtymer (2.9 ± 0.4). Taken together, these results indicated that DnaG_{BA} likely formed a trimer around ssDNA independent of its affinity or K_d. Our earlier studies with *E. coli* showed that DnaB and DnaG proteins formed an unstable complex with three DnaG molecules per DnaB hexamer (or DnaG₃•DnaB₆ complex) during priming.

The binding isotherm from fluorescence anisotropy titration of G4ori sequence binding is shown in Figure 3C. The data points were fitted to equation 2 using Prism 5.0 software. Nonlinear regression analysis gave a K_d of $3.6 \pm 0.5 \times 10^{-8}$ M, which indicated at least seven-fold higher binding affinity than that observed above with other oligonucleotides. The Hill slope determined from this plot was 2.84 ± 0.54 , also suggesting that DnaG_{BA} bound ssDNA as an apparent trimer. Results of the anisotropy analysis of ssDNA binding were closely comparable to that observed with the results of EMSA analysis. Therefore, both ssDNA binding analyses demonstrated that regardless of the binding affinities, DnaG_{BA} formed a trimer upon ssDNA binding.

Glutaraldehyde cross-linking & gel filtration analysis of DnaG_{BA}•DNA complex

In order to determine the size of the DnaG_{BA}•G4ori complex, we used glutaraldehyde cross-linking followed by SEHPLC. The gel filtration column was calibrated using protein markers ranging in size from 17 kDa - 670 kDa (Fig. 4A). Purified DnaG_{BA} eluted with an apparent molecular mass of 66±5 kDa (Figure 4B). We stabilized the DnaG_{BA}•G4ori complex using glutaraldehyde as a cross-linker. This is an efficient amine reactive cross-linker, which binds to the lysine residues of the proteins and creates a somewhat stable complex that can be analyzed by gel filtration. DnaG_{BA}•G4ori complex was cross-linked with 0.2 M glutaraldehyde and was subjected to gel filtration. We found two peaks (Figure 4C). One was at a position expected for DnaG_{BA} monomer. Second was eluted at the position from the gel filtration column earlier than DnaG_{BA} with an elution volume of ~11.5 ml. The apparent molecular mass of the complex was estimated from the linear graph derived from the plot of log[Mw] vs. elution volume for protein standards and was found to be ~242 kDa. A trimeric complex, (DnaG_{BA})₃•G4ori, has a theoretical mass of ~215 kDa and the discrepancy could be due to G4ori ssDNA and crosslinked glutaraldehyde molecules. Therefore, gel filtration results of the cross-linked protein supported the possibility of a trimeric complex.

DNA Priming by DnaG_{BA} was Stimulated by Anthrax DnaB Helicase but Strongly Inhibited by *E. coli* DnaB Helicase

The results described above demonstrated that DnaG_{BA} is active in ssDNA binding as a trimer. Here, we have examined whether the purified DnaG_{BA} is active in primer synthesis. In the ssDNA binding studies, we have identified a sequence containing the origin of replication of bacteriophage G4 (Fl-G4ori) that DnaG_{BA} bound with high affinity. Consequently, we chose to use G4ori oligonucleotide as the substrate for analyzing primer synthesis by DnaG_{BA} *in vitro*.

The primer synthesis assay was used to monitor the primer synthesis from the G4ori template in the presence or in the absence of DnaB proteins. Based on the template sequence, α -³²P UTP was used to label the RNA primers. Primer syntheses by DnaG_{BA} alone (in the absence of DnaB proteins) were carried out for 5, 15, and 30 minutes. An autoradiogram of the RNA products is shown in Figure 5. Primer synthesis was low at 5 minutes and significant amounts of RNA products were observed at both 15 and 30 minute time points (Figure 5A, lanes 1-3). In general, primers were shorter than 20 nucleotides. Overall, primer syntheses by DnaG_{BA} were relatively low but significant compared to other bacterial primases. Perhaps, its binding to ssDNA in the form of a trimer was responsible for its higher primase activity in the absence of DnaB effector. In the presence of DnaB_{BA}, primase activity of DnaG_{BA} was significantly stimulated (Figure 5A, lanes 4-6). Substantial amounts of RNA products were observed at 5 minutes, as well as higher time points. DnaB_{BA} appeared to: (i) reduce the lag time of primer synthesis, and (ii) increase the rate of synthesis of primers significantly. Plots of quantitation of primer syntheses are shown in Figure 5B. At the 0.5 minutes range, the stimulation by DnaB_{BA} was >75-fold. At longer time points the extent of stimulation was reduced.

