Mechanism and Stoichiometry of Interaction of DnaG Primase with DnaB Helicase of Escherichia coli in RNA Primer Synthesis*

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Atanaska V. Mitkova‡, Sujata M. Khopde‡, and Subhasis B. Biswas§
From the Department of Molecular Biology, School of Osteopathic Medicine, University of Medicine & Dentistry of New Jersey, Stratford, New Jersey 08084

Initiation and synthesis of RNA primers in the lagging strand of the replication fork in Escherichia coli requires the replicative DnaB helicase and the DNA primase, the DnaG gene product. In addition, the physical interaction between these two replication enzymes appears to play a role in the initiation of chromosomal DNA replication. In vitro, DnaB helicase stimulates primase to synthesize primers on single-stranded (ss) oligonucleotide templates. Earlier studies hypothesized that multiple primase molecules interact with each DnaB hexamer and single-stranded DNA. We have examined this hypothesis and determined the exact stoichiometry of primase to DnaB hexamer. We have also demonstrated that ssDNA binding activity of the DnaB helicase is necessary for directing the primase to the initiator trinucleotide and synthesis of 11–20 nucleotide long primers. Although, association of these two enzymes determines the extent and rate of synthesis of the RNA primers in vitro, direct evidence of the formation of primase-DnaB complex has remained elusive in E. coli due to the transient nature of their interaction. Therefore, we stabilized this complex using a chemical crosslinker and carried out a stoichiometric analysis of this complex by gel filtration. This allowed us to demonstrate that the primase-helicase complex of E. coli is comprised of three molecules of primase bound to one DnaB hexamer. Fluorescence anisotropy studies of the interaction of DnaB with primase, labeled with the fluorescent probe Ru(bipy)_3, and Scatchard analysis further supported this conclusion. The addition of DnaC protein, leading to the formation of the DnaB-DnaC complex, to the simple priming system resulted in the synthesis of shorter primers. Therefore, interactions of the DnaB-primase complex with other replication factors might be critical for determining the physiological length of the RNA primers in vitro and the overall kinetics of primer synthesis.

During the last few decades, studies on the replication of phage, plasmid, and chromosomal DNA in Escherichia coli and eukaryotic cells have established an understanding of some of the basic mechanisms of DNA replication (1–4). Reconstitution of DNA replication with purified proteins has yielded great insight into the mechanism of DNA replication as well as other aspects of DNA metabolism, such as DNA repair and recombination in prokaryotic and eukaryotic cells (5–10). The replication of the E. coli chromosome requires a large number of proteins that have to work in concert in order to successfully accomplish initiation, elongation, and termination of DNA replication (2, 11–14). Thus, a careful analysis of the interactions between replication factors is of critical importance for gaining further insights into the mechanism and control of the major steps of DNA replication.

Upon DnaA protein activation of the origin, DnaB helicase enters the partially unwound origin. Binding of DnaB to single-stranded DNA (ssDNA) is controlled by its interaction with DnaC. Association with DnaB stimulates the DNA binding activity of DnaC, and with the help of DnaA, it loads DnaB on the ssDNA. In the next step, primase, the DnaG gene product and a single-stranded DNA-dependent RNA polymerase, is recruited to the replication fork through a transient protein-protein interaction with DnaB. Primase initiates synthesis of the RNA primers (18–22). It synthesizes RNA primer only once to initiate leading strand DNA synthesis and repeatedly on the lagging strand in order to initiate Okazaki fragment synthesis (20). It has been shown that primase initiates in vivo Okazaki fragment synthesis in the E. coli chromosome and λ-bacteriophage from unique regions containing predominantly CTG trinucleotide by synthesizing di-ribonucleotide 5′-ppApG-3′ (23, 24). Primase is specific in action during the initiation event, but its specificity is reduced significantly in the presence of DnaB helicase (25). Kinetic analysis suggests that E. coli primase, acting alone, is the slowest RNA polymerase, with an in vitro rate of about one primer every thousand seconds (26). However, the activity of primase on oligonucleotide templates could be stimulated upon the addition of DnaB helicase at an optimum ratio (27). This indicates that the interaction of DnaB with primase stimulates the rate of the primer synthesis. The DnaB helicase is a homohexamer of 52 kDa identical monomers (28, 29), and electron microscopic studies suggest a doughnut-shaped rosette structure (30). In contrast, primase is a monomer of 65 kDa in solution (31). Mutation and deletion studies have demonstrated that the N-terminal domain of primase and the C-terminal region of DnaB are involved in the DNA binding activity (32). More precise studies reported that 16 amino acids at the C-terminal domain of primase are crucial for interaction with DnaB (33), whereas an N-terminal domain of about 12 kDa of DnaB provides the binding site for both primase and DnaC (34, 35).

In the E. coli replication fork, cyclic association and dissociation...
DnaB hexamer complex. Initially we have carried out in vitro purification. The Superdex 200 HR HPLC column (1/1002 × 1 mm) was equilibrated with 100 mM potassium glutamate, pH 7.5, and 10 mM amide-loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue). The column was standardized using BIORAD protein marker. It consisted of the following proteins: thyroglobulin (770 kDa), IgG (160 kDa), ovalbumin (43 kDa), apomyoglobin (16 kDa) and vitamin B12 (1.35 kDa). The column was conditioned with the buffer containing 50 mM HEPES, 5 mM MgCl₂, 1 mM dithiothreitol, and 50 mM KCl, followed by elution with the same buffer. The concentration of protein was calculated at 280 nm, and the extent of labeling was calculated from the absorption at 460 nm. The molar ratio of Ru[bipy]₃ label to the protein was found to be 0.7–0.9.

**steady-state Anisotropy Measurement—Ru[bipy]₃-labeled primitive (1.8 × 10⁻³ M) was titrated against DnaB helicase. Anisotropy of Ru[bipy]₃ was determined using excitation at 460 nm and emission at 670 nm (43) after each addition of DnaB. Excitation and emission slit widths were 8 and 4 nm, respectively. All anisotropy values were corrected for background fluorescence using Equation 1.

\[
A_{corr} = \frac{(A - A_0)(A - A_1)(Q/Q_0)(A_1)}{1 + [(A - A_0)(A - A_1)(Q/Q_0)]}
\]

where \(A_0\), \(A_1\) were the anisotropy values for free and DnaB-bound states, respectively. \(Q_0\) and \(Q_1\) were the fluorescence intensities of the bound and free states, respectively. \(A\) is the measured anisotropy at any point in the titration. The \(A_0\) and \(Q_0\) values for the anisotropy and fluorescence intensity of the completely bound form of primase were calculated from the Y-intercepts of the inverse plots, 1/anisotropy versus 1/DnaB hexamer, and 1/fluorescence intensity versus 1/DnaB hexamer, respectively.

The number of mol of primase per mol of DnaB hexamer \(r\) and concentration of the free form of primase \(L_r\) were calculated using corrected \(A_{corr}\) anisotropy values shown in Equations 2 and 3.

\[
r = \frac{A_0}{(1 - a)A_1}
\]

\[
L_r = \frac{1}{1/(K_r) + n/L_r}
\]

where \(a\) is given as (A/A_0) = A_0/1. \(L_r\) and \(IP\) are the primase and DnaB hexamer concentrations respectively. \(L_r\) is the concentration of free or unbound primase. Scatchard plot was constructed using Equation 4 (44),

\[
v/K_r = (-1/K_r) + n/L_r
\]

where \(n\) is the number of molecules of primase bound per DnaB hexamer, and \(K_r\) is the apparent dissociation constant for DnaB-primase complex.