The C-terminus of DnaG_{BA} and other primases are less conserved compared to the N-terminus region and it is involved in interaction with DnaB helicase. Due to the lack of homology, we anticipated that a heterologous DnaB protein like DnaB_{EC} might not have any interaction with DnaG_{BA}. In order to verify this notion we examined the effects, if any, of DnaB_{EC} on the primase activity of DnaG_{BA}. The results, shown in Figure 5A (lanes 7-9), demonstrated that DnaB_{EC} likely interacted with DnaG_{BA} as DnaB_{EC} completely inhibited the primase activity of DnaG_{BA}. Consequently, it appeared that DnaG_{BA} could interact with both DnaB_{BA} and DnaB_{EC}, except that only cognate DnaB_{BA} can act as a positive effector. A titration of DnaB_{BA} in primer synthesis is shown in Figure 5C. Primer synthesis appeared to increase with

DnaB_{BA} pointing to a clear association between these two proteins leading to an increase in priming.

DISCUSSION

Anthrax DNA Primase is a Highly Soluble Monomeric Enzyme

DNA primases are pivotal enzymes in lagging strand DNA replication as well as in replisomes that carry out bacterial chromosomal DNA replication (1,5,21,23). As described previously, DNA primase appears to form a large protein complex with hexameric DnaB protein encircling the lagging ssDNA strand in the replication fork (1,19). Previous studies (19) indicated that this complex has a stoichiometry of three primase molecules with one DnaB protein hexamer or a 3:6 complex as shown in Figure 6. This complex regulates the site selection as well as the frequency of lagging strand priming, which in turn determines the average length of Okazaki fragments. In *E. coli*, this interaction between DnaG and DnaB proteins is quite subtle and can be demonstrated with difficulty. In addition, it is difficult to measure the interaction between DnaG protein monomers, if any, upon ssDNA binding.

Nonetheless, the *E. coli* DNA replication apparatus remains the most well-investigated system. Because of the large diversity of prokaryotes and prokaryotic pathogens, it is important to understand the mechanism of their DNA replication in order to develop novel strategies to combat them. Thus, it is important to determine which properties of *E. coli* DNA primase are unique only to *E. coli* versus those that are universal among prokaryotes. Except for a few recent reports, very little is known about the replication mechanism in gram-positive bacteria. Even with the application of recombinant DNA technology, it has been difficult to study these proteins in native form due to occasional solubility problems. Surprisingly, both DnaG_{BA} and DnaB_{BA} proteins from *B. anthracis* are highly soluble in recombinant form, providing an opportunity to study these proteins in highly purified forms.

DnaG_{BA} Formed a Trimeric Complex with ssDNA

We have isolated recombinant DnaG_{BA} in highly purified form (Figure 2C) and analyzed the mechanism of ssDNA binding. We have examined a number of synthetic DNA sequences to determine its sequence preference, if any, without success. However, an oligonucleotide with sequence derived from the origin of DNA replication of bacteriophage G4 bound DnaG_{BA} with significantly higher affinity (Figure 3) (21). With a ³²P labeled G4ori oligonucleotide, DnaG_{BA} bound with high affinity, (Figure 3). The K_d value determined from the binding isotherm was $3.35 \pm 0.69 \times 10^{-8}$ M. In addition, the Hill coefficient, N_{App}, derived from the nonlinear regression (equation 2) was 2.76 ± 0.3 (Figure 3B). Therefore, DnaG_{BA} appears to form a trimer during ssDNA binding even though it is monomeric in solution (Figure 2). *E. coli* primase, DnaB_{EC}, is not known to form such a trimer alone but it forms a trimer in a complex with DnaB hexamer (19).

Fluorescein-labeled 27 bp G4 origin sequence, Fl-G4ori, was used to quantitate its binding to DnaG_{BA} using fluorescence anisotropy as a second method of analysis. The K_d was $3.6 \pm 0.5 \times 10^{-8}$ M, which indicated a seven-fold higher binding affinity than that observed above with other oligonucleotides. Even with the G4ori sequence and higher affinity binding, the Hill coefficient was 2.84 ± 0.54 which can be approximated to 3, that also showed that DnaG_{BA} bound the oligonucleotide as a trimer. Therefore, both analyses (Figures 2B and 2C) appeared to demonstrate that regardless of the binding affinities, DnaG_{BA} appeared to form a trimer upon ssDNA binding. Thus, it could be a distinguishing feature of DnaG_{BA} in that it appeared to form a trimer with ssDNA, independent of DnaB protein.

A DnaG_{BA}•G4ori complex, cross-linked with glutaraldehyde and the size of the cross-linked complex was analyzed by SEHPLC (Figure 4). SEHPLC and determination of the molecular weight of the complex indicated a mass of 242 kDa for the cross-linked complex indicating a 3.4:1 ratio of DnaG_{BA}:G4ori in the complex that could be approximated to a trimeric complex. Thus, it appeared that one of the distinguishing features of DnaG_{BA} is its ability to form a trimer, presumably a toroidal trimeric ring, around ssDNA, independent of DnaB protein. The alternative scenario, three primase binding sequentially to three separate sites on one oligonucleotide, seems unlikely due to the small size (27 bp) of the oligonucleotide, and in addition, the binding kinetics does not support this mechanism. In addition, primases are known to interact structurally with DnaB helicases in the replication fork. Because of the toroidal structure of DnaB helicase, this process would require a toroidal ring formation (19,33). In this respect, DNA primase purified from high temperature bacterium, DnaG_{BS}, appear to form a stable complex with its cognate DnaB_{BS} (32). This complex contains three DnaG_{BS} per DnaB hexamer. Bailey et. al. resolved the structure of a complex DnaB_{BS} with the helix binding domain of DnaG_{BS} with a 6:3 ratio (33). However, it is not known whether it can bind ssDNA alone and form a trimer with ssDNA.

Primer Synthesis by DnaG_{BA} was Stimulated by Anthrax DnaB helicase and Inhibited by the *E. coli* DnaB Helicase

DnaG_{BA} synthesized RNA primers on G4ori template in the absence of DnaB_{BA} protein (Figure 5A). However, the rate of primer synthesis was low (Figure 5B). DnaB_{BA} significantly stimulated the rate as well as the extent of primer synthesis. In the initial stage, the stimulation by DnaB_{BA} was ≥ 75 -fold. Beyond this initial stage, the extent of stimulation was reduced as expected. In addition to the stimulation of the rate of primer synthesis, the average size of the primers was also increased and a greater size distribution of RNA primers was observed (Figures 5A & 5C). A titration of DnaB_{BA} in a primer synthesis assay indicated that DnaB_{BA} stimulated RNA synthesis by DnaG_{BA} in a dose-dependent manner (Figure 5D). The amount of DnaG_{BA} in each assay was 0.4 g and half-maximal RNA synthesis was observed at 0.6 μ g of DnaB_{BA}. The amount of DnaB_{BA} needed to reach the half-maximal synthesis appeared somewhat higher than that anticipated from a complex containing three DnaG_{BA} monomers and one DnaB_{BA} hexamer. However, this could be due to weaker interaction between the two enzymes requiring higher level of DnaB_{BA} in order to form the (DnaG_{BA})₃: (DnaB_{BA})₆ complex.

We also examined the effect of a heterologous DnaB, DnaB_{EC}, on the primer synthesis by DnaG_{BA}. The functional interaction between DnaB and primase involves the last 16 amino acids residues of the C-terminal region of the primase (41) and the N-terminal 12 kDa domain of the DNA helicase, corresponding to amino acids residues 14–136 (43). Considering the fact that the C-terminal DnaB binding region of DnaG_{BA} is different from that of *E. coli* DnaG_{EC} (Figure 1), any significant interaction as well as change in primer synthesis due to DnaB_{EC} was not anticipated. Surprisingly, as shown in Figure 5A, DnaB_{EC} completely inhibited primer synthesis by DnaG_{BA}. Thus, even with significant sequence heterogeneity, DnaB_{EC} and DnaG_{BA} appeared to interact functionally or physically and this interaction led to inhibition of primase activity of DnaG_{BA}. Therefore, homologous DnaB_{BA} acted as a positive effector of DnaG_{BA}, whereas heterologous DnaB_{EC} acted as a negative effector. Based on the results presented here as well as previous studies on DNA primases, as discussed above, we propose a hypothetical model for the distributive priming of the lagging strand of bacterial replication forks (Figure 6). The functional DnaB•DnaG complex binds to the lagging strand with a 6:3 subunit-ratio. Once an appropriate DNA sequence comes in contact with a primase subunit of DnaB•DnaG complex, it binds to the ssDNA with higher affinity. The higher affinity binding forces it to break off from the complex as a monomer and it leaves a gap in the primase trimeric ring. This monomeric primase then synthesizes the primer. In the meantime, a new

primase monomer joins the DnaB•DnaG assembly to fill the gap in the trimeric primase ring thereby restoring the 6:3 complex and it starts a new cycle of priming. In this model, DNA primase continually recycles and the priming does not impede the movement of the replisome powered by DnaB helicase. Further studies are required to dissect the proposed model in Figure 6.