**RESULTS**

In the presence of DnaB helicase, E. coli primase is able to initiate primer synthesis from each of the triplets CTN, CNG, and NTG with significant preference for CAG (25). Therefore, we have used this system in order to determine the stoichiometry of primase-helicase complex. Two 23-nucleotide long, single-stranded DNA templates (see “Experimental Procedures”) with CTG (CTG-template) or CAG (CAG-template) as an initiation trinucleotide were used in this study. The templates also contained 16 nucleotides from their 5’ terminus and six residues 3’ to the initiation trinucleotide, which is the minimum required for efficient primer synthesis (25). The primer synthesis assay was used to monitor the primers produced from the templates in the presence or in the absence of DnaB helicase. Because the chosen templates were enriched in adenine, [α-³²P]UTP was used to label the RNA primers, which were visualized on 20% polyacrylamide gel in the presence of 7 M urea (polyacrylamide/urea gel) followed by autoradiography (Fig. 1). When CTG-template was used as a template in the absence of DnaB helicase, we observed a small amount of primers (about 25–26 nucleotides in length) that were longer than the template (Fig. 1, lanes 3 and 7). In contrast, “overlong” primers were not detected under the same conditions by using a CAG-template after 15 or 30 min of reaction (Fig. 1, lanes 1 and 5, respectively). It has been shown that “overlong” primers occurred when the 3’-end of the template forms a hairpin such that the terminal nucleotide joins with another portion of the
template through the base pair (27). In the presence of DnaB helicase, primase demonstrated remarkable primer synthesis from both CTG-template (Fig. 1, lanes 1, 4, and 8) and CAG-template (Fig. 1, lanes 2 and 6) with obvious preferences for CTG-template (Fig. 1, lanes 4 and 8). Longer incubation of the assay did not interfere with the appearance of the synthesized primers or their length (Fig. 1). Since CTG-template was preferable for this coupled in vitro system, further studies were carried out using this oligonucleotide in 15 min primer synthesis. Furthermore, in vivo studies have shown that primer synthesis predominantly initiates from the d(CTG) trinucleotide (18, 19).

Analysis of in Vitro Primer Synthesis Carried out by Primase in the Presence of DnaB Mutants Defective in DNA Binding—The functional interaction between DnaB and primase involves the last 16 amino acids residues of the C-terminal region of the primase (45) and the N-terminal 12 kDa domain of the DNA helicase, corresponding to amino acids residues 14–136 (29). On the other hand, the DNA binding site restricted to the motif RSRARR and the leucine zipper motif that is responsible for DnaB dimerization are localized in the opposite C-terminal domain of the DnaB helicase (46). Our previous studies suggested that DnaBMut1, with specific mutations R324A and R326A in the RSRARR DNA binding motif of the helicase such as translocation on ssDNA template required for successful stimulation of the primase activity. These results were probably a consequence of the fact that mutations R324A and R326A in the RSRARR DNA binding motif of the helicase did not abolish, but significantly reduced the DNA binding and the DNA-dependent ATPase and helicase activities (46). In contrast, DnaBMut1 failed to stimulate the primase activity of the general priming system (Fig. 2B). With increasing concentrations of DnaBMut1, primase continued to synthesize over-long primers and only trace amounts of RNA primers below 20 nucleotides in length were observed (Fig. 2B, lanes 2–6). Therefore, it appeared that mutations R324A and R326A in the RSRARR DNA binding motif of DnaB not only repress the DNA binding and the helicase activity (46), but also attenuate its ability to cooperate with the primase during in vitro primer synthesis.

Analysis of the Primase-DnaB Hexamer Ratio Required for Optimal Primer Synthesis—In the first set of experiments, primer synthesis was studied in the presence of 0.1 μM DnaB helicase (as a hexamer) and increasing concentrations of the primase (as a monomer) from 0.1 to 0.6 μM (Fig. 3A). The synthesized primers were visualized on polyacrylamide/urea gel as described above using [α-32P]UTP as a labeled ribonucleotide (Fig. 2A). The ratio between the components of the general priming system was expressed as the number of primase monomers interacting with one DnaB hexamer (DnaB6) during in vitro primer synthesis. The length of the RNA primers synthesized under these conditions was similar to that observed in Fig. 2A (11–19 nucleotides). Apparently, the 15-nucleotide long primers appeared to be predominant species after analysis of the products on polyacrylamide/urea gel (Fig. 3A, lanes 1–6). It was also observed that the amount of the synthesized primers increased with increasing the concentration of primase from 0.1 to 0.4 μM (Fig. 3A, lanes 1–4) and slightly decreased after exceeding this concentration (Fig. 3A, lanes 5–6). Next, we carried out primer synthesis by keeping the concentration of primase constant (200 nM as a monomer) and increasing concentrations of DnaB helicase (25–200 nM as a hexamer) (Fig. 3B). The appearance of 11–20-nucleotide long RNA primers was observed even in the presence of 25 nM of DnaB hexamer (Fig. 3B, lane 1) and their pattern remained unchanged with increasing concentrations of the helicase (Fig. 3B, lanes 2–7). The predominant species were 14–19 nucleotides in length with significant abundance of the 15-nucleotide long primers. Our results suggested that the optimal primer synthesis was achieved by using 40–65 nM of DnaB hexamer, which correspond to a 5:1, 4:1, and 3:1 primase monomer: DnaB hexamer ratio, respectively (Fig. 3B, lanes 3–5). This finding is in good agreement with results obtained after titra-
tion of DnaB helicase with primase (Fig. 3A). The maximum primer synthesis was achieved with 0.4 ng of primase and 0.1 μM of DnaB, which corresponds to a 4:1 primase monomer:DnaB hexamer ratio (Fig. 3A, lane 4). 

Gel Filtration Analysis of Primase-DnaB Hexamer Complex—Our primer synthesis studies demonstrated that approximately 3–5 (4 ± 1) molecules of primase are required for one DnaB hexamer. In order to determine the exact stoichiometry of this complex, we have carried out further quantitative analysis. The HPLC gel filtration column was size-calibrated with proteins of known molecular weights as described under "Experimental Procedures" (Fig. 4A), and a standard curve of log [mol. wt.] versus corresponding elution volume was generated (inset of Fig. 4A). E. coli DnaB is known to be a hexamer under a wide variety of conditions (47–49). To confirm the oligomeric state of our purified enzymes, we fractionated these proteins using gel filtration column under similar conditions. Purified DnaB and primase were eluted with apparent molecular masses of 300 and 65 kDa, respectively, as expected (Fig. 4B, a and b), which confirmed that DnaB exists as a hexamer and the primase as a monomer in solution. The gel filtration profile of DnaB helicase at 4 °C did not show a peak for a lower order oligomeric form, probably because E. coli DnaB is more stable at lower temperatures and under our experimental concentrations.

When an equimolar mixture of DnaB and primase (in terms of monomers) was subjected to gel filtration under similar conditions, we did not detect any peak additional to the DnaB hexamer and primase peaks (Fig. 4B, c). Although direct interaction of these two replication proteins has been reported (39), Chang and Marians (35) have suggested that this interaction is very weak and hence, very difficult to study quantitatively. It is possible that while passing through the gel filtration column under high pressure, this complex dissociates because of its weak nature. We cross-linked the complex with glutaraldehyde in order to stabilize it. Glutaraldehyde is an amine-reactive cross-linker, which binds to lysine residues of proteins. It is more efficient than other cross-linkers. The only disadvantage is that it forms a stable complex that cannot be denatured and analyzed on SDS-PAGE.

Elution profiles of DnaB hexamer or primase in the presence of glutaraldehyde did not show any change from that observed in the absence of glutaraldehyde (Fig. 5, B and C). When an equimolar mixture (1 × 10^{-5} M of monomers) of DnaB helicase and primase, cross-linked with 0.2 M glutaraldehyde, was sub-