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Abbreviations used

holoenzyme, DNA polymerase III holoenzyme
 ssDNA, single stranded DNA
 G4ori, origin of bacteriophage G4
 B. anthracis, *Bacillus anthracis*
 EMSA, Electrophoretic mobility shift assay
 DTT, dithiothreitol
 TBE, Tris-Borate-EDTA buffer
 SDS, sodium dodecyl sulfate
 PAGE, polyacrylamide gel electrophoresis
 rNTP, ribonucleotide triphosphate
 DnaG_{BA}, DNA primase of *Bacillus anthracis*
 DnaB_{BA}, DnaB helicase of *Bacillus anthracis*
 DnaG_{EC}, DNA primase of *E. coli*
 DnaB_{EC}, DnaB helicase of *E. coli*

REFERENCES

1. Kornberg, A.; Baker, TA. DNA Replication. W.H. Freeman and Co.; New York, NY: 1992.
2. DePamphilis ML. Initiation of DNA replication in eukaryotic chromosomes. J. Cell Biochem. Suppl 1998;30-31:8–17. [PubMed: 9893250]
3. Datta HJ, Khatri GS, Bastia D. Mechanism of recruitment of DnaB helicase to the replication origin of the plasmid pSC101. Proc. Nat'l. Acad. Sci. USA 1999;96:73–78. [PubMed: 9874774]
4. Bramhill D, Kornberg A. Duplex opening by dnaA protein at novel sequences in initiation of replication at the origin of the E. coli chromosome. Cell 1988;52:743–755. [PubMed: 2830993]
5. Bramhill D, Kornberg A. A model for initiation at origins of DNA replication. Cell 1988;54:915–918. [PubMed: 2843291]
6. Fuller RS, Kornberg A. Purified dnaA protein in initiation of replication at the Escherichia coli chromosomal origin of replication. Proc. Nat'l. Acad. Sci. USA 1983;80:5817–5821. [PubMed: 6310593]
7. Biswas EE, Biswas SB, Bishop JE. The dnaB protein of Escherichia coli: mechanism of nucleotide binding, hydrolysis, and modulation by dnaC protein. Biochemistry 1986;25:7368–7374. [PubMed: 3026453]
8. Biswas SB, Biswas EE. Regulation of dnaB function in DNA replication in Escherichia coli by dnaC and lambda P gene products. J. Biol. Chem 1987;262:7831–7838. [PubMed: 3034907]
9. Wickner S, Hurwitz J. Interaction of Escherichia coli dnaB and dnaC(D) gene products in vitro. Proc. Nat'l. Acad. Sci. USA 1975;72:921–925. [PubMed: 1093174]