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**Fig. 2.** *In vitro* primer synthesis in the presence of DnaB mutants defective in DNA binding. Primer synthesis was carried out with 0.8 μM of primase in the absence (lane 1) or in the presence (lanes 2–6) of 0.1–0.3 μM of DnaB helicase. A, primer synthesis with primase and wild-type DnaB helicase. B, primer synthesis with primase and DnaBMut1. C, primer synthesis with primase and DnaBMut2. Positions of the DNA markers are shown on the right.
jected to gel filtration, two peaks were observed (Fig. 5A). One peak was observed at the position expected for the primase monomer. The second was eluted at a position before that of the DnaB hexamer and hence, assigned to the primase-DnaB hexamer complex. We also observed similar elution profiles for samples containing DnaB and primase monomers in different proportions. The molecular mass of the complex was estimated from the standard curve (inset, Fig. 5A) and was found to be 518 ± 25 kDa. The calculated molecular mass values of DnaB and primase complexes were 505 kDa for a DnaB hexamer bound to 3 monomers of primase and 570 kDa for the DnaB hexamer bound to four monomers of primase. The estimated mass of the complex obtained from gel filtration was closely comparable to the theoretical value of 505 kDa for a 6:3 complex of DnaB and primase. Hence, the primase-DnaB hexamer complex, observed in our study was in the form of three primase monomers per DnaB hexamer. Furthermore, we have calculated the areas of the peaks obtained when the primase was subjected to gel filtration alone and in the presence of the DnaB hexamer. This area analysis suggested that in an equimolar mixture of primase and DnaB (in terms of monomers), only ~55% of the primase was complexed to DnaB, and the remainder of the primase was free. These results support three monomers of primase:one DnaB hexamer ratio in the complex.

Scatchard Analysis of DnaB-Primase Complex Formation—Fluorescence anisotropy measurement is a quantitative method to study protein-protein interactions. We have determined the binding affinity and stoichiometry of primase-DnaB hexamer complex using fluorescence anisotropy measurements. This technique was sensitive and allowed us to measure the binding parameter of this weak complex in solution. Primase was labeled with the fluorescent molecular probe Ru[bipy]3. Like other proteins, the Ru[bipy]3-primase (λmax = 640 nm) showed a shift of about 10 nm in emission spectrum compared with free Ru[bipy]3 probe (λmax = 650 nm) (43).

The 1.8 × 10−7 M Ru[bipy]3-primase was titrated with DnaB hexamer and anisotropy was determined at each point using 460 nm as excitation and 670 nm as emission wavelengths, respectively. We selected 670 nm as an emission wavelength to measure the anisotropy, since at this wavelength we found minimal change in the fluorescence intensity. Initial anisotropy of the Ru[bipy]3-primase was 0.1, and it increased with the addition of the DnaB hexamer (Fig. 6A). The base anisotropy value was adjusted to zero by subtracting the initial anisotropy from all anisotropy values. In addition, we corrected all the anisotropy data for change in the fluorescence intensity using Equation 1. The anisotropy data were fitted to the simple hyperbolic equation (Fig. 6A), which gave a Kd of 0.6 × 10−6 M for the primase-DnaB hexamer complex formation. For the construction of the Scatchard plot, we calculated the fraction of the primase in the bound and free states at each titration point using corrected anisotropy values as described under “Experimental Procedures” (Fig. 6B). The linear fit of this plot gave a Kd of 1.2 × 10−6 M and n = 2.77 ± 0.23. Thus, Scatchard analysis demonstrated three primase monomers binding per DnaB hexamer. This result agrees well with our gel filtration data. However, Kd values derived from the Scatchard plot and from anisotropy fit showed a small degree of discrepancy, which could be due to fitting of the anisotropy data by nonlinear regression, considering a one to one interaction model.

Our previous studies have indicated that, in the absence of
DnaB helicase, primase binds to DNA in a sequential dimer pathway without forming a true dimer (41). We observed a very negligible change in the anisotropy when the primase was added to Ru[biipy]3-primase, which supported either a very weak or no direct interaction between primase monomers.

Analysis of in Vitro Primer Synthesis in the Presence of DnaC—The DnaC protein is another major component of E. coli chromosomal DNA replication (15, 17). This replication factor is required for assisting DnaB to bind to the replication origin and appears to have a pivotal role in initiation of helicase function (50, 51). Previous studies demonstrated that in vitro DnaC forms a tight complex with the DnaB helicase in the complete absence of nucleotides and DNA (52). The stoichiometry of the complex is six DnaC monomers (29 kDa each) to one DnaB hexamer leading to a [DnaB:DnaC]6 complex. However, both ssDNA and nucleotides can modulate [DnaB:DnaC]6 complex formation (53).

In vivo, E. coli DnaB helicase unwinds duplex DNA in conjunction with DnaA and DnaC proteins at the replication origin oriC, and allows primase to initiate DNA synthesis on both strands in both directions from the origin (2). On the other hand, E. coli primase is able to synthesize RNA primers in vitro on all of the natural and synthetic templates only in the presence of DnaB helicase, and even does not require SSB (25). Since DnaB helicase is also capable of tightly binding DnaC protein, we examined whether the influence of other replication factor, such as DnaC, would interfere with the activity of the primase, particularly in the DnaB/primase interaction in the general priming system. In these studies, priming on the CTG-template was measured with 0.2 μM of DnaB helicase, 0.8 μM of primase, and increasing concentrations (0.2–2.4 μM) of DnaC protein. Reactions were carried out for 15 min at 30 °C using [α-32P]UTP as labeled ribonucleotide and the products analyzed on polyacrylamide/urea gel, as described above. The autoradiogram of the gel is shown in Fig. 7A. In the absence of DnaC, we observed the usual distribution of the synthesized primers (11–20 nucleotides in length) with significant abundance of the 15-nucleotide long primers (Fig. 7A, lane 1). In the presence of 0.2 μM DnaC protein very short primers (1–7 nucleotides in length) were observed in addition to the primers above 13 nucleotides in length (Fig. 7A, lane 2). The amount of the short primers considerably increased with increasing concentration of DnaC from 0.4 to 2.4 μM, whereas the synthesis of the longer primers was inhibited under the same conditions (Fig. 7A, lanes 3–8). Therefore, in the presence of DnaC, primase was still capable of synthesizing RNA primers, however their length was restricted to 1–7 nucleotides with significant preference of the 4-nucleotide long primers (Fig. 7A, lanes 2–8). In order to examine the extent of the short primers, and the
primers that were typical for the general priming system in the absence of DnaC, we have performed quantitative studies of the predominant 4 and 15 nucleotide primers in Kodak (Fig. 7B). The maximum extent of the 15-nucleotide long primers (\(-6\) arbitrary units) was observed at a 1:1 DnaC:DnaB hexamer ratio, whereas at the same point the extent of the 4-nucleotide long primers was only 0.25 arbitrary units (Fig. 7B). When the DnaC::DnaB hexamer ratio increased to 2:1 and 4:1, the extent of the 15-nucleotide long primers significantly decreased in contrast to the extent of the 4-nucleotide long primers, which considerably increased (Fig. 7B). Further increasing of DnaC concentration did not lead to significant changes in the extent of the synthesized primers (Fig. 7B).

DISCUSSION

DnaB Helicase Binding to DNA Is Necessary for Directing the Primase to the Initiation Sites of the Template—DnaB binds to the replication origin "in vivo" and stimulates primase activity by introducing primase to the replication fork (39). This event includes specific protein-protein interactions between the N-terminal domain of the helicase and the C-terminal domain of the primase (45). Point mutations in the N-terminal domain of the E. coli DnaB helicase that affect the ability of primase to interact with DnaB do not interfere with the primer synthesis but rather, direct the synthesis of Okazaki fragments of altered length. This suggests that the interaction between these two replication proteins is pivotal for the cycle of Okazaki fragment synthesis as well as determination of their length (33). On the other hand, in the absence of DnaB helicase, primase acts alone on 23-nucleotide templates, which stabilizes a 3'-end hairpin template conformation and extends from 3'-hydroxyl of the template to create overlone primers (Ref. 18 and Fig. 2). In contrast, the general priming system consisting of both DnaB and primase demonstrated synthesis of RNA primers with a restricted length of about 11–20 nucleotides (Ref. 16, Figs. 1–3). In our studies with CTG-template, it was the preferred template in comparison to the CAG-template, which is in good agreement with previous studies suggesting that the d(CTG) trinucleotide is the predominant initiation site during replication initiation "in vivo" (23, 25). Therefore, DnaB helicase appears to be important for directing the primase to the initiation trinucleotide site of the template in the reconstituted simple priming system. This observation was confirmed by using DnaB mutants, containing point mutations in the DNA binding motif RSRRARR of the E. coli helicase (Fig. 2). When wtDnaB was substituted for DnaBMut1, containing point mutations R328A and R329A that repress both DNA binding and helicase activity, primase demonstrated a tendency to synthesize overlong primers, obviously as a result of its inability to cooperate with helicase (Fig. 2B). In contrast, DnaBMut2, containing point mutations R324A and R326A with somewhat attenuated binding activity, was capable of cooperating with primase during "in vitro" primer synthesis (Fig. 2B). However, the amount of 11–20-nucleotide long primers was reduced in comparison to wild-type DnaB (Fig. 2A). This result is due to the fact that mutations R324A and R326B do not abolish, but rather significantly reduce, DNA binding and DNA-dependent ATPase and helicase activity (46). It has been shown that the DNA binding motif, RSRRARR, is located in the C-terminal domain of E. coli helicase (45), whereas the primase binding site is positioned in the N-terminal domain (35). Consequently, DnaBMut1 and DnaBMut2 mutants should be able to bind primase, even though they are defective in DNA binding. However, it appears that if the helicase is not capable of binding to DNA, it cannot direct the primase to the initiation d(CTG) site of the template. In conclusion, the ability of DnaB to stimulate primer during "in vitro" primer synthesis requires binding of the helicase to the single-stranded oligonucleotide template.