10. Biswas SB, Biswas-Fiss EE. Quantitative analysis of binding of single-stranded DNA by *Escherichia coli* DnaB helicase and the DnaB x DnaC complex. *Biochemistry* 2006;45:11505–11513. [PubMed: 16981710]
11. Gaylo PJ, Turjman N, Bastia D. DnaA protein is required for replication of the minimal replicon of the broad-host-range plasmid RK2 in *Escherichia coli*. *J. Bacteriol* 1987;169:4703–4709. [PubMed: 2820940]
12. Konieczny I, Helinski DR. Helicase delivery and activation by DnaA and TrfA proteins during the initiation of replication of the broad host range plasmid RK2. *J. Biol. Chem* 1997;272:33312–33318. [PubMed: 9407123]
13. Zhong Z, Helinski D, Toukdarian A. A specific region in the N terminus of a replication initiation protein of plasmid RK2 is required for recruitment of *Pseudomonas aeruginosa* DnaB helicase to the plasmid origin. *J. Biol. Chem* 2003;278:45305–45310. [PubMed: 12952979]
14. Alfano C, McMacken R. Ordered assembly of nucleoprotein structures at the bacteriophage lambda replication origin during the initiation of DNA replication. *J. Biol. Chem* 1989;264:10699–10708. [PubMed: 2525129]
15. Dodson M, Roberts J, McMacken R, Echols H. Specialized nucleoprotein structures at the origin of replication of bacteriophage lambda: complexes with lambda O protein and with lambda O, lambda P, and *Escherichia coli* DnaB proteins. *Proc. Nat'l. Acad. Sci. USA* 1985;82:4678–4682. [PubMed: 2991888]
16. Alfano C, McMacken R. The role of template superhelicity in the initiation of bacteriophage lambda DNA replication. *Nucleic Acids Res* 1988;16:9611–9630. [PubMed: 2847118]
17. Dodson M, Echols H, Wickner S, Alfano C, Mensa-Wilmot K, Gomes B, LeBowitz J, Roberts JD, McMacken R. Specialized nucleoprotein structures at the origin of replication of bacteriophage lambda: localized unwinding of duplex DNA by a six-protein reaction. *Proc. Nat'l. Acad. Sci. USA* 1986;83:7638–7642. [PubMed: 3020552]
18. Arai K, Kornberg A. A general priming system employing only dnaB protein and primase for DNA replication. *Proc. Nat'l. Acad. Sci. USA* 1979;76:4308–4312. [PubMed: 228295]
19. Mitkova AV, Khopde SM, Biswas SB. Mechanism and stoichiometry of interaction of DnaG primase with DnaB helicase of *Escherichia coli* in RNA primer synthesis. *J. Biol. Chem* 2003;278:52253–52261. [PubMed: 14557266]
20. Stayton MM, Bertsch L, Biswas S, Burgers P, Dixon N, Flynn JE Jr, Fuller R, Kaguni J, Kobori J, Kodaira M, Low R, Kornberg A. Enzymatic recognition of DNA replication origins. *Cold Spring Harb. Symp. Quant. Biol* 1983;47(Pt 2):693–700. [PubMed: 6345074]
21. Stayton MM, Kornberg A. Complexes of *Escherichia coli* primase with the replication origin of G4 phage DNA. *J. Biol. Chem* 1983;258:13205–13212. [PubMed: 6355106]
22. Sugino A, Hirose S, Okazaki R. RNA-linked nascent DNA fragments in *Escherichia coli*. *Proc. Nat'l. Acad. Sci. USA* 1972;69:1863–1867. [PubMed: 4558661]
23. Arai K, Low RL, Kornberg A. Movement and site selection for priming by the primosome in phage phi X174 DNA replication. *Proc. Nat'l. Acad. Sci. USA* 1981;78:707–711. [PubMed: 6262762]
24. Lu YB, Ratnakar PV, Mohanty BK, Bastia D. Direct physical interaction between DnaG primase and DnaB helicase of *Escherichia coli* is necessary for optimal synthesis of primer RNA. *Proc. Nat'l. Acad. Sci. USA* 1996;93:12902–12907. [PubMed: 8917517]
25. Yoda K, Okazaki T. Specificity of recognition sequence for *Escherichia coli* primase. *Mol. Gen. Genet* 1991;227:1–8. [PubMed: 1828532]
26. Yoda K, Yasuda H, Jiang XW, Okazaki T. RNA-primed initiation sites of DNA replication in the origin region of bacteriophage lambda genome. *Nucleic Acids Res* 1988;16:6531–6546. [PubMed: 2456527]
27. Tomasz A. Multiple-antibiotic-resistant pathogenic bacteria. A report on the Rockefeller University Workshop. *N. Engl. J. Med* 1994;330:1247–1251. [PubMed: 8139650]
28. Read TD, Peterson SN, Tourasse N, Baillie LW, Paulsen IT, Nelson KE, Tettelin H, Fouts DE, Eisen JA, Gill SR, Holtzapple EK, Okstad OA, Helgason E, Rilstone J, Wu M, Kolonay JF, Beanan MJ, Dodson RJ, Brinkac LM, Gwinn M, DeBoy RT, Madpu R, Daugherty SC, Durkin AS, Haft DH, Nelson WC, Peterson JD, Pop M, Khouri HM, Radune D, Benton JL, Mahamoud Y, Jiang L, Hance IR, Weidman JF, Berry KJ, Plaut RD, Wolf AM, Watkins KL, Nierman WC, Hazen A, Cline R,