Stoichiometry of the Complex [primase-DnaB hexamer] Is 3:1—It has been demonstrated that, at the replication fork, DnaB recruits primase via a protein-protein interaction, which consequently produces primer (26, 27). However, their interaction is assumed to be transient since there is no direct evidence for it. In the case of a simpler system, such as T7 DNA bacteriophage, helicase, and primase are encoded by the same gene and can be studied in detail. During assembly of the T7-coded primosome, primase domains are arrayed as smaller lobes about one face of the toroid against the large hexameric ring of helicase in a 6:6 helicase-primosome arrangement (54). Previous studies have provided information about the binding domains involved in the interaction of these two proteins of E. coli. They demonstrated that the C-terminal 16 amino acid region of primase is crucial for its interaction with DnaB while the N-terminal domain of DnaB is essential for primase binding (23, 24). However, these studies always lack direct evidence of any direct physical interaction between the two enzymes probably due to their weak complex formation. Therefore, the structural arrangement of primase-DnaB hexamer complex in E. coli system is not yet clear.

DnaB alone was found to form a stable homohexameric structure in solution, while primase alone existed as a monomer as seen in gel filtration profiles. Gel filtration data revealed that the stoichiometry of this complex is three primase monomers bound to one DnaB hexamer in the presence or in the absence of ssDNA (Fig. 5). The Scatchard analysis of the fluorescence data also supported this ratio. It demonstrated that DnaB hexamer provides three binding sites for primase (Fig. 6). The high apparent dissociation constant (0.6–1.2 \(\times 10^{-6}\) M) of this complex further affirmed the widely assumed hypothesis that these enzymes have a low affinity for each other and form a transient or weak complex. Primer synthesis carried out in the presence of varying concentrations of DnaB helicase and primase yielded approximately four primase...
monomers per one DnaB hexamer as optimal. Probably, a slight excess of primase is needed to achieve optimal complex formation with a 3:1 ratio due to the transient nature of the complex. Nakayama et al. (29) demonstrated that the DnaB protomer is composed of two main domains. One N-terminal region acts as a binding pocket for DnaC and primase whereas a C-terminal domain provides a DNA binding site and is also responsible for hexamerization. DnaB homohexamer can exhibit a 3-fold as well as 6-fold rotational symmetry (26, 48, 55). San Martin et al. (30) has shown that the DnaB oligomer is a trimer of asymmetrical dimers with a pronounced triangular shape. In addition, DnaB exhibits three high affinity binding sites for ATP analogs, which further supports the 3-fold rotational symmetry of this hexameric molecule (48, 50). In the case of the DnaC-DnaB hexamer complex, each DnaB monomer provides a binding site for a DnaC and thus exhibits a 6:1 stoichiometric ratio (56). Further, electron microscopy of the DnaC-DnaB complex suggests that C-terminal domains of DnaB are arranged in a central ring, which has 6-fold symmetry, whereas the N-terminal domains protrude outward from the central mass in the form of dimers, possessing 3-fold axis of symmetry (57). In the case of the DnaC-DnaB complex, three lobes of the N terminus of DnaB interlock a single dimer of DnaC, thus six DnaC molecules associate per hexamer of DnaB (57). DnaB also provides multiple binding sites for the other interacting replication factors. Consequently, three primase monomers can bind to three N-terminal lobes coming out from the central ring. Probably the inability of primase to form a tight isolable dimer and its size ($M_r \approx 65$ kDa), which is twice that of DnaC ($M_r \approx 29$ kDa), restricts its stoichiometry with DnaB hexamer to 3:1.

Our previous studies on primase-ssDNA binding demonstrated that it associates with DNA as a pseudo dimer (41). However, these studies were carried out in the absence of DnaB and did not preclude the possible formation of a higher order complex. Taken together, we can say that three primase monomers are essential to associate with a DnaB hexamer and, eventually, with ssDNA for efficient primer synthesis.

**DnaC Protein Induces Synthesis of Abbreviated RNA Primers**

Three proteins, DnaA, DnaB, and DnaC, are required for initiation of DNA replication at the chromosomal origin of replication OriC in *E. coli* (52, 58, 59). It has been known that DnaC is required for assisting DnaB helicase to bind to the origin (50–52). Therefore, DnaC protein has a pivotal role in the initiation of DNA replication as well as in DnaB helicase function *in vivo*. DnaB helicase assists primase to prime single-stranded DNA templates *in vitro* in the absence of any other auxiliary proteins (this work, Ref. 16). Results, presented here, demonstrated that DnaB helicase has to bind to the DNA template before directing primase to the initiation trinucleotide (Fig. 2). The size of the primers observed with the general priming system, consisting of *E. coli* DnaB and primase, range from 11 to 20 nucleotides in length, with significant abundance of the 15-nucleotide long primers (Fig. 1–3). It should be noted that a similar observation was made by Bhattacharyya and Griep (25). When the DnaC protein was included in the simple priming system together with the helicase and the primase, the length of the primers synthesized were restricted to 2–7 nucleotides with abundance of the tetranucleotide primers (Fig. 7A).
Furthermore, with increasing concentration of DnaC, the amount of 11–20-nucleotide long primers gradually decreased, while the amount of the shorter primers increased (Fig. 7, A and B). Since, both DnaC and primase are able to form a stable complex with DnaB hexamer (53, 57), it was expected that DnaC would inhibit the primase activity due to its occupation of the DnaB hexamer binding sites. DnaC protein did not appear to inhibit the ability of DnaB to stimulate primase to synthesize RNA primers, except that the primers were shorter in length (Fig. 7A). These results indicate that DnaC protein attenuates the mobility of DnaB helicase on the single-stranded DNA template. The restricted mobility of DnaB helicase limits the translocation of primer necessary for the synthesis of full-length primers. It also appears that both DnaC protein and primase bind on the N-terminal region of DnaB helicase, these proteins can coexist on the DnaB helicase.

We propose that a combination of these two events led to the synthesis of primers that are only 2–7 nucleotides in length. The entire genome of *E. coli* is replicated in ~20 min (2). Consequently, a rapid initiation of DNA replication and priming by primase is required. It was anticipated that removal of DnaC protein from the DnaB-DnaC complex, after DnaB loading onto the DNA, would be necessary before initiation of primer synthesis. Our results demonstrate that primase initiates primer synthesis in vivo due to DnaC dissociation, and the full-length primers (11–20 nucleotides) are likely produced after DnaC protein completely dissociates from the initiation complex. Therefore, this unique primase action allows a shortening of the time required for priming initiation. In addition, it is also probable that shorter primase products can serve as primers for the DNA polymerase III holoenzyme of *E. coli*, further aiding in the process. Further studies are required to assess the possible roles of the shorter primers in *E. coli* DNA replication.

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