- Redmond C, Thwaite JE, White O, Salzberg SL, Thomason B, Friedlander AM, Koehler TM, Hanna PC, Kolsto AB, Fraser CM. The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. *Nature* 2003;423:81–86. [PubMed: 12721629]
29. Takami H, Nakasone K, Takaki Y, Maeno G, Sasaki R, Masui N, Fuji F, Hiram C, Nakamura Y, Ogasawara N, Kuhara S, Horikoshi K. Complete genome sequence of the alkaliphilic bacterium *Bacillus halodurans* and genomic sequence comparison with *Bacillus subtilis*. *Nucleic Acids Res* 2000;28:4317–4331. [PubMed: 11058132]
 30. Koepsell SA, Larson MA, Griep MA, Hinrichs SH. *Staphylococcus aureus* helicase but not *Escherichia coli* helicase stimulates *S. aureus* primase activity and maintains initiation specificity. *J. Bacteriol* 2006;188:4673–4680. [PubMed: 16788176]
 31. Koepsell SA, Larson MA, Frey CA, Hinrichs SH, Griep MA. *Staphylococcus aureus* primase has higher initiation specificity, interacts with single-stranded DNA stronger, but is less stimulated by its helicase than *Escherichia coli* primase. *Mol. Microbiol* 2008;68:1570–1582. [PubMed: 18452509]
 32. Bird LE, Pan H, Soultanas P, Wigley DB. Mapping protein-protein interactions within a stable complex of DNA primase and DnaB helicase from *Bacillus stearothermophilus*. *Biochemistry* 2000;39:171–182. [PubMed: 10625492]
 33. Bailey S, Eliason WK, Steitz TA. Structure of hexameric DnaB helicase and its complex with a domain of DnaG primase. *Science* 2007;318:459–463. [PubMed: 17947583]
 34. Biswas EE, Biswas SB. Mechanism of DNA binding by the DnaB helicase of *Escherichia coli*: analysis of the roles of domain gamma in DNA binding. *Biochemistry* 1999;38:10929–10939. [PubMed: 10460148]
 35. Gryczynski I, Gryczynski Z, Lakowicz JR. Fluorescence anisotropy controlled by light quenching. *Photochem. Photobiol* 1998;67:641–646. [PubMed: 9648529]
 36. Maag D, Fekete CA, Gryczynski Z, Lorsch JR. A conformational change in the eukaryotic translation preinitiation complex and release of eIF1 signal recognition of the start codon. *Mol. Cell* 2005;17:265–275. [PubMed: 15664195]
 37. Sarkar P, Bharill S, Gryczynski I, Gryczynski Z, Nair MP, Lacko AG. Binding of 8-anilino-1-naphthalenesulfonate to lecithin:cholesterol acyltransferase studied by fluorescence techniques. *J. Photochem. Photobiol. B* 2008;92:19–23. [PubMed: 18485727]
 38. Lakowicz, JR. Principles of fluorescence spectroscopy. Vol. 2nd ed.. Plenum Publishers; New York, NY: 1999. p. 10013
 39. Khopde S, Biswas E, Biswas S. Affinity and sequence specificity of DNA binding and site selection for primer synthesis by *Escherichia coli* primase. *Biochemistry* 2002;41:14820–14830. [PubMed: 12475230]
 40. Masai H, Nomura N, Arai K. The ABC-primosome. A novel priming system employing dnaA, dnaB, dnaC, and primase on a hairpin containing a dnaA box sequence. *J. Biol. Chem* 1990;265:15134–15144. [PubMed: 2168401]
 41. Tougu K, Mariani KJ. The extreme C terminus of primase is required for interaction with DnaB at the replication fork. *J. Biol. Chem* 1996;271:21391–21397. [PubMed: 8702920]
 42. Tougu K, Peng H, Mariani KJ. Identification of a domain of *Escherichia coli* primase required for functional interaction with the DnaB helicase at the replication fork. *J. Biol. Chem* 1994;269:4675–4682. [PubMed: 8308039]
 43. Nakayama N, Arai N, Kaziyo Y, Arai K. Structural and functional studies of the dnaB protein using limited proteolysis. Characterization of domains for DNA-dependent ATP hydrolysis and for protein association in the primosome. *J. Biol. Chem* 1984;259:88–96. [PubMed: 6323419]

Figure 1. Sequence Homology of DNA Primases

DnaG proteins from *B. anthracis* (DnaGBA), *B. cereus* (DnaGBC), *S. aureus* (DnaGSA), *E. coli* (DnaGEC) and *Salmonella enteric* (DnaGSE) were aligned and motifs were identified using CLUSTALW2 program of the InterProScan website (<http://www.ebi.ac.uk/Tools/InterProScan/>). The color codings are as follows: red, basic; blue, hydrophobic; green, hydrophilic; orange, neutral; pink, acidic; and light green, proline.

Figure 2. Purification and Analysis of DnaG_{BA}

(A) SDS-PAGE of gel filtration fractions of DnaG_{BA} in buffer A150 as described in Materials & Methods. (B) EMSA analysis of DnaG_{BA} fractions using 32P labeled Sixtymer oligonucleotide (Materials & Methods). (C) SDS-PAGE of DnaG_{BA} fraction V: lane 1, protein marker (M); lanes 2-4, DnaG_{BA} 1, 4 and 15 μg respectively.

Figure 3. Equilibrium ssDNA Binding by DnaG_{BA}

Equilibrium ssDNA binding by DnaG_{BA} were studied using G4ori and Sixtymer oligonucleotides using electrophoretic mobility shift analysis (EMSA). DNA sequences of G4ori and Sixtymer oligonucleotides are given in Materials & Methods. (A) EMSA titration of DnaG_{BA} was carried out with a ³²P-labeled G4ori oligonucleotide and titrated with DnaG_{BA} (10 ng to 1.6 μ g). (B) A plot of quantitative analysis of bands in Figure 3A and its comparison with that obtained in a similar EMSA analysis with a ³²P-labeled Sixtymer oligonucleotide (EMSA data not shown). The data points were fitted to equation 2 as described in Materials & Methods. (C) Fluorescence anisotropy analysis used for measuring ssDNA binding. DNA binding was measured using 6 nM FI-G4ori fluorescent oligonucleotide probe.

Titration was carried out with DnaG_{BA} and fluorescence anisotropy was measured as described in Materials & Methods. Anisotropy values (in millianisotropy or mA) were plotted against log of DnaG_{BA} concentration and the plots were analyzed by nonlinear regression using Prism 5.0.

Figure 4. Cross-linking & Gel Filtration Analysis of DnaG_{BA}•DNA complex

(A) Elution profile of protein markers is shown: thyroglobulin, immunoglobulin gamma, bovine serum albumin (BSA), ovalbumin, and Myoglobin. (B) Elution profile of DnaG_{BA} without DNA. (C) Elution profile of cross-linked DnaG_{BA}•DNA complex. Crosslinking and gel filtration were carried out as described in Materials and Methods.

Figure 5. Analysis of Primer Synthesis by DnaG_{BA} and Differential Effects of DnaB_{BA} and DnaB_{EC}

Primer syntheses and analyses were carried out as described in Materials & Methods. Primers were synthesized using ^{32}P -dNTP. (A) Primer synthesis was carried out with 0.4 μg DnaG_{BA}. Lanes 1-3: without DnaB, for 5 (lane 1), 15 (lane 2), and 30 (lane 3) minutes respectively. Lanes 4-6: with 1.0 μg DnaB_{BA}, for 5 (lane 4), 15 (lane 5), and 30 (lane 6) minutes respectively. Lanes 7-9: with 1.0 μg DnaB_{EC}, for 5 (lane 7), 15 (lane 8), and 30 (lane 9) minutes respectively. (B) Synthesized primers were quantitated by densitometric scanning. (C) Primer synthesis was carried out with 0.4 μg of DnaG_{BA} in the presence of DnaB_{BA} as follows: 0 μg (lane 1), 0.2 μg (lane 2), 0.4 μg (lane 3), 0.6 μg (lane 4), 0.8 μg (lane 5), 1.6 μg (lane 6), and 2.4 μg (lane 7). (D) Primers in (C) were quantitated as described in (B) and plotted. Data points were fitted as described in Materials & Methods.

Figure 6. Primase Recycling Model of Distributive RNA Priming

(1) We have considered here a $(\text{DnaG})_3:(\text{DnaB})_6$ complex formed from one DnaB hexamer and three DnaG primase monomers (P1 to P3); (2) one monomer (P1) identifies and binds to a preferred sequence with a slightly higher affinity, breaks off from the complex, and initiates primer synthesis; (3) a free primase monomer (P4) fills the vacant position in the $(\text{DnaG})_3:(\text{DnaB})_6$ complex; and (4) primase molecule (P1) completes primer synthesis and dissociates from the primed lagging strand and a new cycle of priming begins.